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Regulatory B cells in cancer

Daniel Michaud[1,2,*], **Colleen R Steward**[1,4,*], **Bhalchandra Mirlekar**[1,3], **Yuliya Pylayeva-Gupta**[1,3]

[1]Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA.

[2]Department of Cell Biology and Physiology, The University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA.

[3]Department of Genetics, The University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA.

[4]Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina, USA.

Summary.

Tumorigenesis proceeds through discrete steps where acquisition of genetic lesions and changes in the surrounding microenvironment combine to drive unrestricted neoplastic proliferation and metastasis. The ability of tumor-infiltrating immune cells to promote tumor growth via the provision of signals that enable tumor cell survival and proliferation as well as contribute to immune suppression is an active area of research. Recent efforts have provided us with mechanistic insights into how B cells can positively and negatively regulate immune responses. Negative regulation of immune responses in cancer can be mediated by regulatory B cells and is often a result of increased production of cytokines that can directly and indirectly affect anti-tumor immune function and cancer cell growth. Signals that lead to the expansion of regulatory B cells and the spectrum of their functional roles are not well understood and are the subject of active research by many groups. Here, we elaborate broadly on the history of regulatory B cells in cancer and summarize recent studies that have established genetic models for the study of regulatory B cell function and their potential for therapeutic intervention in the setting of solid cancers.

Keywords

Regulatory B cells; cancer; IL-35; tumor immunology

1. Introduction.

This review highlights the recent studies on the role of regulatory B cells (Breg) in cancer. We provide a brief discussion of the role of regulatory immune cells in development and

Correspondence: Yuliya Pylayeva-Gupta, Ph.D.; The University of North Carolina at Chapel Hill, Lineberger Cancer Center, 450 West Drive, Chapel Hill, NC, 27599, USA., yuliyap1@email.unc.edu, Telephone: 919-962-8296, Fax: 919-966-8212.
*Denotes equal authorship

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disease, followed by a general summary of Breg cell phenotypes and function in solid malignancies. We discuss analysis of preclinical studies that demonstrate the detection methods, genetic models and functional studies on the role of IL-35 producing Breg cells in cancer growth and control of anti-tumor immune responses, and discuss the use of selective targeting of pathogenic B cell subsets. Finally, we highlight translational work describing phenotype, frequency, localization, and gene expression signature of Breg cells in patients with pancreatic cancer, that may enable studies of this B cell subset in many distinct cancer types.

2. Regulatory function in homeostasis.

The immune system serves to provide a potent barrier against invading pathogens. At the same time, there are mechanisms in place that ensure limited reactivity to potentially pathogenic self-antigens. Such protective measures are termed 'tolerance' and encompass a range of mechanisms such as self-antigen-induced apoptosis and anergy, which are largely executed on a cell autonomous level. Extrinsic control of self-reactivity is perpetuated by a collection of peripheral immune cells that suppress unwanted immune cell activity via soluble mediators such as cytokines, direct cell-to-cell contact via membrane bound checkpoint mediators, or via induction of additional regulatory cell types (1, 2). Several distinct regulatory cell types have been shown to contribute to tolerance against abundant self-antigens or those that may be temporarily overexpressed during key developmental processes. These include regulatory T cells (Treg), regulatory B cells (Breg), dendritic cells, macrophages, and myeloid derived suppressor cells (MDSC) among others (3, 4). Their tolerizing activity is shaped by maturation status, often accompanied by low expression of co-stimulatory machinery and microenvironmental cues (5, 6). For example, MDSCs are a heterogeneous population of immature myeloid cells which can suppress T cells, and in normal development are important in maintenance of immune tolerance at the maternal–fetal interface during pregnancy (7, 8). Myeloid cells also play an important role in tolerizing against the vast amounts of self-material that can be carried by apoptotic cells – this occurs via innate checkpoints such Tyro3, Axl and MerTK receptor tyrosine kinases (9).

Adaptive immune cell types also contribute to the maintenance of self-tolerance. Treg cells, characterized by expression of transcription factor *Foxp3*, are well known to be important regulators of peripheral T cell responses and mice that lack regulatory T cells develop severe autoimmune disease (10–12). New T cell subsets capable of regulating autoreactivity have been emerging such as follicular regulatory T cells, and $CD8^+$ Treg; these may be important in restricting emergence of autoreactive B cells and limiting damage in models of type I diabetes respectively (13, 14). B cells can also contribute to tolerance via multiple mechanisms: by producing immunosuppressive cytokines and/or expressing checkpoint ligands that curb activation potential of T cell-mediated immune responses (15). It is well appreciated now that deficiencies in function of regulatory immune cells may lead to unchecked self-reactivity and is closely linked to severity of autoimmune and inflammatory conditions, such as rheumatoid arthritis, lupus erythematosus and multiple sclerosis (3, 4, 16–18).

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3. Regulatory cells in cancer.

The immune contexture that results from continuous exposure of immune cells to tumor-derived factors contributes to cancerous outgrowth in many ways including directly promoting tumor growth, eliciting angiogenesis, supporting recruitment and expansion of additional inflammatory cell types, and fostering metastatic disease. In contrast to autoimmune diseases, the evolution of cancer is linked to changes in the tumor microenvironment that favor suppression of active immune responses (Figure 1) (19–24). This is often achieved by aberrant recruitment and expansion of immunosuppressive immune and stromal cells to the sites of the tumor growth (25–27). In this scenario, the ability of macrophages, MDSC, Tregs and Bregs to execute their normal homeostatic tolerogenic functions is hijacked by the cancer and results in local and systemic immunosuppression that directly counteracts immunotherapy efforts (28, 29).

4. Multifaceted role for B cells in solid cancer pathogenesis.

Cancer immunotherapeutic approaches have been primarily focused on activating T cell-driven immunity, be it through adoptive T cell transfers, immune checkpoint blockade, or chimeric antigen receptor T cell therapy. The need to improve efficacy of such approaches has led to increased focus on other immune cell types within the tumor microenvironment. Mounting evidence supports an important role for B cells in the immunomodulation of cancer. It is clear that both anti- and pro-tumor effects can be attributed to B cells, although more work needs to be done to determine how such effects depend on the type of cancer, tumor microenvironment, and balance of B cell subsets that are present (30–36).

Early studies characterizing tumor-infiltrating lymphocytes (TILs) in non-small-cell lung tumors found that the percentage of B cells was associated with increased survival (37, 38). Additional research has highlighted a positive role for plasma cells in lung adenocarcinoma, as evidenced by associations with increased patient survival (39, 40). Analyses of RNA sequencing data from The Cancer Genome Atlas (TCGA) have also correlated high expression of B cell and plasma cell genes with increased patient survival in bladder, head and neck, melanoma, ovarian, and pancreatic adenocarcinoma (35, 41–48). In a model of pancreatic cancer, B cells were found to upregulate proinflammatory genes that may contribute to an anti-tumor response (49). While there is mounting evidence that B cells alone may provide valuable prognostic insight, biologically, the generation of effective anti-tumor immunity is likely tied to interactions between both B and T cells. In a murine 4T1 breast cancer model, the transfer of both activated B and T cells from tumor-draining lymph nodes yielded greater tumor regression than was observed when either population was transferred individually (50). Tertiary lymphoid structures (TLS), which contain aggregates of B and T lymphocytes in the tumor stroma, have garnered recent attention as a prognostic and predictive factor (44, 51). The presence of activated B cells within TLS have been associated with improved clinical outcome and response to immunotherapy in multiple cancer types. (52–58).

However, despite the promising implications of TLSs and plasma cells in a variety of cancers, a negative role for B cells is also observed across cancer types (35). Higher

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expression of IgG, B cell gene signatures, and plasma cell gene signatures from the TCGA were correlated with poor clinical prognosis in glioblastoma (42, 59, 60). Similarly, elevated expression of B cell-related genes in renal clear cell carcinoma correlated with decreased survival (42, 45). Both pro- and anti-tumorigenic properties have also been described in the same cancer type. In ductal breast cancer, immunohistochemical analysis revealed that high levels of $CD138^+$ plasma cells were associated with decreased survival (61). However, B cell gene expression signatures have been correlated with improved metastasis-free survival using the TCGA datasets (41). Furthermore, analyses of tissue samples from $HER2^+$ and triple negative breast cancer patients support the correlation between increased tumor-infiltrating B cells and improved clinical outcome (62). Collectively, these studies reflect the multifaceted functional properties of B cells. The capacity to promote antigen presentation, cytokine production, antibody-dependent cellular cytotoxicity, formation of TLS, and isotype switching to immune stimulatory antibodies such as IgG1 are thought to help drive an anti-tumoral response, whereas the presence of characteristically immunosuppressive B cell phenotypes and antibody isotypes have been linked to pro-tumor effects (36). The following section addresses several of these functional processes as they relate to anti- and pro-tumoral responses in cancer.

4.1. Antibody production.

Production of tumor-specific antibodies can promote the opsonization and phagocytosis of tumor cells by macrophages and dendritic cells, as well as induce the lysis of cancer cells via complement cascades and antibody-dependent cellular cytotoxicity (ADCC). Tumor-binding IgG antibodies in murine models of melanoma, pancreas, lung, and breast cancer facilitate phagocytosis by dendritic cells, and subsequently lead to enhanced presentation of tumor antigens to T cells (63). *In vitro* studies evaluating IgG antibody responses in melanoma demonstrated effective killing of cancer cells by ADCC as well (64). Furthermore, in a mouse model of triple negative breast cancer, activation of T cells and antibody production by B cells impacted the response to checkpoint blockade immunotherapy (55). It is also important to note that in addition to producing antibodies, plasma cells secrete a myriad of cytokines that can recruit, activate, or suppress other immune cell populations (65). The role of plasma cells in the tumor microenvironment has been reviewed in detail in the excellent review by Sharonov et al. (36).

4.2. Antigen presentation.

Although dendritic cells (DCs) are rightfully hailed as the masters of antigen presentation, DCs are often modified by tumors, hindering their capacity to present antigens. Therefore, the ability of B cells to present tumor-specific antigens to T cells may play a vital role in fostering anti-tumor immunity. For instance, dendritic cells harvested from cervical cancer patients were resistant to α-CD40 treatment and incapable of driving an anti-tumor response, whereas B lymphocytes taken from the same patients responded to α-CD40 treatment and consequently elicited secondary T cell responses (66). Moreover, *in vitro* antigen-presentation assays in a model of non-small-cell lung cancer supported the conclusion that activated B cells within the tumor were not only able to present antigen to $CD4^+$ T cells, but also initiated an effector T cell phenotype (67). It should be noted,

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however, that exhausted tumor-infiltrating B cells in this same study led to the production of suppressive, regulatory FoxP3$^+$ CD4$^+$ T cells (67).

