Chapter 2
B Lymphocytes in Cancer Immunology

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Abstract The role of B lymphocytes in the pathogenesis and treatment of cancer has not received as much attention as the role of T cells. However, most patients with solid tumors harbor circulating antitumor antibodies and most tumors contain a population of infiltrating B cells implying an association between oncogenic events and B-cell activation. B-cell immunity can be beneficial by providing antibody-mediated protection from oncogenic viruses or a source of recombinant tumor-specific antibodies that can be used in combination with chemotherapeutic regimens. However, activation of B cells may also be detrimental to an effective antitumor response. Tumor-reactive antibodies and B cells often recognize antigens that are generated during the unscheduled apoptotic and necrotic death processes, which accompany tumor progression and may be involved in wound-healing processes that promote tumor growth and impair protective T-cell responses. Therefore, methods to eliminate autoreactive B cells, or switch them to a B effector-1 (Be-1) phenotype that amplifies Th1/Tc1-type T-cell responses, which are typically associated with effective antitumor responses, may improve the clinical outcomes of T-cell-mediated immunotherapies. Possible strategies include the administration of B-cell-depleting monoclonal antibodies, use of targeted B-cell stimulatory agents such as Toll-like Receptor agonists, and adoptive transfer of large numbers of *ex vivo* generated tumor-reactive Be-1 cells.

Keywords B lymphocytes • Cancer vaccines • Chronic lymphocytic leukemia • Regulatory B cells • Tumor immunology

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Introduction

Current immunotherapeutic strategies in experimental cancer are focused primarily upon the augmentation of T-cell immunity. Nonetheless, recombinant antibodies, which represent a product of B cells, are playing an increasing role in current clinical cancer therapy [1]. However, B cells themselves have not been studied exhaustively in terms of their potential role in tumorigenesis or suitability as therapeutic targets. One historical reason for this "T-cell-centric" view of cancer biology was the early availability of reagents, such as CD4 and CD8 antibodies, which allowed T cells to be classified into different functional subsets, thereby facilitating detailed studies of T-cell-mediated effects. By comparison, the study of human B-cell biology was delayed for some years due to the lack of similar reagents to clearly differentiate B-cell subsets [2]. In addition, while B cells have long been known to produce antibodies, their ability to act as effector cells in an immune response has only been recognized relatively recently [3, 4]. The following emerging research findings indicate that: (1) B cells have a major impact on tumorigenesis; (2) targeting B cells may improve the efficacy of T-cell-mediated immunotherapy, and (3) B cells themselves may have important antitumor activity in some settings. The purpose of this chapter is to discuss how some of this new information might be incorporated into the design of future cancer immunotherapeutic strategies. Although B cells can clearly undergo malignant transformation into lymphomas and leukemias, the discussion here will focus on the modulatory effects of normal B cells on solid tumor biology, with an additional focus on clinical results in humans.

Peripheral Human B-cell Development

The majority of lymphocytes in the blood are T cells, making up 22–30% of total nucleated white cells. Circulating B cells represent only 7–10% of white blood cells and consist of a number of different subsets that participate in immune responses in secondary lymphoid tissues and at sites of tumor formation [5]. Approximately 75% of circulating B cells do not express CD27, indicating that they have recently emerged from the bone marrow and have not yet encountered antigen in the periphery (see Fig. 2.1). The *Ig* locus of these cells is germ-line indicating they have not yet undergone the somatic hypermutation process in germinal centers that increases the affinity of their B-cell receptors (BCRs) for specific antigens. CD27-negative B cells can be divided into transitional, prenaïve, and naïve B cells on the basis of their expression of CD38 (Fig. 2.1) [6]. Transitional B cells, which have recently emerged from the bone marrow and constitute about 2% of circulating B cells, express high levels of CD5, CD38, IgM, and IgD and are enriched for cells with autoreactive BCRs. Prenaïve B cells comprise approximately 7% of circulating B cells and have lower levels of CD38 but continue to express CD5, IgM, and IgD.
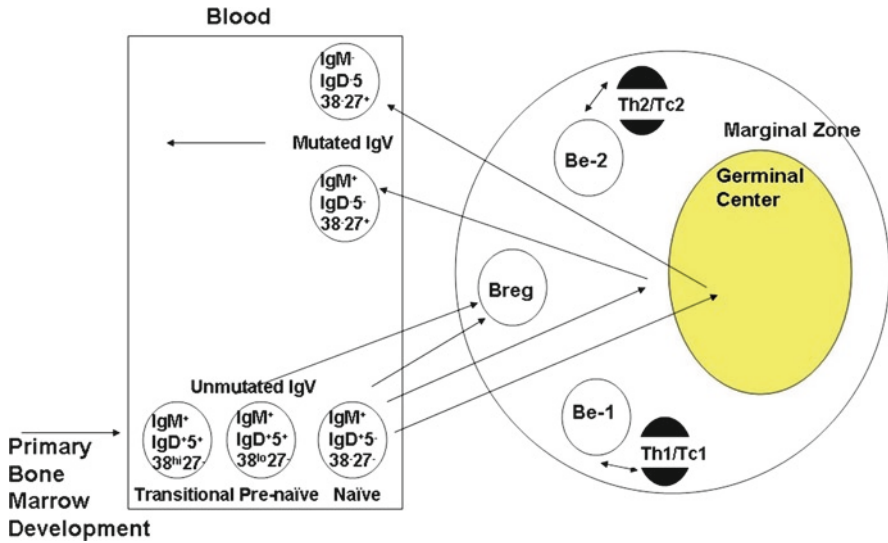
Fig. 2.1 Peripheral B-cell development. As described in the text, antigen-inexperienced primary B cells that have been selected in the bone marrow enter the blood as transitional, prenaïve, and naïve cells that undergo further differentiation in germinal centers and marginal zones of secondary lymphoid organs under the control of antigen. Memory B cells with mutated immunoglobulin variable genes then enter the recirculating pool. Possible sites of development of Bregs and effector B cells are also indicated

