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Review Article

Tertiary lymphoid structures in colorectal cancer - organization and immune cell interactions

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Abstract: Tertiary lymphoid structures (TLS), formerly recognized as Crohn's-like structures, serve as crucial biomarkers for evaluating the progression of colorectal cancer (CRC). Understanding their spatial distribution, cellular composition, and interactions within CRC is paramount for comprehending the immune response in the tumor microenvironment (TME). TLS are comprised of a T-cellular compartment and a B-cellular compartment, the latter encompassing follicular dendritic cells (FDCs), high endothelial venules (HEVs), and lymphatic vessels. While T helper cells predominate in cancer TLS, the specific functions of their subpopulations remain inadequately understood. Notably, T follicular helper (Tfh) cells play a central role in the activation of $CD8^+$ T cells, and both Tfh cells and Tfh-associated genes have been linked to enhanced CRC survival. In stage II CRC TLS, an escalation in the number of $FoxP3^+$ T regulatory cells (Tregs) is regarded as a negative prognostic factor. Moreover, within TLS, T lymphocytes shield B lymphocytes from the immunosuppressive effects of the TME. B lymphocyte activation is succeeded by class recombination (CSR) and somatic hypermutation (SHM). Dendritic cells (DCs) constitute a vital cellular component of the TLS T compartment. During steady state and early stages of CRC, specialized antigen-presenting cells such as DCs migrate to regional lymph nodes through afferent lymphatics. They deliver MHC antigen-derived peptide complexes (tumor antigens) to naïve $CD4^+$ and $CD8^+$ T cells, which subsequently infiltrate the tumor site as antigen-specific T cells. Key DC markers studied in TLS include CD83 and DC-LAMP. Research has indicated that the DC-LAMP gene signature in tumor TLS reflects Th1 cell targeting, cytotoxicity, and T cell activation. This review comprehensively outlines the functions performed by distinct cell subsets within tertiary lymphoid structures (TLS) in tumors.

Keywords: Tertiary lymphoid structures (TLS), T helpers, B cell activation, dendritic cells, colorectal cancer

Introduction

Tumors develop a specific microenvironment consisting in immune cells, endothelial cells, cancer-associated stromal cells and recently found lymphocyte aggregations called tertiary lymphoid structures (TLS), that release tumor-promoting or tumor-suppressing factors in the tumor microenvironment (TME), that govern tumor development [1-3]. An immunoscore assessing different lymphocytes has been created, that together with conventional TNM staging AJCC/UICC TNM classification can lead to better determination of tumor prognosis [4, 5] and the outcome of chemotherapy, radiotherapy and immunotherapy [6, 7].

Colorectal cancer (CRC) and immune biomarkers

CRC is one of the most often diagnosed worldwide [8]. The majority of patients with CRC are usually diagnosed in stage II and III of the disease. Several authors have proposed the use of new immunologic biomarkers that can give information about disease recurrence and the use of adjuvant radio- and chemotherapy [9, 10]. The immune cell infiltration has been evaluated mainly in tumor stroma (TS) and the tumor invasive front (IF) [4, 11]. In CRC the evaluation of the immune infiltrate has been associated with cancer development and prognosis [11-14]. Tumors usually establish an immuno-

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suppressive microenvironment that inhibits effective immune responses. Classically, it has been accepted that tumor-associated antigens (TAAs) are captured and processed by dendritic cells (DCs) that migrate to regional secondary lymphoid organs (SLO) and to TLS and present them to naïve T cells in paracortex and some TAA are accepted by B cell receptor (BCR) of B cells in the cortex [6, 15, 16].

Tertiary lymphoid structures and CRC development

One important immune biomarker of CRC development and prognosis is TLS formation [9, 17], previously known as Crohn's-like structures appeared in the vicinity of the tumor [17-19]. According to me and others TLS present in 78.6% of CRC and are positive prognostic factor for stage II CRC [20]. The peritumoral location of TLS in CRC has been associated with worse prognosis and cancer progression [21, 22]. In a study of large cohort of CRC patients (n=195) authors have found that higher numbers of TLS present in less advanced tumors (TNM - I+II) [23]. Increased TLS in the IF of CRC in node-negative patients are associated with better prognosis [20].

