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Review

The regulatory function of sphingosine-1-phosphate signaling axis on regulatory T cells in colorectal cancer

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Abstract: In tumors associated with inflammation such as inflammatory bowel disease (IBD) and colorectal cancer (CRC), high numbers of regulatory T cells (Tregs) are associated with both favorable and poor prognoses. The functions of Tregs in CRC remain elusive and have yet to be clearly defined. With new evidence supporting many subsets of Tregs, the research on the development and functions of these cells has begun to come to fruition. The sphingosine 1 phosphate (S1P) pathway was recently reported to regulate the development and function of regulatory T cells. This pathway may shine new light into the pleiotropic nature of these cells in cancer. In this review, we will examine current literature on the many functions of Tregs in CRC and highlight the significance of the S1P signaling pathway in Treg development/function with the implication of novel therapeutic strategies in treatment of CRC patients.

Keywords: regulatory T Cells; colorectal cancer; sphingosine-1 phosphate; therapeutic drugs

1. Regulatory T cell development and functions

Regulatory T cells play a key role in maintaining the balance between health and disease and, in so doing, provide a system of checks and balances by: restraining asthma and allergy, preventing cytotoxic lymphocytes from reacting to self-antigens, and controlling inflammatory response. There

are two main types of regulatory T cells; natural Tregs and adaptive/induced Tregs, which function to suppress the immune reaction [1]. Most natural Tregs are found developed in the thymus but they can also be induced in the periphery or in culture [2,3,4]. The natural Treg subset constitutes approximately 5–10% of resident CD4+ T cells and are involved mainly in self-tolerance while adaptive/induced Tregs are important in oral tolerance and inflammation [5,6]. The seminal work by Sakaguchi et al. in 1995 [7] using CD25+ depleted thymic T cells to induce autoimmune disease in nude mice demonstrated that thymic derived CD25+ T cells were the key cells mediating self-tolerance. CD25 is an alpha chain of IL-2 receptor that is expressed in most Tregs; however, a subset of Tregs, such as regulatory T cells 1 (Tr1) may express low levels of CD25 [8]. Signaling from CD25 is required for Treg survival and function [7,9,10]. While CD25 is often used as marker of Tregs, other surface markers such as, CD38, CD62L (L-selectin) and CD103 are sometimes used to identify different subsets of Tregs [11,12,13].

With the discovery of a transcriptional factor Forkhead-box P3 (FoxP3) as a specific Treg marker about a decade ago, the research field in Tregs has exploded. The general idea that FoxP3 regulates immuno-suppressive function of Tregs was formulated after extensive analysis of the scurfy mice [14] and patients with IPEX (Immunodesregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) [15,16] which confirmed that a loss of FoxP3 as a causative factor of multi-organ autoimmune disease. FoxP3 was subsequently shown to control the development and suppressive function of Tregs [17,18]. In the developing thymocytes, T-cell receptor (TCR) engagement with the peptide-MHCII [19,20,21] induces the expression of FoxP3 [22], suggesting that Tregs have self-antigen specificity consistent with the data by Gershon and Kondo [23], showing that adoptively transferred antigen-exposed T cells retain antigen-specific tolerance in naïve mice. FoxP3 forms a complex with nuclear factor of activated T cells (NF-AT) and inhibits the expression of IL-2, IL-4 and Interferon-γ (IFN-γ) cytokines which are important for adaptive immunity [24,25]. The FoxP3/NF-AT complex also upregulates the expression of CD25, cytotoxic T lymphocyte antigen 4 (CTLA4 also known as CD152) and glucocorticoid-induced TNF receptor family-related gene (GITR) in Tregs [24,25]. GITR signaling is necessary for Treg suppressive activity [26]. In conjunction with cytokines, such as IL-10 and transforming growth factor (TGF-β), FoxP3+ Tregs dampen effective cell activity by direct contact via CTLA4, which interacts with CD80 (B7-1) and CD86 (B7-2) on the target cells and inhibits their cellular function [27,28,29]. With CTLA4, Tregs can form an aggregate around DC thereby blocking the ability of DC to interact with effective cells and activation of adaptive immunity [30]. Tregs can also induce DC to produce indoleamine 2, 3-dioxygenase (IDO), an important enzyme required for peripheral tolerance [31]. In short, FoxP3 is a critical intracellular molecule that governs the development and functions of Tregs.

