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Colorectal cancer prevention: immune modulation taking the stage

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Abstract

Prevention or early detection is one of the most promising strategies against colorectal cancer (CRC), the second leading cause of cancer death in the US. Recent studies indicate that antitumor immunity plays a key role in CRC prevention. Accumulating evidence suggests that immunosurveillance represents a critical barrier that emerging tumor cells have to overcome in order to sustain the course of tumor development. Virtually all of the agents with cancer preventive activity have been shown to have an immune modulating effect. A number of immunoprevention studies aimed at triggering antitumor immune response against early lesions have been performed, some of which have shown promising results. Furthermore, the recent success of immune checkpoint blockade therapy reinforces the notion that cancers including CRC can be effectively intervened via immune modulation including immune normalization, and has stimulated various immune-based combination prevention studies. This review summarizes recent advances to help better harness the immune system in CRC prevention.

Keywords

colorectal cancer; prevention; immunosurveillance; immune modulation

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1. Introduction

Despite recent success in screening and early detection, colorectal cancer (CRC) remains the second leading cause of cancer-related deaths in the United States [1]. While colorectal cancer is decreasing overall, studies from the American Cancer Society show that the incidence and mortality rates of CRC among adults 50 in the US is increasing [2, 3]. Prevention or early detection represents a key approach for reducing the mortality and morbidity of cancers including CRC [4].

CRC development occurs in a stepwise fashion, progressing from a benign pre-neoplastic lesion to metastatic disease that has a survival rate of only 11% [5]. This process is driven by a series of well-defined genetic and epigenetic alterations (Fig. 1) that typically take many years or decades to accumulate [6]. This long development process creates a substantial window of opportunity for prevention. CRC also has well-described and readily identifiable precursor lesions in human patients, a process mirrored in animal models [7]. For these reasons, CRC has been extensively evaluated with cancer prevention proof-of-principle studies. Depending on the purpose, CRC prevention can be aimed at suppressing tumor initiation (primary prevention), preventing tumor recurrence or progression (secondary prevention), or management of chronic disease (tertiary prevention) [8]. A variety of pharmacological agents, natural products, and dietary components have been shown to have preventive activity against CRC early lesions [9, 10]. The best example and the most potent is non-steroidal anti-inflammatory drugs (NSAIDs), which have been shown to reduce CRC incidence in animal models and in human epidemiologic studies and clinical trials [11, 12].

While mechanistic studies on CRC prevention have been largely focused on tumor cell intrinsic alterations, activation of local rather than peripheral immune response has been increasingly appreciated as a critical component of CRC prevention. The concept of immunosurveillance, which was coined decades ago, postulates that a normal cell acquiring oncogenic mutations can be recognized as foreign and eliminated by the immune system [13, 14]. This theory is supported by the observation that CRC progression is associated with changes in the composition of tumor infiltrating cells, some of which create an immune suppressive microenvironment [15]. Studies using animal models further support immunosurveillance as a key safeguard mechanism against tumor development. Directly modulating the immune system for cancer prevention, known as “immunoprevention”, through approaches such as vaccines has been successful against malignancies caused by viral infection, such as human papilloma virus (HPV)-induced cervical cancer [16–18]. However, similar approaches have yet to be applied for the prevention of non-viral cancers including CRC.

Chemo/dietary prevention and immunoprevention used to be considered as two different prevention approaches. These approaches have increasingly merged with each other, as accumulating evidence indicates chemopreventive agents also have immune-modulatory effects [19]. Recent success of using antibodies against various immune checkpoints such as Program Death 1 (PD-1), Programmed Death Ligand 1(PD-L1), and cytotoxic T lymphocyte associated antigen 4 (CTLA-4) for cancer immunotherapy provides indisputable evidence that modulating the immune system is a sound approach for cancer treatment. These recent

advances have led to a dramatic increase in prevention studies involving combinations of chemopreventive agents and immune-based biological agents [20]. An improved understanding of the functional role of immune response in CRC prevention likely holds the key for designing more effective prevention strategies.

2. Immunosurveillance as a barrier in early stages of CRC development

2.1 Overview of tumor antigens and cancer immunity

The process of oncogenic transformation produces antigens that can be recognized by the immune system. There are two types of tumor antigens: self-antigens shared by tumor and normal cells, but overexpressed or abnormally modified (or processed) in tumor cells, and mutated neoantigens that are unique to individual tumors [21]. Shared self-antigens are represented by several validated and well-characterized tumor antigens, such as NY-ESO-1, MART-1, MUC1, cyclin B1, CEA, and Her2/neu, which are overexpressed in tumor cells. For example, MUC1 is not only overexpressed, but also hypo-glycosylated in tumor cells, both accounting for its recognition by the immune system [14]. Neoantigens are typically generated by mutations, which include those in oncogenic drivers such as *KRAS*, *TP53*, *BRAF* and *PIK3CA*, but are mostly random ones without an apparent functional role in tumorigenesis. Recent whole-genome exome sequencing studies have identified a plethora of mutations in tumors, some of which are predicted to encode novel antigens. However, most of these mutations have yet to be confirmed as targets of spontaneous immunity and antitumor immune response [22].

Tumor antigens can be presented by major histocompatibility complex (MHC) class I and II molecules on the surface of tumor cells and also by antigen presenting cells, in particular dendritic cells (DCs) that capture, process and present them to T cells [14]. Multiple immune effector mechanisms are engaged in the recognition of abnormal cells (Fig. 2). Natural killer (NK) cells, part of innate immunity, recognize and eliminate nascent tumor cells, a process also contributed to by macrophages and neutrophils [23]. As part of the adaptive immune response activated, CD8+ cytotoxic T lymphocytes (CTLs) that can kill neoplastic cells are considered to be major antitumor effector cells (Fig. 2). This process is regulated by CD4+ T helper (TH) cells with distinct cytokine profiles that promote immune response, or regulatory T cells (Tregs) that can suppress immune response [24]. The adaptive immune response also includes humoral responses, in which soluble tumor antigens are recognized by specific B cells and with the help of CD4+ TH cells, mature and produce tumor antigen-specific antibodies [25]. In contrast to innate immunity, which is non-specific and short-term, the adaptive response is specific and has long-term memory [20]. The components of both innate and adaptive immune response, including particular cell types, effector molecules and pathways, collectively suppress tumor growth [23].

