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Therapeutic Lymphoid Organogenesis in the Tumor Microenvironment

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Abstract

The inflammatory status of the tumor microenvironment (TME) has been heavily investigated in recent years. Chemokine and cytokine signaling pathways such as CCR7, CXCR5, lymphotoxin, and IL-36, which are involved in the generation of secondary lymphoid organs and effector immune responses, are now recognized as having value both as prognostic factors and as immunomodulatory therapeutics in the context of cancer. Furthermore, when produced in the TME, these mediators have been shown to promote the recruitment of immune cells, including T cells, B cells, dendritic cells (DC), and other specialized immune cell subsets such as follicular dendritic cells (FDC) and T follicular helper (Tfh) cells, in association with the formation of "tertiary" lymphoid structures (TLS) within or adjacent to sites of disease. Although TLS are composed of a heterogeneous collection of immune cell types, whose composition differs based on cancer subtype, the qualitative presence of TLS has been shown to represent a biomarker of good prognosis for cancer patients. A comprehensive understanding of the role each of these pathways plays within the TME may support the rational design of future immunotherapies to selectively promote/bolster TLS formation and function, leading to improved clinical outcomes across the vast range of solid cancer types.

Keywords

B lymphocytes; cancer; chemokines; DC; immunotherapy; interleukin-36; lymphoid organs; lymphotoxin; T lymphocytes; vaccine

Introduction

In recent years, a growing body of literature has established important roles for inflammatory immune cells in the etiology of a wide variety of diseases, including infectious virus-associated diseases, autoimmune diseases such as psoriasis and arthritis, and cancer. More recently, work in this field has expanded in an attempt to elucidate critical signaling pathways involved in initiating and fine tuning inflammatory immune cell activity within affected tissue sites. It has become clear that an array of chemokines (i.e. CXCL13, CCL19, CCL21, and members of the TNF family) and cytokines (i.e. IL-36R and LTβR agonists)

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play important roles in the recruitment, activation, and function of immune cells within inflamed/diseased tissue microenvironments. The orchestration of these factors culminates in the development of organized networks of innate and adaptive immune cells within the TME, that have commonly been referred to as tertiary lymphoid structures (TLS). In the following chapter, we will attempt to provide a better understanding of these pathways in order to provide a foundation for the design of next-generation immunotherapies that will allow for the selective targeting of inflammatory pathways at the appropriate time and location during disease evolution in order to prevent, deter or eradicate cancer *in vivo.*

Development of TLS in Chronically-Diseased Tissues

Our understanding of the dynamics of how immune cells infiltrate and persist as an operational unit within the TME has evolved considerably over the past decade, and now encompasses a paradigm in which TLS develop at the periphery or within tumor lesions to limit disease progression or as a consequence of effective treatment intervention. TLS are distinct from primary and secondary lymphoid organs as they do not form during embryonic development and instead can originate in any non-lymphoid tissue that has been subject to prolonged/chronic inflammation (Aloisi & Pujol-Borrell, 2006). TLS express chemokines including CCL19, CCL21, and CXCL13 that recruit naive and effector $CD4^+$ and $CD8^+$ and memory $CD4^+$ T cells, B cells, and NK cells to sites of inflammation (Erica M Pimenta & Barnes, 2014). The primary cell populations found within TLS are dendritic cells (DC), B cells, and naive and memory T cells (Wolf Herman Fridman, Pagès, Sautès-Fridman, & Galon, 2012). For example, lymphocytic infiltrates and an upregulation of associated chemokines have been observed in the affected tissues of individuals with chronic inflammatory diseases such as Sjögren syndrome, rheumatoid arthritis, multiple sclerosis, myasthenia gravis and Hashimoto's thyroiditis (Pitzalis, Jones, Bombardieri, & Jones, 2014), as well as cancer.

TLS: Organizational structure

Interestingly, it has been observed that there is not a uniform distribution of TLS within inflammatory peripheral tissue microenvironments. For instance, in the setting of oral squamous cell carcinoma, approximately one third of tumors presenting with TLS were missed when only one section of the tumor was evaluated by pathologists (Wirsing, Rikardsen, Steigen, Uhlin-Hansen, & Hadler-Olsen, 2014). In Merkel cell carcinoma, most tumor-infiltrating $CD8^+$ T cells and TLS were located at the tumor periphery, with the presence of TLS correlating with an increased $CD8^+$-to-$CD4^+$ T cell ratio at the cortex, but not in the center, of the tumor mass (Behr et al., 2014). Similarly, in breast cancer, TLS are sometimes observed within the tumor core proximal to the stroma, and within an individual lymphoid structure, the number of lymphocytes decreased as a function of proximity to the center of the tumor, with the most actively proliferating lymphocytes localized to a small area adjacent to the stroma (Gu-Trantien et al., 2013). In other models of breast cancer, TIL are observed within the tumor stroma but not embedded in the tumor tissue itself (Mahmoud et al., 2012). In metastatic colorectal cancer, B cell infiltrates were localized to the outer edges of the tumor lesion (Meshcheryakova et al., 2014). These data suggest that the TME is architecturally heterogenous with regard to the presence and localization of TIL/TLS, and

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that diverse signals likely contribute to determining the anatomic locations in which TLS are "seeded". In this regard, studies have shown that the same signals controlling lymphocyte recruitment to sites of inflammation may play drastically different roles under normoxic (characteristic of the tumor outer cortex) versus hypoxic (characteristic of the tumor core) conditions. For example, signaling via the CCL21/CCR7 axis has been shown to promote angiogenesis in inflammatory microenvironments (Pickens et al., 2012), and new blood vessel formation is one mechanism that facilitates both tumor growth and metastasis. In ovarian cancer, hypoxia induced an increase in intrinsic CCR7 expression by tumor cells, with CCL21 signaling in the hypoxic TME contributing to an upregulation of N-cadherin and the matrix metalloproteinase MMP-9, that are known to promote cell migration and invasiveness (Cheng et al., 2014). In patients with non-small cell lung cancer (NSCLC), a similar effect has been observed, as expression of MMP-1 and ADAM metallopeptidase with thrombospondin type 1 motif, 2 (ADAMTS2) in PBMCs was correlated with poor clinical outcome (Kossenkov et al., 2012).

The organizational structure of TLS in tumors can vary substantially. While classical lymphoid structures are comprised of a B cell follicle intertwined with a network of follicular DC, intratumoral B cell infiltrates (BIL) have been observed in which the B cells are heterogeneously "sprinkled" throughout the tissue instead of being localized within focused aggregates. This has been observed in human oral squamous cell carcinoma (Wirsing et al., 2014) and breast cancer (Mahmoud et al., 2012) tissues, and in tissue sections of murine MCA205 fibrosarcomas (Lu Chen, Fabian, Taylor, & Storkus, 2013), in association with more beneficial disease outcomes. Thus, the establishment of higher order structure in TLS in or near tumors *in vivo* may not be a critical factor to the development of effective anti-tumor immune response. It may only be required that the infiltrating effector cells and antigen (cross)-presenting cells interact productively within the TME.