4.3. Spatial organization.

The recent evidence correlating the presence of B cells within cancer-associated TLS with improved patient survival and immunotherapy response highlights that B cells can be a key cell population for facilitating an effective anti-tumor immunity in response to immunotherapy (51, 56–58). Using genomic datasets of soft-tissue sarcoma biopsies, Petitprez et al. were able to create an immune signature that revealed a correlation between increased B cell presence within the tumors and an increase in patient survival. Most notably, the predictive effect on survival was independent of the level of CD8$^+$ T cell presence. In a multi-center clinical trial, patients with a high B cell immune signature had a 50% overall response rate when treated with pembrolizumab (αPD-1), which was significantly higher than patients with low B cell tumor signatures. In melanoma, a similar co-occurrence of CD8$^+$ T cells and CD20$^+$ B cells aggregates correlated with longer patient survival (56). Again, the longer survival was attributed more to the presence of the CD20$^+$ B cells than the presence of CD8$^+$ T cells within the TLS. Melanoma patients that had CD8$^+$CD20$^+$ TLS structures within the tumor also responded better to αPD-1 and αCTLA-4 checkpoint blockade therapy. B cell populations within responding tumors were more clonally diverse and contained significantly more memory B cells and plasma cells indicating a robust immunological response in responding tumors (57). Collectively, these studies indicate that activated B cells in the context of organized immune intratumoral aggregates may confer a predictive outcome to survival checkpoint blockade responsiveness.

5. Immunosuppressive functions of B cells in preclinical cancer models.

Murine models of cancer are powerful preclinical tools for investigating mechanisms of tumor growth and testing the efficacy of therapeutic strategies (Figure 2). Syngeneic models allow for the investigation of the interplay of tumorigenesis and the immune system (68). Such models generally encompass two main approaches: syngeneic cell line implantation and genetically engineered mouse models (GEMM). Syngeneic cell line implantation allows for more flexibility as the cell lines can be injected either subcutaneously, which is more amenable to high throughput *in vivo* experiments, or orthotopically, which better reflects the native tumor microenvironment albeit more technically complicated. GEMMs provide the most translational mimicry of the human tumor microenvironment as cancer evolves from the native host tissue under the guidance of defined genetic alterations. GEMMs, however, can require a long development time for full disease onset. We have successfully utilized both GEMM and orthotopic cancer models to investigate the effects of pancreatic tumorigenesis on the immune response with a particular interest in B cell function (Table 1) (69–72). Both approaches result in the expansion of regulatory B cell and plasma cell subsets. However, the tumor microenvironment that these two systems develop may not be identical and as a result, expose B cells to tumor-associated factors for different durations. GEMMs allow for the development of potential neoantigens *de novo* over time, whereas syngeneic models may grow too rapidly for this process. As a result, B cells in syngeneic models may or may not clonally expand based on a cell line specific antigen which may not

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be translationally applicable to patients. Syngeneic cell lines, however, do have the advantage of being implantable in various genetic knockout models without need for extensive breeding. In the case of B cells, syngeneic tumors can be implanted in mice with deficiencies in cell surface receptors, co-stimulatory molecules, or cytokines. The utilization of both syngeneic and GEMM approaches will provide the most comprehensive understanding of the biology of B cells in cancer.

5.1. Suppressive effects of antibodies.

B cells have been found to play a prominent role in promoting tumor growth by multiple mechanisms including the B cell-specific function of antibody production. This mechanism was first outlined in a mouse model of HPV16-driven epithelial carcinogenesis (73). Interestingly, B cells are not recruited into the premalignant tissues in this model, but instead act from a distance via production of antibodies that circulate to the tumor. The mechanism surrounding the suppressive effect of antibodies in the HPV16 squamous carcinoma model was determined to be the engagement of Fc receptors (FcRy) on infiltrating macrophages and mast cells, overall activating a chronic pro-tumorigenic and pro-angiogenic program to help facilitate tumor growth (74). Depletion of B cells from this model using α-CD20 results in the infiltration of myeloid cells with an M1 anti-tumorigenic phenotype and importantly confers significant sensitivity to chemotherapy in a CD8-dependent fashion (75). A similar FcRy-dependent mechanism driving tumor growth has also been described in pancreatic ductal adenocarcinoma where B cell-dependent activation of macrophage FcRy signaling leads to a M2-type macrophage reprogramming (76). Both the B cell generation of antibodies in response to pancreatic inflammation and FcRy-mediated activation of macrophages were found to be dependent on PI3Ky activation of Bruton's Tyrosine Kinase (BTK). Inhibition of BTK or PI3Ky led to a significant decrease in tumor burden in an orthotopic pancreatic cancer model while also causing an M1-type macrophage conversion and an increased $CD8^+$ T cell response (76).

B cells have also been found to play a prominent role in the promotion of prostate cancer. In a TRAMP model of castration-resistant prostate cancer, $CD20^+$ B cells were found to promote castration-resistant tumor growth in a direct manner through secretion of lymphotoxin which stimulated IKKα and STAT3 in tumor cells (77). B cells in prostate cancer have also been shown to inhibit chemotherapy-induced tumor regression (78). The source of resistance was identified to be infiltrating $IgA^+PD\text{-}L1^+IL\text{-}10^+$ plasma cells, a separate population from the lymphotoxin-producing B cells previously described. The addition of oxaliplatin induced infiltration of the plasma cells into the tumor and their development was dependent on TGF-β receptor signaling and class switch recombination to IgA. Depletion of the plasma cells restored $CD8^+$ T cell activation that is required for oxaliplatin-mediated tumor regression. It should be noted that expression of PD-L1 and IL-10 by B cells were also required to maintain tumor growth after oxaliplatin administration, concluding that IgA expression is not the sole immunosuppressive mechanism in this model. Collectively these and additional recent studies suggest that antibody-producing B cells play a crucial role in promoting tumor growth by suppression of cytotoxic T cell responses and identified B cells as mediators of tumor responsiveness to chemotherapy (79).

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5.2. Immune checkpoints.

The PD-1/PD-L1 immune checkpoint is arguably the most currently studied mechanism in cancer-mediated immunosuppression and a role for this checkpoint has been recently extended to B cells. In a mouse model of hepatocellular carcinoma (HCC), Shalapour et al. identified an IgA^+ population of plasma cells accumulating in the liver that suppressed $CD8^+$ T cell responses through expression of PD-L1 (80). PD-L1 blockade significantly decreased HCC tumor size which correlated with increased T cell activation and decreased IgA^+ plasma cell accumulation in the liver. The $PD\text{-}L1^+$ B cells also expressed IL-10, but the role of IL-10 was not determined. Also, a population of pro-tumorigenic B cells was identified in hepatocellular carcinoma patients with high expression of PD-1 rather than the ligands PD-L1 or PD-L2 (81). These $PD\text{-}1^{Hi}$ B cells exhibit a $CD5^{hi}CD27^{hi}CD38^{dim}CD69^+$ surface marker phenotype and were incapable of producing IL-10 after BCR, CD40, and/or TLR9 stimulation. However, PD-1 activation induced IL-10 expression by HCC tumor B cells and PD-L1 blockade prevented IL-10 expression *in vivo*, decreased T cell suppression, and slowed disease progression. Additionally, $PD\text{-}1^{Hi}$ tumor B cells were incapable of differentiating into plasma cells, suggesting that this B cell subset is separate from previously identified murine regulatory plasma B cells (80). The PD-1/PD-L1 axis may be utilized by B cells in different ways within different tumor types. While PD-L1 mediated immunosuppression is mainly attributed to cancer cells and myeloid cells, these findings demonstrate the need to understand how the axis operates in all cell types within the microenvironment.

5.3. Anti-inflammatory cytokines.

B cell regulation of immune responses in cancer is not limited to the plasma cell compartment as B cell can use non-Ig-based mechanisms, predominantly cytokines. TGF-β is a cytokine that can suppress anti-tumorigenic immune responses. A population of $B220^+CD25^+$ B cells expressing high levels of TGF-β were found in a 4T1 mammary tumor model (82). Provision of TGF-β by B cells converted naïve CD4+ T cells into $FoxP3^+$ Treg cells and suppressed $CD8^+$ T cells (82). Similarly, EMT-6 mammary tumor-educated B cells were reported to suppress $CD4^+$ and $CD8^+$ T cell proliferation and IFNγ secretion both *in vivo* and *in vitro* in a TGF-β-dependent manner (83). The authors also found that $TGF\text{-}\beta^+$ B cells were enriched for PD-L1. Combination blockade of the two inhibitory molecules led to a synergistic decrease in tumor growth (83).

The most cited mechanism for immunosuppression by B cells in cancer has been secretion of the anti-inflammatory cytokine IL-10. IL-10 expression by B cells was upregulated after co-culture of splenocytes with irradiated murine thymoma and melanoma cells (84). The upregulation of IL-10 coincided with a decrease in secreted IFNγ, TNFα, and MCP-1 and was reversed in splenocytes from B cell deficient mice, indicating a B cell-mediated suppression of pro-inflammatory responses. The increase in IL-10 expression was heavily influenced by CD40-CD40L interactions between B cells and cancer cells. This mechanism was not present when B cells were cultured with MCA304 sarcoma cells, suggesting that engagement of IL-10 mediated suppression by B cells in cancer may be tumor-type specific. In a murine tumor model of TPA-induced squamous carcinoma, $CD19^+CD21^{Hi}$ B cells produced IL-10 in a TNFα-dependent manner, which correlated with a decrease in $CD8^+$ T

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cell effector function (85). IL-10 producing B cells were also reported in a model of non-Hodgkin B cell lymphoma and displayed a CD1d^{Hi}CD5^{+} surface marker phenotype. In this case, IL-10 from CD1d^{Hi}CD5^{+} B cells increased tumor growth by suppressing the phagocytic/effector activity of macrophages (86). Considering this evidence, we aimed to understand how IL-10^{+} B cells play a role in the growth of pancreatic tumors. Adoptive transfer of *Il10*$^{-/-}$ B cells into B cell deficient mice bearing orthotopic pancreatic tumors did not affect tumor growth compared to tumor-bearing mice reconstituted with WT B cells (69). Furthermore, orthotopic pancreatic tumors implanted in *Il10*$^{-/-}$ mice did not demonstrate any change in tumor growth compared to WT mice and did not display increased CD8^{+} T cell infiltration or activation, suggesting that IL-10 from B cells does not play in a functional role in this model (72). To this end, the recent discovery of the cytokine IL-35 as a mediator of immunosuppression by B cells in autoimmunity and inflammation has translated into an emerging area of interest in solid cancers and will be discussed in detail below.

