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Targeting the B7 Family of Co-Stimulatory Molecules:

Successes and Challenges

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Abstract

As more patient data is cross-referenced with animal models of disease, the primary focus on T_H1 auto-reactive effector cell function in autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, has shifted towards the role of T_H17 autoreactive effector cells and the ability of regulatory T cells (T_{reg}) to modulate the pro-inflammatory autoimmune response. Therefore, the currently favored hypothesis is that a delicate balance between $T_H1/17$ effector cells and T_{reg} cell function is critical in the regulation of inflammatory autoimmune disease. An intensive area of research with regard to the $T_H1/17:T_{reg}$ cell balance is the utilization of blockade and/ or ligation of various co-stimulatory or co-inhibitory molecules, respectively, during ongoing disease to skew the immune response toward a more tolerogenic/regulatory state. Currently, FDA-approved therapies for multiple sclerosis patients are all aimed at the suppression of immune cell function. The other favored method of treatment is a modulation or deletion of autoreactive immune cells via short-term blockade of activating co-stimulatory receptors via treatment with fusion proteins such as CTLA4-Ig and CTLA4-FasL. Based on the initial success of CTLA4-Ig, there are additional fusion proteins that are currently under development. Examples of the more recently identified B7/CD28 family members are PD-L1, PD-L2, inducible co-stimulatory molecule-ligand (ICOS-L), B7-H3, and B7-H4, all of which may emerge as potential fusion protein therapeutics, each with unique, yet often overlapping functions. The expression of both stimulatory and inhibitory B7 molecules seems to play an essential role in modulating immune cell function through a variety of mechanisms, which is supported by findings that suggest each B7 molecule has developed its own indispensable niche in the immune system. As more data are generated, the diagnostic and therapeutic potential of the above B7 family-member-derived fusion proteins becomes ever more apparent. Besides defining the biology of these B7/CD28 family members in vivo, additional difficulty in the development of these therapies lies in maintaining the normal immune functions of recognition and reaction to non-self-antigens following viral or bacterial infection in the patient. Further complicating the clinical translation of these therapies, the mechanism of action identified for a particular reagent may depend upon the method of immune-cell activation and the subset of immune cells targeted in the study.

1 Background

Successful antigen clearance and the maintenance of immunologic tolerance are both mediated by the successful interplay of multiple components of the immune system. The immune system is functionally divided into two major types of immunity, innate and acquired. Innate immunity provides for the ability to clear antigen in a non-specific manner,

by defense mechanisms that are not altered by previous encounters with that same specific antigen; and is composed of anatomic/physiologic barriers and immune cell-mediated components (i.e., neutrophils, macrophages, dendritic cells and mast cells) [1–3]. Mechanisms by which macrophages and dendritic cells activate cells of the acquired immune system are (1) by acting as antigen presenting cells (APCs) for CD4⁺ helper T cells (T_h cells) via display of antigenic peptides in the context of MHC II interacting in an antigen-specific manner with the T-cell receptor (TCR) expressed on the surface of the CD4⁺ T_h cell [4–6]; (2) providing co-stimulatory or co-inhibitory cell surface receptor/ligand interaction with cells of the adaptive immune system via upregulation of CD80/CD86 that interacts with CD28 expressed on the surface of the CD4⁺ T cell [7]; and (3) the release of cytokines critical for T_h cell differentiation [8–10]. High affinity receptors for specific antigens (i.e., surface Ig and TCR) allow B cells and T cells, respectively, to react and effectively clear low concentrations of antigen. The specificity of the TCR affords a relatively small precursor frequency of antigen-specific CD4⁺ T cells to effectively respond to an antigenic challenge [11]. In addition to specificity, the adaptive immune system also possesses immunologic memory, allowing for a heightened state of response upon a secondary encounter with the same antigen [12, 13]. The adaptive immune system also has the ability to discriminate between self and non-self antigens [14, 15]. The importance of this latter attribute is apparent when there is a break in self-tolerance. Clinically, a break in self-tolerance can have deleterious effects, namely the development of autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and type I diabetes mellitus [16, 17].

The cells of the adaptive immune system exist in either a naïve or effector/memory state. The mature B cell, CD4⁺ T cell, or CD8⁺ T cell is in the naïve or resting state prior to an encounter with antigen. Three major *effector* cell populations are generated when the naïve cell populations become activated, namely the antibody-secreting B cells, CD4⁺ T_h cells [18, 19], and CD8⁺ cytotoxic T cells [20]. For the purpose of the present review, we focus on the activity and function of CD4⁺ T cells. This in no way is to diminish the importance of both B cells and CD8⁺ T cells with regard to autoimmune diseases, such as MS, RA, and type I diabetes; however, the linchpin of these adaptive immune system cell populations is generally agreed to be the CD4⁺ T-cell population. Therefore, considerable effort has been made to modulate CD4⁺ T-cell function to inhibit/decrease disease severity in autoimmunity and increase cellular function in vaccination protocols and cancer therapies. For example, MS is characterized by perivascular CD4⁺ T cell and mononuclear cell infiltration in the central nervous system (CNS) with subsequent primary demyelination of axonal tracks leading to progressive paralysis [21]. The requirement of CD4⁺CD62L^{hi}CD25[−] naïve T cells to receive two signals to become activated was first proposed by Lafferty and Cunningham [22]. This two-signal hypothesis has become the basis for many potential therapies currently under development, such as the extracellular portion of cytotoxic T lymphocyte-associated antigen 4 covalently linked to the Fc portion of an immunoglobulin molecule (CTLA4-Ig) and anti-CD80 monoclonal antibody therapies to be discussed below in Sect. 3.