2.2 Immune-modulating alterations in CRC premalignant lesions

In ~85% cases of CRCs, tumor development engages the well-established adenoma-carcinoma sequence of events (Fig. 1). The precursor lesion adenoma is initiated by gatekeeper genetic alterations in the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor pathway through aberrant Wnt signaling, leading to accumulation and subsequent nuclear

translocation of β -catenin, and activation of oncogenes such as *c-Myc* and *cyclin D1* [6]. Further tumor development is promoted by oncogenic mutations in *KRAS*, *BRAF*, *PIK3CA*, and loss or mutations of other tumor suppressors such as *Tumor Protein p53 (TP53)* [6] (Fig. 1). CRCs arising following the adenoma-carcinoma sequence are often characterized by chromosome instability (CIN) [6].

Tumor antigens involved in early stages of tumor development in premalignant lesions are poorly understood. It remains an open question how gatekeeper changes of CRCs, in particular loss of the tumor suppressor *APC*, can trigger antitumor immune response. The key initial step is to identify potential tumor antigens in premalignant lesions. Several well-characterized tumor antigens, such as MUC1 and CEA, are known to be also upregulated in adenomatous polyps, which is the rationale for using a vaccine approach targeting these antigens for CRC immunoprevention [14]. Recent studies have utilized “omics” approaches to discover other targets. For example, analysis of public gene expression databases identified 160 genes overexpressed in colon adenomas, among which 23 also confirmed at the protein level. Further analysis revealed that serum IgG levels against several of these proteins are elevated in early-stage CRC patients relative to controls [26]. In contrast to the large number of studies in CRCs, only a few studies have analyzed DNA sequence variations by Next-Gen exome sequencing in colorectal adenomas from familial adenomatous polyposis (FAP) and sporadic cases [27–29]. These studies have provided a catalogue of over 200 somatic changes, in addition to *APC* mutations or loss, in colorectal adenomas. Studies of DNA methylation and microRNA expression have identified a large number of hyper- or hypo-methylated genomic sequences and differentially expressed miRNAs, suggesting significant changes in epigenomes [30, 31]. Furthermore, recent studies have compared proteomic profiles in adenomas and identified a large fraction (up to 23%) of altered proteins relative to normal controls [32, 33]. A general trend emerging from these studies is that depending on subtypes, different adenomas have marked heterogeneity in terms of their changes in omics profiles. The levels of overall changes are generally less than those seen in CRCs. However, for each omics profile described, there is a substantial fraction of adenomas that have levels of alterations similar to those seen in CRCs, suggesting a large fraction of the genomic, epigenomic, and proteomic alterations in CRCs are acquired at early stages. The diverse types and significant extent of these alterations suggest they would inevitably be recognized by the immune system. However, very little is known about how these alterations lead to the full activation of the immune response.

The omics studies provided unbiased, global views on alterations in adenomas but are still far from elucidating disease biology or elicited immunity. Candidate approaches have been used to identify potential influences of CRC drivers on anti-CRC immunity. The well-defined gatekeeper alterations and molecular events may provide key clues for understanding the immunobiology of early CRC development. *APC* loss leads to the activation of two critical downstream effectors, *c-Myc* and *cyclin D1*. *c-Myc* functions as a transcriptional factor that promotes cell proliferation, survival, and metabolic reprogramming by transcriptionally regulating hundreds of downstream target genes [34]. A recent study showed that *c-Myc* regulates the expression of two immune checkpoint proteins on the tumor cell surface, PD-L1 that suppresses adaptive immune response, and CD-47 that inhibits the innate immune response [35]. *c-Myc* overexpression could induce a synthetic

lethal interaction with the activation of extrinsic apoptosis pathway that can be utilized by CTLs to kill tumor cells [36]. Similar to cyclin B1 [37], cyclin D1 is also widely overexpressed in tumor cells and has been implicated as a tumor antigen [37]. Peptide sequences derived from cyclin D1 were shown to stimulate activity of CD4+ or CD8+ cytotoxic T cells [38, 39]. It is worth noting that the components of the Wnt signaling pathway that are activated by *APC* loss or β -*catenin* activating mutations, are subject to a variety of post-translational modifications [40]. Delineating the effects on immunity of the gatekeeper alterations in CRC development may require more powerful techniques than those utilized in conventional immunology studies, such as recently developed intestinal crypt culture system and CRISPR/Cas9 genomic editing tools [41].

2.3 Highly immunogenic microsatellite unstable CRCs

Approximately 15% of CRCs display so-called microsatellite instability (MSI), a form of genomic instability characterized by DNA replication errors in microsatellite repeat sequences that generate frame-shift mutations in protein-coding DNA sequences containing such repeats [42]. MSI is caused by DNA mismatch repair (MMR) deficiencies due to mutational or epigenetic silencing of one of the MMR genes, including *MSH2*, *MLH1*, *MSH6* and *PMS2* [6]. Patients with Lynch Syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), have inherited mutations in these genes and are predisposed to MSI CRCs, as well as endometrial, ovarian and stomach cancers [6].