Adaptive/induced Tregs which are generated in the periphery or in culture upon antigen encounter may or may not, however, express FoxP3 [32]. It appears that a suboptimal antigenic stimulation is required as well as signaling from TGF-β to drive the development of adaptive/induced Tregs from conventional CD4+ or CD8+ T cells [4,33,34] (Figure 1). In the gastrointestinal tract, adaptive/induced Tregs are necessary to maintain oral tolerance to enteric flora and food antigens, thus preventing pathological inflammation [6]. FoxP3+ Tregs specific to oral antigens can be induced from mesenteric or enteric lamina-propria CD4+ T cells by gut associated CD103+ dendritic cells [35]. Interestingly, FoxP3+ expression in adaptive/induced Tregs is less stable than in natural Tregs and depends on TGF-β to maintain FoxP3 expression [36].

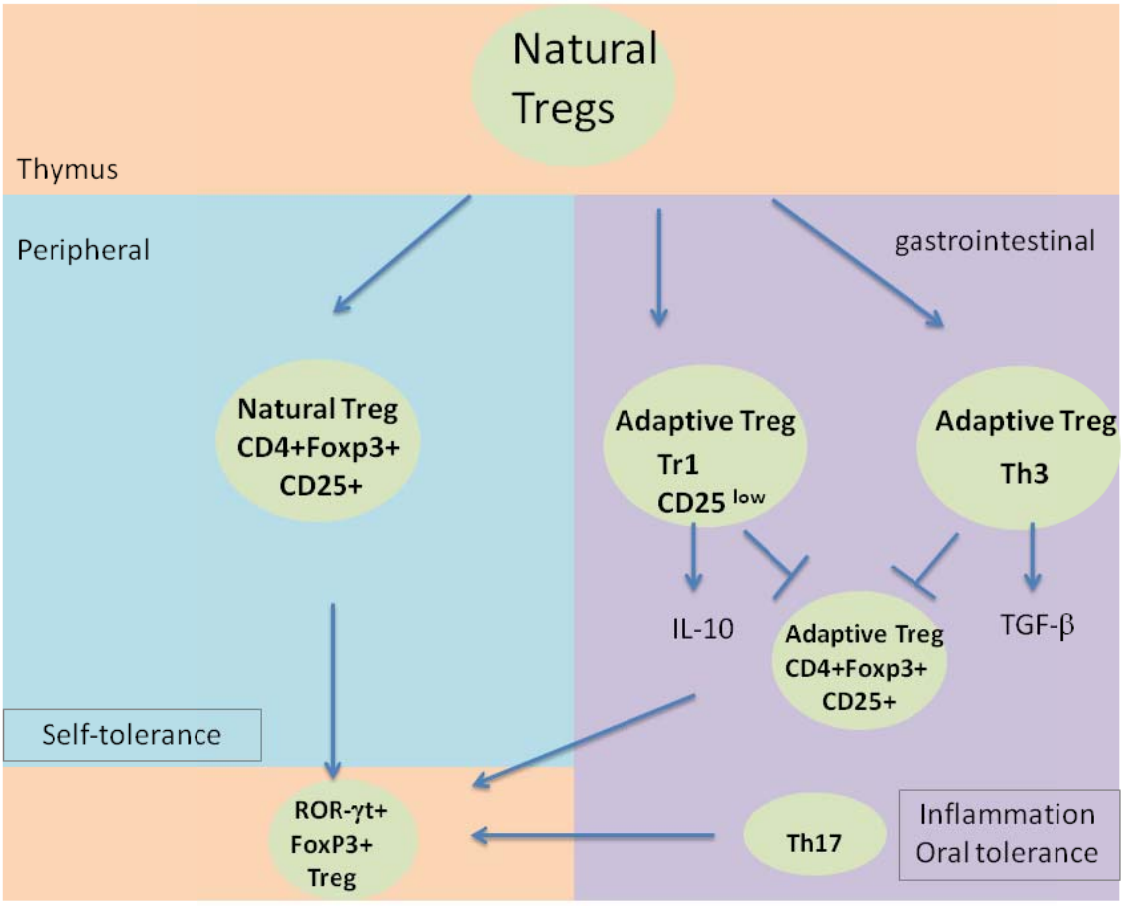

Figure 1. Development of regulatory T cells. Most natural Tregs develop in the thymus, leave the thymus and establish residence in the periphery. These Tregs are important for self tolerance. A subset of Tregs (adaptive Tregs) is induced in the periphery (gastrointestinal tract). These adaptive Tregs are regulated Tr1 and Th3. Another subset of Tregs (ROR-γt+ Tregs) is considered pathological Tregs, since a high number of these cells are associated with increased tumor number and size. ROR-γt+ Tregs may be derived from natural Tregs, Th17 cells, or adaptive/induced Tregs during inflamamation.

Two subtypes of T cells, Tr1 and T helper 3 (Th3), are also known to regulate the development and function of FoxP3+ Tregs. These cells are also considered as regulatory T cells since Tr1 can secrete IL10 [8] and Th3 can secrete TGF-β [37] (Figure 1). While secretory products, IL10 and TGF-β are known for their suppressive functions of mainly effective cell [8,37,38], these cytokines also regulate local FoxP3+ Treg development. Indeed, high CD25+ FoxP3+ Tregs were observed in the periphery of transgenic mice engineered to ectopically express TGF-β in Th3 cells [39]. Tr1 and Th3 differ from adaptive/induced Tregs or natural Tregs in that their suppressive function may be driven by antigen-independent signals [40,41,42]. An