Section 6: Identification and generation of immunosuppressive IL-35^{+}/IL-10^{+} Bregs

6.1. Immunosuppressive cytokine IL-35.

Interleukin-35 (IL-35) is a heterodimeric cytokine within the IL-12 family, which also includes IL-12, IL-23, IL-27, and IL-39 (87, 88). IL-35 is comprised of a p35 alpha subunit, shared with IL-12, and an EBi3 beta subunit, shared with IL-27 (69, 87). The overlap of subunits between these cytokines not only contributes to unique functional interactions, but also influences how experimental models for studying IL-12 family cytokines are designed and interpreted.

The suppressive function of IL-35 was first elucidated within the context of autoimmune and inflammatory conditions (89–91). Collison et al. determined that the EBi3-p35 heterodimer was constitutively secreted by resting wild type Tregs *in vitro* (89). Furthermore, *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Treg cells had reduced regulatory activity *in vitro* and in a mouse model of inflammatory bowel disease (89). Additionally, a protein construct with covalently linked EBi3 and p35 inhibited the proliferation of CD4^{+} CD25^{-} T effector cells and differentiation of Th17 cells *in vitro,* yet expanded CD4^{+} CD25^{+} Tregs (90). Following these studies describing the suppressive role of IL-35 secreted by Tregs, IL-35 production was detected in other cell types, including dendritic cells, B cells, and cancer cells (69, 92–97). IL-35 expression has been found in several human cancers (92, 96, 98–102). Among these studies, IL-35 was detected in patient tumor tissue, plasma, peripheral blood, and in human cancer cell lines using techniques including RT-PCR, enzyme-linked immunoassays, IHC analysis, western blot analysis, and intracellular cytokine staining. The impact of cancer cell autonomous IL-35 on cancer progression, survival rates, and response to treatment is still under investigation: overexpression of IL-35 was implicated in promoting tumor growth by enhancing myeloid cell accumulation and angiogenesis, mediating cell extravasation and increased metastasis, inducing regulatory T cells, and inhibiting apoptosis of cancer cells, although it is still not clear what role cancer cell-production of IL-35 has on immune responses (92, 96, 98–102).

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In addition to identifying the cell populations responsible for secreting IL-35, understanding the mechanisms governing IL-35 signaling has been a key area of interest. One question in the field was whether heterodimer formation was necessary for functional activity. While more work is needed, studies conducted by the Egwuagu lab using models of inflammatory disease, including uveitis and experimental autoimmune encephalitis, helped elucidate the intrinsic immune-suppressive function of each IL-35 subunit (103–105). Unlike other cytokines in the IL-12 family, the alpha and beta subunits of IL-35 and IL-27 are not thought to be linked by a disulfide bond. To combat the consequent challenge of isolating heterodimers of IL-35 *in vivo*, Egwuagu et al. created recombinant mouse IL-35 that enabled expression of both EBi3 and p35 (103). *In vitro* experiments demonstrated that the monomeric and homodimer form of p35 suppressed both T and B cell proliferation, potentially by inducing cell cycle arrest or inhibiting STAT3 and STAT4 activation in IL-6 and IL-12 pathways, respectively (104). In B cells it seems that STAT3 and STAT4 are engaged by IL-35, yet in $CD4^+$ T cells, activation of STAT1 and STAT4 was identified (106). EBi3 also inhibited lymphocyte proliferation, yet the complete heterodimer of IL-35 demonstrated the highest level of suppression (104). Furthermore, although EBi3 had little effect on the expression of *IL-10, p35,* and *Ebi3* mRNAs in B cells, p35 upregulated these genes and consequently, induced the expansion of IL-10 and IL-35 producing regulatory B cells (104). *In vivo*, recombinant p35 and p35-p35 reduced the severity of experimental autoimmune uveitis and experimental autoimmune encephalitis as well (104, 105). Overall, although the suppressive effects were not as strong as heterodimeric IL-35, p35 alone displayed immune-regulatory functions and may have potential as a therapeutic (104). However, the synergy between EBi3 and p35, physiological factors regulating the stability of the heterodimer, and potential activity of monomers or homodimers still remain to be determined, as it is challenging to eliminate more than one subunit at one time without perturbing expression of multiple IL-12 family members.

Although our knowledge and understanding of IL-35 has recently expanded, there is still much to learn about the mechanistic function, regulation, localization, downstream immunosuppressive effects, and therapeutic potential of IL-35. As researchers continue to unravel the unique features of IL-35, the ability to identify relevant cytokine producing populations will be paramount. Many initial studies have relied on PCR to identify IL-35 secreting cell populations. However, gene expression does not always correlate with protein level. It is also difficult to discern cells that express both the p35 and EBi3 subunits of IL-35 by PCR. Therefore, moving forward, more biologically relevant, and sensitive techniques should be incorporated. Specifically, the implementation of ELISA, intracellular flow cytometry, reporter models, and reagents optimized for use in both animal and human samples will enable researchers to obtain a more accurate and detailed representation of IL-35 and its effects.

6.2. Identification of IL-35$^+$ regulatory B cells.

While the identification of IL-35$^+$ B cells is a relatively recent discovery, a suppressive function of B cells has been proposed for decades. B cells have been implicated in suppressing delayed hypersensitivity reactions (107, 108) and recovery from experimental autoimmune encephalitis (EAE) (109). Using models of inflammation, including EAE,

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colitis, and arthritis, the suppressive capability of B cells was linked to the production of IL-10 (110–112). IL-10 then became the hallmark cytokine of a subpopulation of B cells known for their regulatory function, and thus named regulatory B cells (Bregs). However, it is now appreciated that Bregs are a heterogeneous population, and as such, multiple IL-10 and IL-35 producing subsets have been identified. Adoptive transfer of murine transitional 2 marginal-zone precursor cells (T2-MZP) ($B220^{+}CD21^{Hi}CD23^{+}IgM^{Hi}$) was shown to prevent collagen-induced arthritis and diminish established disease through the production of IL-10 (113). Other groups have identified the expansion of IL-10^{+} T2-MZPs, as defined by the markers $CD19^{+}CD21^{Hi}CD24^{Hi}CD23^{Hi}CD1d^{Hi}$, under conditions of inflammation induced by gut flora and arthritis (114). Furthermore, a population of $CD5^{+}CD1d^{Hi}$ B cells characterized by their selective ability to produce IL-10 (B10 cells) (115) was found in murine models of inflammation, EAE, *Listeria* infection, and reported to inhibit effector $CD4^{+}$ T cells, monocytes, and DCs (115–117). Plasma cells ($CD138^{+}MHC\text{-}II^{Lo}B220^{+}$), Tim-$1^{+}$ B cells (Tim-$1^{+}CD19^{+}$), and plasmablasts ($CD138^{+}CD44^{Hi}$) were also observed to secrete IL-10 and mediate immune suppression in mice (93, 118–121).

Recently, the suppressive functions of IL-35^{+} B cells, have been identified. Shen et al. reported IL-35 producing B cells (89, 90, 93). While mice lacking IL-35 expression in B cells survived longer than control mice after both primary infection and secondary challenge with *Salmonella typhimurium,* they were unable to recover from experimental autoimmune encephalomyelitis (93). These results are consistent with the role of IL-35 facilitating an anti-inflammatory response, which would be beneficial in autoimmunity but not in response to infection (93). In these models, $CD138^{+}MHC\text{-}II^{Lo}$ $B220^{+}$ plasma cells were described as the primary source of B-cell-derived IL-35 and IL-10 (93). In a separate study, recombinant IL-35 induced a subset of suppressive $CD19^{+}CD5^{+}B220^{Lo}$ Bregs capable of producing both IL-35 and IL-10, and mice treated with IL-35 were protected from experimental autoimmune uveitis (94). Collectively, these early studies exploring the function of IL-35 in models of autoimmune disease and inflammation detailed a unique immunosuppressive function and ability to induce Treg and Breg populations.

Expanding the role of cytokine-producing Bregs beyond the scope of autoimmune disease, we detected a subset of immunosuppressive IL-10 and IL-35-producing $CD1d^{hi}CD5^{+}$ B cells in murine pancreatic cancer (69). Specifically, pancreatic ductal adenocarcinoma (PDAC), a cancer type with a prominent B cell presence, was found to be heavily dependent on the expression of IL-35 but not IL-10 for proper tumorigenesis in both human PDAC lesions and in a well-characterized oncogenic *Kras*-driven mouse model (69). Orthotopic pancreatic tumor growth in B cell-deficient mice was significantly inhibited compared to WT controls and tumor growth was rescued upon adoptive transfer of wild-type or IL10-deficient but not *p35* deficient $CD1d^{Hi}CD5^{+}$ B cells (69). In our recent studies, we expanded the definition of B regulatory cells to $CD19^{+}CD1d^{Hi}CD21^{Hi}CD5^{+}$ cells (70–72). Bregs under this definition produced both IL-10 and IL-35 and accumulated in the spleen and pancreas of mice from both a spontaneous and orthotopic models of pancreatic tumorigenesis (72). Corroborating these findings, work by Takahashi et al. showed that $CD19^{+}CD1d^{Hi}CD5^{+}$ Bregs and PD-$L1^{+}$ B cells were increased in the spontaneous model of pancreatic cancer with overexpression of IL-1β in the pancreas (122). Treatment with an IL-35 neutralizing antibody led to decreased PanIN formation and a reduced frequency of

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PD-L1^{+} B cells, providing further evidence that IL-35 promote pancreatic tumorigenesis (122).

Our lab also utilized *Il12a*^{GFP} and *Ebi3*^{Tom} reporter mice to detect the subunits of IL-35 *in vivo* (70, 123). Using these models, we demonstrated that expression of both reporters was found in CD19^{+}CD5^{+}CD1d^{hi}CD21^{hi} Bregs, but also, to some extent, in myeloid cells, T cells, and other B cell subsets (70). Additionally, *Ebi3*^{Tom} expression was found to be more tightly regulated than p35 *in vivo* and *in vitro* (70). Our results support previous observations showing low constitutive expression of p35 as opposed to more regulated EBi3 expression (93). Ultimately, stimuli leading to the upregulation of EBi3 expression, such as those fostering coordinated activation of the BCR, CD40, and TLR pathway, as we discuss in detail below, likely confer IL-35 expression (70). Hypothetically, under circumstances of low EBi3 expression, available p35 could be more available to bind p40 subunits, generating IL-12 and facilitating an inflammatory response. In the future, the use of reporter mice that have both *Il12a*^{GFP} and *Ebi3*^{Tom} will enable the identification of cells that express both IL-35 subunits simultaneously and may help address open questions in the field about the regulation, and persistence of this cytokine.