MSI CRCs tend to be much more responsive to immunotherapy than microsatellite stable (MSS) and CIN CRCs [42], which can be explained by a much higher mutational load in these CRCs compared to MSS CRCs and therefore a much stronger immune response [43]. For example, exome sequencing identified a mean of 1,782 somatic mutations per MSI tumor, compared to only 73 per tumor in MSS CRCs, with most (63%) of these MSI mutations predicted to alter amino acids [44]. The mutations in protein-coding sequences can generate a large number of neoantigens in the form of frameshift peptides, which lead to a more robust immune response [42]. The recent success of anti-PD-1 immunotherapy in treating MSI CRCs has stimulated great interest in understanding the immune biology of MSI CRCs [44, 45]. A number of genes with coding microsatellite sequences are known to be mutated in MSI CRCs with high frequency, such as *Transforming Growth Factor β Receptor 2* (*TGFBR2*), *Phosphatase and Tensin homolog* (*PTEN*), *Asteroid Homolog 1* (*ASTE1*), and *Caspase 5* [42]. MSI CRCs provide a useful model for identifying neoantigens and for delineating how these neoantigens elicit antitumor immune response.

2.4 Safeguarding against CRC development by immunosurveillance

The original theory of immunosurveillance postulates that a normal cell acquiring oncogenic mutations can be eliminated by the immune system that functions as a major initial barrier to tumor progression [13, 14]. However, the interplays of tumor cells and immune system are much more complicated than originally thought. It is clear now that the immune response has dual roles in CRC development, with cytotoxic lymphocytes activated by tumorigenic alterations restraining tumor growth, while chronic inflammation creating a microenvironment that fosters tumor cell growth and invasion [46]. The concept of immunosurveillance has now evolved into that of immunoediting [47], which incorporates

the influence of the immuno-suppressive tumor microenvironment [48]. Immunoediting includes three phases (Fig. 1): (1) Elimination: equivalent to immunosurveillance; (2) Equilibrium: upon failure of immunosurveillance, constant immune selection pressure promotes the tumor cell variants with an increased ability to endure an attack by the immune system; (3) Escape: the tumor expands in an unrestrained manner [47, 48].

Epidemiological and clinical studies provide supporting evidence for immunosurveillance. Some immunosuppressed organ transplant patients develop donor-derived cancers, suggesting immune-mediated suppression of tumor development in the cancer-free donors [49]. CRC progression is associated with changes in the composition, density and location of tumor-infiltrating lymphocytes (TILs) that can be used to more accurately predict clinical outcomes than the widely used tumor stage and nodal status [15]. CRCs that are heavily infiltrated with TILs and NK cells are associated with good prognosis, with infiltration of CD3+, CD8+, or CD45RO memory T cells as a strong prognostic factor [15, 50]. Several recent studies showed the infiltration patterns of CD4+, CD8+ TILs, DCs and other immune cells are progressively altered in the normal-adenoma-carcinoma sequence, and also in the low-mid-high grades of adenomas [51–53]. Consistent with the findings on tumor antigens [14], these studies suggest that immunosurveillance and immunoediting occur in very early stages of CRC development in response to tumorigenic gatekeeper alterations.

Genetically engineered mouse models (GEMM) have provided key mechanistic insights into immunosurveillance in colorectal tumorigenesis. Immunocompromised mice, such as *IFN- γ* $-/-$, *RAG2* $-/-$ and Balbc SCID mice, have an increased susceptibility to chemically induced and spontaneous tumors [47]. A number of immune-related studies have been performed on *APC*Min+ mice, which mimic human FAP in intestinal polyp predisposition due to an inherited mutation in *APC*. It was shown that immune cell composition and pro-inflammatory cytokine profiles are altered in the polyps of *APC*Min+ mice, which is correlated with adenoma phenotypes [54, 55]. *RAG2* $-/-$ *APC*Min+ mice on a BALB/c or B6 background have significantly increased number of small intestinal polyps [56]. Adoptive transfer of CD4+ CD25+ Treg lymphocytes can induce tumor regression in B6 *APC*Min+ mice [57]. Modulation of intestinal polyp phenotypes by knocking out several genes, such as *STAT6* and *FasL*, has been shown to be associated with changes in infiltration of neutrophils, myeloid-derived suppressor cells (MDSCs) and TILs [58, 59]. Depletion of Tregs reduces bacterium-induced colonic microadenoma formation in *APC*Min+ mice [60]. A recent study showed that inhibiting autophagy by deleting *ATG7* in *APC*Min+ mice induced polyp regression that required a stress response mediated by CD8+ T cells [61]. Studies of human adenomas and mouse models strongly support that immunosurveillance can be triggered by gatekeeper alterations to inhibit further progression of premalignant lesions.

2.5 Mechanisms of escaping immunosurveillance

The pressure imposed by immunosurveillance selects for rare tumor cells that are able to cope with (to reach equilibrium) and eventually prevail and evade the immune response [62]. There are several ways by which tumor cells can escape immunosurveillance. Loss or reduced expression of class I MHC molecules, also known as Human Leukocyte Antigens

(HLAs), was frequently detected in cancers including CRCs [63]. In addition to HLA downregulation, mutations or downregulation of β 2-microglobulin or the components of antigen processing machinery (APM), such as TAP1 and TAP2, were found to be increased in CRCs with *KRAS* mutations or MSI [46, 64]. Tumor cells defective in APM may avoid detection by CD8⁺ T cells by not presenting their antigens. CRCs express ligands for natural killer group 2D (NKG2D) molecules expressed on NK and some T cells. The expression of NKG2D ligands, which is correlated with NK cell infiltration, was found to be progressively less frequent in tumors with advanced stages, suggesting an immunoediting process selecting for tumor cells that do not trigger recognition by NKG2D⁺ NK cells and T cells [65]. Tumor cells can also produce immunosuppressive molecules, such as Transforming Growth Factor β (TGF- β) and indolamine-2,3-dioxygenase (IDO) [14].

