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 IRAK4 mediates colitis-induced tumorigenesis and chemoresistance in colorectal cancer

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ABSTRACT

Aberrant activation of the NF-κB transcription factors underlies chemoresistance in various cancer types including colorectal cancer (CRC). Targeting the activating mechanisms, particularly with inhibitors to the upstream IκB kinase (IKK) complex, is a promising strategy to augment the effect of chemotherapy. However, clinical success has been limited largely due to low specificity and toxicities of tested compounds. In solid cancers, the IKK kinases is driven predominantly by the Toll-like/Interlekin-1 receptor family members, which signal through the Interleukin-1 Receptor-Associated Kinases (IRAKs), with isoform 4 (or IRAK4) being the most critical. The pathogenic role and therapeutic value of IRAK4 in CRC has not been investigated. We found that IRAK4 inhibition significantly abrogates colitis-induced neoplasm in APC^{Min/+} mice, and bone marrow transplant experiments showed an essential role of IRAK4 in immune cells during neoplastic progression. Chemotherapy significantly enhances IRAK4 and NF-κB activity in CRC cells through upregulating TLR9 expression, which can in turn be suppressed by IRAK4 and IKK inhibitors, suggesting a feedforward pathway that protects CRC cells from chemotherapy. Lastly, increased tumor phospho-IRAK4 staining or IRAK4 mRNA expression are associated with significantly worse survival in CRC patients. Our results support targeting IRAK4 to improve the effects of chemotherapy and outcomes in CRC.
INTRODUCTION

The incidence of colorectal cancer (CRC) is rising worldwide(1, 2). Systemic chemotherapy is critical in improving survival, but clinical response is highly variable(3). Increased efflux, decreased uptake and impairment in enzymatic conversion to active metabolites are strategies utilized by CRC cells to lower the effective intracellular concentrations of chemotherapeutics, but these can be partly overcome by combining different chemotherapeutic agents. However, enhanced survival mechanism driven by NF-κB, MAPK, β-catenin, TNF or PI3K pathways, poses an additional therapeutic barrier(4, 5). Of these, the NF-κB pathway is one of the best characterized. High basal tumor NF-κB expression is present in at least 40% of CRC tumor samples and is associated with aggressive phenotype and resistance to chemotherapy(6-9). Genetic ablation of IκB kinase β-isoform (IKKβ), the master kinase within the IKK complex that activates the canonical NF-κB transcription factors, in intestinal epithelium significantly reduced colitis-induced CRC in mice(10). Besides basal level, NF-κB activity in CRC cells can be further induced upon exposure to chemotherapy(7, 11). Therefore there is strong rationale for targeting the NF-κB pathway in CRC, particularly through inhibiting the IKK complex(12). Unfortunately, success has been limited to date, as most of the tested inhibitors lack specificity and/or are highly toxic, as reflected by embryonic lethality of ablating IKKβ or RelA in mice(13-16). Therefore, novel, less toxic approaches to target the NF-κB pathway should be explored.

In epithelial cells, the IKK-NF-κB axis can be activated by the Toll/IL-1R (TIR) superfamily members, which consist of the Toll-like (TLRs) and IL-1 (IL-1R) families of receptors(17). Ligation of these receptors result in recruitment and assembly of the adaptor protein MyD88 and Interleukin-1 Receptor-associated kinase (IRAK) isoforms 1, 2 and 4, leading to activation of TAK1 kinase and subsequently the IKKK complex and NF-κB(18). Therapeutic value for targeting the IRAK members, specifically the IRAK1 and IRAK4 isoforms, has been elucidated in several cancer types including diffuse large B cell lymphoma (DLBCL)(19, 20), myelodysplastic syndrome(21), T-cell ALL(22), melanoma(23), breast cancer(24), head and neck cancer(25), and in pancreatic cancer by our group(26, 27). In pancreatic cancer, IL-1β secreted by both neoplastic cells and fibroblasts activate the IRAK4-NF-κB axis to foster tumor fibrosis, which
contributes to chemoresistance. Notably, we showed that the kinase activity of IRAK4, but not IRAK1, is essential in driving NF-κB activity in pancreatic cancer cells(27). Contribution of the TIR receptors and IRAK4 in the progression and treatment resistance of CRC has not been investigated in detail. In an elegant study, KRAS-dependent colon cancer cells stimulates BMP-7 secretion to autologously activate BMP receptor, TAK1 kinase and the NF-κB transcription factors(28). To date, safe and effective TAK1 inhibitors remain elusive. TAK1 deletion leads to massive bone marrow and liver failure in mice, again raising concerns for toxicities associated with TAK1 inhibitors(29). Contrastingly, an IRAK4 inhibitor is currently being tested in a clinical trial (NCT03328078) for refractory DLBCL. In this study, we investigated whether IRAK4 is activated, has a pathogenic role, and is a good therapeutic target in CRC.
RESULTS

IRAK4 is required for colitis-induced neoplasm in $APC^{Min/+}$ mice.

We observed robust phosphorylated (denoted p-) IRAK4, p65 and p50 NF-κB subunit staining by immunohistochemistry (IHC) in colon cancer spontaneously formed in $APC^{Min/+}$ mice, whereas these markers were absent or very faint in normal colon epithelium in age-matched wild-type littermates (Figure 1A). While $APC^{Min/+}$ mice formed almost exclusively small intestinal tumors, treatment with 2% dextran sodium sulfate (DSS) in drinking water induces colitis and development of colonic neoplasm at very high penetrance(30), and is a robust model for studying colon cancer progression. Using this approach, we found that mice pre-treated with DSS followed by an IRAK4 inhibitor (IRAK4i) PF06650833 developed significantly fewer visible tumors and microadenomas compared to vehicle-treated mice, and the number of neoplasms in either gender was similar in both treatment groups (Figure 1B, 1C). Intensities of p-IRAK4 and p-p65 IHC staining were drastically diminished in IRAK4i-treated colon, indicating on-target effect of PF06650833 (Supplementary Figure 1). Notably, focused analyses on microadenomas showed that IRAK4i-treated tumors contained significantly less proliferating neoplastic (dual CK+ Ki-67+) cells (Figure 1D). Importantly, IRAK4i protected mice from significant weight loss, with none of IRAK4i-treated mice reaching humane endpoint while many vehicle-treated mice had to be sacrificed (Figure 1E). To delineate the requirement for IRAK4 in hematopoietic cells in this model, we performed bone marrow transplantation to create $APC^{Min/+}$ chimeric mice with IRAK4-null, or as control, wild-type bone marrow (Figure 2A). Following DSS treatment, $APC^{Min/+}$ chimeric mice with IRAK4-null bone marrow developed significantly fewer gross tumors and microadenomas compared to mice reconstituted with wild-type bone marrow, which had similar number of visible tumors and microadenomas as regular $APC^{Min/+}$ mice (Figure 2B). Notably, mice transplanted with IRAK4-null bone marrow had significantly less degree of colitis, as scored based on crypt damage and inflammatory infiltrates (Figure 2C). These results suggest an essential role for IRAK4 function in hematopoietic cells in colitis-induced neoplastic transformation, reflecting the known role of IRAK4 in innate immunity(31). Intriguingly, we did not observe statistically significant difference in the abundance of CD45+ leukocytes in the colon epithelium or tumors in either
group (Figure 2D), strongly suggesting an essential role for IRAK4 in altering the composition, rather than quantity, of immune infiltrates that mediates colitis and neoplastic progression.