As mentioned, the first signal received by a naïve CD4⁺ T cell is from the antigen-specific TCR interacting with an antigenic peptide presented in the context of MHC II on the surface of APCs. The second set of signals is delivered via costimulatory molecules that are expressed on the cell surface of activated APCs, and cytokines that are either produced by the APC and/or by the activated CD4⁺ T cell itself. Classically, CD80 and CD86 expressed on the surface of the APC interact with the co-receptor CD28 that is constitutively expressed on the surface of naïve and activated CD4⁺ T cells [23, 24]. The overall effect of CD28 ligation is to increase the level of proliferation and cytokine production, promote cell survival, and enhance expression of CD40 ligand (CD40L) and adhesion molecules necessary for trafficking, such as very late antigen-4 (VLA-4) (α4β1 integrin) [25–27]. The

costimulatory molecule pairs, CD28-CD80/CD86 and CD40-CD40L, and cellular adhesion molecules, such as VLA-4, represent putative therapeutic targets for blockade of autoreactive CD4⁺ T-cell activation and trafficking to inflammatory sites. In addition to cell-surface-expressed costimulatory molecules, such as CD28, the presence or absence of secreted cytokines affect disease outcome. For example, classically, the production of interleukin (IL)-4 within the local microenvironment leads to naïve CD4⁺ T-cell differentiation toward IL-4-producing T_H2 cells, and locally secreted IL-12 and interferon (IFN)- γ by APCs and activated CD4⁺ T cells respectively induce the IFN- γ -producing T_H1 cell phenotype [27].

Recently, two additional populations of CD4⁺ effector T cells have been identified that secrete IL-17 and IL-9 [28]. These populations are denoted as T_H17 and T_H9 cells, respectively, with T_H17 cells hypothesized to have a role in inflammatory autoimmune diseases such as MS. The T_H17 cell secretes IL-17, IL-6, IL-22, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF), and is hypothesized to differentiate from a naïve CD4⁺ T-cell precursor cell that has been activated in the presence of transforming growth factor (TGF)- β and IL-6 and/or IL-1 β , and is maintained by APC-secreted IL-23 [29–31]. This current hypothesis runs counter to the historical hypothesis that MS, and its mouse model experimental autoimmune encephalomyelitis (EAE), is a predominately T_H1 cell-mediated disease. However, in support of a dispensable role of IFN- γ , both IFN- γ -deficient and IFN- γ receptor-deficient mice present with exacerbated disease scores in EAE [32–34]. In contrast, no disease occurs in T-bet or IL-12 knockout mice and disease symptoms are decreased in the presence of anti-IL-12 mAb [35–38]. This may partly be explained by the decrease in the level of IL-17 produced and the IL-23-induced survival and stability of T_H17 cells due to an absence of IL-23 receptor expression on the CD4⁺ T cell in the absence of T-bet expression [39, 40]. Furthermore, data show that while IL-17-producing cells are important for the initiation of disease in EAE, transferred IL-17 producing CD4⁺ T cell switch to produce IFN- γ upon entry into the CNS [41]. Additionally, while the majority of the data related to autoimmune disease published over the past several years have focused on the deleterious effect of IL-17 during the autoimmune response, more recent data show that in the absence of IL-17 the level of disease severity during EAE is only marginally decreased [42]. In contrast, the more critical cytokine in the induction of EAE disease may be GM-CSF, since in the absence of GM-CSF expression EAE disease induction is eliminated [43]. Therefore, while there is considerable knowledge about immune effector cell populations involved in autoimmune diseases, there still is controversy with regard to the critical cytokine(s) and cell surface molecules mediating self-tissue destruction.