Transitional and prenaïve cells are thought to represent intermediate stages before B cells become naïve cells that are competent to respond to foreign antigens. Possibly because of their expression of CD5, which inhibits signaling through the BCR [7], transitional and prenaïve cells exhibit impaired calcium release and undergo activation-induced cell death in response to BCR cross-linking. In contrast, naïve B cells proliferate upon antigen activation. Unlike naïve cells, transitional and prenaïve B cells also undergo spontaneous apoptosis when placed in culture without exogenous stimulatory signals. This predisposition to die in response to antigenic signaling or absence of trophic factors is thought to ensure that transitional and prenaïve cells have a limited survival *in vivo* unless they encounter an antigen that they recognize and that the process of culling auto-reactive cells, initiated during primary development in the bone marrow, is continued in the periphery [8]. However, transitional and prenaïve cells can receive pro-survival signals via cytokines, such as IL-4, IL-10, and IL-21, and costimulatory molecules, such as CD40 [6]. Accordingly, such B cells may persist at sites of inflammation where their auto-reactivity may influence the outcome of immune responses and contribute to immunopathology [9], which may include antitumor immunity (see below).

Prenaïve cells lose expression of CD38 and CD5 and mature into naïve cells, which constitute around 65% of circulating B cells [6]. CD38$^-$CD5$^-$CD27$^-$IgM$^+$IgD$^+$ naïve cells acquire the ability to respond to antigenic signals through their BCR by proliferating and differentiating into short-lived plasma cells that secrete IgM

antibodies. Other B cells of the activated clone mature into memory cells in the germinal centers through the processes of somatic hypermutation and class-switching, which are under the control of T cells (Fig. 2.1). Memory B cells are long-lived, respond more strongly to subsequent antigenic stimulation compared to naïve cells, are characterized by expression of CD27 in the absence of CD38 or IgD, and comprise approximately 25% of circulating B cells. Some memory cells continue to express IgM and do not undergo class-switching, despite acquiring mutations in their Ig V region genes. Such cells, which are classified as IgM[+] memory B cells [10], are thought to take part in T-cell-independent responses to polysaccharide antigens and represent circulating marginal zone B cells. By comparison, classical memory B cells undergo class-switching in the germinal center, down-regulate IgM expression, use one of the IgG subtypes, IgA, or IgE genes to form the heavy chain of their antigen receptor, and ultimately recognize protein antigens under the control of helper T cells (Fig. 2.1).

B-Cell Effector States

In addition to their well-known ability to differentiate into plasma cells and secrete antibodies, B cells also influence immunity by serving as antigen-presenting-cells (APCs). Naïve B cells are thought to represent an immunosuppressive type of APC because they have been shown to tolerize T cells that interact with them [11, 12]. However, under appropriate conditions that may involve CD40 ligation and cytokine signaling, a naïve B cell can serve as a relatively potent APC that expresses costimulatory molecules such as CD80, CD86, and ICOS, and activates both CD4^{+} and CD8^{+} T cells [13].

B cells exert effector functions not only through the production of antibodies, but also by making cytokines [14]. As a result of interactions with T cells, B cells can be directed to secrete polarized groups of cytokines that parallel those of the dichotomous Th1/Tc1 and Th2/Tc2 differentiation states that exist within T-cell subsets [15]. B effector 1 (Be-1) cells arise through interactions with Th1/Tc1-type T cells and secrete cytokines characteristic of this type of immune response, including IFN-γ, IL-12 and TNF-α. In contrast, B effector 2 (Be-2) cells arise through interactions with Th2/Tc2-type T cells and secrete a polarized pattern of cytokines that includes IL-2, IL-4, IL-6, IL-13, and TNF-α. Through cross-talk with interacting T cells, these polarized B effector states serve to differentially reinforce and amplify Th1/Tc1-type T cells that promote cellular immunity or Th2/Tc2-type T cells that promote humoral immunity [16].