As tumors progress, the microenvironment becomes progressively immunosuppressive (Fig. 1). Immunosuppressive cells including Tregs and MDSCs become more dominant relative to cytotoxic immune cells such as CD8⁺ T cells. These cells normally help to maintain immune tolerance and prevent excessive immune responses. For example, Tregs suppress cytotoxic T cells by producing the immunosuppressive cytokines including TGF- β and interleukin-10 [14]. Several immunosuppressive pathways are commonly upregulated at this stage, such as those involving PD-1 and CTLA-4 [62]. PD-1 expressed on activated T cells, upon binding to its ligands PD-L1 and PD-L2 on the tumor cells, dampens the T cell response by reducing the production of cytokines and T cell proliferation. Similarly, upregulated CTLA-4 on the surface of T cells binds to its ligands CD80/CD86 on antigen presenting cells to prevent co-stimulation via CD28 and continuous activation of T cells [62]. Despite the detection of PD-1⁺ TILs, PD-L1 is rarely expressed in colon adenomas [66]. In contrast, multiple immune checkpoints, including PD-1, PD-L1, CTLA-4, lymphocyte-activation gene 3 (LAG-3), and IDO, are upregulated in invasive fronts and on TILs of MSI CRCs [67]. Blocking antibodies against PD-1, PD-L1, and CTLA-4, known as immune checkpoint inhibitors, have exhibited clinical efficacy in MSI CRCs and various other tumor types by preventing negative signaling to T cells through these molecules and their ligands [44, 45].

Collectively, accumulating evidence suggests that immunosurveillance functions as a critical barrier for CRC development, including at the early and premalignant stages, and represents an attractive target for early intervention and prevention.

3. Immune-modulating effects of CRC preventive agents

A promising approach for reducing the mortality and morbidity of CRCs is chemoprevention, which refers to the use of natural or pharmacological agents to suppress tumorigenesis. For example, regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) has been consistently associated with a reduced risk of CRCs [68, 69]. A number of other agents, such as folic acid, calcium, vitamin D and antioxidants (including vitamin A, vitamin C, vitamin E, selenium and beta-carotene), and ω -3 polyunsaturated fatty acids (PUFAs), have exhibited chemopreventive activity in epidemiological and/or clinical studies, as well as various preclinical models [70, 71]. Increasing evidence indicates that the

chemopreventive effect of these agents is mediated by restoring immunosurveillance and reversing the immune evasive mechanisms that premalignant lesions utilize (Table 1) [19].

3.1 Aspirin and NSAIDs

Recent epidemiological studies in large prospective cohorts show that regular use of aspirin is associated with reduced risk of CRCs with low-level TILs (CD8+, CD3+, and CD45RO T cells), but not those with medium- or high-level TILs [72]. The association of aspirin use with colorectal cancer survival was found to be stronger in patients with PD-L1-low tumors than PD-L1-high tumors [73]. Significant associations were found between aspirin/NSAID use, CRC patient survival, and single nucleotide polymorphisms (SNPs) in immune modulators, such as the components of the JAK/STAT/suppressors of cytokine signaling (SOCS), interferon, and interleukin-8 (IL-8) signaling pathways [74–76]. In a pilot clinical study, short-term pre-operative treatment of CRC patients with the NSAID celecoxib or indomethacin led to increased levels of active TILs and reduced immunosuppressive Tregs in the stroma and tumor tissues [77].

A number of *in vitro* and *in vivo* studies also showed that the antitumor effect of NSAIDs is acting through restoration of antitumor immune response (Table 1). Genetic ablation of cyclooxygenase 2 (COX2), the target of NSAIDs, or its pharmacological inhibition by aspirin in CRC cells promotes immune-dependent tumor suppression [78]. Aspirin and aspirin-like drugs induce HLA-DR expression in CRC cells [79], and alter the balance between pro- and anti-inflammatory cytokines generated by interaction between colon cancer and immune cells [80]. Tumor suppression by the NSAID sulindac was shown to require CD8+ T cells, and is accompanied with significantly diminished M2 macrophage recruitment [81]. The NSAIDs celecoxib and indomethacin affect the expression of PD-L1 and PD-L2 in specific bone marrow cells [82, 83]. Additionally, NSAIDs have been shown to alter the phenotype of tumor associated macrophages (TAMs) from the pro-tumor M2 phenotype to the antitumor M1 phenotype in *APC* Min/+ mice [84]. The stimulatory effect of NSAIDs on antitumor immunity could also be working by suppression of COX2-mediated production of prostaglandin E2 (PGE2) in tumor and stromal cells, which induce immunosuppression through their inhibitory effects on DCs, macrophages, neutrophils, CTL, TH1, and NK cells, as well as its stimulatory effects on MDSCs, Tregs and TH2 [85, 86].

Recent studies revealed selective killing of *APC*-deficient intestinal stem cells through apoptosis induction as a key effect of NSAIDs in CRC chemoprevention [36, 87]. This effect is mediated by a synthetic lethal interaction of *APC*-deficiency-dependent c-Myc accumulation and NSAID-induced activation of the death receptor pathway, leading to mitochondria-mediated apoptosis via the proapoptotic Bcl-2 family member Bid [36]. The immune effect of NSAIDs could be explained by apoptosis induction. Several types of cell death, referred to as “immunogenic cell death” (ICD), could activate antitumor immunity [88]. NSAID-induced apoptosis was found to involve induction of endoplasmic reticulum (ER) stress [89] that can induce ICD markers such as cell-surface translocation of calreticulin (CRT) and activation of DCs [88]. The detailed mechanisms by which NSAIDs

and other chemopreventive agents restore immunosurveillance through ICD remain to be further elucidated.

3.2 Other chemopreventive agents

Vitamin D has been shown to have a protective effect on the risk of cancers including CRC [90]. Vitamin D deficiency has also been linked to inflammatory bowel disease (IBD), a risk factor of CRC [91]. A recent epidemiological study revealed the association of high level of plasma 25-hydroxy-vitamin D with lower risk of CRCs with intense immune infiltrate, including MSI CRCs [92]. This could be explained by the regulation of vitamin D metabolism by the immune cells including B cells, macrophages, and T cells, and by the role of vitamin D in T-cell antigen receptor signaling and T-cell activation [92].