**IRAK4 is constitutively activated and drives NF-κB activity in human CRC.**

We next evaluated activation status of the IRAKs and NF-κB pathway proteins in human CRC. We detected robust p-IRAK1, a direct substrate of IRAK4, in 11 out of 12 CRC lines, whereas p-IRAK1 signals were faint in normal colon cell lines FHC and CCD-18Co. On the other hand, p-IRAK4 is detectable at various intensities in both normal and CRC lines (Figure 3A). In these CRC lines, we did not detect an N-terminally truncated, inactive form of IRAK4 protein using an antibody raised against the C-terminus of IRAK4, as reported in myeloid malignancies(32) (Supplementary Figure 2A). Notably, p-IKKα/β, p-p65 and p-p50 are detected predominantly in CRC lines. In this limited panel of cell lines, we did not observe any correlation between known genetic mutations (KRAS, PIK3CA and BRAF) and activation status of these NF-κB pathway molecules, indicating that NF-κB activity cannot be contributed to these oncogenic events. Next, we performed IHC analyses using a pool of five commercial human CRC tissue microarrays (TMAs). We found p-IRAK4 IHC staining to be significantly stronger in CRC (N=220) compared to normal colon tissues (N=49, Figure 3B), although a fraction of normal colon mucosa also stained robustly with p-IRAK4. The staining intensity of p-IRAK4 did not differ among CRC from various clinical stage (Supplementary Figure 2B). Perhaps relevant, expression of IRAK4 mRNA is significantly higher in colon cancer than normal colon tissues from analysis of Oncomine(33), a public microarray database (Supplementary Figure 2C). Upregulated p-IRAK4 staining was also present in benign colonic polyps, although the number of samples was low (N=10). Interestingly, p-IRAK4 staining in tumor-adjacent normal colon samples was higher compared to non-cancerous colon samples. These results suggest that IRAK4 can be activated as early as pre-cancerous stage and participates in neoplastic progression, echoing our observation in APC<sup>Min/+</sup> mice. Notably, we observed strong, significant correlation between the p-IRAK4 and p-p65 IHC staining in colon cancer samples (Figure 3C). We did not use p-p50 for correlation because the distribution of p-p50 is almost exclusively nuclear, precluding accurate
interpretation of staining intensity. Together, these results establish strong correlation between activation of IRAK4 and NF-κB pathway during CRC progression.

**IRAK4 drives NF-κB activity in human and murine CRC cells**

To investigate the causal relationship between IRAK4 and NF-κB activity, we stably silenced IRAK4 in two CRC lines, DLD-1 and KM12, using two different small hairpin RNAs (shRNAs). We observed decreased p-IRAK1, p-IKKα/β, p-p50, p-65 and nuclear p65 levels in IRAK4-silenced cells (Figure 4A, 4B). NF-κB activity in IRAK4-silenced cells can be rescued by re-expression of wild-type, or more potently, activated mutant of IKKβ (Supplementary Figure 3A), placing IRAK4 as the upstream event of NF-κB activity. Notably, IRAK4-silenced CRC cells were also defective in anchorage-independent growth and tumorigenesis (Figure 4C, 4D). To complement our findings in murine model, we also investigated the cell-autonomous role of IRAK4 in MC38 cells, a murine CRC line derived from a C57BL/6J mouse (34). Using CRISPR/Cas9 technique, we generated polyclonal MC38 lines stably expressing Cas9 and two different IRAK4-targeting sgRNAs. Compared to vector control line, IRAK4-ablated MC38 lines are severely defective in clonogenic growth, anchorage-independent growth and tumorigenesis (Figure 4E, 4F, 4G). As a result, mice bearing IRAK4-ablated MC38 tumors survived significantly longer than those bearing scramble control tumors (Figure 4H). Notably, IRAK4 sgRNA-transduced MC38 tumors eventually grew but when re-cultured were all found to have restored IRAK4 expression (Supplementary Figure 3B). Together, we conclude that IRAK4 is necessary for both the hematopoietic and epithelial compartments in CRC development and progression. Together, our results strongly support IRAK4 as the driver of the canonical NF-κB pathway and is required for tumorigenesis of CRC cells.

**IRAK4 Inhibitors suppress NF-κB activity in CRC cells**

To translate our findings, we tested two different IRAK4 inhibitors (IRAK4i). Of these, PF06650833 is being tested in clinical trial (35), and was shown to protect inflammation-associated renal failure in rats (36). We previously showed that AS2444697 blocks IL-1β induced NF-κB activation in pancreatic cancer cells (27, 36). Here, we observe varied susceptibility of different CRC lines to both IRAK4i in
standard 2D cultures (Figure 5A). Importantly, we observed strong positive correlation in IC₅₀ values between the two IRAK4 inhibitors across 13 CRC lines, cross validating the specificity of these two compounds (Figure 5B). Supporting our results using shRNAs, both IRAK4 inhibitors suppressed clonogenic growth of CRC cells (Figure 5C). Notably, while IRAK4 inhibitors did not suppress growth of SW80 and SW620 cells in monolayer culture, they potently suppressed clonogenic growth of these cells, implying engagement and need for IRAK4 and NF-κB activation in stress-induced conditions, as we previously reported in pancreatic cancer cells(26). Both IRAK4 inhibitors potently blocked p-IRAK1, p-p50, nuclear translocation of p65 and NF-κB driven luciferase reporter activity dose-dependently in CRC cells (Figure 5D, 5E, 5F). Notably, PF06650833 was also able to dose-dependently suppress p-IRAK4 and p-IRAK1 in normal colon cell line FHC (Supplementary Figure 3C), suggesting IRAK4 as the upstream activator of IRAK1 in non-neoplastic colon cells. As further validation of their specificity, the inhibitory effects of PF06650833 and AS2444697 on NF-κB reporter can be completely abolished by ectopic expression of constitutively active IKKβ mutant(37), but not wild-type IKKβ (Figure 5G). These results validate the utility of these IRAK4 inhibitors as NF-κB suppressors in CRC.

