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Pillars of Immunology

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Development of techniques allowing production of mAbs in 1975 (1) ultimately led to an explosion of commercial and medical applications, as well as the creation of many valuable tools for addressing important questions in basic scientific research. The enhanced precision in Ag recognition offered by mAbs made these reagents key to the discovery of new molecules with major roles in immune cell functions. The featured *Pillars of Immunology* article by Clark and Ledbetter (2) provides an excellent illustration of the successful application of this approach. This study exploited a novel mAb to begin to identify the important immunological functions of a newly identified B cell surface protein, Bp50, now well known as the TNF receptor superfamily member CD40.

To search for new B cell-specific Ags, the authors immunized mice with human lymphocytes depleted of T cells and prepared individual hybridoma cultures from recipient mouse splenocytes. Flow cytometric analysis was used to identify mAbs in the hybridoma culture supernatants that bound to the surface of human B cells, but not T cells. The anti-Bp35 (CD20) mAb the authors had previously produced (3) was used as a positive control for B cell specificity. In this manner, they isolated the now well-known human CD40-specific mAb G28-5. G28-5 is a high-affinity mouse IgG1 mAb that binds robustly to human, but not mouse, CD40 and is useful in CD40 detection via flow cytometry as well as immunoprecipitation. An additional important attribute of G28-5 is its strong agonist activity toward human CD40. A large number of laboratories (including my own) benefited—and continue to do so—from the use of this versatile mAb as an experimental tool. Its influence upon CD40 research was greatly expanded by the authors' deposit of the G28-5 hybridoma with the American Type Culture Collection, thereby allowing the mAb to be used by all. Early progress in characterization of immune cell surface Ags was also greatly facilitated by a series of international workshops on human leukocyte Ags to which participants brought their reagents to share, compare, and discuss. The first of these was held in Paris in 1982, at which the current unifying CD nomenclature (for "cluster of differentiation") was proposed.

This initial characterization of G28-5 in the *Pillars* article reports that the ~50-kDa Ag it recognizes is expressed only by $CD20^+$ B cells and is not found on T cells, granulocytes, monocytes, or platelets (2). It is now realized that, under various conditions, CD40 can be expressed by all these cell types and more (for recent reviews, see the special issue of *Seminars in Immunology,* Ref. 4). Interestingly, the first report of the molecule ultimately called CD40 identified it as a tumor Ag expressed by a bladder carcinoma (5), and it was subsequently confirmed that CD40 expression can be induced on various types of carcinomas (6). However, because the expression of CD40 is constitutive and most robust on $CD20^+$ B cells, the initial focus was on CD40 functions in B cells. This focus resulted in rapid accumulation of information about how engagement of CD40 impacts B cell activation, which revealed the key functions of CD40 in effective T cell–B cell interactions (reviewed in Ref. 7).

The Clark and Ledbetter study presents initial identification of Bp50/CD40 expressed by human B cells. The report focuses upon the question of whether this membrane molecule is involved in the initial activation steps of resting B cells (increase in cell size, de novo RNA synthesis, G_1 cell cycle entry) and/or the later postactivation events of B cell DNA synthesis and cell proliferation. An important additional question addressed was whether this new Ag could cooperate with additional known B cell receptors to enhance any of these measures of B cell activation. The authors found that treatment with G28-5 alone could not stimulate B cell proliferation but could enhance, by 4- to 5-fold, proliferation induced by signals delivered via the BCR or CD20.

A particularly novel and interesting finding of the study was that engagement of CD40 had the greatest effect when delivered *after* an initial activating signal from another receptor such as the BCR (2). This important result foreshadowed the biological role of B cell CD40 in cognate B cell–T cell interactions. The B cell performs poorly in nonspecific uptake of Ag, compared with highly phagocytic APCs such as dendritic cells. However, B cells can concentrate very small quantities of Ag with great efficiency via BCR-mediated uptake and can then present this Ag quite effectively to activated T cells. Thus, Ag-presenting B cells have typically received initial signals delivered by BCR engagement before they attract the attention of an activated cognate T cell that provides surface CD40L (CD154) to bind

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the B cell CD40. The relatively small effect of CD40 ligation on a naive B cell may thus serve to guard against promiscuous noncognate B cell activation. A more general principle that was reinforced by the *Pillars* study was the finding that activation and proliferation signals can be delivered by distinct but cooperating receptors, and that these signals may be most effectively delivered sequentially, rather than simultaneously.

Although it was subsequently shown that sufficient quantities of human CD154 alone can induce increased B cell proliferation in vitro (8), the basic finding that CD40 cooperates with a variety of additional activation signals has been reproduced by many investigators. Cooperation with the BCR emerged as key to maximizing the effectiveness of the CD40 activation signal (9), whereas interaction with cytokine signals is important to the impact of CD40 on promoting Ig isotype switching (8, 10) and also contributes to CD40-mediated enhancement of B cell proliferation (11, 12). CD40 also cooperates with various additional immune-regulating receptors, including class II MHC (13), TLRs 7 and 9 (14–16), Fas apoptosis inhibitory molecule (17), and Notch (18).