Further research will be required to define completely the precursor cells that give rise to effector Be-1 and Be-2 cells and characterize the molecular mechanisms that drive B cells into these states. Not surprisingly, in view of the association with T-cell interactions, effector B cells are thought to originate from recently activated naïve B cells that enter germinal follicles to begin the processes of somatic hyper-mutation and class-switching [14]. Subsets of recirculating memory B cells may be

already programmed to develop into cytokine-producing Be-1 or Be-2 cells [17]. Be-1 differentiation is thought to result from signaling through IFN-γ receptors on B cells [18] which induces the transcription factor T-bet to regulate gene expression in Be-1 cells in a manner analogous to the role it plays in regulating gene expression in Th1/Tc1-type T cells [19]. In contrast, signaling through the IL-4α receptor is thought to control B-cell differentiation towards Be-2 cells [20]. Because pro-inflammatory cytokine production by human B cells is enhanced by phorbol esters [13, 21], strong activation of mitogen-activated protein kinase (MAPK) signaling pathways may be needed for effector B-cell differentiation [22]. This MAPK activation may be contributed by a variety of signaling complexes on the B-cell surface, including the antigen receptor, MHC molecules [19, 20], and concomitant signaling through multiple toll-like receptors (TLRs) [23] or through a combination of TLRs and cytokine receptors [14, 21].

B cells can differentiate into regulatory cells (Bregs) that are characterized by production of immunosuppressive cytokines such as IL-10 and TGF-β [24]. In contrast to effector B cells, which amplify T-cell responses, IL-10 secreting B cells have been demonstrated to dampen effector T-cell responses in a variety of experimental situations [24], including the inhibition of immune responses against tumors [25]. The cellular origins and molecular mechanisms accounting for Breg differentiation are incompletely understood. Unlike effector B cells, which differentiate in the germinal follicle, it has been reasoned that Bregs develop from marginal zone B cells, or perhaps from CD5+ transitional or prenaïve cells [26]. In mice, CD5-expressing cells of the so-called B1-B cell lineage are thought to give rise to Bregs [14]. However, the existence of the analogous cell lineage in humans remains uncertain. Production of IL-10 by some human B cells is associated with strong activation of the transcription factor, STAT-3 [21]. The tone and duration of MAPK signaling may also determine if B cells acquire regulatory functions. When B cells of marginal zone origin are treated only with IL-2 and a TLR-7 agonist, they produce little TNF-α but make the high levels of IL-10 associated with the Breg phenotype. However, if the cells are concomitantly treated with diacylglycerol mimetics, which activate ERK via Ras guanyl nucleotide-releasing proteins (RasGRPs) [27], IL-10 is "switched off," both TNF-α and-β production are increased, and the B cells acquire strong T-cell stimulatory capabilities [21].

In addition to their ability to make antibodies and cytokines and serve as APCs, activated B cells can acquire cytotoxic capabilities that may be of importance for antitumor immunity. For example, an Epstein-Barr Virus (EBV)-infected B cell line established from a breast cancer biopsy was shown to lyse breast cancer cells *in vitro* [28]. However, other activated B cells can kill activated T cells and may thereby inhibit T-cell-mediated responses [29]. Killer B cells often express molecules that are characteristic of Breg cells, including CD5, IL-10, and TGF-β. These observations suggest that Bregs may exert their inhibitory effects via both immunosuppressive cytokine secretion and direct lysis of T cells. The mechanism of killing can occur through diverse TNF and TNF receptor (TNFR) family members such as Fas ligand (CD178) and Fas, TRAIL (CD253) and its death receptors such as DR5 (TNFRSF10B or CD262), and programmed death ligands 1 and 2 (PDL1:CD274

and PDL2: CD273) [14]. In addition, some human B cells stimulated by IL-21 together with TLR or BCR agonists express granzyme B [30] and may thereby kill through perforin-mediated mechanisms typically associated with cytotoxic CD8+ T cells or NK cells [31].

B Cells and Cancer

Evidence that B-cell activation is connected to cancer progression comes from an extensive literature on the presence of antibodies that recognize tumor antigens in cancer patients and a much smaller literature on the infiltration of tumors by B lymphocytes.

Serology

Circulating antibodies that recognize antigens expressed by cancer cells have been found in most patients with solid tumors [32, 33]. Using SEREX technology, where patient sera is used to screen recombinant cDNA libraries obtained from tumors, over 2,500 different proteins are listed in the Cancer Immunome database [http://ludwig-sun5.unil.ch/CancerImmunomeDB/] from breast, gastric, renal, lung, prostate, hepatic, and ovarian cancer, as well as melanoma, mesothelioma, sarcoma, neuroblastoma, lymphomas, and leukemias. Most of these antigens are ubiquitous cytoplasmic proteins such as actin, cytokeratin, DNA polymerases, and heat-shock proteins. They are not tumor-specific and would be mainly protected from circulating antibodies by their predominantly intracellular location, although such antigens can be externalized during inflammatory and apoptotic processes that accompany tumor growth (see below) [34]. Accordingly, antibodies that target these antigens would not seem capable of mediating therapeutic antitumor responses. It is possible that the relative inability to detect cell surface antigens that are more accessible to antibodies relates in part to the use of bacteria to express mammalian cDNA in SEREX assays. Bacteria lack glycosylation enzymes and are therefore unable to make glycoproteins found on the plasma membranes of eukaryotic cells [35].