interesting note is that colonic mesenchymal fibroblasts from patients who underwent colectomy for colon cancer or isolated from IBD patients can stimulate the development of CD4+CD25+FoxP3+ Tregs [43]. Collectively, these data support the idea that adaptive/induced Tregs can be generated in the periphery by local concentration of cytokines or from stimulation by resident cells or/and recruited immune cells.

2. Colorectal cancer, regulatory T cells and S1P signaling

Colorectal cancer is the second leading cause of cancer death in the United States [44]. Approximately 15% of all colorectal cancers are related to one of the two inherited forms: Familial Adenomatous Polyposis (FAP), which is caused by a mutation in the adenomatous polyposis coli (*APC*) gene and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) which is caused by mutation in any of the DNA mismatch repair protein such as: the *msh2* gene, Mut homolog 1, *mlh1* gene, or *hMSH2* gene [45-49]. Other sporadic colorectal carcinomas have been linked to mutations in p53 genes, K-ras, SMAD4, 18q21 allelic loss associated with or without chromosomal or microsatellite instabilities [50]. Numerous studies have indicated that inheritance of the mutated *APC* gene is not sufficient to cause colorectal cancer; additional genetic alterations or environmental changes are required for tumor formation [51]. In a murine model of APC mutation known as Min mice, the development of polyps, a precursor to CRC development in these mice, is influenced by a mutation in the *Mom1* (modifier of Min) gene. *Mom1* encodes for a secreted phospholipase, A2, which catalyzes the formation of arachidonic acid, an important mediator of inflammation. Indeed, Min mice with mutated *Mom1* gene were shown to have lower polyp and tumor numbers [52,53]. These data suggest that the microenvironment affects polyp development and may genetically and epigenetically alter tumor cell growth in inflamed/immune environments [54]. Collectively, these data established that multiple mutations are necessary for colorectal cancer development, and that inflammation is an important environmental factor modifying gene mutations and tumorigenesis.