Unlike Tregs, which express FoxP3, a single transcription factor facilitating the development of Bregs across the distinct spectrum of pathological conditions has been elusive. This, and the myriad of phenotypic markers of Breg subpopulations, support the idea that Bregs are not lineage specific, but are instead derived from B cells at multiple stages of development in response to stimuli (124). As such, different inflammatory environments may provide unique stimuli and foster the development of distinct Breg populations. For instance, upregulation of the transcription factor aryl hydrocarbon receptor was shown to promote the generation of IL10^{+}CD19^{+}CD21^{Hi}CD24^{Hi} Bregs and suppress pro-inflammatory gene expression in a model of murine arthritis (125). Furthermore, the conditions surrounding immune activation, including those driven by microbial contexture, may lead to differentially up- or down-regulated surface markers, potentially giving rise to inconsistencies between experimental models (124). In addition to characterizing distinct surface markers for the wide range of Breg subsets, discerning the localization of these populations is also critical. An understanding of cellular localization, be it within the tumor, spleen, pancreas, lymph nodes, or peripheral blood, can not only help elucidate local or systemic effects, but also provides valuable insight about potential targets for the development of therapeutics.

In line with the hypothesis that any B cell can become a Breg, some groups have identified populations of suppressive plasmablasts (93, 121). As discussed above, Shen et al. reported IL-35 and IL-10 producing CD138^{+} B cells that facilitated immune suppression (93). Furthermore, Matsumoto et al. documented the presence of suppressive IL-10 secreting plasmablasts in the draining lymph nodes of mice during EAE (121). We assessed whether plasma cell formation and immunosuppressive cytokine function may exist in the same B cell subset in cancer. To do this, we profiled splenic B220^{lo}CD138^{+} plasma cells from healthy mice and mice with orthotopically derived pancreatic cancer for p35 and IL-10 using intracellular cytokine staining. Treatment with α-CD40 and LPS did not upregulate p35 or IL-10 in plasma cells, suggesting plasma cells do not account for a cytokine-producing B

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cell subset in murine PDA (71). Overall, in our mouse models and human samples of pancreatic adenocarcinoma, production of IL-35 and IL-10 by murine plasma cells or human plasmablasts was not observed (71). These differences not only highlight the diversity of Breg phenotypes but may also support the idea that specific stimuli within the inflammatory environment induce differentiation of distinct Breg subsets.

6.3. Function of IL-35$^+$ regulatory B cells.

To understand the mechanisms of IL-35-mediated suppression in cancer, we analyzed established mouse models of PDAC with a deficiency for IL-35 (72). We determined that genetic ablation of IL-35 in mice results in reduced orthotopic pancreatic tumor growth by way of increased T cell activation (Figure 1) (72). Murine pancreatic tumors in *Il12a*$^{-/-}$ and *Ebi3*$^{-/-}$ mice had a significant increase in intratumoral CD4$^+$ and CD8$^+$ T cells that express TNFα and/or IFNγ, and concurrently contained significantly fewer FoxP3$^+$ CD4$^+$ T cells, overall resulting in significantly smaller tumors. While these models provide insight into the B cell specific role of IL-35, it is important to consider the subunit overlap within the IL-12 family cytokines. We utilized *p40*$^{-/-}$ and *IL27Rα*$^{-/-}$ mice as controls to account for the overlapping use of p35 and Ebi3 in IL-12 and IL-27 heterodimers (72). Furthermore, administration of aPD-1 in *Il12a*$^{-/-}$ mice resulted in a synergistic reduction in pancreatic tumor growth and increased T cell activation compared to *Il12a*$^{-/-}$ controls. Treatment of WT mice with aPD-1 did not result in decreased pancreatic tumor growth, similar to clinical findings, suggesting IL-35 inhibition confers sensitivity to checkpoint blockade (72).

Next, to understand the role of B cell-specific IL-35 expression in T cell suppression, orthotopic pancreatic tumors were implanted in mice with a genetic ablation of either *p35* ($B^{p35-/-}$) or *Ebi3* ($B^{Ebi3-/-}$) specifically in B cells, generated by bone marrow chimerism and CD19-Cre recombination, respectively (71, 93, 123, 126). Using $B^{p35-/-}$ and $B^{Ebi3-/-}$ mice, we demonstrated that B cell ablation of IL-35 resulted in smaller tumors, whereas Treg specific ablation of IL-35 ($Treg^{Ebi3-/-}$) did not affect tumor growth (71). Furthermore, the combination of α–PD-1 with B cell–specific loss of IL-35 significantly reduced tumor growth compared to $B^{Ebi3+/-}$ mice treated with either isotype or α–PD-1 or $B^{Ebi3-/-}$ mice treated with isotype control. Additionally, tumors in B cell IL-35 knockout mice contained a significant increase in IFNγ$^+$ and TNFα$^+$ CD4$^+$ and CD8$^+$ T cells, and had decreased frequencies CD4$^+$FoxP3$^+$ T cells (71). This data supports prior studies demonstrating the functional role of IL-35 to suppress the proliferation and activation of CD4$^+$ T effector cells and induce the expansion of Tregs (89, 90, 126–130). Previous work also demonstrated that IL-35 signals through STAT1/STAT4 heterodimers in CD4$^+$ T cells, yet interestingly, we observed phosphorylation of STAT1, STAT3, and STAT4 in CD8$^+$ T cells in the presence of recombinant IL-35 (71, 106). To elucidate the impact of IL-35 on CD8$^+$ T cell function, we analyzed the expression of IFNγ and two chemokine receptors expressed on effector T cells, CXCR3 and CCR5, that have been linked to intratumoral CD8$^+$ T cell infiltration (Figure 3) (131, 132). Recombinant IL-35 suppressed expression of IFNγ, CXCR3, and CCR5, in OVA-specific CD8$^+$ T cells, supporting a role for IL-35 in reducing CD8$^+$ T cell activation and tumor infiltration (71). To clarify the signaling mechanism driving STAT3 activation in CD8+ T cells, T cells were sorted by singular expression of the IL-35 receptor chain subunits, IL12Rβ2 or gp130 (71). Treatment with rIL-35 revealed STAT1 and STAT3

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activation in gp130^{+}CD8^{+} T cells, but only STAT4 activation in the IL12Rβ2^{+}CD8^{+} subset (71). Moreover, the gp130+ T cells downregulated of IFNγ, CXCR3, and CCR5, supporting the conclusion that the mechanism of IL-35 mediated inhibition of CD8+ T cells is driven by STAT3 (Figure 3) (71). Inhibition of STAT3 activity in CD8^{+} T cells resulted in decreased pancreatic tumor growth with a significant increase in the infiltration of CD8^{+} T cells (71). Treatment of tumor-bearing mice with a neutralizing IL-35 antibody resulted in a significant decrease in pSTAT3^{+} CD8^{+} T cells within the tumors and an increased frequency of CXCR3^{+} and CCR5^{+} CD8^{+} T cells (71). Collectively, this evidence connects B cell-specific expression of IL-35 to activation of STAT3 followed by decreased infiltration and activation of CD8^{+} T cells and decreased tumor growth suggests that this axis may be a promising therapeutic target to augment anti-tumor T cell responses in combination with checkpoint blockade (71).

6.4. IL-35 vs IL-10: functional redundancy and divergence.

IL-10 and IL-35 have each been shown to contribute to immune suppression, however, the functional interplay between these cytokines in Bregs remains unclear. Mounting evidence suggests subpopulations of cells secreting either IL-35 or IL-10 may utilize distinct mechanisms to facilitate immune suppression (133, 134). Tumor growth in B cell-deficient mice that received adoptively transferred *Il10*$^{-/-}$ B cells was similar to that of WT mice, yet we observed decreased tumor growth in B cell-deficient mice that received *Il12a*$^{-/-}$ B cells (69). Moreover, recent data demonstrated that IL-10 and IL-35 may be functionally divergent with only partially overlapping and non-redundant regulation (134). Separate populations of Treg cells expressing IL-10 or IL-35 were found using triple reporter mice (*Il10*^{GFP};*Ebi3*^{Tom};*Foxp3*$^{Cre\text{-}YFP}$) (134). This supports previous data from IL-35 reporter mice (Ebi3-Dre-Thy1.1) that characterized distinct populations of IL-10 and IL-35 producing Treg subsets with different transcription factors, activation status, geographic location, and effector functions (133). Furthermore, human Treg cells from healthy donors and those with non-small cell lung cancer showed divergent expression of IL-10 and IL-35, as opposed to being double positive for each cytokine (134). However, TCR stimulation of purified Tregs *in vitro* was sufficient to induce both *Il10* and *Ebi3* expression, providing evidence for a model of developmental plasticity with transitory states instead of discrete subsets (134). Functionally, while IL-10 exhibited a more dominant role limiting effector function and IL-35 limited memory differentiation, both cytokines showed overlapping ability to induce inhibitory receptors such as PD-1, LAG3, TIM3, TIGIT, and 2B4 (134). Collectively, these results highlight an adaptive plasticity in which Tregs may be intrinsically capable of producing either IL-10 or IL-35 yet are influenced by environmental cues to favor the expression of one or the other (134). Similarly, Shen et al. found three distinct subpopulations of plasma cells differentiated by their level of CD138 and CD22 that expressed mRNA for either IL-10 or the two subunits of IL-35 during *Salmonella* infection, yet rarely expressed transcripts for both cytokines (93). Moving forward, the use of reporter mice to distinguish IL-10 and IL-35 expression in B cells will provide further insight into the coordinated mechanisms of IL-10 and IL-35 in the tumor microenvironment, and may help inform the development of combination therapies to enhance the efficacy of immunotherapy.

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7. Cancer-associated inflammation as a prerequisite for Breg expansion and function

The release of cancer-associated inflammatory molecules within the tumor microenvironment are key initiators of the immune responses and recruitment of many immune cell types. We are beginning to understand how these microenvironmental cues may trigger differential responses from B cells within tumors and secondary lymphatic organs. Cancer-associated inflammation appears to increase the number of regulatory B cells from either the existing population or inducing differentiation of naïve B cells into a regulatory phenotype. Additionally, secretion of IL-10 and IL-35 is unlikely constitutive, and is instead induced by inflammation (135). It is still unclear what mechanisms cancer-associated inflammatory molecules cause these events to occur. B cells are equipped with multiple innate and adaptive antigen receptors as well as a plethora of inflammatory cytokine receptors which drive their differentiation and differentiation. In this section, we will discuss how these receptors dictate regulatory B cell function in cancer.