Other techniques, distinct from SEREX methods, have been used to characterize naturally arising anticancer antibodies in human patients. Using a "candidate" antigen approach, antibodies to cell surface receptors, such as the HER-2/*neu* epidermal growth factor receptor (EGFR) which is overexpressed on 25–50% of breast tumors, are found in the sera of nearly a quarter of patients [36]. By making hybridomas from B cells in draining lymph nodes, or from tumor-infiltrating B cells (TIBs), antibodies that recognize cell surface glycoproteins and cytoplasmic proteins have been identified [35]. More recently, the specificities of B cells that infiltrate solid tumors have been identified by amplifying Ig V regions, cloning and sequencing these rearranged genes, constructing combinatorial libraries of single-chain variable region gene fragments (scFVs), and then selecting for tumor-binding capacity [37]. Using such approaches, it has been shown that some antitumor responses are directed

against glycolipid antigens. However, even these sophisticated techniques continue to demonstrate that many antibodies made by tumor-infiltrating B cells recognize intracellular autoantigens, such as actin [38], that become externalized during apoptotic processes or are oxidized or proteolytically degraded during apoptosis [34]. Taken together, these observations suggest that intratumoral B cells, as well as B cells in organized lymphoid tissues that make circulating antibodies in cancer patients, often recognize structures associated with apoptosis and cell death which are processes that accompany tumor progression [39, 40].

Tumor-Infiltrating B Cells

Lymphocytic infiltrates are found in most solid tumors. The dominant cell population is usually T cells; in general, the more T cells that are found in a tumor, the better the prognosis [41, 42]. B cells are also a component of intratumorallymphocytic infiltrates, albeit usually a minor population compared to T cells. However, in early ductal breast carcinoma in situ, infiltrating B cells are found in excess of T cells and form the predominant intratumoral lymphocyte population [43]. It is also interesting to note that medullary breast cancer, which constitutes 3–7% of all breast cancers and has a favorable prognosis compared with other types of infiltrating ductal carcinomas, is characterized by infiltrates of B cells and plasma cells [38], along with T cells [37]. Tumor-infiltrating B cells (TIBs) are also found in other types of breast cancer [44] and other cancers including melanoma [45], lung cancer [46], and mesothelioma [47].

   B cells can enter tumors in response to chemoattractants produced during the inflammation that accompanies, and may even cause, tumor progression [48]. However, by cloning rearranged immunoglobulin genes in tumor biopsies and comparing VH gene usage and the mutation status of *Ig* genes, it appears that intratumoral B cells are related and selected by antigen responses *in situ*, rather than being recruited nonspecifically from the blood into the tumor [38]. Given the antigen-specificity of the antibodies made by some TIBs described above, it seems possible that intratumoral B cells may often be responding to antigens on apoptotic bodies or to intracellular proteins that have been degraded by proteases or oxidized during the inflammatory processes inside a tumor. However, there is little information as to whether these intratumoral B cells are Be-1 cells, Be-2 cells, or Bregs (see above).

B Cells and Cancer: Friends or Foes?

While the evidence that B cells and their antibody products are associated with cancer seems clear, whether this association is protective, causal, or simply incidental has not been clarified. The answer seems to depend in part on how early the cancer is in its development. Most evidence suggests that, once the tumor is established, B cells probably have a negative effect on protective antitumor responses and may even facilitate tumor progression.

While cancers are characterized by collections of aberrant genetic events that corrupt signaling pathways and interfere with normal cell death processes [49], cancer progression is also intimately intertwined with inflammation [48]. Agents that cause cancer, such as cigarette smoke in lung cancer [50], ultraviolet light in skin cancers [51], ulcerative colitis in colon cancer [52], and micro-organisms such as *Helicobacter pylori* in intestinal cancers [53], *Hepatitis* B and C in hepatomas [54], and Human *papilloma virus* (HPV) in cervical cancer [55], are also associated with chronic inflammation. Inflammation may provide signals that promote growth of genetically-aberrant cells and may further select for more aggressive tumor cells by increasing genetic instability [48].

As cancers grow, inflammatory processes seem to become self-sustaining. Because of the break-down in control mechanisms that prevent unrestrained cellular proliferation, tumor cells continue to grow beyond the limits that are normally supported by environmental nutrients and blood supply [56]. However, even tumor cells with impaired cell death pathways cannot grow indefinitely in nutrient-poor conditions and undergo "unscheduled" apoptotic or necrotic death [39]. Apoptosis has important consequences for antitumor T-cell responses as it has been associated with peripheral tolerance mechanisms and the deviation of immune responses away from protective Th1/Tc1-type responses [57]. By comparison, necrosis causes inflammation, which leads to production of chemokines and cytokines associated with wound repair. These repair mechanisms can then be used by the tumor cells for further growth and another round of the wound-repair cycle [58]. This type of biology has led to the idea that tumors are analogous to "wounds that do not heal" [59, 60]. Although this model is clearly oversimplified, these general principles of how cancers develop are of some relevance in trying to better understand the role of B cells in tumor progression.