Therefore, we decided to examine the precise cellular composition of TLS, possible immune cell collaboration in TLS and to delineate the axis of DCs Ag presentation to naïve CD4 and CD8 T cells in T-zone, T follicular helper cells (Tfh) in the T zone and at the border of germinal centers (GC), and B cell activation. We try to represent in schemas the complex efforts of DCs, Tfh cells and B cells to activate $CD8^+$ T cells and send them to the tumor area.

TLS origin and formation

In the recent 20 years, a new lymphoid structure has been investigated in some tumors such as bronchial cancer, CRC, gastric cancer, etc. [9] and has been called tertiary lymphoid structure. TLS has been situated closer to the tumor and mirror SLO organization and function. TLS are formed mainly at the tumor margin or in the tumor stroma [2]. Moreover, TLS are detected at sites of chronic inflammation [20] or autoimmunity [24]. The term TLS has been mentioned first in 1992 by Louis Picker and Eugene Butcher [25]. Authors call these structures extra-lymphoid sites, where lymphocyte precursors (or memory lymphocytes) are restimulated by antigen. TLS are structures that arise in non-immune tissues and initiate adaptive immune response [24].

TLS contains immune cells organized as SLOs [26]. The development of SLOs is a genetically programmed process that takes place during embryogenesis. SLOs are lymph nodes (LN), white pulp of the spleen, human appendix, mucosal-associated lymphoid tissue (MALT) that includes also Payer's patches (PP) and tonsils [27-29]. LNs and PPs develop prenatally, while bronchial-associated lymphoid tissue (BALT), isolated lymphoid follicles in the intestine and gallbladder occur postnatally [30, 31]. The latter are induced and preprogrammed during ontogeny but appear after birth [32]. Primary lymphoid organs are bone marrow and thymus. The liver loses its hematopoietic function postnatally and it can form TLS in adult life [33]. Other organs like kidneys, heart, salivary glands, synovium, etc. form lymphoid aggregates in disease, considered as TLS [24].

TLS closely resemble SLO and are structures from single cluster of lymphocytes to structures resembling LNs but without capsule [34]. SLOs are organized by lymphoid tissue inducer (LTi) cells and by lymphoid tissue organizer (LTo) cells that produce CXCL13, CCL19 and CCL21 chemokines that attract recruitment of B and T lymphocytes. Moreover, LTo cells change into follicular dendritic cells (FDCs) and form fibroblastic and reticular cell network in SLO [35]. Morphologists define TLS by the following criteria: TLS have T and B cell compartments; FDCs in B cell zones are associated with enzyme-activation-induced cytidine deaminase (AID) required for initiation of somatic hypermutation (SHM) and immunoglobulin class switching recombination (CSR) [6]. TLS have also high endothelial venules (HEV) and lymphatic vessels that are still not well studied [32]. TLS formation is induced by chronic inflammation even in the absence of LTi cells [36]. Some authors report that T helper 17 (Th17) cells [36, 37], B cells [38], DCs [7, 39], M1 macrophages [3, 40], and T follicular helper cells (Tfh) [29, 41] may initiate TLS organization. Therefore, immune cells may substitute for LTi cells in order to stimulate TLS neogenesis [42] (**Figure 1**).