The function of FoxP3+ Tregs in colorectal cancer is paradoxical. On one hand, a high density of FoxP3+ Tregs in tumors is associated with a good prognosis [55,56]. As a strong correlation has been shown between inflammation and CRC outcomes [57,58] and when Treg function is to suppress the inflammatory response, CRC tumorigenesis develops slower and the prognosis is most likely be more favorable. However, other studies have shown that FoxP3+ Tregs favor colon tumor growth [59,60,61] consistent with reports in other tumors such as breast cancer and hepatocellular carcinoma where increased numbers of Tregs correlate with reduced overall survival [62-65]. This is particularly true if Treg function is to suppress effective T cells involved in immuno-surveillance. Tregs may promote tumorigenesis by suppressing the Th1 antitumorigenic response or by interfering with the inhibitory function of cytotoxic T cells [56,66]. An important distinction should be noted, however, between CRC and other epithelial cancers. In many solid tumors, infiltrating T lymphocytes have specificity for tumor-specific antigens or self-antigens that allow them to destroy cancer cells [67,68]. In the case of CRC, in addition to cytotoxic CD8+ T with specificity to tumor antigens, there are many inflammatory T cells with specificity to the commensal miroflora [69,70] which could affect the functions of Tregs and cytotoxic T lymphocytes. These data add to the complexity of our understanding of the roles of Tregs in CRC.

As such, direct analysis of Tregs in tumors may not be a reliable biomarker for prognosis of CRC [71-74]. Instead, others have examined the ratio of CD3+ or CD4+ to FoxP3+ Tregs and have determined that this may be a better predictor of clinical outcome in patients with colon carcinoma [75,76,77]. Yet, there are still many facets to Tregs, CRC, and tumor progression that have not been explored in detail. For example, how significant is the ratio of CD3+/FoxP3+ in the stromal vs. tumor region or the ratio of CD8+/FoxP3+ in the surrounding regions of the tumor as predictive values of clinical outcomes? What is the predictive value of the number of FoxP3+ Tregs in association with metastasis? What is the relationship between Tregs and chemotherapy? What are the

densities of FoxP3+ and disease outcomes in other types of CRCs such as: DNA mismatch repair (MMR), PIK3CA mutation, and sporadic CRC? Lastly, what specific antigens do CRC-associated Tregs have? These questions need to be addressed further to determine the function of Tregs in CRC in human.

Recent identification of a subtype of Tregs in a mouse model and in some human colon cancer helps to explain the paradoxical functions of these cells [78]. One differentiating factor between distinctive Tregs is the expression of RAR-related orphan receptors (ROR)-γt, which have previously been shown to be vital for Th17 differentiation [79] (Figure 1). Th17 cells were shown to preferentially express IL17, IL-17F and IL-22, and induce inflammation [79]. Using $APC^{\Delta 468}$ mice containing a mutation leading to a loss of APC function that develops benign adenomatous polyps, Blatner et al. [78] observed a significant number of CD4+, ROR-γt+, FoxP3+ Tregs surrounding the polyps consistent with their analysis of biopsied samples isolated from colon cancer patients. Interestingly, these ROR-γt+ FoxP3+ Tregs share many features similar to Th17 cells, suggesting that these cells may be derived from either 1) Th17 cells or 2) induced/activated Tregs responding to a polyp-environment rich in cytokines (IL-1β, IL-6 and IL-23) or 3) natural Treg homing into the colonic mucosal layer (Figure 1). Nevertheless, targeted deletion of ROR-γt in Tregs of $APC^{\Delta 468}$ mice prevents the development of polyposis and increases mouse antitumor immunity suggesting that these pathologic ROR-γt+ Tregs actively contribute to polyp and tumor growth. It remains necessary to examine what triggers the switch in these cells to become ROR-γt+ Tregs. Dissecting the mechanisms of this transition state could have implications in future immunotherapy targeting Tregs.