Evidence for a Protective Effect of B Cells in Antitumor Responses

As described above, B cells can potentially inhibit the development and progression of cancers by making antitumor antibodies or by differentiating into appropriate effector B-cell states. B-cell-derived antibodies play an essential role in protection against viral infections. In this context, B cells can protect against tumor development by helping to clear oncogenic viruses before they can become established and initiate tumor development. An excellent example of this is the use of HPV vaccines to prevent cervical cancer [61].

Recombinant antibodies have clearly been shown to contribute to the clearance of established tumors in patients. The efficacy of antibodies against CD20 (Rituximab®) in lymphoma [62] or HER-2/*neu* (Herceptin®) in breast cancer [63] have resulted in almost paradigmatic changes in treatment strategies for these cancers. Similarly, antibodies against angiogenic factors, such as VEGF, slow progression of metastatic disease [64] and antibodies against glycolipid gangliosides that are

overexpressed on cancer cells, particularly melanoma, are under clinical investigation [65]. The therapeutic activity of these antibodies can be increased even further by coupling them to toxins such as radioactive isotopes or cytotoxic proteins of bacterial or plant origin [66]. Antibodies with similar specificities as the recombinant antibodies can be demonstrated to arise naturally in cancer patients [35]. The levels of naturally arising antibodies are generally very low and well below the therapeutic concentrations that can be obtained by injecting recombinant antibodies. Accordingly, it seems unlikely that naturally arising antitumor antibodies can be effective in clearing established tumors and, as discussed below, may even promote tumor growth as a result of their low concentrations. However, vaccines that enhance endogenous production of these antibodies might have therapeutic potential, a concept that has been validated in an experimental model where vaccination with a recombinant adenovirus expressing a truncated HER-2/*neu* antigen resulted in sufficient antibody production to block HER-2/*neu* function and clear subcutaneous HER-2/*neu*-expressing breast cancers in mice [67].

Several experimental models demonstrate a possible protective role for B cells against tumors that may be attributable to effector B cells. Lung metastases caused by intravenous injection of the chemically induced rat mammary adenocarcinoma, MADB106, are significantly increased when host B cells are depleted by specific antibodies [68]. The mechanism in this model seems to be a local effect of pulmonary B cells, which promote IFN-γ production and facilitate killing of tumor cells by NK cells [69]. It is possible that the protective cells in this model may represent Be-1 cells. In a mouse model, J558L plasmacytoma cells engineered to overexpress lymphotoxin (TNF-β) were cleared in syngeneic BALB/c mice through B-cell-dependent mechanisms because the tumors were significantly infiltrated with lymphocytes that expressed B220 (a B-cell marker) and failed to grow in nude mice (which lack T cells but contain B cells) but did grow in SCID mice (which lack both T and B cells) [70]. These findings are again suggestive of a role for effector B cells in tumor clearance. Similarly, a fusion of a tumor-specific antibody (directed against the human EGFR) and lymphotoxin prevented pulmonary metastases following intravenous injection of the human melanoma cell line, M24met, in nude mice (but not SCID mice). This therapeutic effect was accompanied by infiltration of B220+ cells into the metastases [71]. Taken together, these observations suggest that B cells can protect against cancers under certain conditions. However, the experimental models may have limited application to the clinical setting, which typically involves treating established tumors rather than preventing tumor initiation.

Evidence for a Negative Effect of B Cells on Antitumor Responses

In principle, naturally arising antibodies against cell surface proteins, carbohydrates, and lipids might be expected to kill tumor cells by activating complement, causing antibody-mediated cellular cytotoxicity, or initiating signaling events that cause

apoptosis [72]. Such scenarios may occur in the early stages of cancer but it is almost impossible to study these types of "negative" situations in the clinic without the presence of an actual tumor. In practice, tumors progress despite the presence of circulating antitumor antibodies. A simple explanation for this situation is that the cell surface structures on tumor cells that are targeted by antibodies are autoantigens and, as a result of tolerance mechanisms, high-affinity antibodies cannot be made in sufficient titers to mediate an effective antitumor response.

Weak, humoral immune responses that fail to clear tumor cells may actually have a detrimental effect on the clinical outcome by contributing to the inflammatory responses that drive tumor progression [73]. For example, transgenic mice that express HPV early region genes under the control of a human keratin 14 promoter exhibit multistage development of invasive squamous cell carcinoma of the epidermis. When they are crossed to Rag1$^{-/-}$ mice, which have a complete absence of functional B and T cells, tumorigenesis is markedly delayed and associated with reduced inflammation. Adoptive transfer of B cells or sera (which presumably contained antitumor antibodies) from the wild-type transgenic mice restored inflammatory cell infiltrates and tumor progression in premalignant lesions. These results suggest that antitumor antibodies cause inflammation that promotes the growth of cancer cells [74]. Similar concepts have been invoked to explain the role of antibodies to the foreign ganglioside, *N*-glycolylneuraminic acid (Neu5Gc), which accumulates in metabolically active cancer cells [75]. Injection of large amounts of anti-Neu5Gc antibodies slowed progression of Neu5Gc-bearing tumor cells but low amounts of antibodies promoted tumor growth. Tumor progression resulting from low levels of antibodies could be inhibited by cyclooxygenase-2 (COX-2) inhibitors, thereby suggesting that the antibodies induced an inflammatory state that promoted tumor growth.