Chemotherapy induces TLR9-IRAK4 circuitry to sustain CRC cell survival

Besides basal activity, DNA damage such as that induced by chemotherapy can activate ATM, which can bind and activate TAK1 and IKKγ (NEMO) subunit, resulting in further activation of the canonical NF-κB cascade which allows cells to survive and undergo DNA repair(38). Therefore, abrogating NF-κB induction represents a promising strategy to potentiate the cytotoxic effect of chemotherapy(39). As expected, we found that exposure of CRC cells to chemotherapy significantly upregulates NF-κB reporter activity in CRC cells (Figure 6A). While oxaliplatin and 5-FU alone were able to modestly activate NF-κB activity, the combination of both agents led to a more significant upregulation, indicating that the degree of NF-κB induction is not specific to any class of chemotherapeutic but is more likely associated with the extent of DNA damage. To explore whether IRAK4 is required for chemotherapy-induced NF-κB activation, we tested CRC cells with oxaliplatin or 5-FU separately in the absence or presence of PF06650833 or AS2444697. Both IRAK4 inhibitors significantly suppressed NF-κB activity and enhanced...
apoptosis of CRC cells treated with either chemotherapy agent, indicating that the suppressive effect of IRAK4i is not specific to any chemotherapeutic agent (Supplementary Fig 4A, 4B, 4C). Similarly, IRAK4-silenced CRC cells are significantly more sensitive to 5-FU, oxaliplatin and SN-38 (a metabolite of irinotecan, Supplementary Figure 4D). Because IRAK4 is typically activated by TLRs or IL-1R, we examined expression levels of these receptors, as well as IL-1α and IL-1β following chemotherapy (5-FU plus oxaliplatin) exposure. Of these receptors, TLR9 is the most significantly upregulated in all three CRC lines following exposure to chemotherapy (Figure 6B). Notably, 5-FU and to a lesser degree, oxaliplatin, were able to induce TLR9 expression and p-IRAK4, but the combination of both agents was at least additive (Figure 6C, left panel). Notably, chemotherapy induced-TLR9 coincides with upregulated p-IRAK4 and p-IKKα/β (Figure 6C, right panel). Because TLR9 signals through IRAK4 and IKK(40), we postulate that TLR9 may play a role in chemotherapy-induced NF-κB activation. Supporting this notion, we observed significantly increased interaction between TLR9 and p-IRAK4 in chemotherapy-treated CRC cells by proximal ligation assay (PLA) using TLR9 and p-IRAK4 antibodies (Figure 6D). Interestingly, some level of interaction was already present in control cells, indicating TLR9 as an activator of IRAK4 and NF-κB at baseline. Knockdown of TLR9 by two different shRNAs increased apoptosis, as determined by PARP cleavage, and completely abrogated chemotherapy-induced NF-κB activation (Figure 6E, 6F). Conversely, IRAK4i or IKKβi (IMD-0354) partially suppressed chemotherapy induced-TLR9 mRNA and protein expression and promoted apoptosis (Figure 6G, 6H). These results suggest that TLR9 is a transcriptional target of NF-κB during DNA damage. Intriguingly, chemotherapy also enhanced cleavage of TLR9, a marker for TLR9 activation, in 293T cells (Supplementary Figure 5). Because TLR9 is known to sense double-stranded DNAs, which presumably are enriched following DNA damage, these data depict a scenario where damaged DNA may bind and activate TLR9, leading to IRAK4-IKK-NF-κB activation which in turn further upregulates TLR9 expression, forming a feedforward circuitry to sustain cellular survival. Therefore, breaking this circuitry with IRAK4i or IMD0354 can enhance the pro-apoptotic effect of chemotherapy. Together, these results provide strong rationale for testing IRAK4 inhibitors in combination with chemotherapy in vivo.
**IRAK4 inhibition synergizes with chemotherapy in vitro and in vivo**

We next quantified the drug effect interactions between the two IRAK4 inhibitors and oxaliplatin, SN38 and 5-FU, across six fixed-ratio doses in three CRC cell lines, using the widely adopted Chou-Talalay method (Compusyn software)(41). Of the three CRC lines tested, most combination indices (CI) values fell below 0.9, the defined cutoff for synergism(41) (Figure 7A, 7B). Next, we investigated whether IRAK4i will potentiate chemotherapy in xenograft mouse models. We chose to focus on oxaliplatin and 5-FU because these two agents (in the form of FOLFOX) are the most common first line agents administered in advanced CRC and in the postoperative setting. To this end, we focused on DLD-1 and KM12 cells, which readily form tumors in immunocompromised mice with very short latency (~14 days). Established (~100mm³) subcutaneous KM12 and DLD-1 tumors growing in nude mice were treated. Although neither AS2444697 nor chemotherapy alone was able to significantly suppress tumor growth, in both models combo-treated tumors were significantly slower in growth kinetics and volume (Figure 7C, Figure 7G). Combination-treated tumors showed significantly larger areas of necrosis, more apoptosis (by cleaved-caspase 3⁺ area) and less proliferation (Ki-67⁺) of neoplastic cells (dual pan-CK⁺/Ki-67⁺ cells) compared to the other treatment arms (Figure 7D, 7E, 7H, 7I). Notably, p-IRAK4 and p-p50 IHC staining were reduced in AS2444697-treated tumors, confirming on-target effects (Supplementary Figure 6A). To further confirm and potentially translate our findings, we repeated the experiment using PF06650833, the IRAK4 inhibitor now being tested in clinical trials for patients with rheumatoid arthritis(35) (NCT02996500). In this experiment, CRC-bearing mice were sacrificed when their subcutaneous xenograft tumors exceeded 2000mm³, or when any humane endpoints pre-defined in the animal protocol were met. In both models, we observed significant slower tumor growth kinetics and prolongation of survival in mice treated with PF06650833 plus chemotherapy (Figure 7F, 7J). Combo-treated mice had slower weight gain compared to mice in other arms over the treatment course but did not exhibit any noticeable signs of distress (Supplementary Figure 6B and 6C). Together, our results strongly support testing IRAK4 inhibitor with chemotherapy in clinical trials for CRC patients.

**High p-IRAK4 is associated with poor overall survival in colon cancer patients**
For colon cancer patients with Stage III (lymph node-positive) and Stage II disease with high risk histopathologic features (particularly T4 disease, or Stage IIb, IIc), 5-FU plus oxaliplatin (FOLFOX) is routinely administered after surgery to lower the chance for recurrence. However, up to 30% of treated patients still develop relapse, indicating resistance of micro-metastatic cells to chemotherapy. Since our preclinical studies showed that activated IRAK4 may be a mechanism of chemoresistance, we reasoned that high IRAK4 activity may be associated with poorer prognosis in patients with Stage IIB-III disease who are routinely treated with FOLFOX. To this end, we analyzed p-IRAK4 IHC staining on two commercial CRC TMAs with annotated overall survival data. In these CRC samples, p-IRAK4 staining was present predominantly in the cytoplasm of neoplastic cells, while some staining was seen in infiltrative leukocytes and a subset of stromal cells (Figure 8A) (42). We found that high p-IRAK4 staining in neoplastic cells was significantly associated with poorer overall survival in patients with Stage IIB-III disease, but not in patients with Stage I or II disease (Figure 8B, 8C, 8D). Notably, analysis of the TCGA database showed that Stage IIb-III colon cancer patients with higher IRAK4 mRNA expressing tumors, as defined by mRNA Z score >0.5, had statistically significant, poorer overall survival compared to the remaining patients with lower IRAK4 mRNA expression (Figure 8E). In patients with Stage IV CRC, FOLFOX in combination with an anti-VEGF or anti-EGFR antibody is frequently administered as 1st line treatment, but response rate is about 50-60%. To probe the prognostic impact of p-IRAK4 in metastatic CRC patients, we collected metastatic liver samples from 225 patients who underwent synchronous or metachronous liver and colon resection for curative intent at our institution. TMAs were constructed from the metastatic liver tumors from these patients and stained with p-IRAK4 antibody. All samples were categorized as high, medium or low in p-IRAK4 staining by H-score. Thirty-two patients had paired primary and metastatic liver CRC tumors for comparison. We did not observe significant correlation in p-IRAK4 staining intensity between paired primary versus metastatic liver samples (Figure 8F). The remaining 204 patients went on to receive systemic chemotherapy either at Washington University or other institutions. Notably, when controlling for other clinicopathological factors (Supplementary Table 1), we found that patients with high tumor p-IRAK4 IHC staining had significantly poorer overall survival compared with those with medium to low p-IRAK4 levels (Figure 8G). Overall, these data show that high
IRAK4 activity is a poor prognostic factor for CRC patients, lending further support for targeting IRAK4 in combination with chemotherapy in CRC to improve outcome.