Immune infiltrate in TLS

T helper cells predominate in TLS in cancer and Th2 cells and their cytokines mainly present in

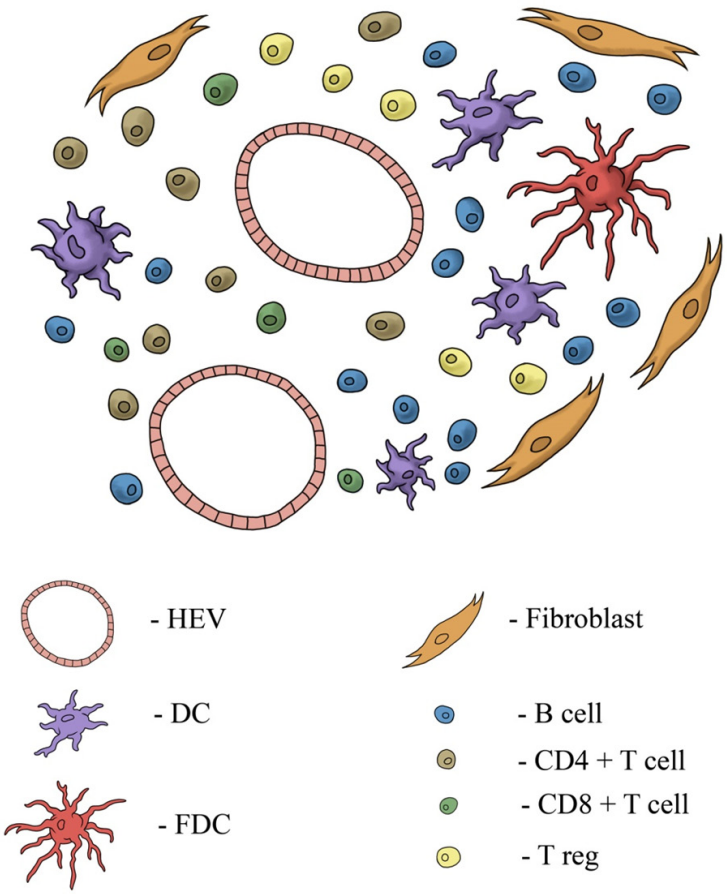

Figure 1. Scheme of TLS. Cells in T zones are CD4⁺ and CD8⁺ T cells, Tregs and dendritic cells. High endothelial venules (HEVs) carry immune cells i.e. T, B cells DCs, from blood, coming from bone marrow. B cells gather in the germinal centers around Follicular dendritic cells (FDC). TLS have no collagen-rich capsule (adapted after Munoz-Erazo et al., 2020) [29].

TLS in advanced CRC [8]. Th17 cells have recently emerged as key factors that trigger TLS development [29, 37]. Tfh cells and FDCs in the germinative centres (GCs) of TLS can produce chemokines like CXCL13 and IL-6 that promote B cell activation, their antigen-presentation and the initiation of humoral immune response [3, 34].

T helper cells T helper 1 (Th1), T helper 2 (Th2), T helper 17/22 (Th17)/(Th22), T regulatory cells (Tregs) and Tfh cells comprise T cell infiltrate in TME and TLS [43-46]. Th1, Th2 and Tfh cells have been first investigated in SLO and TLS immune responses [46]. Some studies have demonstrated that Th17/Th22 cells participate in shaping of TLS [24]. The presence of Th cell subsets in TLS is reported by a few authors [8, 20, 47, 48].

T follicular helper cells are defined in 1980 and are specialized T cells that provide help to B cells [41, 49]. Tfh cells activate other immune cells such as B lymphocytes and CD8⁺ T cells [45]. Tfh cells stimulate affinity maturation and production of antibodies of B cells. CCR5 chemokine ensures movement of naïve T cells from T cell zone to B cell follicle. Tfh cells express STAT3 and Bcl6 transcription factors (TF) and IL21R, CXCR5, CD28, CD40L, CD84, TCR, ICOS and secrete cytokines like IL-10, IL-21 and IL-6 [49, 50]. Bcl6 represses the differentiation and formation of Th1, Th2, and Th17 cells [50]. Recent investigations suggest that Tfh can differentiate into IL-17 producing Tfh cells independent of retinoid-orphan receptor (RORγt) expression [50, 51]. In CRC, increased numbers of T helpers in TLS and of Tfh-related genes, such as *CXCL13* and *IL-21* has been associated with improved survival [8, 47]. In SLO naïve T cells (CD4⁺) activated by TGFβ and IL12/23 change to Tfh cells CXCR5⁺ PD-1⁺ Bcl6⁺ ICOS⁺ Sox4⁺ in GCs [52]. In peripheral tissues CXCL13 induces TLS formation and the appearance of T peripheral helper (Tph) cells that are CXCR5⁻ PD-1⁺ Sox4⁺ CCR2⁺ and that target B memory cells [41] (**Figure 2**).