While many Tregs are observed in CRC [75,77,80], little is known about the mechanism regulating their entry into CRC, though cytokine-mediated regulation of Tregs in inflammation has been suggested [81]. Recently, several chemokine-signaling axes have been shown to mediate Treg recruitment to inflamed sites and tumors [82,83]. In addition to G protein coupled receptor (GPCR) chemokine receptors, sphingosine-1 phosphate receptors ($S1P_1$–$S1P_5$) are also important regulators of immune cells including Tregs [84,85,86]. Of the five S1P signaling receptors, signaling from $S1P_1$ is most studied and has been shown to regulate immune cell migration and activation of specific T cell subsets [87,88]. S1P, the agonist of these receptors, is a sphingolipid that is formed from sphingosine, a product of ceramide degradation, by two known sphingosine kinases SphK1 and SphK2 [89,90,91]. S1P and $S1P_1$ regulating the differentiation of Th2, Tregs, Th17 and to some extent Th1 cells have been shown [88]. Moreover, experiments using transgenic mice carrying CD4+ T cells overexpressing $S1P_1$ provides a first clue that this pathway may regulate Treg recruitment in extrathymic tissues [92]. The $S1P_1$ transgenic mice expressed high level of IL-4, an important cytokine required for the selective development of CD4+ CD25+ Tregs [86]; thus, high numbers of CD4+CD25+FoxP3+ Tregs were observed in $S1P_1$ transgenic compared to wild type mice [86].

More recent studies, have shown that $S1P_1$ signaling restrains thymic Treg development, affects their peripheral numbers, and inhibits Treg suppressive functions by interfering with the function of TGF-β [87,93]. Using transgenic mice with Treg overexpression of $S1P_1$ signaling or transgenic mice with $S1P_1$ ablation in T cells, Liu et al. demonstrated $S1P_1$ signaling controls T cell lineage specification [94]. A reduced number of Tregs were observed in the lymphoid tissues and in the colon of mice with T cells over expressing $S1P_1$ while Th1 subset was increased in the same mice [94]. Interestingly, another group had reported that there was an increase of Tregs in the periphery of $S1P_1$-transgenic mice [92]. The discrepancy between these data may be due to the pleiotropic

function of S1P which is known to orchestrate many intracellular and extracellular physiological and pathophysiological processes including: cell proliferation, migration and immune regulations [95-98]. Extracellularly, S1P functions as chemotactic agent, thus, Tregs with overexpression of $S1P_1$ signaling may exhibit an increase in their homing mechanism. At the same time, S1P may function intracellularly to block the differentiation of Tregs [94].

In the context of CRC, we have recently shown that S1P and $S1P_1$ receptor are also important mediators of cancer progression in a conditional knock-out Stat3 mouse model of colitis associated colorectal cancer [99]. Our paper along with a few other publications demonstrated that the S1P pathway mediated a dynamic loop between tumor cells and recruited immune cells. S1P secreted by tumor cells recruited immune cells whereby those cells secreted interleukin-6 (IL-6) which in turn stimulated tumor cells via IL-6 receptor (GP130) and STAT3 to make more S1P. Thus, S1P maintains the IL-6/STAT3/Sphingosine-kinase signaling in tumor cells [99-102] (Figure 2). We demonstrated