Curcumin, a plant product, has been shown to be effective against CRCs in various preclinical models [70]. It has been shown to be effective in reducing aberrant crypt foci (ACF) in pilot clinical studies [93], and it is currently being tested in clinical trials for polyp prevention [70]. Curcumin has several stimulating effects on the immune system, including inhibiting immune suppressors such as COX2, TGF- β , and IDO, reducing MDSCs and redirecting them to a M1 macrophage phenotype, increasing B-cell frequency, activating NK cells and T cells, and restoring memory T cells [94–97]. The antitumor activity of curcumin was found in *APC*^{Min} mice where there was an increase in both B and T cell populations in the intestinal mucosa of these mice [98].

Marine ω -3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) are known to have an anti-CRC effect in preclinical models [99]. EPA was found to reduce the numbers and size of polyps in FAP patients [100]. A recent epidemiological study showed that high intake of ω -3 PUFAs is associated with reduced risk of CRCs with a high level, but not those with a low level of FOXP3⁺ T-cell density, suggesting modulation of Tregs as a key mechanism of CRC prevention [101].

Metformin, the most widely used anti-diabetic drug, was shown to decrease ACFs and suppress cell proliferation in patients in a pilot clinical study [102]. Metformin was found to enhance circulating T memory cell numbers via modulation of fatty acid metabolism [103], and rescue of cell surface expression of HLA in breast cancer cells [104].

4. CRC immunoprevention

The notion of immunosurveillance suggests that directly modulating the host immune response can be a powerful way to intervene cancer initiation, an approach known as “immunoprevention”. The success of immunotherapy is limited by a large tumor burden and highly immunosuppressive microenvironment. Immunoprevention is a more favorable anticancer approach as it focuses on early malignancies with minimal or lower levels of tumor burdens and heterogeneity, and in a less immunosuppressive microenvironment that would lead to immunoediting. Identification of a plethora of tumor antigens in early lesions has further established the feasibility of this approach for CRC prevention. A number of preclinical studies and several clinical studies on CRC immunoprevention have been described (Table 2).

4.1 Vaccination for CRC immunoprevention

Vaccination is by far the best example for cancer immunoprevention. Hepatitis B virus (HBV) and HPV vaccines are very effective in primary prevention of virus-associated hepatocellular carcinoma and cervical cancer, respectively [105]. Compared to drug-based chemoprevention approaches, immunization has several advantages, including safety, long-term memory, cost-effectiveness, and ease of delivery and possibility to be combined with other preventive approaches [20]. The effectiveness of the vaccine approach in viral-infection-induced cancers provides a strong proof-of-principle for immunoprevention of non-viral cancers including CRCs.

Vaccination as primary prevention for non-viral cancers is in the very early stages of development. MUC1 represents an attractive target for CRC immunoprevention due to its overexpression and hypo-glycosylation in different stages of CRCs including pre-malignant lesions [14]. It was among the most prioritized tumor antigens by the National Cancer Institute (NCI) based on several criteria, such as immunogenicity, specificity, and oncogenicity [106]. Animal studies provided proof-of-principle for developing MUC1-based vaccines for CRC prevention. Vaccination with a MUC1 peptide reduced the size and number of existing polyps in *APC* Min+ mice expressing *MUC1* transgene [107]. Similarly, a MUC1-based vaccine was found to ameliorate inflammation and prevent progression to colitis-associated colon cancer (CACC) in the *MUC1* transgenic/*IL10* knockout mouse model [108].

Based on the preclinical studies, a MUC1 vaccination clinical trial was carried out in patients with a history of advanced premalignant colorectal adenomas for evaluating immunogenicity and safety [109]. Among 39 evaluable patients, 17 (44%) developed a marked (2-fold) anti-MUC1 IgG antibody response. The vaccine also induced long-term memory, with 12 (75%) of the 16 patients who received a booster at 52 weeks responding with an increase in antibody titers. Interestingly, the non-responder patients had a significantly higher percentage of circulating MDSCs than the responder patients and healthy donors. These findings suggest the presence of immunosuppressive mediators in premalignant stages of tumor development, and provide a rationale for vaccinating at an even earlier stage or incorporating MDSC-targeted immune modulation in later premalignant stages. No significant toxicity was observed among the patients who responded to the vaccine. A multi-center, placebo controlled randomized phase II clinical trial for testing the efficacy of this vaccine in patients with the history of advanced colon adenomas has been initiated (NCT02134925; clinicaltrials.gov). The association between the presence of MDSCs, immunologic vaccine response, and polyp recurrence will be evaluated.

In addition to MUC1, prophylactic vaccines for other tumor antigens have also been tested in preclinical models. A vaccine regimen for CEA was shown to generate strong CEA-specific host immune responses, reduce the number of intestinal tumors, and improve overall survival of *CEA* transgenic *APC* Min+ mice [110]. Phase I/II clinical trials have also been undertaken using the vaccine Ad5 that encodes CEA. Cell-mediated immunity was seen in 61.3% of advanced CRC patients who received the vaccine. However, the overall CRC patient survival of 48% was the same across all groups irrespective of vaccination status [111]. Therapeutic vaccines for neoantigens such as mutated KRAS and TP53 have been

tested in animal models and in clinical trials for advanced tumors [17, 112], but have yet to be applied in early and premalignant settings.