Using multiplex immunohistochemistry it is shown that higher Tfh cells and macrophages densities in sixty-seven CRC patients are associated with relapsed patients [8]. The prognostic value in 603 patients with CRC liver metastasis of TLS in the tumor stroma and peritumorally is established. It is shown that Tfh cells prevail in TLS in the tumor [53]. In three endoscopic ortothopic colon-cancer mouse models the densities of Tfh cells decrease with tumor progression [54]. Recently, Tfh cells have emerged as the key cell type, required for the formation of GCs in SLO and TLS [55].

Tregs are detected in CRC TLS [56]. FoxP3⁺ Tregs are studied in TLS in stage II of CRC and

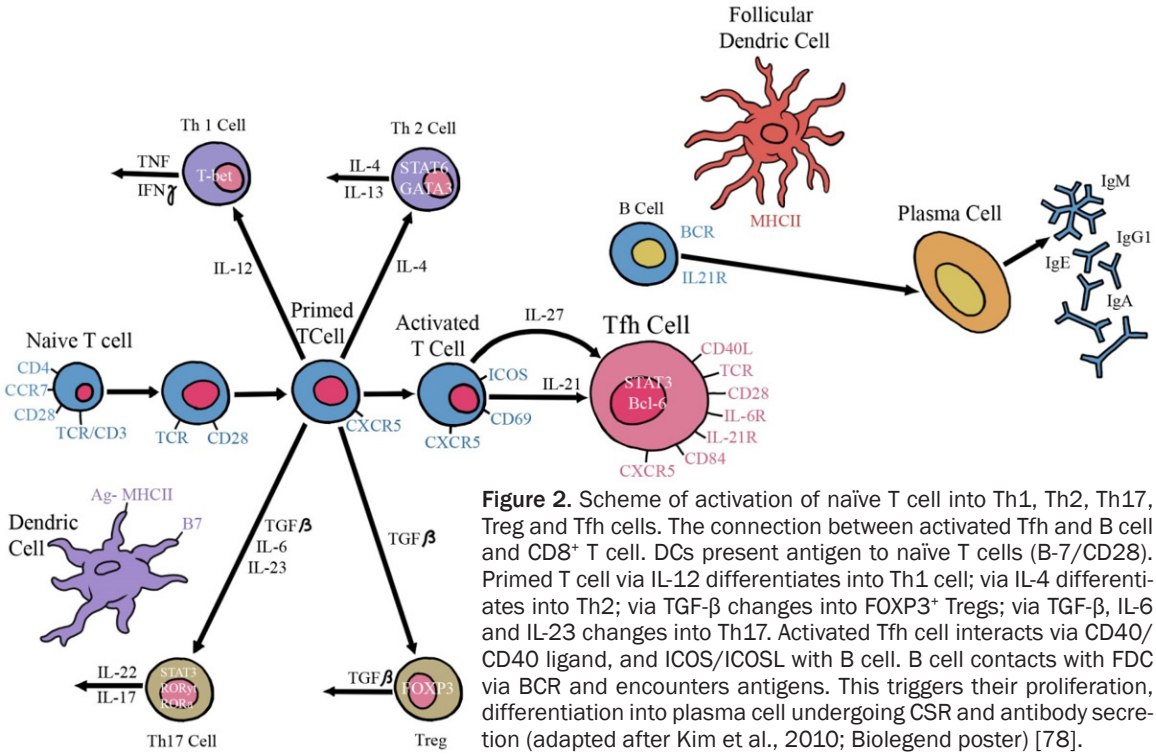

Figure 2. Scheme of activation of naïve T cell into Th1, Th2, Th17, Treg and Tfh cells. The connection between activated Tfh and B cell and CD8⁺ T cell. DCs present antigen to naïve T cells (B-7/CD28). Primed T cell via IL-12 differentiates into Th1 cell; via IL-4 differentiates into Th2; via TGF-β changes into FOXP3⁺ Tregs; via TGF-β, IL-6 and IL-23 changes into Th17. Activated Tfh cell interacts via CD40/CD40 ligand, and ICOS/ICOSL with B cell. B cell contacts with FDC via BCR and encounters antigens. This triggers their proliferation, differentiation into plasma cell undergoing CSR and antibody secretion (adapted after Kim et al., 2010; Biolegend poster) [78].

in LNs, where the increased number of Tregs is a negative prognostic factor of overall survival [57]. On the contrary, the increased number of FoxP3⁺ Tregs in the tumor tissue of stage II CRC has been associated with better survival [58]. Decreased number of FoxP3⁺ Tregs in tumor stroma of CRC has been found in MSS CRCs [11].