**DISCUSSION**

In the present study, we provided evidence, for the first time, that IRAK4 is activated and drives IKK-NF-κB activity and is a novel promising therapeutic target in CRC. Using pharmacologic and genetic approaches, we showed that targeting IRAK4 stifles colitis-induced neoplastic progression in genetic mouse model and lowers NF-κB activity in human CRC cells. We provided new mechanistic insight that chemotherapy induces a TLR9-IRAK4-NF-κB feedback loop in CRC cells that when disrupted, can enhance chemotherapy-induced apoptosis. On this premise, IRAK4 inhibitors synergize with chemotherapy and prolong survival of CRC bearing mice. Notably, activation or high expression of IRAK4 is predictive of poorer overall survival in colon cancer patients who receive chemotherapy. As opposed to IKK or TAK1, targeting IRAK4 may be more clinically tractable, based on lack of observed toxicities in our treated mice and that *IRAK4-null* mice are viable and have normal lifespan(43). However, both IRAK4 inhibitors that we used had no single agent activity in CRC growth in vivo, indicating the need to combine with chemotherapy in future clinical trial design for CRC patients. In addition, all combo-treated mice eventually still succumbed to disease progression, suggesting emergence of resistance mechanisms which remain to be investigated. Importantly, because *IRAK4-null* mice are defective in mounting innate immunity when challenged with microbes(43), and IRAK4-deficient patients suffer from severe bacterial infections before puberty(44, 45), infection will be a concern for IRAK4 inhibitors especially when co-administered with chemotherapy. Therefore, proper anti-microbial prophylaxis should be considered in future clinical trial design.

In this study, we showed TLR9 to be the driver of IRAK4 and NF-κB in CRC cells upon chemotherapy exposure. Interestingly, we also observed significantly upregulated TLR9 mRNA expression in pancreatic cancer cells (MIA Paca-2) following exposure to oxaliplatin, SN38, 5-FU and gemcitabine, suggesting TLR9 upregulation may be a common adaptive response following DNA damage at least in these two
cancer types (Supplementary Figure 7). Based on these premises, we propose that acute DNA damage response recruits ATM, which directly triggers the NF-κB cascade, while damaged DNA may also activate the preexisting pool of TLR9. Activation of the NF-κB cascade results in transcriptional upregulation of TLR9, which subsequently engages the IRAK4-NF-κB cascade to amplify and sustained the feedforward circuitry (Figure 9). Besides TLR9, it is reasonable to hypothesize that one or more TLRs, and their natural ligands originating from surrounding damaged cells or even gut microbes within the tumor microenvironment, to be the trigger of IRAK4 and NF-κB in CRC. Supporting this notion, manipulation of gut microbiome composition with fecal transfer or antibiotics can enhance or lower colitis-induced CRC formation in mice(46, 47), establishing microbiota dysbiosis as a pro-tumorigenic event. More provocatively, transfer of fecal samples from CRC patients promotes intestinal inflammation and tumorigenesis in germ-free mice (48). Because IRAK4 is the master kinase downstream of most TLRs, it will be very interesting to determine whether IRAK4 inhibition can suppress CRC formation in these models.

The actual role of MyD88, the critical adaptor that recruits and activate IRAK4, during different stages of colitis-induced CRC development is divergent, largely dependent on mouse models and experimental conditions(49). We showed that IRAK4 inhibitor is highly effective in blocking DSS-induced neoplastic progression in APCMin/+ mice but has no effect on tumorigenic growth of established CRC tumors in vivo. To clearly determine the role of epithelial IRAK4 during neoplastic progression, we pre-treated APCMin/+ mice with DSS to allow enough time for inflammation to occur before introduction of IRAK4i, which is known to have anti-inflammatory effect. The near complete obliteration of tumor formation in IRAK4i-treated mice suggests epithelial IRAK4 to be required in neoplastic transformation, which was further supported by the requirement of IRAK4 in MC38 model. These results are in agreement with work by others which showed an essential cell-intrinsic role of MyD88 in spontaneous intestinal and AOM-induced colon cancer formation in APCMin/+ mice (50-52). As opposed to MyD88−/− APCMin/+ model, MyD88−/− C57BL/6 mice develop more severe colitis upon acute AOM and/or DSS treatment, leading to more DNA damage and higher incidence of CRC development(50, 53, 54). On the contrary, in a chronic oxazolone-
induced colitis model, *MyD88*−/− mice develop robust inflammation but not colon cancer, largely due to loss of MyD88-dependent pro-tumorigenic M2 macrophages(55). Nonetheless, co-administration of MyD88 inhibitor (TJ-M2010-5) with DSS after AOM treatment attenuated colitis and prevented CRC development in Balb/c mice(56). Importantly, we showed that IRAK4 function in hematopoietic cells is critical in DSS-induced colitis and CRC progression, although no difference in leukocyte abundance was seen throughout the colon of mice transplanted with wild-type or *IRAK4*−/− bone marrow. This finding, while surprising, was in agreement with another report which showed that *LysM*-Cre driven deletion of IKKβ in myeloid cells reduces colitis-induced tumor burden without affecting the abundance of myeloid infiltrates(10). We envision the following possibilities. First, loss of IRAK4 may attenuate DSS-induced/or and subsequent tumor-associated inflammation, allowing faster healing of injured epithelial cells. Second, loss of hematopoietic IRAK4 reduces/abrogates production of tumor-promoting cytokines such as IL-1β and TNFα, as shown to be regulated by IKKβ (10), which may directly impact epithelial cell transformation and proliferation. Third, loss of hematopoietic IRAK4 may shift the composition of intra-tumoral immune infiltrates including subtypes of macrophages, granulocytes and lymphocytes subsets, to an anti-tumorigenic phenotype thereby impeding neoplastic progression. Generation of conditional *IRAK4-null* mouse will be essential in dissecting the role of IRAK4 in various subsets of hematopoietic cells during CRC development and to definitively address these possibilities. Nonetheless, our results suggest that systemic inhibition of IRAK4 appears to be an effective strategy in curbing CRC development.