7.1. Toll-like receptors.

Toll-like receptors (TLRs) are a class of receptors that can recognize bacterial and viral pathogen-associated molecular patterns and direct expression of regulatory and/or inflammatory genes in response. *In vitro* stimulation of splenic B cells with TLR2, 4, or 9 agonists is sufficient to significantly upregulate MyD88-dependent IL-10 expression, suggesting that engagement of TLRs on B cells can drive their regulatory properties (93, 136–138). Prior research also implicates TLR stimulation in the expression of IL-35 as well. *Il12a* in naïve splenic B cells is constitutively high *in vitro,* but not *Ebi3* (93). Using splenic *Il12a*^{GFP} and *Ebi3*^{Tom} B cells, we also reported a similar finding of constitutive *Il12a*^{GFP} expression and absent *Ebi3*^{Tom} expression *in vitro* (70). TLR stimulation of *Il12a*^{GFP} B cells resulted in the upregulation of GFP, but only to a moderate amount due to the high basal expression level (70, 93). TLR stimulation of *Ebi3*^{Tom} B cells, however, revealed a highly significant upregulation of *Ebi3*^{Tom} expression indicating that the EBi3 subunit is more tightly regulated in B cells than p35 (70). The role of TLRs in IL-35 expression should also be considered in the context of simultaneous CD40 or BCR stimulation as both pathways synergistically increase Il12a and more significantly EBi3 beyond TLR stimulation alone (70, 93). (70). The requirement for this proposed synergy in disease has been demonstrated in an infection model, but the application to other diseases is currently not known (93). The role of TLRs in activation of Bregs in cancer has not been robustly addressed *in vivo*, but TLR activation by cancer-associated byproducts is entirely plausible. Endogenous TLR ligands are highly abundant within the tumor microenvironment in multiple forms. Traditionally, TLR ligands acting as pattern associated molecular patterns (PAMPs) are present on the cell surface of bacteria and fungi. Recent discoveries into the role the microenvironment plays in tumor growth and immune modulation implicate microbiota-driven stimulation of B cell TLRs within tumors or secondary lymphoid organs (139). Pancreatic cancer models have recently revealed that bacteria infiltrate the pancreas during tumorigenesis and accelerate disease progression (140). Cell-free gut extracts from mice bearing pancreatic tumors revealed a significant increase in the amount of TLR ligands present. The effect that such cancer microbiome-associated TLR ligands have specifically on

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B cells still needs to be explored but could serve as a source of TLR-dependent induction of regulatory B cell function in cancer. TLR ligands also take on the form of damage associated molecular patterns (DAMPs), which are molecules released by damaged or dying cells capable of binding various TLRs. Two prominent DAMPs, HMGB1 and S100A9, have been found in pancreatic ductal fluid from patients with pancreatic adenocarcinoma and could hypothetically serve as a B cell TLR agonist as well (141). It will be important to examine the extent of TLR function on B cell function in cancer going forward as many current therapies such as radiation and chemotherapy result in the generation of DAMPs within tumors. The use of preclinical mouse models with conditional B cell deletion of specific TLRs or key signaling proteins such as MyD88 or TRIF can provide insight into these functions *in vivo*.

7.2. B cell receptor.

The role of the BCR in the suppression of pro-inflammatory responses in cancer has yet to be clearly defined, but existing data from autoimmune disease models suggests that the BCR plays a critical role in this process. MD4 mice, which harbor a transgenic BCR for hen egg lysozyme, are unable to resolve MOG-induced EAE (110). The resolution of EAE was found to be dependent on the production of IL-10 by B cells. MD4 mice have significantly fewer splenic IL-10$^+$ B cells in homeostatic conditions, suggesting that antigen recognition by the BCR is critical for the development of regulatory B cells as well (136). Stimulation of the BCR alone *in vitro* is not sufficient to induce IL-10 expression, however, combination of BCR , CD40 and TLR4 stimulation significantly increases both IL-10 and IL-35 expression in B cells, suggesting that BCR recognition of antigen alone is not sufficient to regulate an anti-inflammatory B cell response (70, 93, 142).

The role of the BCR in various cancers has been focused on the recognition of antigen and subsequent production of antibodies, however, it is still not well understood if the secretion of immunosuppressive cytokines by B cells in cancer is based on cognate interactions with the BCR. A B cell could release these cytokines after direct interaction with a tolerizing self-antigen released by a tumor and/or interaction with a T cell specific for the same self-antigen to quell inflammation. Better understanding of the antigenic strength, the nature of the antigen (mutated tumor antigen or tolerizing self-antigen), timing of antigenic exposure and local environment may provide insight into why some Breg cells present as plasma cells and some do not. Moving forward it will be important to examine the phenotype of B cells that produce immunosuppressive cytokines in response to cancer and what peptide sequences the BCR is able to recognize. This could be approached using syngeneic tumor implantation in cytokine reporter models for IL-10 (143, 144) and IL-35 (70, 123) to isolate activated regulatory B cells and decipher their characteristics via surface markers, transcriptomes, and BCR sequencing.

7.3. CD40.

The co-stimulatory molecule CD40 is essential for proper maturation of B cells to eliminate pathogens and accumulating evidence suggests a role for CD40 in Breg cell function as well. While CD40 is not essential for IL-10 production by splenic B cells, transgenic expression of CD40L on B cells can significantly increase the generation of IL-10$^+$ B cells (136).

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Furthermore, splenic Transitional 2 (T2) B cells isolated from MRL/lpr lupus-prone mice significantly upregulate IL-10 production in response to CD40 stimulation *ex vivo* (145). Mounting evidence suggests that CD40 engagement is a key factor in whether a B cell expresses IL-10 or IL-35. Splenic B cells isolated from *Il10*-eGFP reporter mice and stimulated with LPS display a significantly larger frequency of GFP+ B cells when compared to LPS+α-CD40 stimulated B cells (93). Interestingly, *Il10* mRNA expression in LPS+α-CD40 treated B cells was similar to that of naïve B cells, but *Ebi3* expression was significantly upregulated (93). These findings collectively suggest that CD40 stimulation regulates IL-10 vs. IL-35 expression in B cells. CD40 agonism alone is sufficient to significantly induce EBi3 production in B cells, which agrees with prior data demonstrating that *Ebi3* is a direct target gene of CD40 (70, 93, 146). *Il12a* expression, however, is not significantly upregulated after CD40 agonism (93). Collectively these data suggest that CD40 stimulation strongly promotes IL-35 expression by primarily upregulating *Ebi3*.

What remains uncertain is how CD40 stimulation affects different B cell subsets in the context of disease. LPS+α-CD40 stimulation *in vitro* consistently induces IL-35 expression specifically in $CD1d^{Hi}CD5^{+}$ B cells, but not in $CD1d^{Lo}CD5^{-}$ B cells (71, 72, 93). Bone marrow chimeric mice containing *Cd40*$^{-/-}$ B cells display more severe EAE pathology than controls suggesting a loss of regulatory potential during inflammation (110). Furthermore, myelin oligodendrocyte glycoprotein (MOG) immunized mice supplemented with *Cd40*$^{-/-}$ $CD1d^{Hi}CD5^{+}$ B cells experienced more severe EAE than mice supplemented with WT $CD1d^{Hi}$ $CD5^{+}$ B cells (147). These data suggest that CD40 signaling promotes regulatory function within $CD1d^{Hi}CD5^{+}$ B cells and contributes to limiting disease severity, but the effect of B cell CD40 loss on regulatory function and cancer outcome are currently unknown. B cell deficient mice supplemented with *Cd40*$^{-/-}$ splenic B cells do not display overall altered subcutaneous MC38 tumor-growth compared to mice supplemented with WT splenic B cells suggesting that B cell-specific CD40 is not essential in tumor growth (148). However, this finding may be model-specific, as $CD40L^{+}$ EL4 thymoma cells co-cultured with *Cd40*$^{-/-}$ B cells produce significantly less IL-10 than co-cultured WT B cells (84). Suppression of co-cultured $CD8^{+}$ T cells and $NK1.1^{+}$ T cells with B cells and EL4 cells was found to be IL-10 dependent, but the *in vivo* effect of CD40 deletion on tumor immunity was not examined. Use of agonistic CD40 to promote the anti-inflammatory properties of B cells has been successfully seen in a mouse model of Lupus. MRL/lpr lupus mice treated with α-CD40 experienced significantly increased survival attributed to increased IL-10 producing B cells, an increased frequency of T2 B cells, and a decrease in circulating α-dsDNA immunoglobulins (145). In preclinical models of pancreatic cancer, use of agonistic CD40 combined with immune checkpoint blockade resulted in the complete regression of tumors (149). The effect of CD40 agonism on B cells specifically was not determined, but the resolution of disease leads one to hypothesize that α-CD40 did not strongly activate the immunosuppressive functions of B cells. Activation of splenic B cells *ex vivo* with CD40L and subsequent transfer to mice bearing B16.F10 melanoma or E.G7 lymphoma tumors displayed slowed tumor growth compared to unstimulated controls further suggesting that B cell CD40 agonism is anti-tumorigenic (150). These studies suggest that engagement of CD40 on B cells is important for their regulatory capacity, but this is also disease specific. While CD40 is important in IL-10 and IL-35 function, B cell differentiation into antibody-

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secreting cells is also heavily dependent on CD40, so future investigation into what molecular mechanisms determine B cell fate in response to CD40 stimulation is incredibly important.

7.4. Cytokines.

Resolution of the immune response by anti-inflammatory immune cells limits the extent of the response and evidence suggests that pro-inflammatory cytokines can directly regulate regulatory B cell function. This was demonstrated in a murine model of arthritis, where IL-10 producing B cells capable of suppressing auto-reactive T cell function arise in response to increased environmental IL-6 and IL-1β (114). Many cancers are highly dependent on pro-inflammatory cytokines for the recruitment of pro-angiogenic and mesenchymal cell types necessary for proper tumor growth. These cytokines have both autocrine effects within the tumor microenvironment, but also paracrine effects as well. In orthotopic pancreatic cancer mouse models, knockdown of tumor cell-derived IL-1β results in smaller tumors containing fewer $CD1d^{Hi}$ $CD5^{+}$ regulatory B cells and concurrently more $CD8^{+}$ T cells expressing IFNγ and Granzyme B (151). Additionally, pancreatic tumor-bearing mice administered with a neutralizing IL-1β antibody displayed similar results while also synergizing with α-PD-1 treatment to further reduce tumor burden (151). *In vitro*, IL-6 and IL-1β stimulation in combination with agonistic α-CD40 results in a significant increase in the expression of IL-35 subunits *Il12a* and *Ebi3* in addition to IL-10 suggesting that cancer-associated inflammatory cytokines promote regulatory B cell function and expansion in pancreatic cancer (152).