Th17 cells (CD3⁺ RORγt⁺) are necessary for the development of TLS [8, 29, 59]. In fact, Th17⁺ cells has been dispersed around TLS (my observation). IL-17 is secreted by a lot of cells such as γδ T cells, macrophages and lymphocytes [11]. It has been supposed that Tregs attenuate Th17 cell-dependent pro-inflammatory and tumor-enhancing responses that could be possible explanation for the favourable prognosis in CRC [60] (**Figure 3**).

Follicular dendritic cells (FDC) contained opsonized antigens on their surface. FDCs are described firs in 1968 by Andras K Szakal and Michel G Hanna [61]. FDCs are cells of mesenchymal origin, that lack phagocytosis, lysosomes or Birbeck granules. They have many fine dendritic processes forming a network in the GC and retain antigen-antibody-complement immune complexes for a long time. They retain mobile B cells and T cells in GC [62]. FDCs are located in the light zone of GCs. These cells have complement receptors (CR1/CR2 - CD35/CD21), immunoglobulin gamma Fc region receptor (FcγRIIB/CD32 and Fc2εRII/CD23) - epsilon Fc region receptor [63]. FDCs are induced in TLS.

Unlike T lymphocytes in T zone of TLS, B lymphocytes has been acknowledged recently as a patent immune cell that organizes anti-tumor immunity [63, 64]. Since 1970s, it has been considered that B cells possess only pro-tumor function, while recently it has been shown that B cells take part in anti-tumor immunity [59, 65]. B lymphocytes develop in liver during fetal life (B-1a lineage) and secrete “natural IgM antibodies”. In adults, B lymphocytes arise in bone marrow (BM) from common lymphoid progenitor (CLP). These B cells are of B-1b and B-2 lineages [66]. It is proven that B cells in TME, outside TLS can acquire suppressive function and contribute to tumor growth [59, 67]. In TLS the mantle of T lymphocytes can physically protect B cells from tumor immunosuppressive molecules [67]. In mature germinal centers of TLS and SLO, there are two zones i.e. dark zone (DZ) in the periphery and light zone (LZ) in the center. Naïve B cells first are concentrated in the