TLS in cancer: Clinical Correlates of Disease Progression and Response to Treatment

In the cancer setting, the presence of TLS in the TME correlates with increased disease-free survival in patients, with similar results obtained in murine tumor models (see **Table I**). These structures allow for activation, expansion and differentiation of tumor antigen-specific B and T cells within the tumor itself, leading to more effective anti-tumor immune response even in the absence of therapeutic intervention (de Chaisemartin et al., 2011; Erica M Pimenta & Barnes, 2014). In melanoma, a 12-gene signature has been characterized that predicts both the presence of TLS within a tumor and increased survival. This signature includes genes that encode for CCL19, CCL21, and CXCL13, as well as CCL4, CXCL9, CXCL10, and CXCL13 (Messina et al., 2012). In patients with oral squamous cell carcinoma, the presence of TLS is associated with a decrease in tumor-associated death (Wirsing et al., 2014). In Merkel cell carcinoma, the presence of TLS correlated with significantly increased recurrence-free survival compared with patients whose tumors did not contain TLS (Behr et al., 2014). Even in patients with metastatic disease, particularly metastatic colorectal cancer, an increased number of discrete TLS within the TME correlates with an increase in overall survival and a decrease in disease recurrence compared with

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patients presenting with less immune cell infiltrates. These groups can be stratified based on the presence of TLS or the level of $CD45^+$ or $CD20^+$ tumor-infiltrating cells (Meshcheryakova et al., 2014), indicating that the interactions between B cells and other lymphocyte populations play a role in mediating anti-tumor immunity. This paradigm is also present in lung cancer, as patients with intratumoral TLS have an increased likelihood of survival compared to those who do not (Dieu-Nosjean et al., 2008; Germain et al., 2014). In lung cancer, TLS arise spontaneously and confer a beneficial phenotype to patients (de Chaisemartin et al., 2011). In these patients, both the density of mature DC (Dieu-Nosjean et al., 2008) and follicular DC (Germain et al., 2014) can be used as markers for increased survival. Tumors containing less mature DC demonstrate a corresponding decrease in Type 1-polarized $CD4^+$ T cells (Dieu-Nosjean et al., 2008), suggesting that TLS within the TME are crucial locations for generating effective Type 1 anti-tumor immune responses and that a diminished ability to prime a Type 1 response allows for tumor growth. Supporting this contention, in lung cancer, the presence of mature DC within TLS was a better predictor of patient survival than the presence of $CD8^+$ T cells in TLS, with high densities of mature DC also correlating with increased expression of genes related to Type 1 effector cell polarization and cytotoxicity in the TME (Goc, Fridman, Hammond, Sautès-Fridman, & Dieu-Nosjean, 2014; Goc, Germain, et al., 2014). In primary $HER2^+$ breast cancer, infiltration of lymphocytes corresponded to a decrease in the recurrence rate of tumors and a more favorable patient outcome. This was marked by an increase in intratumoral levels of chemokines associated with the development of lymphoid structures- including CCR7, CCL19, CXCL9, CXCL10, CXCL13, and LIGHT- and levels of genes associated with lymphocytes- such as ZAP70, CD8, CD28, and Lck (Alexe et al., 2007). B cell infiltration also corresponded with a more favorable prognosis in breast cancer. The number of B cells found within the TME correlated with an increase in cancer specific survival and disease free survival in patients (Mahmoud et al., 2012). B cells in the TME undergo antigen-driven proliferation, somatic hypermutation, and affinity maturation within the tumor, and these cells co-localize with T cells, follicular DC, and plasma cells into structures resembling tertiary lymphoid organs (Nzula, Going, & Stott, 2003). In a subsequent study of breast cancer patients, an additional marker of overall survival related to the presence of TLS was determined to be the presence of T follicular helper cells, a subset of $CD4^+$ T cells that produces CXCL13 and recruits B cells to sites of inflammation (Gu-Trantien et al., 2013). In this study, the presence of CXCL13 was also positively correlated with IFN-γ expression, suggesting that T follicular helper cells play a role in initiating Type 1 immune responses. B cells have independently been shown to be required for the generation of anti-tumor CTL responses, especially in melanoma. Depletion of B cells before inoculation of B16 melanoma into mice led to an increase in primary tumor burden and in number of lung metastases, indicating that B cells are important for the initial immune response generated against a tumor. The increase in tumor growth was concurrent with a decrease in IFN-γ- and TNF-α-secreting T cells in the TME as well as a decrease in the number of T cells found in the periphery and in the tumor draining lymph node (DiLillo, Yanaba, & Tedder, 2010). Of note, it was also observed that the T cells present within tumors with high numbers of tumor infiltrating lymphocytes were more likely to be T follicular helper cells or Type 1-polarized $CD4^+$ cells (Gu-Trantien et al., 2013). These data support the idea that orchestrated interactions between immune cell subtypes within the tumor are critical to the generation of

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protective anti-tumor immune responses. Interestingly, some tumors that arise in highly inflammatory microenvironments benefit from the infiltration of regulatory T cells (Tregs) as opposed to effector immune cells into the TME. In particular, in the cases of head and neck cancer and colorectal cancer, the presence of intratumoral Treg has been reported to convey good clinical prognosis (Wolf H Fridman et al., 2011; Salama et al., 2008).

Furthermore, the presence and magnitude of tumor infiltrating lymphocytes (TIL) in the TME has been strongly associated with the effectiveness of a range of chemo- and immuno-therapeutic agents. Many immunotherapy strategies currently being investigated in clinical trials involve immune checkpoint blockade, including the use of antibodies capable of inhibiting signaling through CTLA-4 or PD-1 into T cells. Interestingly, it appears that these therapies may work, in part, via increasing the ability of newly-arrived $CD8^+$ TIL to be primed and then mature into protective anti-tumor T effector cells. Priming of $CD8^+$ T cells in the tumor draining lymph nodes or the trafficking of circulating T effector cells into the TME appear less important to clinical outcome than the priming of resident TIL after blockade of CTLA-4, PD-L1, or IDO (Spranger et al., 2014). Similarly, the presence of T follicular helper cells in breast cancer TME predicts for superior responsiveness to pre-operative chemotherapy (Gu-Trantien et al., 2013). More broadly, the presence of TLS or CXCL9 expression in the TME of patients with breast cancer is a statistically significant predictor of a higher incidence of complete response to neoadjuvant chemotherapy (Denkert et al., 2010).

Cues for TLS Development

While the precise sequence of signals that control TLS development has yet to be completely elucidated, especially in the context of the TME, certain signaling pathways classically known to recruit immune cells into inflammatory tissue microenvironments appear to be involved.

The requirement for lymphotoxin signaling in the Evolution of TLS in the TME

Lymphotoxin (LT)-α/-β signaling through the LTβR is required for the establishment and maintenance of lymphoid structures. LTα and LTβ are members of the TNF family and share common receptors and signaling pathways with other members of their family. The lymphotoxin ligands are expressed predominantly by immune cell subsets while the receptors are found on epithelial cell populations. The receptor-ligand interactions promote the organization of immune cells and stromal cells within lymphoid structures (K. Schneider, Potter, & Ware, 2004). The LTα/LTβ subunits exert their biologic function by forming three distinct trimeric molecules, each with different receptor specificity. The homotrimer of lymphotoxin alpha, $LT\alpha_3$, is a secreted protein that signals through TNFR1, TNFR2, and HVEM (Mauri et al., 1998; K. Schneider et al., 2004). Two membrane bound heterotrimers can also form from the subunits: $LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$, with $LT\alpha_1\beta_2$ being the predominant form. $LT\alpha_1\beta_2$ signals through the lymphotoxin beta receptor, while $LT\alpha_2\beta_1$ is able to bind TNFR1 and TNFR2 but does not have a clear biologic role in signaling through these receptors (K. Schneider et al., 2004). The receptors TNFR1 and TNFR2 have broad expression throughout the body. The LTβR is expressed by stromal cells, epithelial cells,