Cell-mediated immunity, involving cytotoxic T cells, is generally thought to be the most important arm of the immune system for clearing established tumors [76]. Antibody production is primarily the result of humoral immunity that is promoted by Th2/Tc2-type T cells and B cells can promote antigen-driven responses to deviate towards Th2/Tc2-type responses [77]. Since Th2/Tc2 cells are not as efficient as Th1/Tc1 cells at clearing tumor cells, B cells are often considered detrimental to effective antitumor responses [25, 78]. However, the recent identification of Be-1 cells, which amplify Th1/Tc1-type T-cell responses [3], challenges this idea and suggests that only some B-cell effector states, presumably Be-2 cells and Bregs, are detrimental to effective antitumor immunity.

B cells have recently been found to play important roles in wound-healing [79]. Although B cells are not prominent components of cutaneous wounds, their removal by genetic means [79, 80] impeded the wound healing process by decreasing the production of cytokines, including TGF-β and IL-10. Furthermore, wound-healing was improved by adoptive transfer of IL-10 secreting B cells [80]. Given the concept of cancer as a "wound that doesn't heal" [59], these findings suggest that the small numbers of B cells found in cancer stroma might have properties of IL-10-secreting Bregs that promote tumor growth by both inhibiting local antitumor T-cell responses and promoting the processes of wound-healing [81].

B cells that participate in wound-healing are likely those that recognize antigens on apoptotic bodies and cytoplasmic proteins that have been oxidized or degraded by proteases, as such processes are associated with tissue damage. Such B cells would then secrete antibodies that cause apoptotic bodies to be cleared rapidly by monocytes and dendritic cells, limiting the presence of free autoantigens and inflammatory signals which cause immune responses and also ensuring these important APCs tolerize T cells rather than activate them [82]. A teleologic explanation for why B cells behave in this manner during normal wound healing might be to prevent toxic type 1 T-cell responses and scarring. While this behavior may preserve normal tissue functioning once a wound is repaired, analogous processes in a tumor microenvironment would inhibit clearance of tumor cells by T cells. Moreover, activation of B cells by apoptotic bodies could result in production of cytokines such as IL-10 and TGF-β that can inhibit T-cell responses and promote tumor growth. B cells can also be activated by adhesion molecules in an antigen-independent fashion [83], which could also lead to cytokine production, T-cell suppression, and tumor growth. For example, CD5$^+$IgM$^+$ B1-B cells that express the glycoprotein, MUC18 (also known as melanoma cell adhesion molecule), were found to bind B16 melanoma cells that also expressed MUC18 *in vivo* via MUC18/MUC18 interactions [84]. This heterotypic cell–cell interaction led to enhanced metastasis of the melanoma, perhaps by increasing ERK-signaling in the tumor cells. While the existence of B1-B cells in humans is still unclear, intriguingly, it was found that CD5$^+$IgM$^+$ cells (which may represent transitional or prenaïve B cells as described above) accumulated in biopsies from melanoma patients and correlated with MUC18 expression on human melanoma cells [84]. Taken together, these observations suggest that some types of intratumoral B lymphocytes may promote cancer progression by direct interactions with tumor cells.

Chronic Lymphocytic Leukemia as a Paradigm for Tumor Promotion by B Cells

Experiments in mice can be used to examine the role of B cells in tumorigenesis by removing B-cell populations via genetic or pharmacological means and adoptively transferring B-cell populations. Such experimental approaches can accentuate the typical effects of B-cell, allowing them to be uncovered in a "background" of competing physiological phenomena [79, 80]. In humans, this approach is obviously not feasible. However, a specialized clinical condition, chronic lymphocytic leukemia (CLL), may serve to illustrate some of the negative effects of B cells on solid tumors in humans.