IL-21 is another pro-inflammatory cytokine shown to play a role in the promotion of regulatory function in B cells. Recombinant IL-21 significantly upregulates the frequency of IL-10 producing B cells *in vitro*, specifically B cells with a $CD1d^{Hi}CD5^{+}$ phenotype (147). Using a MOG-induced model of EAE, mice receiving adoptively transferred *Il21r*$^{-/-}$ B cells developed more severe EAE pathology and increased MOG-TCR CD4+ T cell proliferation suggesting that IL-21 drives autoimmune T cell suppression *in vivo*. IL-21, however, is an essential cytokine for the maturation of B cells from the germinal center to plasma cells. The differential effects of IL-21 on different B cell populations may be explained by the observation that $CD1d^{Lo}CD5^{-}$ B cells do not upregulate IL-10 in response to IL-21 (147) and could perhaps follow a conventional plasma cell developmental path. In a mouse model of triple negative breast cancer, IL-21 provided by follicular $CD4^{+}$ T cells results in the development of tumor-specific plasma cells that in turn resulted in increased long-term survival (55). This, however, is in stark contrast to studies in pancreatic cancer, skin cancer, and prostate cancer where plasma cells play a pro-tumorigenic role and increase tumor burden. Although the role of IL-21 in these models was not examined, the potential of IL-21's effect on B cell fate may not only be B cell population dependent, but also cancer type dependent as well. Additionally, the location of IL-21 stimulation may play an important role as Lindner et al. demonstrated that IL-21 activated B cells within tumors of various human carcinomas present a regulatory phenotype of IL-10^{+} $CD25^{+}$ and IDO^{+} (153).

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8. Mechanisms & markers of immune suppression by B cells in human cancer.

Corroborating data from murine models, studies in human cancer have described multiple regulatory B cell phenotypes. Without evidence for a distinct lineage marker, the origin of human Bregs remains unclear. It is not fully understood whether the suppressive function of Bregs is intrinsic to pre-defined subsets, or if instead, any B cell has the capacity to become regulatory in the presence of certain environmental stimuli. To date, human Breg subsets have been identified in a wide variety of B cell subsets (Table 2). Furthermore, production of immunosuppressive cytokines has been consistently observed in Bregs derived from immature B cells as opposed to mature cells like plasmablasts (71, 96). Differences in sampling location (peripheral blood versus intratumoral) may contribute to the observed variation in Breg phenotype, although more work is needed to clarify potential site-specific dependence. Additionally, the assortment of techniques used to identify and purify Breg subsets may contribute to the described phenotypic variation. Iwata et al. first identified a population of IL-10 secreting B cells in human blood samples from patients with various autoimmune diseases (154). This distinct subset of B cells was characterized by the ability to express IL-10 following *ex vivo* stimulation and by the presence of $CD5^{+}CD24^{hi}CD27^{+}$ surface markers (154). IL-10 secreting B cells have since been described in numerous human cancers and are oftentimes linked to cancer progression. For instance, peripheral blood samples from patients with esophageal cancer contained elevated levels of IL-10 producing $CD19^{+}CD5^{+}$ B cells compared to healthy controls (155). Higher frequencies of IL-10 producing B cells were observed in late-stage disease samples than early-stage samples suggesting a role for regulatory B cells in the progression of esophageal cancer (155). Similarly, increased levels of $CD19^{+}CD24^{+}CD38^{+}$ B cells were detected in the peripheral blood and bone marrow of patients with acute myeloid leukemia and linked to reduced overall survival (156). IL-10 producing B cells were also detected in the tumors of patients with gastric cancer, breast cancer, head and neck squamous carcinoma, and esophageal squamous carcinoma (157–160). However, functional analysis of this subpopulation still needs to be conducted. Enhanced cancer progression was also attributed to B regulatory cells in a cohort of chronic lymphocytic leukemia patients treated with rituximab (161). Since TGF-β producing tumor-evoked Bregs express were $CD20^{Lo}$, Bodogai et al. noted that treatment with rituximab enriched for the immunosuppressive $CD20^{Lo}$ Bregs in leukemia patients and consequently led to enhanced cancer progression (161).

The correlation between high frequencies of regulatory B cells and increased cancer progression, may also be due to IL-10's role in facilitating regulatory T cell (Treg) development. In ascites from ovarian cancer patients, increased IL-10 producing B cells were not only associated with advanced disease, but were also positively correlated with the frequencies of $FoxP3^{+}CD4^{+}$ T cells and negatively correlated with the frequency of IFNy $^{+}CD8^{+}$ T cells (162). Zhou et al. detected increased infiltrating $CD19^{+}IL\text{-}10^{+}$ Bregs in tongue squamous cell carcinoma tumors compared to the surrounding tissue via IHC and the increased Breg frequency was associated with decreased patient survival (20). Additionally, Bregs induced by tongue squamous cell carcinoma cells *in vitro* were able to convert

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effector $CD4^+CD25^-$ T cells into Tregs through an IL-10 driven mechanism (20). As seen with IL-10, TGF-β has been shown to facilitate the conversion of effector T cells into Tregs in the context of cancer (20, 162, 163). Wang et al. reported that although Bregs did not hinder the proliferation of T cells, Bregs were positively correlated with Treg frequency and suppressed $CD4^+$ T cell IFNγ and TNFα secretion (163). Increased TGF-β mRNA expression and TGF-β1 was detected in $CD19^+CD24^{Hi}CD38^{Hi}$ Breg cells and Treg conversion was inhibited by the addition of neutralizing α-TGF-β (163). Together, these studies highlight the importance of further elucidating the crosstalk between Breg and Treg cells, and their role in shaping the tumor microenvironment.

B cells in human tumors are heterogeneous. $CD38^+CD1d^+IgM^+CD147^+$ B cells uniquely characterized by granzyme B expression have been identified in breast, cervical, and ovarian tumors (153). Linder et al. demonstrated that these granzyme B^+ cells were induced by IL-21, and functionally inhibited T cell proliferation by degrading the T cell receptor zeta chain *in vitro* and were found adjacent to IL-21^+ T cells in tumors (153). Overall, these findings support a role for IL-21 in Breg induction and draw attention to granzyme B's immunomodulatory capacity within the tumor microenvironment. Another distinct subset of PD-1^{Hi} $CD5^{Hi}CD24^{-/+}CD27^{Hi/+}CD38^{Dim}$ Bregs was identified in human hepatocellular carcinoma (81). Like other conventional Breg subsets, increased infiltration of PD-1^{Hi} Bregs was significantly correlated with disease progression in patients (81). Additionally, PD-1^{Hi} Bregs were shown to secrete IL-10 and suppress tumor-specific immunity upon activation of the PD-1/PD-L1 axis (81). Understanding of the development, and functional significance of the myriad of Breg subpopulations is still an active area of research.

To understand which subsets of human B cells may contribute to the regulatory function in pancreatic cancer, we characterized B cells from the blood of from treatment-naïve patients with pancreatic cancer and healthy volunteers. (71). QPCR analysis of sorted B cells identified two distinct Breg subpopulations: an IL-10 producing $CD19^+CD24^{Hi}CD27^+$ B10 subset and an IL-10^+IL-35^+ producing $CD19^+CD24^{Hi}CD38^{Hi}$ immature B cell subset akin to identified murine Bregs (71). The frequency of $CD19^+CD24^{Hi}CD38^{Hi}$ cells was determined to not only be higher in patients with PDAC, but also to produce elevated levels of IL-10 and IL-35 compared to healthy controls (71). Moreover, immature B cells and $CD4^+$ T cells isolated from PDAC patient blood were determined to be the main producers of IL-35 as determined by flow cytometry (71). To assess $CD8^+$ T cell and IL-35^+ immune cell infiltration, the expression of $CD8^+$ T cell-specific pSTAT3 and CXCR3 was examined using multiplex immunofluorescence in EBi3 high and EBi3 low areas of 11 PDA patient samples (71). Most IL-35^+ B cells were in tertiary lymphoid aggregates, implicating these regions as potential sites of active immune suppression (71). Corroborating the observed activation of pSTAT3 in $CD8^+$ T cells, the percentage of $pSTAT3^+CD8^+$ T cells was higher in areas rich with $EBi3^+$ immune cells (71). Inversely, lower percentages of $CXCR3^+CD8^+$ T cells were found in regions with increased IL-35 production, suggesting IL-35 may inhibit $CD8^+$ T cell infiltration by downregulating CXCR3 through a STAT3-mediated pathway (71).

To elucidate the correlation between IL-35^+ B cells and cytotoxicity in human pancreatic cancer, RNAseq was performed on regulatory and conventional B cells from both PDAC

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patient and healthy volunteer PBMCs. A tumor-associated Breg signature was calculated using expression values of genes differentially upregulated in Bregs compared to conventional B cells, and was found to inversely correlate with the cytotoxic $CD8^+$ T cell index in The Cancer Genome Atlas pancreatic adenocarcinoma cohort (71). The Breg signature was also negatively correlated with anti-tumor T cell activity in many other cancer types (71). Taken together, this data highlights a subset of $CD19^+CD24^{Hi}CD38^{Hi}$ IL-35 and IL-10 producing B cells in human pancreatic cancer and identifies a STAT3-mediated signaling pathway that may facilitate $CD8^+$T cell exclusion from the tumor microenvironment (71).

9. Therapeutic potential.

Reflecting on the dual functionality of B cells, therapeutic strategies involving the activation and depletion of B cells have each been explored (Figure 2) (164). One branch of recent therapeutic strategies has centered around enhancing B cell stimulation of T cells. Vaccination with antigen-loaded $CD40^+$ B cells resulted in the induction of specific T cell responses (150). Similarly, α-CD40 activated B cells from cervical cancer patients triggered anti-tumor T cell responses (66). Additionally, CpG-ODN (165), a fusion cytokine of GM-CSF and IL-4 (166), and tumor-derived autophagosomes known as “DRibbles” (167, 168), have all been implemented as therapies to activate B cells and foster the successful activation of T cells.