4.2 Immune modulators as potential CRC preventive agents

The recent success of checkpoint inhibition for cancer immunotherapy has generated tremendous enthusiasm for cancer prevention by directly targeting the immune system using immune modulating agents. The information on the efficacy and toxicity profiles of anti-PD-1, anti-PD-L1, and anti-CTLA-4 antibodies is being rapidly accumulated from various clinical studies. It may be possible to utilize these antibodies for CRC immunoprevention in high-risk populations such as those with MSI or Lynch syndrome. The recent findings that PD-1 and CTLA-4 are upregulated to a greater extent in MSI CRCs suggest that these high-risk individuals would benefit from immune checkpoint inhibition [67]. For the purpose of immunoprevention, it is particularly important to define the biomarkers of effectiveness and properly manage potential toxicities. It is anticipated that the ongoing preclinical and clinical studies will soon reveal whether it is feasible to use the immune checkpoint blocking antibodies for cancer immunoprevention.

In addition to checkpoint blocking antibodies, other targets such as cytokines have been explored for immunoprevention of cancers including CRCs [48]. It was shown that interleukin-6 (IL-6) produced by hematopoietic cells promotes adenoma growth and ablation of IL-6 reduces the number and size of colonic adenomas in a model of CACC [113]. Tocilizumab, an FDA-approved humanized anti-IL-6 receptor monoclonal antibody, was found to be effective in reducing tumor burden and increasing animal survival in Nlrp1-deficient *APC*^{Min} mice [114]. Blocking antibodies for IL-17 and IL-23 receptors could also suppress colonic hyperplasia and tumor formation caused by bacteria-induced colitis [115]. Similarly, neutralizing antibodies against IL-22 could suppress bacteria-induced CRCs [116]. However, it remains to be determined whether these cytokine blocking antibodies can be used for tumor prevention in individuals with high risk of developing CRCs.

4.3 Combinations of immunoprevention and chemoprevention

A major obstacle in cancer chemoprevention is toxicity associated with long-term and regular drug administration. While a handful of anticancer agents have been shown to be effective in CRC chemoprevention, many promising agents could not be applied to CRC prevention due to toxicity. On the other hand, immunoprevention in particular vaccination, potentially offers a safer tumor prevention approach. But various immune modulating agents have yet to demonstrate efficacy in human clinical studies. Combinations of different classes of anticancer agents represent a promising approach to overcome these limitations and achieve safer and more effective cancer prevention. For example, a combination of low oral doses of the NSAID sulindac and difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, could markedly suppress recurrence of adenomatous polyps with few side effects [117]. Based on a preclinical study [118], a randomized clinical trial was carried out to test the combination of sulindac and the epidermal growth factor receptor (EGFR) inhibitor erlotinib on FAP patients with duodenal polyps. This combination resulted in a lower duodenal polyp burden after 6 months of treatment [119]. These studies involved

reduced dosage of the chemopreventive agents with different mechanisms and non-overlapping toxicities for safer and more effective chemoprevention.

Combinations of chemopreventive drugs with immune-modulating agents have been tested in preclinical studies and may offer improvement of CRC prevention [20]. It is rational to combine NSAIDs with immune checkpoint blockers due to the immunosuppressive effects of COX2/PGE2 [85], as well as NSAID-induced upregulation of PD-L1 and PD-L2 [83]. Preclinical data demonstrate that COX2 inhibition by NSAIDs such as aspirin synergizes with anti-PD-1 blockade in inducing eradication of tumors, implying that COX2 inhibitors could be useful adjuvants for cancer immunoprevention [120]. Similarly, a combination of anti-CEA vaccine with celecoxib had a synergistic effect in *CEA*-transgenic *APC*^{Min} mice [121]. A number of preclinical studies showed that NSAIDs such as celecoxib and indomethacin could synergistically or additively enhance the antitumor efficacy of vaccines and cytokines [122]. Furthermore, various combinations of immune modulating agents and chemotherapeutic agents have been explored for the therapeutic purpose [123]. The results of these studies will provide rationales and roadmaps for testing such combinations in a prevention setting.

5. An emerging role of gut microbiota in CRC prevention

The gastrointestinal tract closely interacts with 80% of the host immune system and contains trillions of microorganisms that have co-evolved with it for millions of years [124]. This symbiotic relationship is critical for the maintenance of normal intestinal physiology, barrier function, and mucosal immunity [125, 126]. The intestinal microbial composition is dynamic and highly responsive to antibiotic treatment, dietary changes, exercise, and gastrointestinal pathogens. Large shifts in microbial composition can lead to community imbalance, a state known as dysbiosis. This can be triggered by deficiencies or dysregulation of the intestinal immune system itself [124], and sensed by pattern recognition receptors including Toll-like receptors (TLRs) expressed in the GI epithelial and immune cells that specifically recognize pathogen-associated molecular patterns (PAMPs) from microbial pathogens, as well as damage-associated molecular patterns (DAMPs) released from dying cells [127, 128].

Dysbiosis is associated with metabolic and cardiovascular diseases, inflammation, and cancer [124]. Reduced abundance of gut protective short-chain fatty acids (SCFA; i.e. butyrate), which are produced by Lachnospiraceae family members, have been reported in CRC patients [129, 130]. Dysbiosis leads to prolonged NF- κ B activation, intestinal barrier dysfunction, inflammation, and increased cancer risk [124, 131]. Several mechanisms can be involved, including TLR-MyD88-mediated NF- κ B and ERK activation [132, 133], defective-barrier-driven IL-23/IL-17 production [134], and IL-17C upregulation in intestinal epithelial cells [135]. Given the highly interactive nature of mucosal immunity, challenges remain to define the direct contribution of specific bacterium (or bacteria) on CRC risk using well-controlled animal models and human prevention trials required for translation. It was shown that a specific human colonic bacterium, enterotoxigenic *Bacteroides fragilis* (ETBF), can induce IL-17- and Treg-dependent colon tumor formation in *APC*^{Min} mice [60, 115].