Lastly, we showed that activated IRAK4 (p-IRAK4) is associated with poor prognosis in CRC patients with Stage IIb-IV disease after resection. These patients are routinely treated with chemotherapy (mainly FOLFOX) following surgery and therefore high p-IRAK4 is a potential indicator for lack of benefit from chemotherapy. Our results are in congruent with another report which showed that overexpression of MyD88 is associated with poor prognosis in colon cancer(57). Importantly, it is well-appreciated that only about 20% patients with Stage III CRC benefit from adjuvant FOLFOX(58). While various combinations of markers have been developed to aid prognostication and patient selection for chemotherapy(59),
enhancing the effect of chemotherapy, as can be achieved by co-targeting IRAK4 and the IKK-NF-κB, represents an equally important endeavor to improve overall patient outcome.

In summary, our study provides preclinical rationale for testing IRAK4 inhibitors in combination with chemotherapy in CRC. We demonstrate that chemotherapy can induce IKK-NF-κB and sustain CRC cell survival by upregulating TLR9-IRAK4 signaling, providing justification for targeting IRAK4. Further work is needed to elucidate potential resistance mechanisms and to enable development of more effective and durable combinatorial regimens for this promising treatment paradigm.
METHODS

Cell lines. All human cell lines were purchased from ATCC, which performs its own authentication by short tandem repeat DNA profiling. MC38 cell line was a kind gift from Dr. David DeNardo (WUSTL, St. Louis) SW620, SW620-L1 and SW620-L2 were kind gifts from Dr. Mien-Chie Hung (MDACC, Houston), were published (60) and not further authenticated. All cells were cultured in DMEM/10% FBS/1% Pen-Strep at 5% CO₂, 37°C incubators. Mycoplasma testing was performed every 6 months using MycoSEQ Detection kit (Applied Biosystems). All cell lines were used for fewer than 6 months after receipt or resuscitation from cryopreservation.

Human, murine CRC samples and Tissue Microarrays (TMA). All studies were performed per ethical principles of both in Declaration of Helsinki. Commercial CRC TMA sections were purchased from US BioMax (C0702a, C0952a, BC05002b, BC051110b, HCol-Ade180Sur-08). All cases were de-identified. Human colorectal tumor tissue microarray (TMA) was established at Washington University from 225 patients with metastatic colorectal cancer who underwent synchronous or metachronous resection for curative intent from year 2000 to 2010. The TMA contains two cores (1.0mm diameter) from each patient. Clinical information of these patients has been maintained in a retrospective clinical database. For survival analyses of Washington University CRC TMA, 21 patients were excluded due to poor sample quality (>50% area shredded or necrotic, N=8), demise within 3 months after surgery from postsurgical complications (N=5) or had no documentation on subsequent chemotherapy (N=8). The remaining 204 patients went on to receive systemic chemotherapy either at Washington University or other institutions.

Mouse experiments. For xenograft experiments, 5 million cells/flank were injected subcutaneously into 7-8 weeks old nude mice (for human cells) or C57BL/6J mice (for MC38 cells). Both mouse strains were purchased from JAX lab. Treatment was started when tumors reached a volume of ~100mm³. APCMin/+ mice were a gift from Dr. Nicholas Davidson, WUSTL, originally from JAX lab. IRAK4-null mice were purchased from EUCOMM. Both strains were maintained and genotyped per vendor’s protocol. For colitis-induced CRC model, 6- to 8-week-old APCMin/+ mice (gift from Dr. Nicholas Davidson, WUSTL,
originally from JAX lab) were treated for one week with 2% DSS in drinking water, which was then replaced with regular drinking water. All mice were euthanized 4 weeks later, and colons were prepared and analysed as published(61). Histologic analyses of mouse colons were independently performed by KHL, QL and GI pathologist MBR in blinded manner. For bone marrow transplantation, 6-week-old APC<sup>Min/+</sup> recipient mice were given a split dose (~4-hours apart) of lethal irradiation totalling 10.5 Gy prior to transplantation via retro-orbital injection of 2x10<sup>6</sup> unfractionated whole bone marrow cells harvested from 8-week-old wild-type of IRAK4-null mice.

**Immunohistochemistry (IHC), Immunofluorescence (IF) and scoring.** Antibodies used for IHC and IF staining were provided in Supplementary Table 2. Unless otherwise specified, all IF quantification was performed under fluorescence or light microscopy on ten 20X power fields/tumor, and data were presented as mean ± SEM, as described(26). For all TMA image analysis, whole slide tissues scans were obtained at 20X magnification on Zeiss Axio Scan Z1 Brightfield/Fluorescence Slide Scanner, (resolution of 0.645 µm per pixel). Whole tissue slide scans were then analyzed with HALO software (Indica Labs Perkin Elmer) using TMA module with Area quantification FL V1.2. P-IRAK4 IHC intensity for each sample was calculated using H-score (1×(% of lightly stained cells)+2×(% of intermediate stained cells)+3×(% of darkly stained cells). Tissue cores with exhausted epithelium were excluded. For APC Min/+ mouse experiments, histologic analyses and colitis scoring were conducted using published method(62). Briefly, 16-point system consisting of crypt damage (scale, 0–4), crypt damage extent (scale, 0–4), inflammatory infiltrate (scale, 0–4), and inflammatory infiltrate extent (scale, 0–4) was applied to three random 400X fields in mid- and distal colons of each mouse. Staining intensity obtained from scanners were independently verified and concurred by GI pathologist MBR. Survival analysis was performed by JL in blinded manner.

**Plasmids and creation of stable cell lines.** Stable knockdowns were generated using pSuperRetro as described(26). Target sequences: shIRAK4#1: TTCAGTAGTAATGTCAACC; shIRAK4#2: GCCTAATGGTTCATTGCTA, shTLR9# GAGCTAAACCTGAGCTACAAC; shTLR9#2:
GCACG GTGCCACCTCCACACT. Mouse CRISPR sequences: sgIRAK4#1: GTCGCCAACTGTGCAGTTCG; sgIRAK4#2: TGGCGACCTTGTGGATAC.

**Drugs and reagents.** 5-FU and oxaliplatin were purchased from the Siteman Cancer Center Pharmacy. SN-38 was purchased from Pfizer. Details of inhibitors were: IRAK4 inhibitors PF06650833 (Tocris, cat#6373), AS2444697 (Tocris, cat#5430) and IMD-0354 (Tocris, cat#2611).

**NF-κB reporter assays.** These were done using Dual-Glo® Luciferase Assay System and read with Synergy H4 Hybrid Microplate Reader.