On the other hand, depletion of B cells as a means of mitigating their regulatory function in cancer has also been evaluated. While the broad depletion of $CD20^+$ cells using α-CD20 was deemed advantageous in select studies on colon cancer, melanoma, and cutaneous T cell lymphoma, other studies identified small, or in some cases, detrimental effects (161, 169–173). This potentially reflects ability of B cells to both promote and/or inhibit tumor growth (174, 175). CD20 antibodies do not reliably discern between effector and regulatory B cells. Attempts at targeted reduction of Breg populations has been the recent focus of several therapies, but seem to target mechanisms common to all B cells (161, 176–181). With mounting evidence supporting an immunosuppressive role for B cells in cancer, tailored treatments targeting Breg function have promising clinical implications. Implementing an IL-35 blockade in combination with immunotherapy may be another therapeutic avenue to explore (71). Additionally, based on our findings supporting a role for STAT3 activation in facilitating reduced $CD8^+$ T cell infiltration, incorporation of a STAT3 inhibitor may help enhance $CD8^+$ T cell mediated responses (71). While more work is needed to develop these strategies, selectively targeting Bregs and their regulatory functions, has the potential to increase efficacy of cancer immunotherapies.

10. Conclusion and outstanding questions.

While recent findings suggest that rational targeting of B cell function may be beneficial for cancer immunotherapy, there is still a lot to be understood. There is a clear need to identify tumor-associated drivers that promote effector versus regulatory B cells. This could be enabled by engineering pre-clinical models that make use of well-defined antigens, allow enough time to generate both conventional and innate B cell responses, and test the

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functional relevance of specific B cell subsets in cancer. Use of regulatory cytokine reporter mice will help to discern the timing and localization of Bregs and could be further fleshed out in the patient population using multiplex staining on existing tumor samples. Studies should also be designed that address the role of the tumor microenvironment composition on the polarization of B cells.

Available patient data needs to be harnessed to understand how B cell function may be regulated in distinct cancer types by studying relevance of driver mutations, mutational load, and microenvironment contexture. Identification of tumor-associated antigens and an enhanced investigation into immunoglobulin repertoire data will enable better understanding of how tumors drive functional specification of B cell subsets. This could be achieved by single cell sequencing in combination with B cell receptor profiling to identify unique markers, transcription factors, and/or chemokine pathways that define Breg populations. Better modeling and mechanistic understanding of how tertiary lymphoid structures form in cancer will allow for vaccination strategies that favor formation or expansion of functional TLS without eliciting Breg responses. It would also be informative to understand to which extent activated Bregs cycle in and out of tumor bed to influence systemic anti-tumor immune responses. In addition, B cells express a multitude of receptors that respond to environmental cues, such as changes in microbial composition. Thus, the influence of bacterial and viral history with regards to shaping of B cell responses in cancer should be carefully considered. Commonly used cancer treatments, such as chemo and radiation therapy likely affect B cell numbers and functionality, and these effects are not well understood. Investigations into how current immunotherapeutic approaches affect the balance of pro- and anti-tumor B cell responses will also be of tremendous benefit. The field of regulatory B cell biology in cancer has a lot of work left to do, but the momentum is clearly here, and we should look forward to more mechanistic insights into contributions of B cells to cancer emergence and progression.

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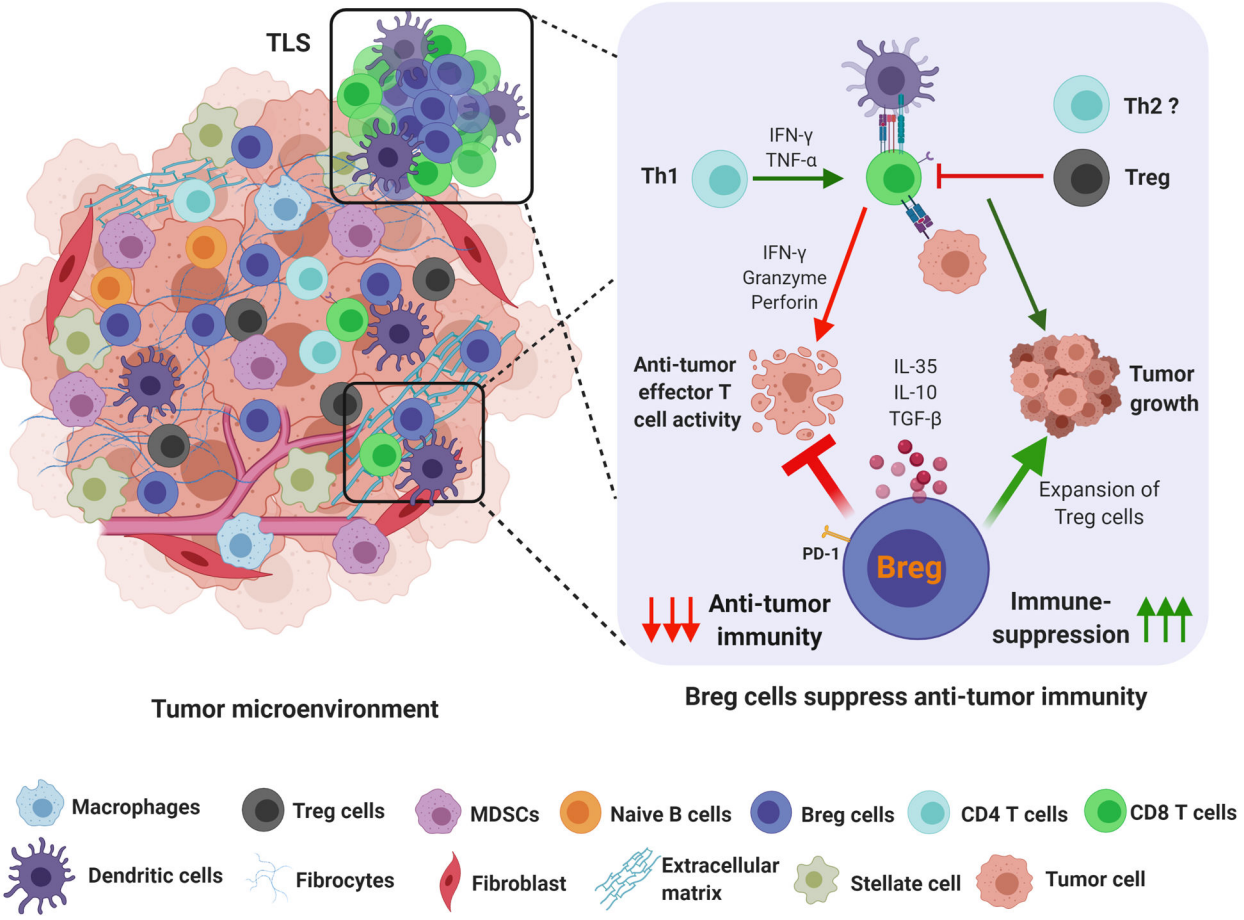

Figure 1. Breg cells are suppressors of anti-tumor immunity.

The tumor immune microenvironment is highly immunosuppressive. Recent reports indicate that apart from MDSC and regulatory T cells, subsets of immunosuppressive B cells (Breg) are important for controlling anti-tumor immune responses. Bregs expand intratumorally, in tertiary lymphoid structures, draining lymph nodes, and major secondary lymphoid organs, and can produce immune suppressive cytokines such as IL-35 and IL-10 in response to tumor-associated inflammation. B cell-derived IL-35 can dampen the effector T cell response by inhibiting effector $CD4^+$ and $CD8^+$ T cell responses and enhance the proliferation of Treg cells to simultaneously affect multiple arms of the anti-tumor immune response.

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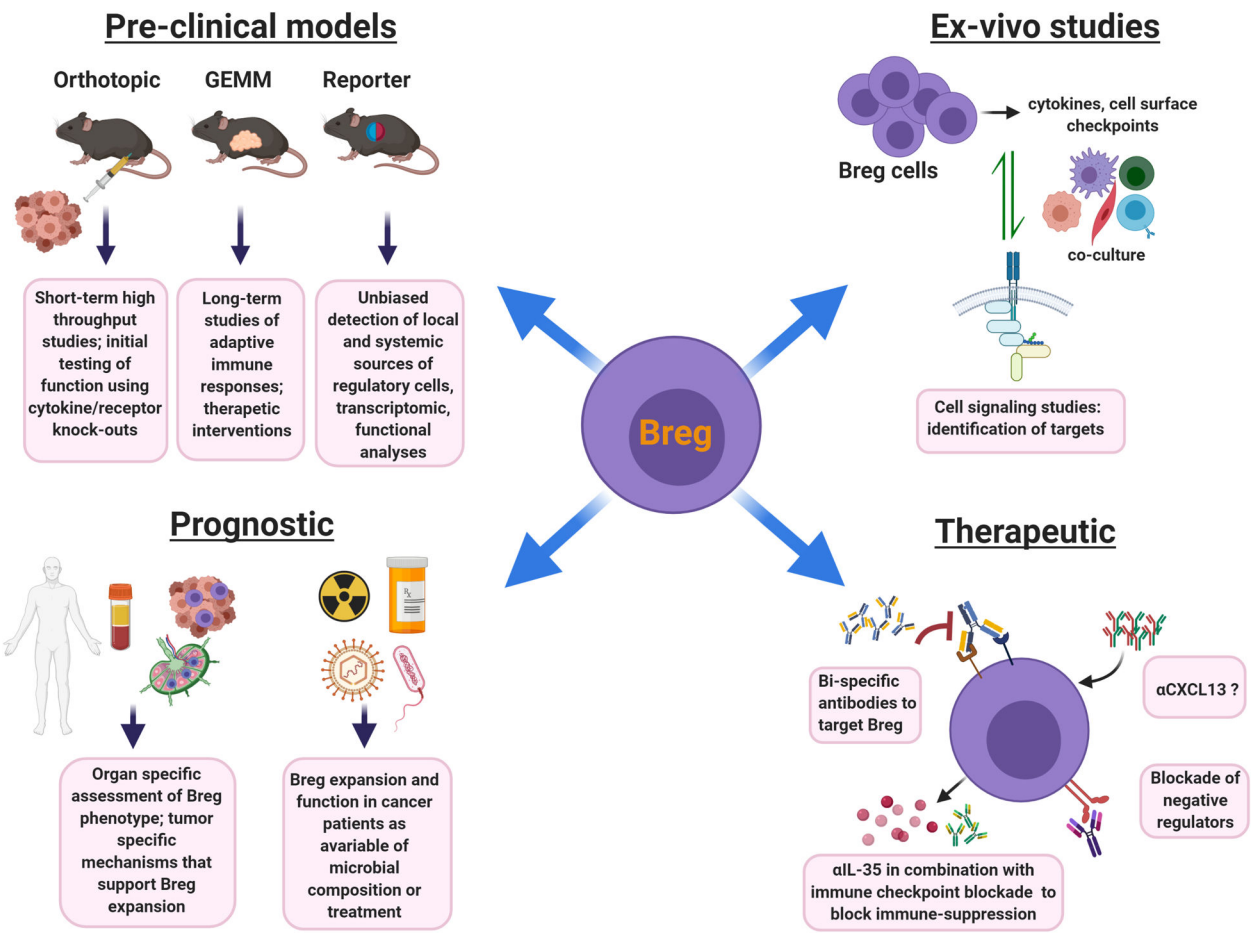

Figure 2. Understanding Bregs: pre-clinical models and translational potential.