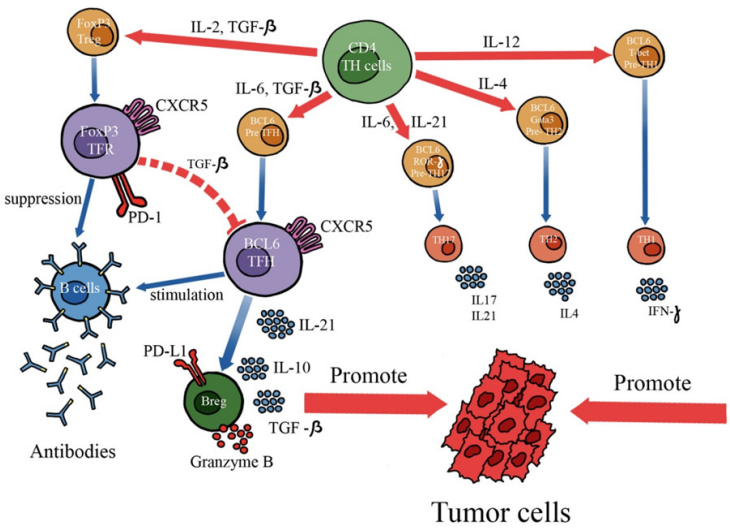

Figure 3. Schema of Tfh cells and CRC promotion. Different cytokines and transcription factors govern T helper appearance in TLS. T follicular regulatory (TFR) cells originating from thymic T-reg cells express CXCR5, PD-1 and ICOS. TFR cells suppresses germinal center B cells and the production of anti-tumor antibodies. Tfh cells via production of IL-21 stimulate Bregs proliferation. Bregs production of TGF-β and IL-10 regulate the immune tolerance in TME promoting tumor development (adapted after Hetta et al., 2020) [46].

DZ, where they contact with Tfh cells coming from T zone, after priming by DCs. Tfh cells through CD40L molecule interact with B cells (CD40 molecule) and promote B cell activation and differentiation [66-68]. In LZ of GC B cells capture antigens through B cell receptor (BCR). Antigens (complement-bound peptides) come from peripheral tissues via lymph and are captured by follicular dendritic cells (FDC) [63]. Later B cells process and present antigen to $CD4^+$ and $CD8^+$ T cells through MHC class II and class I molecules, respectively [69]. So B cells act as APCs such as DCs and promote T cell-mediated immune responses. Activated B cells then undergo class switch recombination (CSR) and generate IgG, IgA and IgE antibodies. B cells also undergo somatic hypermutation (SHM) of the Ig variable region and so ensure antibody diversification and affinity maturation [70, 71] (**Figure 2**).

In TME B cells mainly gather in TLS. In addition, B cells significantly correlate with prognosis of CRC [70]. The role of B lymphocytes in CRC is less explored and in matters of prognostic significance consensus has yet to be reached [72]. The favorable patient prognosis in a large cohort of CRC probes is shown for $CD20^+$ B cells [73]. The neutral and negative impact of $CD20^+$ B lymphocytes on CRC patient's prognosis has been also detected [74]. Patients with CRCs highly infiltrated by $CD20^+$ B lymphocytes show favorable outcome [72]. However, the function of B lymphocytes in tumor development still remain unclear.

Dendritic cells are professional antigen-presenting cells (APCs), that are a component of TLS [39, 75-77]. Tumor-infiltrating macrophages can activate T cells in tumor stroma, while DCs encounter antigen and migrate to TLS or to the draining LNs via various lymph vessels [78], where they prime naïve CD4 and CD8 T lymphocytes [79]. DCs together with B cells present antigen to T cells. At present four main types of DCs are determined i.e. cDC1, cDC2, migratory DC3 (mDC3) and plasmacytoid DCs (pDCs). A fifth DC subset DC5 has been recognized and considered to be inflammatory (DC5) that contained mostly cDC2 state [77, 79, 80]. The cDC1s have been considered to be main cells in cross-presentation of antigens [76]. The cDC1s are major source of IL-12, activate NK cells and drive CD4 cells towards Th1 responses [76, 81]. The cDC2s are the most numerous DC population in tumours. They provide licensing signal to cDC1s, which activate $CD8^+$ T lymphocytes (CTLs). The cDC2s activate $CD4^+$ T cells via MHC class II and provide help to cDC1s to maximize CTL responses [75, 76]. $CD4^+$ T lymphocytes primed by DCs are mainly of Th1, Th2, Treg and Tfh phenotypes in TLS and in LNs. Tfh contact with B lymphocytes and activate them to present new antigens (accepted via TCR) to naïve $CD4^+$ and $CD8^+$ T cells or to modify into plasma cells [75] (**Figure 4**).