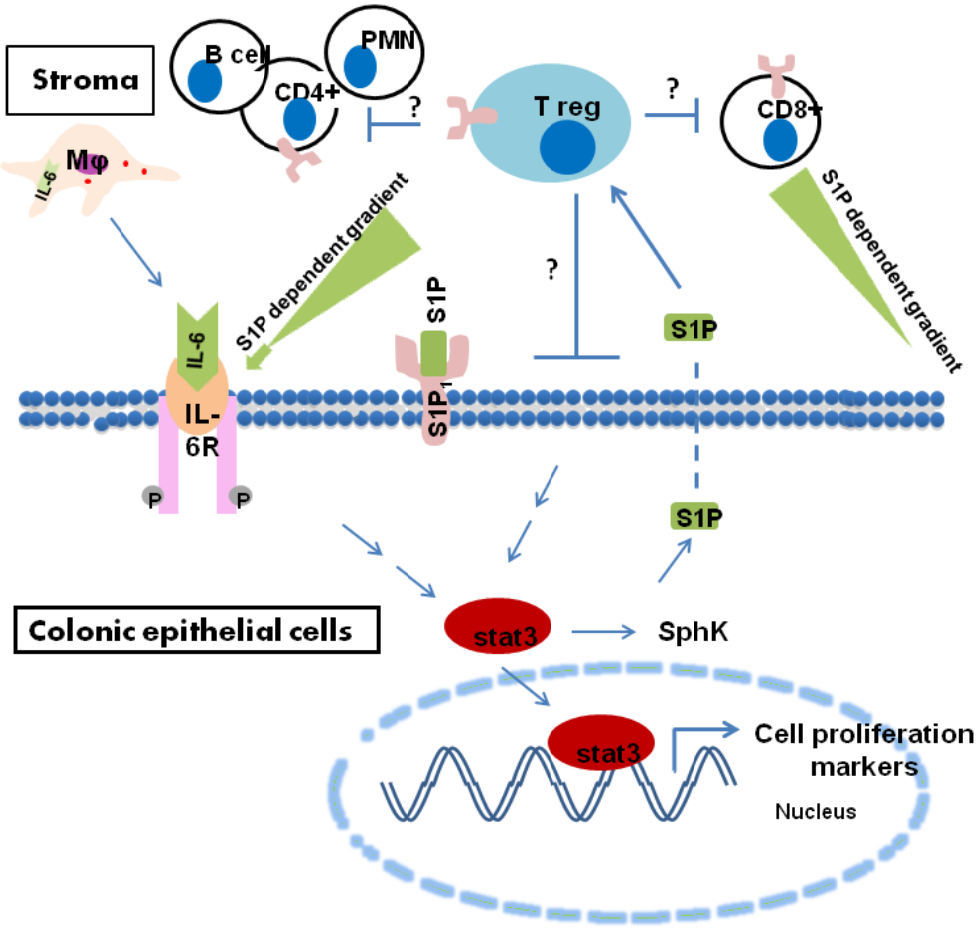

Figure 2. Functions of Tregs in inflammation associated colorectal cancer. The S1P pathway is known to regulate Treg development. S1P is produced by sphingosine-1 phosphate kinases which are activated by STAT3. IL6 produced by recruited immune cells maintains the persistent activation of STAT3. Tregs may function during colorectal cancer progression by suppressing cytotoxic CD8+ T cells or by directly inhibiting tumor cell growth. A lack of Tregs development in the peripheral due to S1P signaling may result in increased inflammation, which favors tumor growth.

that blocking this signaling pathway inhibits tumor progression [99]. However, whether $S1P_1$ regulates Treg development or their function in the inflamed environment remains to be determined. We observed a reciprocal relationship between S1P signaling (tumor progression) and the Treg population [99]. Interestingly, in a recent study published by Liang et al. [100] using $SphK2^{-/-}$ mice (one of the two kinases responsible for making S1P) in a colitis associated colon cancer model, $SphK2^{-/-}$ mice, exhibited severe inflammation and developed larger tumors in greater numbers after AOM/DSS treatment. The phenotype was shown to be due to a compensatory mechanism of upregulation of SphK1 after the loss of SphK2. The group also used FTY720 (Fingolimod), a prodrug that is converted to the active FTY720-P form, to down regulate $S1P_1$ signaling and demonstrated that FTY720 alleviates colitis reducing tumor size and number [100]. While the group did not report on the effect of FTY720 on Treg development, others have shown that FTY720 induces activation of CD4+CD25+ Tregs [94,103]. These findings suggest that modulation of $S1P_1$/IL6/STAT3 signaling in transformed/cancer cells or in immune cells, including Tregs, may affect the tumor microenvironment and thereby alter CRC tumor progression.

3. Therapeutic strategies targeting Tregs and S1P in CRC

Among many immune cells recruited to the CRC tumor site, Tregs appear to orchestrate the immune reaction that affects clinical outcomes. In light of Blatner's data [78], the identification of ROR-γt+ Tregs provides a new immnunotherapy targeting Tregs. An important consideration should be noted in regards to the time of Treg recruitment during tumor development. Tregs which are ROR-γt- tend to arrive at tumor site very early during the inflammation [104]. These Tregs have specificity to the non-self-antigen and would most likely function in controlling inflammation [35]. High number of ROR-γt- Tregs, especially during early stage of tumor development, may indicate a good prognosis. This hypothesis is consistent with the findings that treatment targeted inflammatory response in CRC would have a positive clinical outcome [105]. As such, in a recent retrospective study of over 900 patients, those with colorectal cancer on a regular regimen of aspirin were significantly likely to have reduced mortality rates [105]. This study demonstrates the crucial role of inflammation, especially the interaction between inflammation and tumor progression, and the benefit of controlling some CRCs at the early stage with anti inflammatory drugs.