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monocytes, and DC. HVEM is expressed by T cells, DC, macrophages, and epithelial cells (Zhu & Fu, 2011; **Table II**). Specifically, signaling through the LTβR is required for high endothelial venule differentiation and for the formation of organized secondary lymphoid organs. Blockade of signaling through the LTβR results in decreased lymphocyte migration into lymph nodes in a model of collagen-induced arthritis, and this appears due to impaired expression of adhesion molecules PNAd and MAdCAM on high endothelial venules (HEV; Browning et al., 2005). A similar decrease in lymphocyte trafficking is observed in Peyer's patches in the absence of LTβR-mediated signaling (Dohi et al., 2001). In the spleen, signaling through the LTβR is required for the segregation of B cells and T cells into distinct zones, and for the generation of follicular DC and the formation of B cell follicles (Gonzalez, Mackay, Browning, Kosco-Vilbois, & Noelle, 1998). LTβR-associated signaling also plays a key role in TLS formation. In mice that constitutively express both LTα and LTβ in the pancreas, the T and B cell chemokines CCL19, CCL21 and CXCL13 were more predominantly expressed, L-selectin$^+$ (aka CD62L$^+$; binds PNAd and MAdCAM) cells were more abundant, and T and B cell zones within the immune infiltrate were more pronounced than in mice only expressing LTα (Drayton, Ying, Lee, Lesslauer, & Ruddle, 2003). In the TME, PNAd and L-selectin expression is exclusively found within TLS and not elsewhere in the stroma or the tumor tissue, and the two co-localize with each other (de Chaisemartin et al., 2011). L-selectin is important for the recruitment of central memory T cells (Y. W. Jung, Rutishauser, Joshi, Haberman, & Kaech, 2010), in conjunction with CCL19 and CCL21 gradients that signal through CCR7 on these cells (Y. W. Jung et al., 2010; Erica M Pimenta & Barnes, 2014). LTα/LTβ induce the production of CCL19, CCL21, and CXCL13 (Ansel et al., 2000). Specifically, lymphotoxin alpha is required for CCL19, CCL21, and CXCL13 expression, while LTβ is most critically required for CXCL13, but less so CCL19 or CCL21, expression (Wang et al., 2002). This appears to be a result of the differential receptor binding ability of the lymphotoxins and the downstream signaling components being activated (K. Schneider et al., 2004). Such pathways appear to involve a positive feedback loop in the spleen, where cells expressing the LTβR and CXCL13 recruit B cells, with activated B cells subsequently expressing $LT\alpha_1\beta_2$, begetting further expression of CXCL13 by stromal cells (Ansel et al., 2000). A similar biologic circuit has been shown to exist between $LT\alpha_1\beta_2$ and CCL19 and CCL21 in both secondary and tertiary lymphoid organs (Ansel et al., 2000; S. A. Luther et al., 2002). Blockade of signaling through the LTβR using a soluble decoy receptor blocks the recruitment of both B cells and CD8$^+$ T cells into lymphoid organs (Browning et al., 2005).

Surprisingly, tumor and stromal cells may also express and respond to signaling through the LTβR. Induction of this signaling pathway by the natural ligand $LT\alpha_1\beta_2$ or by cross-linking with LTβR-Ig can induce the secretion of the pro-inflammatory mediators IL-8 and CCL5 by both melanoma (A375) and fibroblast (WI38VA13) cell lines *in vitro* (Degli-Esposti et al., 1997). The effect of LTβR signaling on non-immune cell subsets is predicted to have an additional role in promoting anti-tumor immunity in addition to the role of this signaling axis in the formation of TLS.

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EnLIGHTening Protective Immunity in TLS

Both stromal and immune cell populations respond to LIGHT (also known as TNFSF-14). LIGHT, expressed by T cells, immature DC, and macrophages (M. Zhu & Fu, 2011), is related to the lymphotoxins and is also able to signal through multiple receptors in the TNF superfamily, including the LTβR on stromal cells and HVEM (also known as TNFRSF-14; Mauri et al., 1998) on T cells. In regard to the generation of an immune response, LIGHT is required for $CD8^+$ but not $CD4^+$ T cell proliferation and differentiation (Tamada et al., 2002). Mice lacking LIGHT were shown to have impaired $CD8^+$ T cell responses to bacterial infection (Tamada et al., 2002), indicating a requirement for LIGHT in the generation of successful Type 1 immune responses. Furthermore, LIGHT is able to synergize with IFN-γ to enhance the production of CXCL9, CXCL10, and CXCL11, which serve to recruit and polarize $CXCR3^+$ Type-1 immune responses (Miyagaki et al., 2012).

In the context of tumor immunology, expression of LIGHT appears to have a broadly beneficial role in reducing tumor burden and improving survival. In human breast cancer patients, LIGHT expression correlates with the generation of TLS- specifically, de novo formation of lymph nodes- in the TME. Expression of LIGHT mRNA was 5 times greater in the newly-formed lymph nodes than in normal lymph nodes isolated from the same patient (Gantsev et al., 2013), suggesting that LIGHT may be a driver in the formation of TLS in the cancer setting. Introduction of LIGHT into the TME has been shown to promote the development of anti-tumor immunity in numerous models of cancer. Forced expression of LIGHT in a fibrosarcoma model ($Ag104L^d$) resulted in increased signaling through the LTβR on stromal cells in the TME, leading to upregulated expression of CCL21 and MAdCAM-1 by these cells. Treated tumors also exhibited increased $CD8^+$ T cell infiltration that ultimately led to the rejection of established disease (Yu et al., 2004, 2007). A similar result was observed in established primary melanoma (B16) and colon (MC38) cancers (Yu et al., 2007). Intratumoral vaccination with a recombinant adenovirus encoding the cDNA for LIGHT (Ad.LIGHT) is able to mount anti-tumor immune responses against B cell lymphoma (Hu et al., 2010), cervical cancer (Kanodia et al., 2010), and breast cancer (Yu et al., 2007). In this B cell lymphoma model, treatment with Ad.LIGHT induces expression of CCL21 and recruitment of T cells into the TME, increases overall survival from primary tumor challenge, and protects treated mice against normally lethal tumor re-challenge (Hu et al., 2010). When applied in combination with a HPV16-VRP vaccine in cervical cancer models, Ad.LIGHT is able to increase circulating levels of anti-tumor T cells, promote $CD8^+$ T cell infiltration into the TME, regulate tumor growth and increase overall survival, when compared with vaccines alone (Kanodia et al., 2010). Ad.LIGHT injected into established murine 4T1 breast cancers results in diminished lung metastasis after surgical removal of the primary tumor (Yu et al., 2007). These results suggest that Ad.LIGHT is able to initiate a systemic, tumor antigen-specific immune response that is protective. Work evaluating treatment with mesenchymal stem cells engineered to overexpress LIGHT (MSC-L) has also been shown to inhibit tumor growth in both gastric (X. Zhu et al., 2013) and breast (Zou et al., 2012) cancer models. In the breast cancer model, the efficacy of MSC-L was dependent on the ability of lymphocytes to be recruited into the TME via LTβR-dependent signaling events (Zou et al., 2012). Interestingly, another study evaluating LIGHT/HVEM signaling determined that advanced stage gastric cancer patients expressed

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significantly lower levels of HVEM on the surface of their leukocytes, and higher serum levels of soluble HVEM shed from leukocytes when compared to healthy controls (Heo et al., 2012). Prior studies reported the presence of robust levels of soluble HVEM in the serum of patients with autoimmune diseases such as psoriasis, dermatitis, and arthritis (H. W. Jung et al., 2003), suggesting that soluble HVEM may represent a marker of ongoing chronic inflammatory conditions. In addition, low levels of HVEM have been shown to drive the generation of dominant Type-2-polarized immune responses in patients with cutaneous T cell lymphoma, in association with disease progression (Miyagaki et al., 2012). Low levels of LIGHT production in metastatic colorectal cancer lesions has been linked to a decreased number of intratumoral T cells compared to normal tissue (Qin, Upadhyay, Prabhakar, & Maker, 2013). This could support a mechanism of immunosuppression that could be reversed by introducing LIGHT into the system as an interventional strategy. In support of this possibility, forced expression of LIGHT in the mouse TRAMP-C2 model of prostate cancer overcomes Treg-mediated immunosuppression and synergizes with a biologic vaccination strategy (PSCA TriVax) to activate DC and recruit effector T cells into the TME (Yan et al., 2015). In particular, this pancreatic tumor model expresses “self” antigens, suggesting that LIGHT plays a role in the generation of autoimmune responses, which in the context of cancer may promote a reduction in tumor burden. HVEM, the receptor for LIGHT, is also able to bind BTLA, a molecule found on T effector cells that enhances their ability to be suppressed by Tregs (Tao, Wang, Murphy, Fraser, & Hancock, 2008). Thus, in the immunosuppressive TME, LIGHT may compete with BTLA for binding of HVEM, thereby limiting the ability of Tregs to suppress immune effector cells (Tao et al., 2008). Furthermore, LIGHT has the capacity to bind to decoy receptor 3 (DcR3), a soluble receptor that is expressed by many tumors, including esophagus, stomach, colorectal, pancreatic, lung, brain, renal, ovarian, blood, hepatocellular, and oral cancers (Lin & Hsieh, 2011). DcR3 is related to two other decoy receptors of the TNF family that bind but do not induce signaling upon binding their ligand. Since DcR3 sequesters LIGHT, an additional avenue of translational research aims to engineer a mutant LIGHT that is unable to bind DcR3 but retains its ability to signal through HVEM and LTβR in order to induce more potent anti-tumor immune responses. Of note, LIGHT that is unable to bind DcR3 is better able to induce the apoptotic death of tumor cells (Morishige et al., 2010), with its effects on immune cell subsets to be determined. LIGHT does appear to have detrimental effects in tumors that arise as a result of chronic inflammation. In livers affected with hepatitis or virally-induced hepatocellular carcinoma, levels of LTA, LTB, LIGHT and LTβR are increased, and chronic hepatitis in this model can be alleviated by treatment with LTβR-Ig, which serves as a sink for LTβR ligands (Haybaeck et al., 2009).