**Western blots.** Cell lysates were harvested using radioimmunoprecipitation assay (RIPA) cell lysis buffer [10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.05% sodium dodecyl sulfate, 10 mM Na2EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate], supplemented with protease and phosphatase inhibitors (Roche)]. Cell lysates were resolved in Tris-glycine sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Membranes were probed with primary antibodies diluted in blocking buffer (1X TBST with 5% w/v bovine serum albumin) overnight at 4°C. The primary antibodies are listed in Supplementary table 2. Membranes were washed three times in 1X TBST buffer and probed with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Membranes were developed with the Pierce ECL Western Blotting Substrate and detected using a ChemiDoc XRS+ system (Bio-Rad).

**In vitro cell viability assay and calculation of combination indices.** 5,000 cells/well were plated in triplicates in 96 well plates one day prior to addition of the inhibitors at the indicated final concentrations. After 5 or 7 days of culture, viability assay was measured using Resazurin colorimetric analysis as described (26). For drug interaction studies, cells were cultured in triplicates in the presence of six fixed-ratio concentrations for 72 hours followed by Alamar Blue viability assay. Combination indices were
calculated using Compusyn software as described (41). All experiments were done at least three times in triplicates and one most representative set of data was presented.

**PLA (Proximal Ligation Assay)**

PLA was performed using Duolink in situ starter kit (DUO92101, Sigma) per manufacturer’s protocol. Number of puncta per field was quantitated using NIS-element software using Nikon C2+ fluorescent confocal microscope. Ten random 400X fields per condition were analysed and quantitated.

**Public database.** For TCGA data, matching clinical and IRAK4 mRNA expression data on Colorectal Adenocarcinoma (TCGA, Provisional, 640 samples) were downloaded from the cBioPortal website(63). For IRAK4 expression, RNAseq V2 RSEM dataset was used. Cases with missing survival data, clinical stage, IRAK4 expression data, who are known to not receive adjuvant treatment or died < 3 months from initial diagnosis were excluded. Clinical stage was determined based on provided information, or the provided T, N and M information. Rectal cancer was excluded since these patients typically received neoadjuvant chemoradiation, which poses additional variable. Kaplan-Meier survival analysis was independently performed by biostatistician JL, after separating patients based on IRAK4 mRNA expression level (Z-score cut off 0.5, which roughly separates all cases to upper one and lower three quantiles). Expression profiles of IRAK4 in normal colon versus cancer were downloaded from the Oncomine database(33).

**Statistics.** All results, when applicable, were expressed as the means ± SEM. Statistical analysis was performed using the Prism 6 software program. Unpaired student’s two-tailed t-tests were used to compare two groups when appropriate. For multiple groups, one-way ANOVA analysis with Tukey’s post-test were used. P values <0.05 were considered as statistically significant. Cox proportional hazards models were used to evaluate the relationships between clinical characteristics and overall survival. The upper quantile of p-IRAK4 H score in TMA and IRAK4 mRNA in TCGA are used as cut point. Survival probabilities were calculated using Kaplan-Meier method. Differences between strata of p-IRAK4 H score
and IRAK4 mRNA were determined by log-rank tests. All statistical tests were two-sided using an \( \alpha = 0.05 \) level of significance. SAS Version 9.4 (Cary, NC) was used to perform all survival analyses.

**Study Approval**

All mouse experiments were conducted under approval by WUSTL IACUC protocol (#20160142). We adhered to the ARRIVE guidelines for reporting on animal studies. All human tissue studies were approved by the Washington University School of Medicine Ethics Committee (IRB # 201108117).
AUTHOR CONTRIBUTIONS

QL and KHL designed the studies and wrote the manuscript. QL, YC, DZ, LL, NK, HJ conducted the experiments, acquired and analyzed data. GAC performed bone marrow transplant experiments. JG and RCF constructed the CRC TMA and provided clinical data on patients. JMH and DGD performed automated TMA data acquisition and computation of H-scores of all TMAs. MBR independently analyzed all TMAs and tissue sections in blinded manner. JL perform survival analysis on TCGA and TMA datasets in blinded manner. KHL supervised the entire project.
ACKNOWLEDGEMENTS

This study was supported by R21 CA223112 (K. Lim, M. Ruzinova), BJHF-ICTS Clinical and Translational Research Program (K. Lim), Concern Foundation Conquer Cancer Award #388329 (K. Lim), the SIP Award funded by the Siteman Cancer Center and The Foundation for BJH (K. Lim, M. Ruzinova), Surgical Oncology NCI T32 training grant (J. Grossman), and the NIH CTSA Grant Number UL1 TR000448 (K. Lim). Q. Li is supported by the National Natural Science Foundation of China (grant 81401735). We acknowledge the WUSTL DDRCC (grant P30 DK052574) for providing technical support.
REFERENCES


FIGURE LEGENDS

Figure 1. IRAK4 is required for colitis-induced neoplasm in $APC^{Min/+}$ mice

(A) Representative consecutive H&E and IHC (400X) images of the indicated markers in colon from a 6-month-old C57BL/6J $APC^{Min/+}$ mice and wild-type littermates bred in the same cage. Three pairs of mice were examined showing identical results.

(B) Treatment scheme of vehicle or IRAK4i (PF06650833) in $APC^{Min/+}$ mice after DSS treatment.

(C) Representative pictures and quantification of visible colon tumors and microadenomas (200X) of treated $APC^{Min/+}$ mice. (Mann Whitney test, ***p<0.001)

(D) Representative immunofluorescence pictures of dual pan-CK+ (green) and Ki-67+ (red) cells from colonic neoplasms of $APC^{Min/+}$ mice. Quantification of Ki-67+ areas were calculated from 5 random 400X fields containing pan-CK+ cells of 10 colon per arm. (Scale bars: 50μM, two-tailed t test)

(E) Serial measurements of body weight of $APC^{Min/+}$ mice treated as indicated. Data presented as means ± SEM (ANOVA, *p<0.05, **p<0.01, ***p<0.001)
Figure 1

A

Aged-matched normal colon

APC\textsuperscript{min}+ colon cancer


B

Treatment scheme

22 APC\textsuperscript{min}+ mice (10M, 12F)

2\% DSS in drinking water X 1 week

5M, 6F vehicle

4 weeks

sacrifice

5M, 6F IRAK4i (PF06650633) 10mpk/day

C

Gross tumor

Microadenoma

D

Ki-67, pan-CK, DAPI

vehicle

IRAK4i

\textit{p}<0.0001

\%Ki-67 area/HPF

vehicle (n=100) IRAK4i (n=100)

E

Mouse body weight (gm)

2\% DSS

Vehicle (n=11) IRAK4i (n=11)

Days After Treatment start

25 30

15 20 25 30 35 40
Figure 2. Bone marrow IRAK4 is required for colitis-induced neoplasm in $APC^{Min/+}$ mice

(A) Treatment scheme of $APC^{Min/+}$ mice

(B) Representative picture and quantification of visible colon tumors and microadenomas from DSS-treated $APC^{Min/+}$ mice pre-transplanted with wild-type or $IRAK4$-null bone marrow. (Mann Whitney test, **$p<0.01$, ***$p<0.001$)

(C) Representative IHC pictures and quantification of degree of colitis of colonic tissues from DSS-treated $APC^{Min/+}$ mice pre-transplanted with wild-type or $IRAK4$-null bone marrow. For each group of six colons, three random 400X fields were scored and presented as mean ± SEM (two-tailed t test, ***$p<0.001$).