Pre-clinical models: Several types of *in vivo* modeling approaches can be utilized to study Breg biology in cancer. Orthotopic mouse models involving transplantation of syngeneic tumor cells are quick to generate and B cell-specific knock-out strains can be used to study gene functionality in tumor growth. Limitations include short-life span and the need to characterize the ability of existing cancer cell lines to initiate B cell responses. GEMM models take longer to develop and can be useful for long-term studies of adaptive immune responses, tertiary aggregate formation, and pre-clinical testing of Breg targeted therapeutics. Limitations are time-intensive breeding and the potential tolerization of the immune system to certain cancer-associated antigens. The reporter models are useful for unbiased identification of Breg subsets, as well as studies on timing, migration patterns, and localization of Bregs in cancer. Limitations include differential lifespan of reporter protein versus that of the cytokine. **Signaling studies**: *Ex vivo* analysis using co-culture of Breg cells, T cells, cancer cells, and/or other cellular components of the tumor microenvironment allows for the testing of crosstalk mechanisms between cell types. **Prognostic studies**: IL-35^{+} Breg frequency in peripheral blood of cancer patients can serve as a potential prognostic determinant of the immunosuppressive nature of the tumor microenvironment. More research needs to be done to determine how Breg phenotype and functional attributes may be adjusted based on their localization in blood, tumor, and/or lymphoid aggregates and secondary lymphoid tissues. Exposure to immuno-, chemo-, and radiation-therapy as well as

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the microbial contexture of the cancer patient may influence frequency and function of Bregs and should be further studied. **Therapy**: Strategies that specifically target Bregs and their functional outputs are more likely to boost the anti-tumor immune response. Neutralizing IL-35 antibody treatment could decrease Breg function and enhance the infiltration and cytotoxic activity of $CD8^+$ T cells within tumors as well as synergize with checkpoint blockade. A better understanding of Breg-specific markers may lead to the development of antibodies to deplete these immunosuppressive subsets. Neutralizing α-CXCL13 can potentially block B cell infiltration into the tumor microenvironment, however, α-CXCL13 may also have prominent effect on conventional B cell function and formation of productive tertiary lymphoid structures, therefore such treatment may need to be tailored to tumors with prominent Breg activity.

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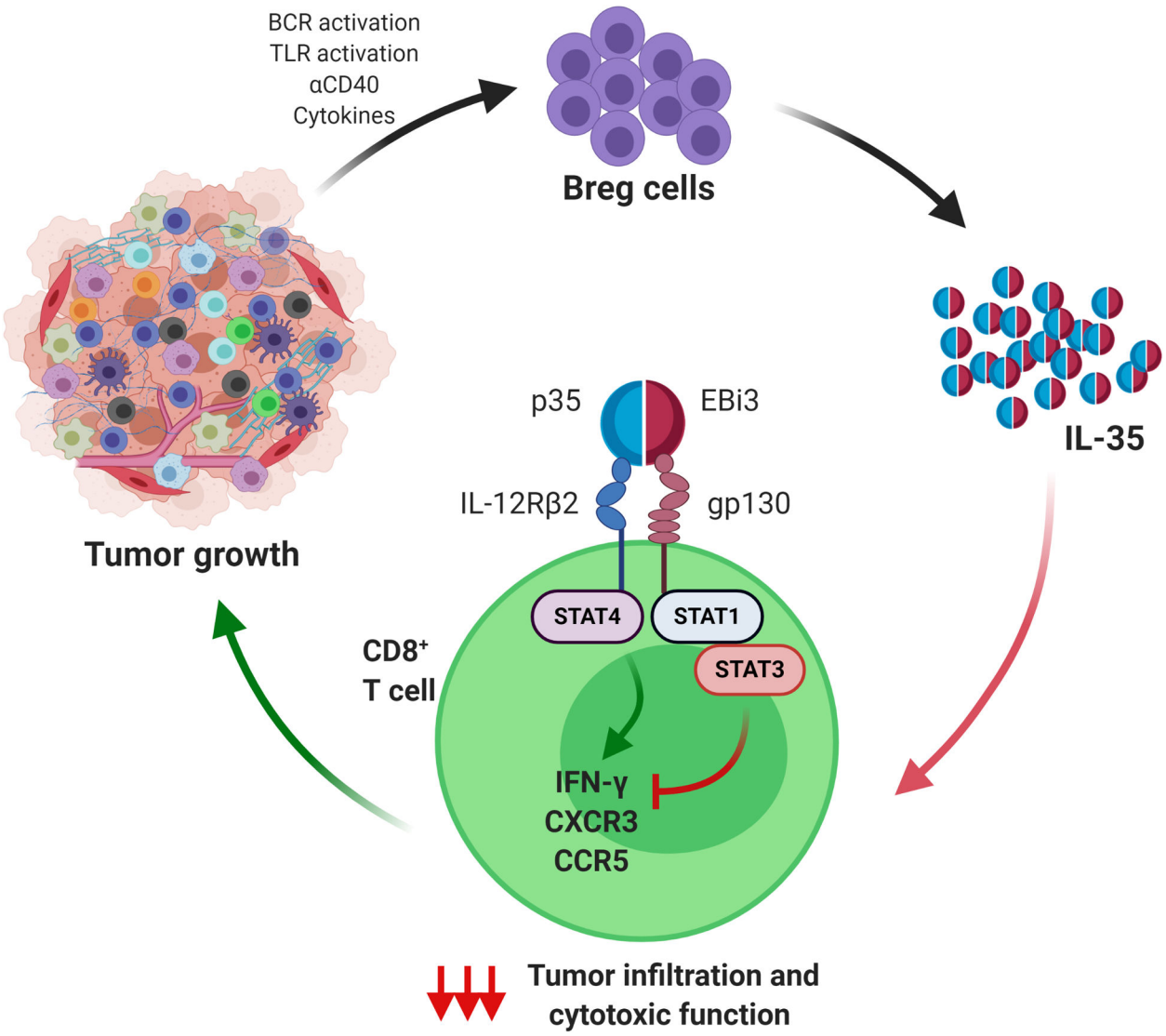

Figure 3. IL-35$^+$ Bregs control CD8$^+$ T cell activity in pancreatic cancer.

Breg-derived IL-35 (formed by the dimeric subunits p35 and EBi3) signals to CD8$^+$ T cells via IL-12Rβ2 and gp130 heterodimeric receptors. IL-35-gp130-mediated signaling enhances STAT3 activity in CD8$^+$ T cells. Activated STAT3 dampens expression of chemokine receptors CXCR3 and CCR5 and expression of the effector cytokine IFNγ in CD8$^+$ T cells. This results in decreased ability of CD8$^+$T cells to infiltrate tumors and produce functional responses. While stimulation of IL-12Rβ2 is known to activate STAT4 and potentiate cytotoxic function of CD8$^+$ T cells, the presence of IL-35-activated STAT3 leaves the STAT4 signaling arm ineffective and the overall result is the reduction of cytotoxic capacity of CD8$^+$ T cells.

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Table 1.

Regulatory B cells identified in murine models of cancer

Cancer Type	Surface Markers	Location	Effector Function	Reference
Pancreatic Ductal Adenocarcinoma	$CD19^{+}CD1d^{Hi}CD5^{+}$	Tumor	IL-35	69
Pancreatic Ductal Adenocarcinoma	$CD19^{+}CD1d^{Hi}CD5^{+}CD21^{Hi}$	Tumor	IL-35	70, 71, 72
Prostate Cancer	CD20+	Tumor	Lymphotoxin	77
Prostate Cancer	$CD19^{+}CD20^{Lo}B220^{Lo}IgA^{+}PD\text{-}L1^{+}$	Tumor	IL-10	78
Hepatocellular Carcinoma	$CD19^{+}B220^{Lo}CD138^{+}IgA^{+}$	Tumor	PD-L1	80
Breast Cancer	$CD19^{+}B220^{+}CD25^{+}CD69^{Hi}$	Tumor	TGF-B	82
Breast Cancer	$PD\text{-}L1^{Hi}$ $CD86^{Hi}$ IAd^{Hi} $CD62L^{Hi}$ $LAP^{+}CD44^{Lo}$	Tumor	TGF-B, PD-L1	83
Squamous Carcinoma	$CD19^{+}CD21^{Hi}$	Tumor	IL-10	85
Non-Hodgkin Lymphoma	$CD19^{+}CD1d^{Hi}CD5^{+}$	Tumor	IL-10	86

Instances of regulatory B cells observed in murine models of cancer.

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Table 2.

Regulatory B cells identified in human cancer

Cancer Type	Surface Markers	Location	Effector Function	Reference
Pancreatic Ductal Adenocarcinoma	$CD20^{+}$	Tumor	IL-35	72
Pancreatic Ductal Adenocarcinoma	$CD19^{+}CD24^{Hi}CD38^{Hi}$	Peripheral Blood	IL-35, IL-10	71
Hepatocellular Carcinoma	$CD19^{+}CD5^{Hi}CD27^{Hi}CD38^{Dim}$	Tumor	PD-1, IL-10	81
Oesophageal Carcinoma	$CD19^{+}CD5^{+}$	Peripheral Blood	IL-10	157
Ovarian Carcinoma	$CD19^{+}CD20^{+}$	Ascites	IL-10	160
Ovarian Carcinoma	$CD19^{+}$	Tumor	Granzyme B	155
Tongue Squamous Carcinoma	$CD19^{+}$	Tumor, Lymph Node	IL-10	20
Gastric Cancer	$CD19^{+}CD24^{Hi}CD38^{Hi}$	Peripheral Blood, Tumor	IL-10, TGF-β1	161
Gastric Cancer	$CD19^{+}CD24^{Hi}CD27^{+}$	Peripheral Blood, Tumor	IL-10	162
Gastric Cancer	$CD19^{+}$	Peripheral Blood	IL-35	101
Breast Carcinoma	$CD19^{+}$	Tumor	Granzyme B	155
Breast Carcinoma	$CD19^{+}$	Tumor	IL-10, CD25	159
Cervical Carcinoma	$CD19^{+}$	Tumor	Granzyme B	155
Head & Neck Squamous Carcinoma	$CD19^{+}CD24^{Hi}CD38^{Hi}$	Tumor	IL-10, CD25	160
Esophageal Squamous Carcinoma	$CD19^{+}$	Tumor	IL-10, PD-1	161

Instances of regulatory B cells observed in human cancer.