LIGHT has also been shown to play a role in NK cell involvement in anti-tumor immunity. NK cells constitutively express HVEM, and forced expression of LIGHT in the TME mediates recruitment of NK cells to the tumor from the periphery and activation of these cells, including secretion of IFN-γ. Strikingly, this study observed a requirement for both activated NK cells and IFN-γ in inducing an anti-tumor CTL response at later time points: peak NK cell infiltration was observed at 10 days post tumor inoculation, while peak $CD8^+$ T cell levels occurred 22 days post tumor inoculation. This unique role for NK cells in $CD8^+$ T cell activation is not observed in secondary lymphoid organs such as the spleen (Fan et al.,

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2006). NK cells have also been shown to induce the maturation of DC using a similar pathway. Upon recognition of target cells, NK cells upregulate their cell surface expression of LIGHT. LIGHT-expressing NK cells were able to induce upregulation of CD86 on the surface of autologous DC in a cytokine-independent manner (Holmes et al., 2014). Thus in addition to playing a role in the recruitment of lymphocytes to the TME, LIGHT appears to also have a direct role in priming anti-tumor immune responses, pointing to LIGHT as a potential therapeutic agent to be explored in the clinic.

The Importance of CCR7 Agonists for TLS Evolution in the TME

CCL19 (i.e. EBV-induced molecule 1 ligand chemokine/ELC or MIP-3β) and CCL21 (i.e. secondary lymphoid chemokine/SLC) are constitutively expressed by stromal cells and serve to recruit $CCR7^+$ cells to sites of inflammation (Legler, Uetz-von Allmen, & Hauser, 2014). Within the immune repertoire, CCR7 is expressed on naive and memory T cells, B cells, DC, and NK cells (Badr, Borhis, Treton, & Richard, 2005; Ohmatsu, Sugaya, Kadono, & Tamaki, 2007; Erica M Pimenta & Barnes, 2014). In a mouse model involving the forced expression of CCL19, CCL21a, or CCL21b in peripheral tissues, each single chemokine was sufficient to induce immune cell infiltration into the pancreas (S.-C. Chen, Vassileva, et al., 2002; S. A. Luther et al., 2002), but not into the skin or the central nervous system (S.-C. Chen, Leach, et al., 2002; S.-C. Chen, Vassileva, et al., 2002). CCL19/CCL21-induced infiltrated tissue contained high endothelial venules (HEV) and organized networks of stromal cells (S. A. Luther et al., 2002), consistent with the TLS paradigm. Some cancers have evolved mechanisms to antagonize the host-protective effects of CCR7-mediated immune cell chemotaxis. HPV-induced cervical cancer manipulates its local microenvironment by secreting IL-6, which inhibits NFκB and CCR7 expression by mature DC and instead upregulates the pro-tumorigenic MMP-9 metalloproteinase. Such effects are reversible as a consequence of treatment with neutralizing anti-IL-6 antibodies (Pahne-Zeppenfeld et al., 2014). However, CCR7 has also been reported to have detrimental effects in certain cancers. In hepatocellular carcinoma, signaling by both CCL19 and CCL21 promoted the proliferation and invasion of tumor cells, while CCRL1/CCX-CKR, a naturally occurring receptor sink for the CCR7 ligands, was able to mitigate these effects (Shi et al., 2015). CCRL1 is unable to induce intrinsic intracellular signaling pathways, but it mediates the internalization and degradation of CCL19 and disallows its agonism of CCR7 (Comerford, Milasta, Morrow, Milligan, & Nibbs, 2006). In melanoma, cells have been shown to express CCL19 and CCR7, and expression of CCR7 correlates with metastasis, especially to the liver (Dobner, Riechardt, Joussen, Englert, & Bechrakis, 2012). Thus in certain cases, it appears that tumor cells have established mechanisms to use the body's natural chemokine gradients to benefit their own survival. While in many instances their roles are considered as parallel or redundant, expression of CCL19 and CCL21 in different organs may be under the control of different signaling pathways. For example, blockade of signaling through the LTβR causes a decrease in CCL19, but not CCL21, levels in lymph nodes (Browning et al., 2005). These results suggest that signaling pathways involving these chemokines include both shared and differential components and that these differences may be organ-dependent.

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Chemotaxis of (naive) B cells towards a CCL21 gradient is mediated in part by Type-1 IFN-α. Specifically, IFN-α is able to diminish the ligand-induced receptor internalization of CCR7 in the presence of CCL21, allowing for B cells in pro-inflammatory microenvironments to traffic more efficiently during the generation of antigen-specific humoral immune responses (Badr et al., 2005). In mice, three isoforms of CCL21 exist. CCL21a differs from CCL21b and CCL21c based on the presence of a serine instead of a leucine at position 65, whereas the exon sequences of CCL21b and CCL21c are identical and may represent splice variants (Nakano & Gunn, 2001). Humans express just one isoform of this protein, which contains a leucine at position 65 but performs the same functions as all three mouse isoforms. The tissue distribution of CCL21 varies between mouse and humans as well: in humans, CCL21 expression is found predominantly in lymphoid tissues (lymph node, spleen, and appendix) while in mice, CCL21 is more broadly expressed and is found at the highest levels in spleen and lung (Hedrick & Zlotnik, 1997). Within lymphoid structures, CCL21 is expressed by stromal cells and endothelial cells, especially those that make up high endothelial venules (Ohmatsu et al., 2007), and allow for the recruitment of CCR7-expressing immune cells towards a gradient. In melanomas treated with DC engineered to express recombinant CCL21 (i.e. DC.CCL21), TLS developed at the site of vaccination, and expression of IFN-γ by $CD4^+$ and $CD8^+$ T cells was observed, concurrent with a reduction in tumor burden in treated patients (Lu Chen et al., 2013; Mulé, 2009). In this situation, generation of anti-tumor effector T cells takes place within the TLS: DC.CCL21 (i.e. DC transduced to express CCL21) do not migrate to the tumor draining lymph node. Instead, naive T cells are recruited to the TME from the peripheral circulation, and begin to express CD25 (IL-2Rα) within 24 hours of organ-site arrival (Mulé, 2009). Interestingly, some tumors intrinsically express CCL21, in association with an immunosuppressed TME. This may be the result of CCL21 recruitment of $CCR7^+$ Tregs that can mitigate the clinical benefits of inflammatory immune effector cells (M. A. Schneider, Meingassner, Lipp, Moore, & Rot, 2007). In the setting of melanoma, tumor cell secretion of CCL21 promotes tumor immune escape through the production of TGF-β and the recruitment of Tregs and myeloid derived suppressor cells (MDSC; Zlotnik, Burkhardt, & Homey, 2011). A similar result has been observed in a pancreatic islet beta cell tumor model, in which forced overexpression of CCL21 in the tumor cells led to enhanced tumor progression and significantly higher numbers of Tregs found within the TME (Shields, Kourtis, Tomei, Roberts, & Swartz, 2010). This latter result was dependent on host tissue expression of CCR7. CCL21-CCR7 signaling has also been reported to have a proangiogenic effect. In a model of rheumatoid arthritis, a CCL21 gradient caused migration of $CCR7^+$ micro-vascular endothelial cells. CCL21 also leads to the secretion of pro-angiogenic factors, such as VEGF, Ang-1, and IL-8, by fibroblasts and macrophages. Neutralization of CCL21 or blockade of CCR7 abrogated micro-vascular endothelial cell migration *in vivo* (Pickens et al., 2012). Thus in the context of cancer, the pro-angiogenic capability of CCL21 signaling may mediate tumor progression, as *de novo* blood vessel formation is required for tumor growth and metastasis.