As a tumor progresses, ROR-γt+ Tregs were shown to be pro-tumorigenic since they suppress the cytotoxic T cells. Drugs targeting the ROR receptor to interfere with the function of ROR-γt+ Tregs would most likely alter disease progression. Along this line, drugs that target the S1P pathway to prevent Tregs as well as other lymphocyte egression from the lymph nodes are especially important. In our recent paper, we demonstrated that the S1P pathway is associated with IL-6 signaling pathway which maintains the persistently activated STAT3 and sphingosine kinases in tumor cells. The cycle between S1P, immune cell recruitment, IL-6, activated STAT3 and sphingosine kinases and S1P is required for tumor progression and has been demonstrated by other groups [100,101]. Interestingly, we observed that the epithelial STAT3 status in tumor cells correlated with the reciprocal Treg population in the tumor environment of colitis associated CRC; while conditional knock-out mice of epithelial STAT3 showed a significantly higher CD4+CD25+ Tregs [99]. We advocate the usage of FTY720 as well as other classes of drugs to perturb the S1P metabolism in treatment of late stage CRC [106].

Understanding the mechanisms by which Tregs mediated immune suppression would help in

designing more appropriate drugs to inhibit Treg activity. As mentioned previously, Tregs mediate the suppression of effective T cells by direct contact via CTLA4. Antibodies targeting the interaction between CTLA4 and CD80 have been developed to inhibit the suppression function of Tregs on cytotoxic T cells; thereby allowing those cells to destroy tumors [107,108,109]. In an animal model of colitis using adoptively transferred of CD4+CD25− T cells into SCID mice infected with the protozoan parasite Leishmania, transferring CD4+CD25+ T cells can reverse colitis. The effect of CD4+CD25+ was blocked if anti-CTLA4 antibody was used [110]. These data suggest that antibody against CTLA4 can block the function of Tregs. Antibodies against CD25 targeting Tregs have also been used to improve immunotherapy responses in cancer [111,112]. In short, different modalities targeting Tregs in CRC should be considered as an additional strategy to the current treatment options.

4. Conclusion

Since the discovery of FoxP3+, many subsets of regulatory T cells have been identified. Their main function is to suppress the immune system thus preventing the innate and adaptive immune responses from going awry. Functions of Tregs in CRC are beginning to unfold with the identification of non-pathological and pathological Tregs (ROR-γt+). Understanding the development of these cells is pivotal in CRC treatment. Several mechanisms have been proposed for their presence in CRC: 1) they could represent pre-existing Tregs 2) arise from CD4+ effective cells and 3) be recruited from natural Tregs. The general consensus of pathological Tregs in CRC is pro-tumorigenic since they suppress immunosurveillence and antitumor immunity. As such, regulation of these cellular functions with antibodies against CTLA-4 antibodies would provide an attractive alternative to CRC treatment. Another strategy would be to block lymphocytes recruitment to mucosal layer of the colon by FTY720, prodrug of FTY720-P that binds to $S1P_1$. Prolonged exposure of the cells to FTY720-P prevents the recycling of $S1P_1$ to the surface after internalization. Hence, S1P signaling is blocked in the presence of FTY720 and FTY720-P. S1P pathway is unique in that it is not only involved in lymphocyte egression, but also that its intracellular signaling regulates the differentiation of CD4+ T cells into one type of immune cells that favor adaptive immunity, or another type (Tregs) that dampens it. The S1P pathway is also a critical component of the interaction between colon cancer cells and stromal cells. The IL-6/STAT3/SphK/S1P pathway promotes the persistent activation of STAT3 in tumors, which upregulates S1P to recruit immune cells which secrete IL-6. Thus, the development of Treg-centric therapeutics with S1P signaling inhibitor is likely to affect multiple cells and would add a novel and more effective therapeutic strategy against colorectal cancer especially those cancers associated with inflammation.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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