Epidemiological studies indicate intake of foods rich in dietary fiber, such as fruits, vegetables and whole grains, reduces CRC risk [136]. Certain food-derived metabolites have been shown to increase the abundance of gut protective bacteria that may prevent inflammation and CRC development [137]. It would be interesting to determine if modulation of microbiota via probiotics, foods, and other means will help "normalize" gut mucosal immunity to reduce CRC risk [138].

6. Conclusions and future directions

CRC prevention represents a paradigm for cancer prevention in general, but faces several significant challenges. For example, NSAIDs are still not FDA-approved for cancer prevention and are mostly used off-label due to toxicity. Some key issues in CRC prevention, mechanistic insights in particular, have remained unresolved, despite a large number of published studies. The molecular mechanisms for the specificity of NSAIDs in eliminating emerging tumor cells remains poorly understood. Chemoprevention and immunoprevention studies described in the literature have not sufficiently addressed how the observed efficacy is related to the targeting of disease underpinning, in particular loss of *APC*, MSI, and subsequent alterations.

Several research areas will likely drive the advances in CRC prevention. Delineating the molecular mechanisms of immunosurveillance and escape in early tumor development will provide important clues and rationale for harnessing the immune system. Combinations of multiple omics approaches and cutting-edge bioinformatics tools will identify neoantigens in early stages of tumor development including CRCs [139], and will provide a pre-cancer atlas necessary for better design of preventive agents and for precision prevention clinical trials. The types of immune cells and their spatial and temporal changes in response to gatekeeper alterations remain to be defined. The role of cancer stem cells in shaping the immune response needs to be examined. Identification of key antigenic targets for CRC prevention will empower the development of prophylactic vaccines and other immune modulating agents including those involving the gut microbiota.

Proper management of toxicity for immune modulating agents is a key issue for applying these agents for CRC prevention. It is conceivable that new agents and strategies will also be developed for mitigating the side effects of systematic activation of the immune system by agents such as immune checkpoint blockers. Testing of different combinations of preventive agents including chemopreventive and immunopreventive agents will continue to be explored for dose reduction and efficacy improvement.

Applications of emerging new tools and technologies will enable mechanistic investigations and accelerate clinical translation. Analysis of anticancer immune response currently relies on syngeneic CRC transplantable tumor models such as MC38 and CT26, which do not recapitulate cancer development through pre-neoplastic lesions. Additional and improved animal models are urgently needed. Application of the CRISPR/Cas9 genomic editing technique will also facilitate development of new animal models that can be used to study CRC prevention [140]. Organoid culture from pre-neoplastic lesions provides a powerful tool for dissecting early tumorigenic events applicable to mouse and human tumors with

diverse drivers [41]. Liquid biopsies using circulating tumor DNA (ctDNA) [141], are useful for non-invasive and early detection of pre-neoplastic lesions in prevention trials.

In conclusion, prevention or early detection represents one of the most promising approaches for reducing the mortality and morbidity of cancers including CRCs. Immune modulation has emerged as a major player in CRC prevention due to the increased appreciation of immunosurveillance as a critical barrier for tumor development, the immune-modulating effects of chemopreventive agents, and the recent success of cancer immunotherapy. With many tasks ahead, now is the best time for cancer biologists and immunologists to work together to harness the human immune system to effectively prevent the development of CRCs.

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Abbreviations

ACF	aberrant crypt foci
APC	Adenomatous Polyposis Coli
APM	antigen processing machinery
ASTE1	Asteroid Homolog 1
CACC	colitis-associated colon cancer
CIN	chromosome instability
COX2	cyclooxygenase 2
CRC	colorectal cancer
CRT	calreticulin
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
DFMO	difluoromethylornithine
EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum

ETBF	enterotoxigenic <i>Bacteroides fragilis</i>
FAP	familial adenomatosis polyposis
GEMM	genetically engineered mouse models
GI	gastrointestinal
HBV	hepatitis B virus
HLA	Human Leukocyte Antigen
HNPCC	hereditary non-polyposis colorectal cancer
HPV	human papilloma virus
IBD	inflammatory bowel disease
ICD	immunogenic cell death
IDO	indolamine-2,3-dioxygenase
LAG-3	lymphocyte-activation gene 3
MDSCs	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MMR	mismatch repair
MSI	microsatellite instability
MSS	microsatellite stable
NCI	National Cancer Institute
NK	natural killer
NKG2D	natural killer group 2D
NSAIDs	non-steroidal anti-inflammatory drugs
PAMPs	pathogen-associated molecular patterns
PD-1	Program Death 1
PD-L1	Programmed Death Ligand 1
PGE2	prostaglandin E2
PTEN	Phosphatase and Tensin homolog
PUFAs	polyunsaturated fatty acids
SNPs	single nucleotide polymorphisms
SCFA	short-chain fatty acids

SOCS	suppressors of cytokine signaling
TAMs	tumor associated macrophages
TH	T helper
TLRs	Toll-like receptors
TGF-β	Transforming Growth Factor β
TGFBR2	Transforming Growth Factor β Receptor 2
TILs	tumor-infiltrating lymphocytes
TP53	tumor protein p53
Tregs	regulatory T cells

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Figure 1. Immunoediting and immunoprevention along adenoma-carcinoma progression in colorectal tumor development

Development of colorectal cancer is driven by genetic alterations in the *APC/β-catenin* pathway, leading to aberrant Wnt signaling and formation of hyperplastic cryptic foci and early adenomas. Accumulation of genetic alterations in oncogenes such as *KRAS/BRAF* and tumor suppressors such as *TP53* results in formation of late adenomas and carcinomas. Emerging tumor cells generate mutated neoantigens or tumor associated antigens (TAAs) that can trigger antitumor immune response initiated by dendritic cells (DCs) and mediated by CD8+ T cells, CD4+ T cells, and other immune cells as indicated. This process selects for tumor cell variants that are capable of enduring the attack by the immune system, and also creates an immunosuppressive microenvironment due to activation of the PD-1/PD-L1 and CTLA4 pathways, as well as immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). During this process, antitumor immune response and immunosuppression reach equilibrium, and eventually allow tumor cells to escape from immunosurveillance. Both chemoprevention and immunoprevention modulate antitumor immune response and restore immunosurveillance through various effects on immune cells and effector molecules and pathways

Figure 2. Overview of antitumor immune response

Tumor antigens are captured by dendritic cells (DCs) and B cells and presented to T cells in the draining lymphnodes. This will lead to priming of humoral (antibody) and cellular responses (helper and cytotoxic T cells). Interferon- γ (IFN- γ)-producing CD4+ T cells, cytotoxic CD8+ T cells, and natural killer (NK) cells are the main effectors for this antitumor response. These effects can be attenuated via the actions of regulatory T cells (Tregs).