CCL19 is expressed by stromal cells in lymphoid organs, as well as by mature DC (Sanchez-Sanchez, Riol-Blanco, & Rodriguez-Fernandez, 2006). Like CCL21, the transcription of CCL19 is regulated by two NFκB binding sites and one interferon-stimulated response

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element in its promoter region. The inhibition of NFκB activation partially down-regulates transcription of IFN-γ and CXCL10 by DC (Pietila et al., 2006). However, CCL19 also stimulates the proliferation and metastasis of breast cancer cells, which can only be alleviated by interfering with CCR7 receptivity on tumor cells (Su et al., 2014). Increased levels of CCL19 and CCR7 are also known to be expressed by prostate cancer tissues, and signaling by CCL19 through CCR7 expressed on prostate cancer cells induces cell proliferation (Peng, Zhou, An, & Yang, 2015). CCR7 is expressed by gastric cancer cells, with higher levels of CCR7 expression associated with lymph node metastasis, higher stage tumor, and poor overall survival. Treatment of human gastric cancer cells with CCL19 induced the expression of MMP-9 and decreased levels of E-cadherin, consistent with a shift towards a pro-metastatic phenotype (Cheng, Guo, Yang, & Yang, 2015). In ovarian cancer, $CCR7^+$ tumor status was also correlated with advanced disease stage and with lymph node metastasis, and these clinical parameters were linked to increased expression of MMP-9 and N-cadherin (Cheng et al., 2014) that were subsequently determined to be dependent upon CCL19 signaling (Cheng et al., 2015).

Interestingly, however, in a metastatic melanoma model, co-administration of CCR7 at the time of adoptive T cell therapy resulted in increased survival, proliferation, and effector function of transferred T cells and led to prolonged survival of $CCR7^+$ T cell-treated mice compared to mice treated with transferred T cells alone (Thanarajasingam et al., 2007). In a lung cancer model, intratumoral injection of recombinant CCR7 led to complete tumor regression concurrent with trafficking of $CD4^+$ and $CD8^+$ T cells to the tumor and to the tumor draining lymph node (S. Sharma et al., 2000). This effect was dependent upon T cell recruitment (S. Sharma et al., 2000) and the recruiting cytokines/chemokines CXCL9, CXCL10, and IFN-γ (Sherven Sharma et al., 2003). In the LoVo model of human colorectal cancer, treatment with recombinant CCL19 suppressed tumor growth *in vivo* concurrent with increased serum levels of IL-12 and IFN-γ via a T cell-independent mechanism that involved DC and NK cells (Lu et al., 2015). Furthermore, results from non-tumor models support a beneficial role for CCL21 in generating protective Type 1 immune responses. CCL21 can co-stimulate effector $CD4^+$ and $CD8^+$ T cells, induce T cell proliferation, and induce a Type 1 polarized immune response characterized by secretion of IFN-γ but not IL-4 or IL-5 (Flanagan, Moroziewicz, Kwak, Hörig, & Kaufman, 2004). When taken together, these results suggest a dual function for CCR7 and its ligands CCL19 and CCL21 in tumor immunity, as CCR7 signaling may promote the formation of TLS and the recruitment and survival of immune effector cells in an inflammatory microenvironment, or the recruitment of suppressive immune cells such as Tregs and MDSCs and the secretion of regulatory cytokines, depending on context. The precise signals involved with determining the pro- vs. anti-tumor impact of CCR7 and its ligands have not yet been elucidated.

DC are also recruited to lymphoid organs and activated via CCR7 ligand gradients. After the acquisition of antigen in its local microenvironment and the provision of activation (danger or maturation) signals, DC upregulate CCR7 on their surface and become competent to migrate in response to secondary lymph node chemokines, CCL19 or CCL21 (Ashour, Turnquist, Singh, Talmadge, & Solheim, 2007; Clatworthy et al., 2014). The ability of DC to migrate in response to a CCR7 ligand gradient was found to be partially dependent on MMP-9 expression by the DC (Clatworthy et al., 2014). Subcutaneous injection of CCL21

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led to the recruitment of lymphocytic infiltrates into the skin at the site of injection 4 days later. CCL21 injection also led to an increased DC and T cell recruitment to the draining lymph node, and this recruitment also peaked 4 days post-injection (Ashour et al., 2007). In addition to recruiting DC to sites of inflammation, CCL21 has the ability to boost the T cell-priming function of DC. Human DC treated with recombinant CCL21 and subsequently peptide pulsed and co-cultured with $CD8^+$ T cells were better able to stimulate IFN-γ release from the T cells than peptide-pulsed DC that were not treated with CCL21. The T cells also expressed slightly elevated levels of perforin, granzyme B, and FasL. Interestingly, this benefit of CCL21 treatment required CXCL10 signaling during the T cell priming phase (Hong, Lee, Kim, & Lee, 2014).

The Importance of CXCR5 agonists in TLS Evolution

CXCL13, also known as BLC, is critical for the formation of SLO and TLS. CXCR5 is expressed on the surface of B cells, and these cells migrate towards gradients of CXCL13 expressed by follicular DC or stromal cells in lymphoid organs (Ohmatsu et al., 2007). Mice deficient in either CXCL13 or its receptor, CXCR5, lack structured lymphoid organs including lymph nodes, Peyer's patches and spleen, which appears due to a lack of follicular DC networks that are required for the organization of recruited B cells into follicles/germinal centers (Ansel et al., 2000). CXCL13 is under the transcriptional control of LTβR-mediated signaling in all secondary lymphoid organs (Browning et al., 2005), and signaling by CXCL13 through CXCR5 leads to increased cell surface expression of $LT\alpha_1\beta_2$ (Ansel et al., 2000). Interestingly, the CCR7 ligands CCL19 and CCL21 also promote elevated expression of $LT\alpha_1\beta_2$ on the surface of B cells, though to a lesser extent than does CXCL13 (Ansel et al., 2000). In response to infection, DC and $CD4^+$ T cells are able to upregulate CXCR5, and this promotes the recruitment of B cells to sites of immune priming in lymphoid organs (León et al., 2012). Forced expression of CXCL13 in non-lymphoid organs leads to the formation of lymphoid structures and the recruitment of immune cells into affected tissue sites (Sanjiv A Luther, Lopez, Bai, Hanahan, & Cyster, 2000). Interestingly, CXCL13 expression within lymphoid organs appears to be required not only for B cell migration to lymphoid organs, but for antigen presentation to B cells at these sites as well (Coelho et al., 2013).