(D) Representative IHC pictures and quantification of CD45$^+$ cells from colon of DSS-treated chimeric $APC^{Min/+}$ mice. For each group, 5-6 random 400X pictures were taken and CD45$^+$ cells counted using ImageJ software, and presented as mean ± SEM (two-tailed t test).
Figure 2

A. Treatment scheme

12 APCmin mice (6 weeks old)

Lethal irradiation

then BM transplant

WT BM (N=6)

IRAK4+ BM (N=6)

5 wks

2% DSS in drinking water for 1 week

5 wks

sacrifice

B. Colon

WT BM

IRAK4+ BM

Number per mouse

***

N=6/group

Gross tumor

Microadenoma

C. Colitis histologic score

WT BM

IRAK4+ BM

***

D. colon epithelium

colon tumor

CD45+ cells/100X field

p=0.05

WT BM

IRAK4+ BM

31
**Figure 3. IRAK4 is constitutively activated in human CRC**

(A) Western blots showing phosphorylation (denoted p-) status of IRAK1, IRAK4, IKKα/β, p65 and p50 in normal colon versus colon cancer cell lines with different oncogenic background.

(B) Representative p-IRAK4 IHC images and H-scores based on epithelial compartment of normal colon, normal colon tissues adjacent to cancer, polyp and colon cancer pooled from five different commercial TMAs (ns: not significant, ANOVA **p<0.01, ***p<0.001, scale bars: 50μM).

(C) Representative p-IRAK4 IHC images and assigned scores of p-IRAK4 and p-p65 IHC staining on colon cancer samples on a commercial tissue microarray (TMA, US Biomax CO702a). Staining intensity and scoring were interpreted by GI pathologist (MBR) and Spearman correlation between intensity of both markers analyzed using GraphPad Prism 6.0.
Figure 3

A  

<table>
<thead>
<tr>
<th>Genotype</th>
<th>KRAS</th>
<th>BRAF</th>
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<tr>
<td>Adjacent</td>
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B  
Pooled TMA samples (US Biomax)  
p-IRAK4 (T345) IHC scored by scanner

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal colon</th>
<th>Normal adjacent</th>
<th>Inflammation</th>
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<tr>
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<td>Inflammation</td>
</tr>
<tr>
<td>Primary colon cancer</td>
<td>Polyp</td>
<td>Primary colon cancer</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Metastasis (Lymph node)</td>
<td>Metastasis (Lymph node)</td>
<td>Metastasis (Lymph node)</td>
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C  
IHC score (manually scored by GI pathologist)

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<td>p-IRAK4</td>
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Graph showing correlation between p-p65 and p-IRAK4 IHC scores.
Figure 4. IRAK4 drives NF-κB activity in human and murine CRC cells

(A) Western blots showing the effect of stable IRAK4 knockdown on the indicated markers, compared to scramble (S) control cells.

(B) Western blots showing diminished nuclear p65 following IRAK4 knockdown in two CRC lines. Treatment with IKKβ inhibitor IMD-0354 overnight served as control.

(C) Relative quantification of soft agar colonies formed by DLD-1 and KM12 cells stably expressing scramble or two IRAK4 shRNAs grown over three weeks. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (ANOVA, ***p<0.001).

(D) Tumor kinetics of the indicated KM12 tumors grown subcutaneously in bilateral flanks of nude mice (N=5 mice or 10 tumors/group). Data represents mean tumor volume ± SEM (ANOVA, ***p<0.001).

(E) Western blots showing IRAK4 protein levels in MC38 cells infected with pLentiCRISPR/Cas9 alone or with two sgRNAs targeting IRAK4. Two different polyclonal batches of cells were made and pooled for colony formation assay (14 days) and soft agar assay (21 days). Data represent one of three sets of soft agar experiment each done in triplicates and presented as mean ± SEM. (ANOVA, ***p<0.001)

(F) Growth kinetics of the indicated MC38 cells grown in C57BL/6J mice (ANOVA, ***p<0.001).

(G) Kaplan-Meier survival analysis of the indicated MC38 cells grown in C57BL/6J mice (Log-rank, ***p<0.001).
Figure 4

A

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B

DLD-1

- p55
- Histone H3

KM12

- p55
- Histone H3

C

<table>
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<tr>
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<td>100</td>
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<tr>
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<tr>
<td>sIRAK4#2</td>
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</table>

D

KM12

- Tumor volume (X 100 mm³) vs. Days after tumor inoculation

E

MC38

- Total IRAK4
- β-tubulin

Batch 1

Batch 2

F

MC38

- Tumor volume (X 100 mm³) vs. Days after tumor inoculation

G

MC38

- Percent survival vs. Days after tumor inoculation

N=10/arm
Figure 5. IRAK4 inhibitors suppress NF-κB activity in CRC cells

(A) Alamar blue assay showing the viability of thirteen colon cancer lines cultured in serial concentrations of two different IRAK4 inhibitors for 5 days, and the respective IC$_{50}$ values. Data represent one of three sets of experiment each done in triplicates.

(B) Correlative analyses of IC$_{50}$ values between the two indicated IRAK4 inhibitors in 13 colon cancer cell lines.

(C) Quantification of clones formed by the indicated CRC lines treated with DMSO or two different IRAK4 inhibitors over three weeks. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (ANOVA, *p<0.05, **p<0.01, ***p<0.001).

(D) Western blots showing the suppressive effect of two different IRAK4 inhibitors on the indicated markers after overnight treatment.

(E) Western blots showing diminished abundance of nuclear p65 in two different CRC cell lines treated with both IRAK4 inhibitors overnight. IKKβ inhibitor IMD-0354 served as positive control.

(F) NF-κB luciferase reporter assay of five different CRC lines incubated with DMSO or the indicated inhibitors overnight. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (ANOVA, *p<0.05, **p<0.01, ***p<0.001).

(G) NF-κB luciferase reporter assay of KM12 cells transfected with empty vector, wild-type (WT) or constitutively activated (S177E/S181E) IKKβ incubated with DMSO or the indicated IRAK4 inhibitors overnight. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (Tukey’s multiple comparison test, ns: not significant, *p<0.05, **p<0.01).
Figure 6. Chemotherapy induces autologous TLR9-IRAK4-NF-κB circuitry

(A) Luciferase reporter assay showing the effect of chemotherapy on NF-κB activity in two different CRC lines. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (ANOVA, *p<0.05, ***p<0.001).

(B) Quantitative PCR showing changes in expression of the indicated genes in three CRC lines following exposure to chemotherapy (5-FU and oxaliplatin, both 10μM) overnight. Data represent one of two sets of experiment each done in biological duplicates and technical triplicates and presented as mean ± SEM.