Table 1

Effects of common chemopreventive agents on the immune system

Chemopreventative agents	Immune cells/effector molecules	Effects	Study systems	References
Aspirin and NSAIDs	TAMs	Decreased PD-L1 expression	<i>In vitro</i>	[82]
		Decreased M2 recruitment	<i>In vivo</i> (Balb/c syngeneic model)	[81]
		Switching from M2 to M1 phenotype	<i>In vitro</i> and <i>in vivo</i> (APC Min+ mice)	[142]
	DCs	Reduced ability to produce IL-12	<i>In vitro</i>	[86]
	MDSCs	Decreased MDSC population	<i>In vivo</i> (C57BL/6 <i>Ccl2</i> -deficient mice)	[19, 86]
		Decreased PD-L1 expression	<i>In vitro</i>	[82]
	Tregs	Reduced population or inhibitory effect	Clinical (CRC and other cancer patients)	[77, 122]
	T cells	Increased T cell population and function	Epidemiological; clinical (CRC and other cancer patients)	[72, 73, 77, 122]
	NK cells	Enhanced NK cell-mediated cytotoxicity	<i>In vivo</i> (Balb/c syngeneic model); clinical	[19]
	HLA-DR	Induced expression	<i>In vitro</i>	[79]
	Cytokines	Altered balance of pro-and anti-inflammatory cytokines	<i>In vitro</i>	[80]
Vitamin D	T cells	T-cell antigen receptor signaling and T-cell activation	<i>In vitro</i>	[92]
Curcumin	B cells	Increased B cell frequency	<i>In vivo</i> (APC Min+ mice)	[19, 98]
	T cells	Increased T cell population	<i>In vitro</i> ; <i>in vivo</i> (APC Min+ mice)	[98]
		Restoring central memory T cell (TCM) and effector memory T cell (TEM) populations	<i>In vivo</i> (Swiss albino mice)	[95]
		Stimulating cytotoxicity	<i>In vitro</i>	[19]
	NK cells	Upregulation of NK activation	<i>In vitro</i>	[95]
	TAMs	Switching from M2 to M1 phenotype	<i>In vitro</i> and <i>in vivo</i> (xenograft model)	[19, 94]
	MDSCs	Decreased interaction with cancer cells	<i>In vitro</i>	[94]
		Reduced MDSC population and mobilization inhibition	<i>In vitro</i> and <i>in vivo</i> (xenograft and allograft Balb/c mouse models)	[19, 94]
	Tregs	Reduced inhibitory effect	<i>In vivo</i> (Swiss albino mice)	[95]
ω -3 PUFAs	Tregs	Reduced inhibitory effect	<i>In vitro</i> ; epidemiological	[101]
Metformin	HLA-DR	Induced expression	<i>In vitro</i>	[19, 104]

Chemopreventative agents	Immune cells/effector molecules	Effects	Study systems	References
	T cells	Enhanced memory T cell numbers	<i>In vitro</i> and <i>in vivo</i> (<i>Cbl-b</i> ^{-/-} and B6.Ly5.2/Cr CD45.1 congenic mice)	[103]

Abbreviations: DC, dendritic cells; HLA, Human Leukocyte Antigen; MDSCs, myeloid derived suppressor cells; NK, natural killer cells; PD-L1; Programmed Death Ligand 1; TAM, tumor associated macrophages; Tregs, regulatory T cells

Table 2

Agents for CRC immunoprevention

Immunopreventative agents	Targets	Prevention studies	Study system	Major findings	References
Vaccines	MUC-1	Preclinical	<i>MUC1</i> transgenic <i>APC</i> ^{Min} mice	Reduced number of polyps	[107]
		Clinical	<i>MUC1</i> transgenic/ <i>IL10</i> - KO mice	Ameliorated inflammation and CAC progression	[108]
			Patients with history of advanced premalignant colorectal adenomas	44 % with a marked MUC1-specific antibody response 75% of those with induced long-term immunity Significantly higher percentage of circulating MDSCs in non-responders	[109]
	CEA	Preclinical	<i>CEA</i> transgenic <i>APC</i> ^{Min} mice	Reduced tumor burden and increased survival	[110]
		Clinical	CRC patients	61.3% had cell mediated immunity Overall patient survival of 48% was the same across all groups irrespective of vaccination status	[111]
	TP53/KRAS	Clinical	Patients with several types of cancers inclusive of CRC	26% with detectable CTLs against mutant TP53 or KRAS 42% with positive IFN- γ responses	[17, 112]
Immunomodulators	Tocilizumab (humanized anti-IL6R antibody)	Preclinical	<i>Nlrp1</i> -deficient <i>APC</i> ^{Min} mice	Reduced tumor burden and increased survival	[114]
	Blocking antibodies for IL-17, IL-23, and IL-22	Preclinical	<i>APC</i> ^{Min} mice; 129SvEv <i>RAG</i> ^{-/-} mice with Hh infection and AOM treatment	Suppressed colonic hyperplasia and tumor formation	[115, 116]