Like CCR7 ligand chemokines, CXCL13 appears to play multiple roles within the TME. Although this chemokine is crucial for the recruitment of immune cells into sites of inflammation, it can also mediate the invasion and metastasis of many types of cancer. Colon cancer cell lines commonly express the CXCR5 receptor, and are able to proliferate and migrate in response to CXCL13 gradients in a CXCR5-dependent manner (Z. Zhu et al., 2015). Interestingly, the migratory capacity of these colon cancer cells appears to be mediated downstream of CXCR5 signaling by the matrix metalloproteinase MMP-13 (Z. Zhu et al., 2015), consistent with results observed in human colon cancer patients (Zucker & Vacirca, 2004). A similar result has been observed in prostate cancer, as primary prostate cancer tissues and prostate cancer cell lines both express CXCR5. After treatment with CXCL13, prostate cancer cell lines upregulate MMP-1, MMP-2, and MMP-9, and are able to migrate towards a CXCL13 gradient in a CXCR5-dependent manner (S. Singh et al., 2009). In the Myc-CaP and TRAMP prostate cancer models *in vivo*, CXCL13 is upregulated

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by myofibroblasts in the tumor stroma downstream of TGF-β in response to hypoxia, and this promotes metastasis and the development of more aggressive, hormone-independent tumors (Ammirante, Shalapour, Kang, Jamieson, & Karin, 2014). Many subtypes of human lung cancers also express CXCR5. Specifically, adenocarcinomas and squamous cell carcinomas, but not healthy lung tissues, express CXCR5 (R. Singh, Gupta, Kloecker, Singh, & Lillard, 2014). Furthermore, patients with lung adenocarcinomas or squamous cell carcinomas present with increased serum levels of CXCL13 when compared to healthy patients, and CXCR5-expressing lung cancer cell lines are able to migrate towards a CXCL13 gradient *in vivo* (R. Singh et al., 2014). In colorectal cancer, both CXCR5 and CXCL13 may be upregulated in malignant compared to normal tissues, and patients with higher expression of CXCR5 and CXCL13 present with a lower 5-year overall survival and lower 5-year progression free survival when compared to disease stage-matched patients whose tumors were negative for CXCR5 and CXCL13 (X.-W. Qi, S.-H. Xia, Y. Yin, L.-F. Jin, Y. Pu, D. Hua, 2014). Some breast cancer patients have been shown to express elevated levels of CXCL13 in both the TME and systemically in the peripheral blood (Panse et al., 2008). In particular, patients with metastatic breast cancer present with significantly higher serum levels of CXCL13 when compared to normal controls and to patients whose tumors were resected, suggesting that CXCL13 may be a potential biomarker capable of detecting early metastatic disease in these patients (Panse et al., 2008). Both CXCR5 and CXCL13 have also been shown to be expressed by patients with infiltrating ductal carcinoma, and that their co-expression correlates with lymph node metastasis and an up-regulation in expression of MMP-9 in these patients, supporting a role for the CXCR5-CXCL13 axis in promoting epithelial to mesenchymal transition of breast cancer cells (Biswas et al., 2014).

In a clinical trial, the presence of CXCL13 on tumor infiltrating lymphocytes was associated with a lower occurrence of complete response to treatment in HER2 positive breast cancer patients (Denkert et al., 2014). However, other groups have reported a beneficial role for CXCL13 and CXCR5 in breast cancer. In particular, there was a positive correlation observed between high intratumoral CXCL13 or CXCR5 expression and increased disease free survival in high risk HER2-positive, estrogen receptor-low breast cancer patients (Razis et al., 2012). In hormone receptor-positive invasive ductal carcinoma, patients with grade I non-triple negative breast cancer presented with higher intratumoral levels of CXCL13 compared to patients with higher grade (grade II/III) or triple negative tumors (Erica Maria Pimenta et al., 2014). Interestingly, interferon regulatory factor 5 (IRF5), a regulator of CXCL13, is present in some but not all breast cancers, and media conditioned *in vitro* by IRF5-positive tumors is able to recruit B- and T-cells while IRF5-negative tumor conditioned media is not (Erica Maria Pimenta et al., 2014), suggesting that these tumors may secrete CXCL13 *in vivo*, generating a gradient to recruit lymphocytes into the TME. Thus in the cancer setting, increased CXCL13/CXCR5 expression- especially in patients with metastatic disease- may in fact represent a mechanism by which protective immune responses are actively recruited into disease sites.

Overall, work evaluating the role of chemokine signaling in the context of tumor progression suggests that chemokine/chemokine receptor expression may not be entirely beneficial or entirely harmful to the patient. Instead, expression may coordinately mediate immune cell recruitment into the TME, as well as, promote the metastasis of tumor cells, as many forms

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of tumor/tumor cell lines have been shown to express chemokines or chemokine receptors. Thus in the context of generating novel therapeutics to cancer, it will be important to balance the positive and the negative effects of enhancing or inhibiting signaling through these pathways. Specifically, it may be useful to stratify patients based on expression of chemokines/chemokine receptors in order to optimize the benefits of targeted (immuno)therapies.

Therapeutic Manipulation of TLS in Cancer Patients: Establishing a Paradigm for Anti-Tumor Efficacy

Our own work in murine models suggests that forced overexpression of cDNA encoding the Type 1 transactivator Tbet within the TME is therapeutic in the cancer setting. In particular, we have shown that DC engineered to (over)express Tbet (i.e. DC.Tbet) inhibit the growth of CMS4 (BALB/c) and MCA205 (C57BL/6) sarcomas *in vivo* after intratumoral injection, leading to the prolonged overall survival of treated tumor-bearing mice (L Chen et al., 2013; Qu, Chen, Lowe, Storkus, & Taylor, 2012). This result is dependent upon the presence of host lymphocytes and NK cells, as RAG1$^{-/-}$ mice and mice depleted of either CD8^{+} T cells or NK cells were not protected from tumor growth by treatment with DC.Tbet (L Chen et al., 2013). As T cells and NK cells must be able to traffic to the site of the tumor, it stands to reason that early chemokine signaling plays a key role in the mechanism by which intratumoral delivery of DC.Tbet leads to the recruitment of Type 1-polarized immune cells into the TME. In support of this hypothesis, DC.Tbet (but not control DC) cells express increased transcript levels for numerous chemokines, including CCL1, CCL4, CCL8, CCL12, CCL17, CCL25, CCL28, and CXCL12 (L Chen et al., 2013). Furthermore, DC.Tbet treatment leads to an upregulation of CXCL9 and CXCL10 in the TME as late as 21 days post tumor inoculation (Qu et al., 2012). As a set, these chemokines are attractants for T cells, B cells, and NK cells as well as for DC and monocytes. These results provide a framework by which DC.Tbet promotes a rapid (hours) and sustained (days to weeks) chemokine response to actively recruit and retain immune cells in TLS within the effectively treated TME.

Importance of IL-1 Family Member Cytokines in Establishing Therapeutic TLS

The IL-36 family of cytokines is comprised by a recently identified IL-1 sub-family that supports the generation of pro-inflammatory immune responses. These cytokines share sequence similarities and three-dimensional structures with known IL-1 family members such as IL-1α and IL-1β, and were in fact identified using genomics approaches to identify homologous sequences to IL-1 and IL-1Ra (Barton, Herbst, Bosisio, Higgins, & Nicklin, 2000; Busfield et al., 2000). The IL-36 subfamily consists of three agonists (IL-36α, IL-36-β and IL-36γ, previously referred to as IL-1F6, 8 and 9, respectively) and one antagonist (IL-36Ra/IL-1F5) that signal through a heterodimeric receptor consisting of IL-1Rrp2, a unique receptor, and IL-1RAcP, a co-receptor shared with the IL-1 and IL-33 receptors. Like all IL-1 family members, the IL-36 agonists and IL-1F5 require processing before they become fully active biologically. Indeed, specific truncation of the N-terminus of each of

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these proteins results in a 10^3-10^5 fold increase in biologic activity when compared to unprocessed, full-length protein (Towne et al., 2011).