CLL is the most common leukemia in the developed world. Chemotherapy is indicated for symptomatic disease but CLL patients are often asymptomatic and initial clinical management typically consists only of observation, sometimes for

long periods of time [85]. The disease consists of an expansion of monoclonal B cells that express CD5 and low levels of IgM. The originating cell-type of CLL is not clear but the presence of somatic hypermutation in the *Ig* locus and low expression of CD38 in about half the cases suggests a postgerminal center origin, possibly in memory IgM$^+$ cells (Fig. 2.1) [10]. On the other hand, the absence of somatic hypermutation and high CD38 expression in the remaining cases suggests an origin in transitional or prenaïve cells [86]. Regardless, BCRs on CLL cells-often recognize autoantigens such as rheumatoid factor, DNA, actin, and myosin, many of which are generated during inflammation and apoptosis [34, 87] and have been shown to be recognized by B cells that infiltrate solid tumors [38]. Moreover, CLL cells express high levels of IL-10 and TGF-β and characteristically suppress T-cell responses by a variety of mechanisms which include inhibiting CD40L signaling in T cells [88], killing T cells via Fas/FasL interactions [89], or disrupting immune synapses [90]. These properties have led some scientists to speculate that CLL may be a tumor of regulatory B cells [91]. Accordingly, insights into the effects of Bregs on solid tumor progression in humans may be provided by studying the behavior of solid tumors that arise in CLL patients.

Compared to other people, CLL patients have more than double the risk of developing solid tumors. These cancers are mainly squamous cell skin cancers but also include melanoma, prostate, breast, gastrointestinal, lung, and other tumors [92, 93]. This increased risk is independent of specific treatment for CLL and solid tumors often arise in patients who are being managed only by observation, suggesting that some intrinsic property of the increased monoclonal B cell population is responsible. It is possible that the regulatory properties of the CLL B cells may be preventing effective antitumor T-cell responses or perhaps may be encouraging inflammatory processes (from uncontrolled viral infections, for example) which promote tumor progression.

However, another clinical observation is that, when solid tumors arise in CLL patients, they are often much more virulent than usual [94, 95]. The explanation for this phenomenon is also not clear but may again be related to impairment of protective antitumor T-cell responses. However, it is interesting that CD5$^+$ B cells have been implicated in promoting melanoma progression in both mice and humans through direct interactions with melanoma cells [84]. CLL cells characteristically express CD5 and perhaps CLL cells use their autoreactive BCRs to bind to solid tumor cells, become activated, and produce cytokines that promote the growth of solid tumors.

Interestingly, the regulatory phenotype of CLL B cells seems to be somewhat plastic. For example, primary CLL cells can be grown in tissue culture in the presence of cytokines and TLR-agonists and maintain their suppressive features, such as IL-10 production and inability to stimulate T cells. However, in the presence of strong ERK-activation, which occurs with signaling through the BCR or with diacylglycerol agonists, the CLL cells acquire features of Be-1 cells, shut off IL-10 production, make high levels of inflammatory cytokines such as TNF-α, and strongly stimulate proliferation of Th1/Tc1-type T cells [13, 21]. Importantly,

under these conditions, CLL cells are able to kill model tumors, such as MCF-7 breast cancer cells, *in vitro* (F. Wen, D. Spaner, unpublished data). Taken together, these clinical observations support the concept that B cells, particularly regulatory cells, may have a-major negative impact on the development and progression of solid tumors. However the *in vitro* results also raise the possibility that the phenotypic state of tumorigenic B cells may be manipulated to convert them into antitumor effectors.

B-Cell-Directed Cancer Immunotherapy

The above discussion suggests that B cells may play a positive role in preventing the development of cancer but have mainly negative effects on successful clearance of established tumors. These concepts suggest that depleting or enhancing specific B-cell populations may be of use in curative immunotherapy strategies.

Eliminating Negative B-Cell Effects

If B cells are inhibiting antitumor T-cell responses and promoting tumor growth, then B-cell depletion may potentially improve the results of cancer immunotherapy for established solid tumors. Interestingly, although most cancers are incurable, many are responsive to radiation therapy and chemotherapy that are highly toxic to lymphocytes, especially B cells. Although usually considered a side-effect, it is possible that depletion of B cells removes a source of trophic factors for tumor cells. As such, the B-cell depletion that occurs with these modalities may represent one mechanism that is partly responsible for their therapeutic benefits [96]. In addition, removal of B cells may promote the activity of the remaining antitumor T cells and lead to better control of the tumor, as evidenced by the abscopal effect of radiotherapy [97] or the increased activity of antigen-reactive T-cell clones injected into B-cell deficient hosts [98, 99].

The immunostimulatory properties of conventional chemotherapy are being actively investigated [22, 100] and involve other cell populations in addition to B cells. Specific depletion of B cells can be achieved with recombinant antibodies. While conventionally used to treat B-cell malignancies, these antibodies could also be used in solid tumor patients to eliminate nonmalignant B cells that produce trophic factors for tumors and immunosuppressive factors for T cells. The CD20 antibody, Rituximab®, eliminates B cells quite effectively and safely [101] and other CD20 antibodies, such as Ofatumumab® [102], CD23 antibodies such as Lumiliximab® [103], and antibodies against CD22 (Epratuzumab®) [1] are becoming available for clinical use. In an experimental murine model, CD20 antibodies slowed the growth of established CD20⁻ solid tumors but did not induce tumor regression. However, in combination with vaccines, monoclonal

antibody-mediated B-cell depletion led to enhanced antitumor responses associated with both increased numbers of activated CD8^{+} splenic T cells and tumor regression [104]. Consistent with these findings, treatment of colorectal cancer patients with Rituximab® as a single agent led to regression of metastases in 4 of 8 evaluable patients [105]. These findings suggest that it may be advantageous to use B-cell-depleting antibodies to improve the results of cancer vaccines [106] or adoptively transferred tumor-reactive T cells [107].