Interestingly, in Table I and Fig. 5 of the *Pillars* paper, CD40 shows no cooperation with B cell growth factor (BCGF, now known as IL-4) in stimulating cell cycle progression or B cell proliferation, whereas both CD40 and BCGF enhance the anti-μ (BCR) signal that induces both events. It is now known that both IL-4 and CD40 provide stronger survival than proliferation signals to B cells (19, 20), and this may contribute to how each cooperates with BCR signals. However, the important role of CD40 in inducing Ab class switch recombination is now appreciated (21). The combination of CD40 + IL-4 robustly promotes switching to IgE in human B cells (22) and also induces activation of the transcription factor NFAT (23).

Fig. 4 and Tables 1 and 2 of the *Pillars* article demonstrate that CD40 signals also cooperate with prior or simultaneous signals delivered via CD20 to stimulate human B cell proliferation. CD20 is now the target of a variety of successful therapeutic mAbs used in the treatment of both B cell malignancies and autoimmune diseases (reviewed in a recent special issue of *Seminars in Hematology*, Ref. 24). Evidence for a physical association between CD40 and CD20 was reported in human B cell lines (25). CD40 signaling in normal human B cells induces the internalization of CD20, which in turn increases its ability to stimulate calcium flux (26). This interaction between the two receptors can have either stimulatory or inhibitory effects on transformed B cells, depending upon the tumor type and the order of signals. CD40 prestimulation enhances the death of chronic lymphocyte leukemia cells induced by anti-CD20 mAbs (27), and simultaneous delivery of CD40 + CD20 signals enhances the in vitro proliferation inhibition of transformed human B cell lines and non-Hodgkin lymphoma cells (28). However, prestimulation of CD20 was found to enhance the proliferative response of B cell acute lymphoblastic leukemia cells to CD40 stimulation (29).

The context-dependent outcome of B cell responses to CD40 engagement is now well appreciated. High levels of CD40 engagement on Burkitt lymphoma cells can suppress their proliferation, whereas low levels are stimulatory. In addition, ligation of CD40 expressed by aggressive B cell lymphomas and myelomas typically inhibits proliferation, whereas the same treatment of more indolent or chronic lymphomas and leukemias can enhance B cell survival and growth (reviewed in Ref. 30). CD40 signaling also has differential effects on human B cells at different stages of development (31).

CD40 is implicated in the pathogenesis of multiple human diseases and disorders. In addition to various B cell malignancies as discussed above, CD40 signals contribute to a variety of autoimmune diseases (reviewed in Ref. 32), atherosclerosis (reviewed in Ref. 33), and transplant rejection (reviewed in Ref. 34). The prophetic statement "It may be possible to use appropriate combinations of mAb to direct and regulate human B cell proliferation or differentiation" made in the concluding paragraph of the *Pillars* paper has been realized in the decades since by a large number of in vitro studies, as well as in animal models. In reports too numerous to cite in this article, a variety of mAbs to CD40, both agonistic and antagonistic, as well as other agents that affect the interaction of CD40 with its ligand CD154, have been employed to either mediate or block CD40-induced immune cell activation. Although promising results have been obtained in many of these studies, including preclinical models, to date no CD40-specific mAbs have been employed as both safe and efficacious treatments for specific human diseases, although pursuit of this desirable goal continues.

A very important experimental advance provided by agonistic CD40-specific mAbs, such as G28-5, is that they allowed the acquisition of valuable information on the function of CD40 on B cells well before its natural ligand was known. It would be an additional 6 y before the initial published reports identifying CD40L/CD154, expressed by activated T lymphocytes of both mouse and human (35–37). Knowledge of how CD40 engagement affects B cells provided important clues to the likely location of its natural ligand. In addition, characterization of CD40 permitted by mAbs allowed use of the CD40 extracellular domain as a probe for the identification of its ligand in all of these studies, several of which involved Dr. Clark (35) or Dr. Ledbetter (36). Identification of CD40L was followed quickly by the realization that mutations in the gene encoding CD40L, found on the human X chromosome, explain the pathological features of most cases of the X-linked form of the human immunodeficiency disease hyper-IgM syndrome (38–40). This disease is characterized by a striking defect in the production of "switched" Ig isotypes, and the lack of an effective humoral memory response, resulting in increased susceptibility to various infections (41). Reports characterizing mouse strains genetically deficient in either CD40 or its ligand rapidly followed. The lack of either member of this receptor-ligand pair caused an in vivo phenotype remarkably similar to that of the human disorder (42–45). Ongoing efforts of many laboratories continue to discover new roles for CD40 and define the molecular mechanisms of its function in a variety of cell types. These early studies highlighted the importance of CD40 in mediating effective interactions between B and T lymphocytes that regulate key aspects of the adaptive humoral immune response. It is impressive that these major breakthroughs in understanding built upon the ability to isolate valuable new mAbs that could both identify new receptors and provide important clues to their biological functions.

Disclosures

The author has no financial conflicts of interest.

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