Similar to other IL-1 family members, the IL-36 family cytokines lack a signal sequence that directs classical secretion from the cell (Busfield et al., 2000). Instead, both the cleavage and secretion of the IL-36 cytokines occurs through a pathway that is also involved in the secretion of IL-1β and IL-18, i.e. pyroptosis. This is a pro-inflammatory form of cell death that is distinct from apoptosis and requires the processing of the pro-forms of these cytokines by the non-canonical inflammasome, with the participation of caspase-1 (Lian, Milora, Manupipatpong, & Jensen, 2012; van de Veerdonk, Netea, Dinarello, & Joosten, 2011). The cytokines themselves are not involved in activating the cell death pathway; instead, recognition of pathogens or danger signals through Toll-like receptors and NOD-like receptors on antigen presenting cells activate caspase-1, with the secondary release of these pro-inflammatory cytokines from producer cells undergoing pyroptosis propagating Type-1 immune responses (Bergsbaken, Fink, & Cookson, 2009). In immune cells, IL-36 expression is also induced downstream of IL-18 and Tbet (Bachmann, Scheiermann, Härdle, Pfeilschifter, & Mühl, 2012); in epithelial cells, IL-36 is classified as an alarmin and is induced by cathelicidins such as LL37 (Li et al., 2014). Once released from cells, the IL-36 cytokines have effects on many IL-36R$^+$ cell types throughout the body. In addition to expression on immune cells, in humans, predominant IL-36R expression occurs in the skin, while in mice, receptor expression is more broadly distributed throughout organs including the prostate, esophagus, uterus, seminal vesicle, and paw (Towne et al., 2011). Numerous immune cell subsets also express the IL-36R. In mice, these subsets include DC, CD4$^+$ T cells, macrophages, and neutrophils but not B cells (Vigne et al., 2011). CD8$^+$ T cell expression of IL-36R is equivocal, with reports either supporting the presence or lack of expression on these immune effector cells (Vigne et al., 2011; Weinstein et al., unpublished). In humans, the receptor has been reported to be expressed by antigen presenting cells (Foster et al., 2014; Mutamba, Allison, Mahida, Barrow, & Foster, 2012) but not neutrophils, T cells, or B cells (Foster et al., 2014).

Despite speciation in the range of cell types expressing the IL-36 receptor (i.e. between mice and humans), IL-36 ligands appear to induce similar immune responses in both species. Downstream effectors of IL-36 signaling include NFκB, MAPK, ERK1/2, and Jnk. This leads to secretion of IL-6, IL-8 and GM-CSF by IL-36-treated mouse and human transformed cell lines (Towne et al., 2011). Interestingly, in a mouse model of fibrosarcoma (MC57-SIY), NFκB signaling is required in both CD4$^+$ and CD8$^+$ T cells for priming of a Type 1 immune response (as measured by secretion of IFN-γ and TNF-α, and specific lysis of target cells) and control of tumor growth (Barnes et al., 2015). This suggests a possible mechanism by which signaling through the IL-36 receptor via NFκB may promote anti-tumor immunity.

Amongst primary cells, IL-36 induces the activation and maturation of human and mouse DC. In response to treatment with IL-36 agonists, murine DC upregulate CD80, CD86, and MHCII (Vigne et al., 2011), and human DC upregulate CD83, CD86, and HLA-DR. Furthermore, IL-36 signaling leads to increased secretion of IL-1β and IL-6 by human DC (Foster et al., 2014) and IL-1β, IL-6, and IL-12 by mouse DC (Vigne et al., 2011), strong

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indications that IL-36 plays a critical role in promoting states of both acute and chronic inflammation. Reports conflict as to which subsets of human DC express the IL-36 receptor. However, transcript analyses and functional assays suggest that both human plasmacytoid (Mutamba et al., 2012) and myeloid (Foster et al., 2014) DC may express high levels of IL-36R mRNA and increase pro-inflammatory cytokine secretion (pDC: IFN-α; mDC: IL-1β, IL-6) in response to signaling through the IL-36 receptor.

Naive murine $CD4^+$ T cells constitutively express the IL-36 receptor and mature in response to IL-36 signaling. In particular, IL-36 (but not other IL-1 family members or IL-12) specifically induces IL-2 secretion and the proliferation of naive $CD4^+$ T cells (Vigne et al., 2012). Treatment of $CD4^+$ T cells with recombinant IL-36β in the presence of antigen leads to the secretion of pro-inflammatory cytokines/chemokines and the induction of a canonical Type-1 effector cell phenotype characterized by expression of Tbet and secretion of IFN-γ (Vigne et al., 2012). In the presence of IL-12, IL-36 can induce secretion of IFN-γ by $CD4^+$ T cells (Vigne et al., 2012). However, the local cytokine milieu plays a role in conditioning the cellular response to IL-36R agonism. In the absence of IL-12, signaling through the IL-36 receptor instead leads to transcription of GATA3 and secretion of IL-4 in T cells (Vigne et al., 2012). Notably, mice deficient in expression of IL-36R exhibit impaired IFN-γ, IL-6, TNF-α, and nitrite responses to bacterial challenge (Vigne et al., 2012), suggesting that IL-36 plays a crucial role in the initiation of adaptive immunity *in vivo*. It is worth noting that IL-36R expression is lost in Th1-, Th2-, and Th17-polarized $CD4^+$ T cells after they have matured (Vigne et al., 2012).

Although human T cells do not express the IL-36R (Foster et al., 2014), DC treated with rhIL-36 induce effects on the responding human T cell repertoire that appear similar to those induced directly on murine T cells by rmIL-36. For example, IL-36α-treated DC enhance allogeneic $CD3^+$ T cell proliferation to a degree greater than mitogen-activated T cells (Mutamba et al., 2012), with human T cells expanded with IL-36-conditioned DC also secreting increased levels of IFN-γ (Mutamba et al., 2012). Human T cells primed in the presence of IL-36 are likely to be Type 1 polarized, since IL-36β treatment of DC leads to their secretion of IL-12 and IL-18, which then prompt the transcriptional activation of Tbet in responder T cells (Mutamba et al., 2012).

IL-36 as an Early Inflammatory Mediator of Lymphoid Organogenesis in Tissues, Including Cancer

In the context of disease, IL-36 family cytokines have been implicated in the pathogenesis of several autoimmune diseases. Most notably, IL-36 signaling plays a major role in skin autoimmune diseases such as psoriasis and dermatitis. Pustular psoriasis may arise from DITRA, a deficiency in the IL-36R antagonist IL-1F5 (Renert-Yuval et al., 2014). A murine model of this disease is characterized by massive immune cell infiltrate into skin lesions of IL-1F5-deficient mice. This infiltrate consists of $CD45^+$ lymphocytes, neutrophils, and macrophages that are recruited into the diseased skin in an IL-36R-dependent manner (Tortola et al., 2012). Mutations in IL-1F5 have also been observed in patients presenting with acute generalized exanthematous pustulosis (AGEP), a drug-induced side effect that presents with skin lesions containing robust lymphocytic infiltrates (Nakai, Sugiura,

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Akiyama, & Katoh, 2014; Navarini et al., 2013). It has been hypothesized that individuals with loss-of-function mutations in IL-1F5 are more likely to develop lesional skin diseases. Similar immune infiltrates have been observed in patients presenting with pustular psoriasis, although in this disease setting, the dominant driver of pathogenesis appears to be locoregional overexpression of IL-36R agonist cytokines that leads to increased activation of MAPK and NFκB signaling in lesional skin (He et al., 2013).

The role of IL-36R agonists in the pathogenesis of arthritis remains contentious. Clinical reports suggest that patients with psoriatic arthritis and rheumatoid arthritis express elevated levels of IL-36α in their synovial lining when compared to patients with osteoarthritis, with IL-36α expression correlated with increased production of IL-6 and IL-8 in the affected joints (Frey et al., 2013). Expression of both the IL-36R and IL-36α is increased in the human TNF transgenic mouse model of inflammatory arthritis (*hTNFtg*) concurrent with lymphocytic infiltration into the joints, however, blockade of signaling through the IL-36 receptor using an antagonist antibody did not relieve inflammation (Derer et al., 2014). A similar result was observed in a collagen-induced arthritis (CIA) model (Lamacchia et al., 2013). Thus, in arthritis, IL-36 is likely a contributing factor in disease pathogenesis, but not necessarily a dominant driver as it is in skin autoimmune conditions.