A number of problems can be anticipated with these approaches. One potential limitation is that the antibodies do not readily distinguish between different effector B-cell classes. Elimination of Be-2 cells and especially Bregs are probably desirable but elimination of Be-1 cells, which amplify Th1/Tc1-type immune responses, may be detrimental to a successful T-cell-mediated antitumor response. In addition, B-cell depletion leads to increased numbers of transitional B cells that enter the circulation from the bone marrow [108]. It is not yet known if these cells might be more easily recruited into the Breg compartment and negate an otherwise therapeutic benefit.

Promoting Positive B-Cell Effects

Vaccines and Recombinant Antibodies

Vaccines that increase protective antibody titers and prevent infections with oncogenic viruses represent one modality by which B cells can be effectively manipulated for meaningful antitumor activity. The HPV vaccine, which prevents cervical cancer, is one of the best examples of this [61]. More universal use of the hepatitis B vaccine would likely prevent many cases of hepatoma [109] although vaccines capable of preventing the development of viral escape mutants in immunocompromised patients are needed to deal with the problem of HBV vaccine failure in a minority of subjects. An effective vaccine against *Helicobacter pylori* would similarly be expected to prevent the development of many gastric cancers [110]. Given that viruses have been estimated to be involved in 15–20% of cancers world-wide [111], continued development of prophylactic vaccines is likely to play an important role in cancer prevention.

Similarly, the development of recombinant antibodies to cell surface structures expressed predominantly by cancer cells will continue to be an important area for cancer therapy. The ability to generate libraries of single chain variable fragments (scFvs) overcomes many of the laborious steps associated with traditional methods of making hybridomas and offers a way to rapidly generate therapeutic antibodies to any desired antigen [37]. However, more detailed understanding of how these antibodies exert their antitumor effects *in vivo* [72] is still needed in order to develop strategies to improve the clinical results, such as increasing complement activation [112] or antibody-dependent cellular cytotoxicity [113].

Enhancing B-cell Activity In situ

Since B-cell effector states seem to be somewhat plastic (see above), an alternative approach to deleting inhibitory B cells in order to improve the therapeutic efficacy of antitumor T cells might be to turn intra-tumoral and intra-nodal B cells into Be-1 effectors in situ. The expected outcome of such an approach would be to amplify and prolong a Th1/Tc1-type of antitumor T-cell response sufficiently to clear tumor cells.

At least three different signals may be necessary to cause B cells to turn off production of immunosuppressive cytokines such as IL-10 and express the costimulatory molecule pattern required for strong stimulation of Th1/Tc1-type T cells [21]. These signals are provided by cytokines, such as IFN-γ [19] or IL-2 family members [114], TLR-agonists [115] or TNFR family members such as CD40 [116, 117], and strong MAPK activation, such as provided by diacylglycerol analogs [22] or possibly HLA-class II antibodies [83, 118]. While these reagents are not absolutely specific for B cells [119], clinical efficacy of such combinations is likely to depend on meaningful differentiation of B cells into the Be-1 phenotype *in vivo*. A more important stumbling block may be the well-known difficulties of extrapolating *in vitro* observations to *in vivo* settings [120]. Problems of hypoxia and poor vasculature with incomplete drug penetration into tumor microenvironments [121] may prevent immmunomodulatory agents from being able to increase the immunogenicity of intranodal and intratumoral B cells sufficiently to promote effective antitumor activity in situ [122].

Adoptive B-Cell Transfer

B cells turn out to be relatively easy to culture and expand to large numbers *in vitro* [123]. A relatively unexplored area of B-cell immunotherapy is "tissue engineering" with activated B cells that have been generated *in vitro*. For example, immunogenic B cells can be used as a vaccine platform to present tumor antigens to T cells [124] with significant potential advantages over dendritic cells because of the ease of generating large numbers for the repeated injections thought to be necessary for vaccine efficacy [125].

Adoptive T-cell therapy is another active area of cancer immunotherapy research [107]. As described in this chapter, B cells are capable of killing tumors [29], and may also be able to elicit antitumor responses following injection of large numbers into patients [4]. More importantly, coinjection of large numbers of Be-1 cells might amplify the effects of adoptively-transferred tumor-reactive T cells. It is not clear which peripheral blood subsets would be most suitable for initiating B-cell expansion cultures. Transitional and prenaïve cells are enriched in B cells with auto-reactive BCRs and thus may be more easily activated by cancer autoantigens to mediate killing of tumor cells. As with T cells, antigen-specificity and enhanced

immunogenicity may be genetically engineered into B cells before infusion [126]. Regardless, the availability of methods to rapidly grow large numbers of B cells offers the opportunity to explore the potential benefits of adoptive B-cell therapy for cancer.

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