(C) Western blots confirming increased protein expression of TLR9 following overnight treatment with the indicated chemotherapeutic agent (all at 10μM) in HCT-116 (left panel) and three different CRC lines (right panel).

(D) Representative (400X) confocal images and quantification (mean±SEM) of PLA puncta (red) formed between TLR9 (CST #D9M9H) and p-IRAK4 (ABNOVA #A8A8, 1:500) in three colon lines treated with vehicle or chemotherapy (5-FU and oxaliplatin, both 10μM) overnight. (two-tailed t, **p<0.01)

(E) Western blots showing the effect of TLR9 knockdown on chemotherapy-induced PARP cleavage in HCT-116 cells.

(F) Luciferase reporter assay showing the effect of TLR9 knockdown on chemotherapy-induced NF-κB activity in HCT-116 cells (ANOVA, *p<0.05, **p<0.01).

(G) Western blots showing suppressive effects of IRAK4 and IKK inhibitors on chemotherapy induced-TLR9 expression and apoptosis.

(H) Quantitative PCR showing suppressive effect of IRAK4 inhibitor (PF06650833 4μM overnight) on chemotherapy induced TLR9 expression. Data represent one of two sets of experiment each done in biological duplicates and technical triplicates and presented as mean ± SEM. (Tukey’s multiple comparison test, ns: not significant, *p<0.05, **p<0.01, ***p<0.001).
Figure 7. IRAK4 inhibition potentiates the cytotoxic effect of chemotherapy in vivo

(A) Combination indices between AS2444697 or (B) PF06650833 with chemotherapeutic agents 5-FU, oxaliplatin or SN38 in three different CRC lines calculated using Compusyn software. Cells were cultured in six fixed-ratio concentrations in triplicates over 72 hours and viability assayed by Alamar blue. IRAK4 inhibitor: 5-FU/oxaliplatin/SN-38: (8:50, 4:25, 2:12.5, 1:6.25, 0.5:3.125, 0.25: 1.56). Experiments were done three times in triplicates and one set of data presented as mean±SEM.

(C) Final weight and picture of DLD-1 tumors harvested simultaneously when vehicle-treated mice reached maximum volume of ~2000mm³. Data represents mean ± SEM (Tukey’s multiple comparison test, ns: not significant, *p<0.05, ***p<0.001).

(D) Quantification of cleaved caspase-3+ area per 200X field and (E) dual CK+ and Ki-67+ cells per 400X field of DLD-1 tumors treated as indicated. Ten random pictures were taken from each ten tumors/arm and data represents mean ± SEM (Tukey’s multiple comparison test, **p<0.01, ***p<0.001).

(F) Kaplan-Meier survival of mice bearing DLD-1 tumors treated as indicated when tumors reach 100mm³ and until tumor volume exceeded 2000mm³ or whenever humane endpoints are reached (N=10/group, Log-rank test).

(G) Final weight and picture of KM12 tumors harvested simultaneously when vehicle-treated mice reached maximum volume of ~2000mm³. Data represents mean ± SEM (Tukey’s multiple comparison test, ns: not significant, *p<0.05, ***p<0.001).

(H) Quantification of cleaved caspase-3+ area per 200X field and (I) dual CK+ and Ki-67+ cells per 400X field of KM12 tumors treated as indicated. Ten random pictures were taken from each ten tumors/arm and data represents mean ± SEM (Tukey’s multiple comparison test, ns: not significant, *p<0.05, **p<0.01, ***p<0.001).

(J) Kaplan-Meier survival of mice bearing KM12 tumors treated as indicated when tumors reach 100mm³ and until tumor volume exceeded 2000mm³ or whenever humane endpoints are reached (N=10/group, Log-rank test).
Figure 7

A

AS2444697 and 5-FU

AS2444697 and oxaliplatin

AS2444697 and SN38

Combination index (CI)

0.0 0.2 0.4 0.6 0.8 1.0 1.2

Fraction affected by dose

DLD-1

KM12

HT-29

B

PF06650833 and 5-FU

PF06650833 and oxaliplatin

PF06650833 and SN38

Combination index (CI)

0.0 0.2 0.4 0.6 0.8 1.0 1.2

Fraction affected by dose

DLD-1

KM12

HT-29

C

DLD-1 Model

Vehicle

AS2444697

5-FU

Combo

Final tumor weight (g)

% Caspase-3+ area

Ki-67+ cells/HPF

F

Percent survival

Days after treatment start

Median survival (days)

Vehicle: 24
PF06650833: 25
5-FU + oxali: 31
Combo: 43

Log-rank p<0.0001

G

KM12 Model

Vehicle

AS2444697

5-FU

Combo

Final tumor weight (g)

% Caspase-3+ area

Ki-67+ cells/HPF

J

Percent survival

Days after treatment start

Median survival (days)

Vehicle: 35
PF06650833: 41
5-FU + oxali: 46
Combo: 62.5

Log-rank p<0.0001
Figure 8. Prognostic impact of IRAK activation or expression in colon cancer

(A) Representative IHC images showing different intensities of p-IRAK4 staining as determined by H-score from two commercial colon cancer TMAs (US Biomax: BC051110b, HCol-Ade180Sur-08). (Scale bars: 100μM)

(B), (C), (D) The overall survival (OS) by Kaplan–Meier analysis of patients with different stages of colon cancer as stratified by the H-score of p-IRAK4 staining of two commercial colon cancer TMAs (US Biomax: BC051110b, HCol-Ade180Sur-08).

(E) Overall survival (OS) by Kaplan–Meier analysis of Stage IIb-III colon cancer patients as stratified by the IRAK4 mRNA expression (Z-score cut off at 0.5) from TCGA (provisional) database.

(F) Correlation (Wilcoxon signed rank test) of p-IRAK4 IHC staining intensity between matched primary and liver metastatic CRC samples from a cohort of 32 patients who underwent surgery at Washington University from year 2000-2010.

(G) Representative IHC images showing different intensities of p-IRAK4 IHC staining in liver metastasis samples, and Kaplan–Meier overall survival, as stratified by p-IRAK4 H-score from a cohort of 204 patients who underwent liver resection surgery at Washington University from year 2000-2010.
Figure 9. Schematics depicting a proposed model in which DNA damage incurred by chemotherapy triggers a TLR9-IRAK4-NF-κB circuitry to sustain CRC cell survival. At basal state, IRAK4 and NF-κB are activated through mechanisms that remain to be determined, but likely through upstream TIR family members including TLR9. Upon exposure to chemotherapy, damaged DNA may activate NF-κB through ATM and engaging the pre-existing pool of TLR9, which in turns activates IRAK4 and NF-κB, resulting in survival and further upregulation of TLR9. These chained events result in enhanced survival which enables CRC cells to evade chemotherapy-induced apoptosis.
Figure 9

Model: DNA damage induces TLR9-IRAK4-NF-κB survival mechanism

Basal state

Colon cancer cell

Chemotherapy

Colon cancer cell