The main DCs markers investigated in TLS are CD83 and DC-LAMP [76, 77, 81]. $CD123^+$ pDCs or BDCA-2 are searched [76, 82]. It has been shown that gene signature of DC-$LAMP^{hi}$ TLS tumors presents Th1 orientation, cytotoxicity and T cell activation [81].

When exploring the presence of DCs in CRC, it has been reported that DCs are detected in all CRC specimens [83, 84]. Recently, we have

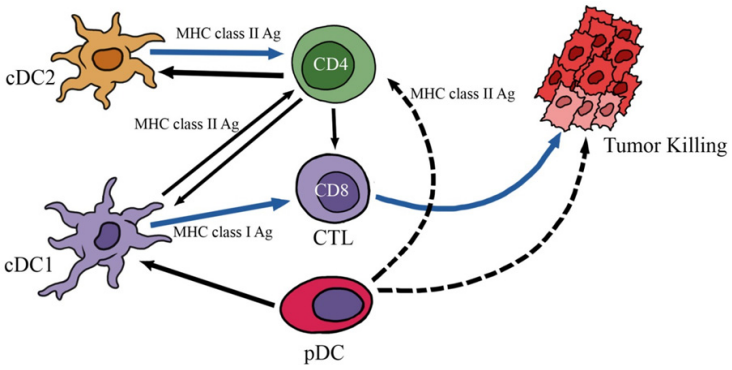

Figure 4. Schema of cDC1 and cDC2 role in anti-tumor immunity. The cDC2 cells activate $CD4^+$ T cells. The cDC1 cells predominantly activate $CD8^+$ T cells. Activated $CD4^+$ T cells provide help to maximize CTL responses. pDCs additionally activate cDC1 through type I interferon and $CD4^+$ T cells (adapted after Noubade et al., 2019; Rossi et al., 2022) [67, 69].

shown that CD1a and CD83 DCs are significantly higher in TS and IF of MSI CRC patients [85]. Limited data existed about DCs in TLS of CRC specimens [86]. In early stage of lung cancer DC-$Lamp^+$ DCs home selectively in TLS and are associated with longer survival [87]. DCs from colon and rectal cancers mature differently, while colon cancer inhibits the levels of five DC markers (CD54, CD80, HLA-DR, CD86, and CD83), rectal cancer enhanced the levels of three DC markers (CD80, CD86, and CD83) [88]. Typically, TLS of the periphery of the tumor have more organized and distinct DC/T cell and B cell zones than the intratumoral TLS, which contained mostly B cells [89]. In rectal cancer the neoadjuvant CRT stimulates the recruitment of pDCs in comparison without such therapy [82]. The cDC2 ($CD1c^+$) DC phenotype has been considered to be the most abundant, while other DC subtypes vary in each cancer type [90]. That means that the investigation of DC subtypes in TLS in CRC, will reveal the fine contexture of these immune structures.

TLSs in the invasive front of node negative CRCs have been characterized with increased density of $CD3^+$ T lymphocytes and $PNaD^+$ high endothelial venules (HEVs), $CD20^+$ B lymphocytes and $CD21^+$ FDCs, two chemokines CCL21 and CXCL13 and Lyve-1^+ lymph vessels that indicate favorable outcome. Presence of these chemokines indicates that TLSs mediate active recruitment of lymhphocytes and dendritic cells in the tumor. Colon and rectal cancers have different distribution of immune cells and react differently to therapy. Noteworthy, another understudied issue is the impact of standart treatment (surgery, chemotherapy and radiotherapy) on the functional properties and prognostic significance of immune cells in TLS. It will be important to assess B cells, T cells in the context of Th1/Tfh/Treg/Th17 subsets and DCs in TLS as this undoubtedly influences their functional attributes in CRC.

Therefore, TLSs are induced structures by inflammation, autoimmune processes and cancer. They are formed as a site of immune battle next to pathologic area. A more detailed analysis of their immune cell content is needed to clarify the fine mechanisms of anti-tumor cellular and molecular response.

Disclosure of conflict of interest

None.

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