Notably, IL-36 is a downstream target of Tbet (Bachmann et al., 2012), with a positive-feedback loop allowing for IL-36 to induce secondary transcription of Tbet as well (Vigne et al., 2011). In human myeloid cells, silencing of Tbet by siRNA decreased expression of IL-36γ, and expression of IL-36γ by DC was also dependent upon IL-18/IL-18R signaling via MAPK- and NFκB-dependent pathways. Specifically, the promoter region of IL-36γ contains both Tbet and NFκB binding sites, with IL-18 signaling inducing Tbet binding to the IL-36γ promoter (Bachmann et al., 2012). Tbet is also expressed in both human and murine DC, where it has been shown to be critical to DC1 functional polarization and the ability of these APC to activate Type-1 T cell responses *in vivo* (Lugo-Villarino, Maldonado-Lopez, Possemato, Penaranda, & Glimcher, 2003). Interestingly, DC.Tbet generated from CCR7$^{-/-}$ hosts appeared most effective in preventing tumor growth, strongly suggesting that their preferred biology was manifest in the TME and not the TDLN (L Chen et al., 2013). Indeed, the anti-tumor efficacy of i.t.-delivered DC.Tbet appears critically dependent upon TIL recruitment, activation, expansion and differentiation within the TME. In this context, IL-36 has been shown to bolster T cell proliferation and cytokine secretion, including secretion of IFN-γ (Vigne et al., 2011). Furthermore, CCL1 and CXCL10-chemokines observed to be upregulated by DC.Tbet cells- are known to be upregulated by wild-type DC after stimulation with IL-36R agonists (Vigne et al., 2011). Thus we believe that IL-36 is a key early mediator of TLS development in inflamed tissues and that purposeful instigation of IL-36 delivery or production in the TME (via administration of DC.Tbet or an equivalent modality) will have the potential to evolve both humoral and cellular immunity that is protective and/or therapeutic to the cancer-bearing host.

Consistent with the observation that overexpression of IL-36 in tissues correlates with increased immune cell infiltration, results from our laboratory suggest that IL-36 plays a role in the induction of chemokines that can rapidly recruit T and B cells into the inflammatory microenvironment of therapeutically-managed tumors. In contrast to concerns for pathologic

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autoimmunity resulting from such immune infiltrates in psoriasis and arthritis models, this is a highly-preferred biologic outcome in the context of cancer.

Conclusions and Future Directions for Clinical Translation

Chemokine expression within the TME and the development of TLS can often, but not always, represent a positive prognostic marker in patients with solid tumors. Due to the differential requirements for effector and regulatory immune cell subsets within the TME of a diverse array of cancer types, immunotherapies designed to promote the recruitment of immune cells into the TME, or those targeting chemokine pathways, must be evaluated on an empirical “case-by-case” basis. Examples of controversial effects of TLS include the positive effect of TLS presence in metastatic, but not primary, colorectal carcinoma, or the benefit of both Treg and effector T cell infiltration in head and neck cancer (Balermpas et al., 2013; Wolf H Fridman et al., 2011), which traditionally arises as a result of prolonged inflammation at the site of disease. Future avenues of research must elucidate whether the presence or absence of certain chemokines, cytokines, or cell populations within the TLS can predict a patient's ability to mount a successful anti-tumor immune response secondary to treatment. Although trends in the prognostic value of TLS are seen within cancer subtypes, it is likely that a better method of stratifying patients for immunotherapy will be to evaluate the specific immune cell infiltrates and chemokines expressed at the time of diagnosis, in order to determine whether enhancing or suppressing the immune response therapeutically is the best course of treatment.

Therapeutic agents targeting TLS-relevant chemokine pathways have thus far been evaluated in mouse tumor models, with forced expression of LIGHT or CCL21 both mediating beneficial therapeutic outcomes against a variety of solid tumors. Further analyses of the pathways involved in beneficial immune responses within the TME have identified additional immunomodulatory agents that may prove to be clinically important targets of immunotherapy. One such chemokine is IL-36, a pro-inflammatory member of the IL-1 family and potent inducer of NFB activation in IL-36R^{+} responder cells and an apparent orchestrator of TLS development in the TME of tumor-bearing mice treated with DC.Tbet cellular therapy *in vivo*. We are currently evaluating the ability of IL-36 to drive Type 1 anti-tumor immune responses and TLS formation *in vivo*, when used as a single agent or in the context of combination immunotherapies (i.e. vaccines and adoptive T cell transfer).

In summary, recent advances in our understanding of chemokine and cytokine pathways and their role in the generation of lymphoid organs have allowed for a greater appreciation of the dynamic crosstalk between immune cell types that occurs within TLS that form in or proximal to the TME. This paradigm provides a set of biologic endpoints that should be achieved in order to render improved clinical benefit as a consequence of (immuno)therapeutic intervention in cancer patients. Specifically, intratumoral delivery or promotion of TLS-facilitating factors, applied as single agents via viral vectors or transduced cells (i.e. DC) or direct injection of recombinant proteins into accessible tumor lesions, may allow for the preferential manipulation of protective-over-regulatory TIL within tumor-associated TLS. Under such conditions, TLS-primed/expanded anti-tumor immune effector cells may confer systemic clinical benefits (i.e. locoregional treatment of a single

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lesion may beget circulating immune-mediated regulation of disseminated disease) with minimal anticipated off-target toxicities.

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Table I

Presence and prognostic significance of TLS in cancer.

Cancer subtype	Location of TLS	Inflammatory infiltrates	Anti-inflammatory infiltrates	Predictive outcome of TIL presence	References
Melanoma	peri-tumoral stroma; intratumorally	B cells; T cells; $CD86^+$ DC	N/A	increased survival	(DiLillo, Yanaba, & Tedder, 2010; Messina et al., 2012)
Oral squamous cell carcinoma	peri-tumoral stroma	B cells; follicular DC	N/A	increased survival	(Wirsing, Rikardsen, Steigen, Uhlin-Hansen, & Hadler-Olsen, 2014)
Merkel cell carcinoma	tumor periphery	$CD8^+$ T cells; B cells; APC	N/A	increased recurrence-free survival	(Behr et al., 2014)
Lung cancer (NSCLC)	tumor stroma	mature DC; follicular DC; $CD62L^+$ and naive $CD4^+$ and $CD8^+$ T cells; B cells; follicular DC	N/A	increased survival	(de Chaisemartin et al., 2011; Dieu-Nosjean et al., 2008; Germain et al., 2014; Goc, Fridman, Hammond, Sautès-Fridman, & Dieu-Nosjean, 2014; Goc, Germain, et al., 2014)
Metastatic colorectal cancer	tumor periphery	$CD45^+$ T cells; $CD20^+$ B cells	N/A	increased overall survival; decreased disease recurrence	(Meshcheryakova et al., 2014)
Breast cancer	proximal to/within tumor stroma	lymphocytes; B cells; T cells; follicular DC; plasma cells; Tfh	N/A	decreased disease recurrence; increased response to chemotherapy	(Alexe et al., 2007; Denkert et al., 2010; Gu-Trantien et al., 2013; Mahmoud et al., 2012)
Colorectal carcinoma	tumor periphery	B cells; Type 1-polarized memory T cells; CD8+ T cells; CD45RO+ T cells	Tregs	inflammatory cells: disease progression and recurrence; Tregs: positive prognosis	(Fridman et al., 2011; Pimenta & Barnes, 2014; Salama et al., 2008)
Head and neck cancer	tumor stroma; tumor periphery; intratumorally	macrophages; $CD4^+$ T cells; $CD8^+$ T cells	$FoxP3^+$ Tregs	effector T cells: increased overall survival; Tregs: decreased local recurrence	(Balermpas et al., 2013; Fridman et al., 2011)

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Table II

Lymphotoxin/LIGHT receptors and their cell expression profiles.

Receptor	Expression	Ligands	References
TNFR1	Widespread	TNF-α; LTα3; $LT\alpha_2\beta_1$	(Schneider, Potter, & Ware, 2004; Zhu & Fu, 2011)
TNFR2	Widespread	TNF-α; LTα3; $LT\alpha_2\beta_1$	(Schneider et al., 2004; Zhu & Fu, 2011)
LTβR	Stromal cells; epithelial cells; monocytes; DC	$LT\alpha_1\beta_2$; LIGHT	(Mauri et al., 1998; Zhu & Fu, 2011)
HVEM	T cells; DC; macrophages; NK cells; epithelial cells	LIGHT; $LT\alpha_3$	(Zhu & Fu, 2011)