During immune homeostasis there is a balance between the activities of pro-inflammatory and anti-inflammatory T cells such that immune surveillance is maintained, while autoimmunity is avoided. Evidence has emerged that TGF- β is a critical differentiation factor that regulates this balance dependent upon the absence or presence of IL-6. The cytokine TGF- β is a critical differentiation factor for the generation of T_{reg} cells in the presence of IL-2 [44, 45]. On the opposing side of this balance, if the naïve CD4⁺ T cells are activated in the presence of both TGF- β and IL-6, the resulting cells differentiate into a T_H17 cell phenotype [30] as stated previously. Therefore, the *in vivo* situation is more complex than a simple 'yes' or 'no' situation. For example, a protective role for T_{reg} cells has been reported in multiple autoimmune diseases. CD4⁺CD25⁺FoxP3⁺ T_{reg} cells (nT_{reg} cells) are the best documented T_{reg} cell population. Depletion of this cell population in neonatal mice results in the spontaneous induction of autoimmune diseases [46–48]. For example, multi-organ autoimmune diseases (such as thyroiditis, gastritis, and insulinitis) are induced in BALB/c athymic nude mice receiving syngeneic CD4⁺ T cells depleted of CD25⁺ T cells, while reconstitution of CD4⁺CD25⁺ T cells within a limited period of time after the transfer

of CD25⁻ T cells inhibited disease development [49]. Similarly, CD4⁺CD25⁺ T cells from non-obese diabetic (NOD) mice delayed/prevented development of spontaneous diabetes in CD28-deficient NOD mice [50]. It is noteworthy that regulation of self-reactive T cells most likely involves multiple populations of immunoregulatory T cells, including nT_{reg} cells, IL-10-producing induced (iT_{reg} or Tr1) cells and TGF- β -producing T_{reg} cells (T_h3) [51–53].

2 Currently Approved Therapies for MS

The majority of current FDA-approved autoimmune disease therapies (e.g., interferon β [54, 55], glatiramer acetate [56–58], the topoisomerase inhibitor mitoxantrone [59], and natalizumab, an antibody targeting VLA-4 [60]) function via immune deviation or antigen non-specific immunosuppression, and require administration over the remainder of the patient's life. Beside the blockade of the adhesion molecules necessary for inflammatory cell entry into the CNS, blockade of secretory chemoattractants and biolipid receptors, such as sphingosine 1-phosphate receptor (S1P1) blockade via fingolimod (FTY720) treatment [61], are also under investigation. However, while treatment of autoimmune disease models like EAE with FTY720 show efficacy, the issue remains that upon cessation of FTY720 treatment, mice experience disease relapse within days [62]. Furthermore, the inhibition of total immune cell trafficking for an extended period of time is likely to lead to the recurrence of a latent viral infection, such as JC virus in MS patients receiving anti-VLA-4 [63], or eventually other opportunistic infections. In the example of glatiramer acetate, a drug composed of the amino acids L-glutamic acid, L-alanine, L-tyrosine, and L-lysine, which is administered via daily subcutaneous injection for the treatment of relapsing-remitting MS [56–58], the mechanism of action is believed to be the elicitation of a low level of TCR signaling in the absence of costimulatory molecule signaling (signal 1 in the absence of signal 2). Treatment with glatiramer acetate is hypothesized to induce immune deviation toward a T_h2 phenotype (potentially disease-regulatory) compared with a T_h1/17 phenotype (disease-promoting) by inducing a low level of TCR stimulation. In an attempt to further test the two-signal hypothesis, several groups have investigated the therapeutic potential of anti-CD3 mAb treatment in the absence of costimulatory signals in the treatment of various autoimmune diseases. However, treatment with an unaltered anti-CD3 mAb is potentially a double-edged sword such that while treatment eliminates pathogenic autoreactive CD4⁺ T cells, thereby ameliorating autoimmune disease progression, this therapy may also induce non-specific side effects through the activation of bystander T cells [64–66]. For example, the induction of general immunosuppression which increases the patient's susceptibility to opportunistic infection and the common occurrence of cytokine-release syndrome in which treatment recipients suffer severe side effects due to the non-specific production of inflammatory cytokine. Furthermore, cross-linking of CD3 may in some cases initiate a signal of sufficient strength that it eliminates the need for a costimulatory molecule-induced signal normally required for T-cell activation. Additionally, immunotherapeutic strategies have been shown to work, at least in part, through the alteration of signal one and/or inhibition of costimulatory molecule stimulation (signal 2), thereby inducing CD4⁺ T cell anergy [25]. For example, short-term blockade of CD28-CD80/CD86 interactions is hypothesized to induce long-term unresponsiveness of T cells undergoing activation at the time of treatment, the use of CD28-CD80/CD86 inhibitory reagents, such as CTLA4-Ig, which will be discussed in the following section, is a viable option to achieve the goal of re-establishing tolerance to self-antigens.

3 Therapeutic Use of CTLA4-Ig

The mechanism of action for CTLA4-Ig treatment is believed to block CD28-CD80/CD86 interaction by CTLA4-Ig binding to and blocking CD80/CD86 on the APC. In this manner the autoreactive CD4⁺ T cell would receive signal 1 in the absence of signal 2. For example,

CD80/CD86 are expressed on the surface of dendritic cells within the synovium of RA patients [67]. Experimentally, CTLA4-Ig treatment has been successfully used to inhibit the level of disease severity in the collagen type II-induced arthritis (CIA) model. Both the role of T-cell costimulation in disease development and progression has been studied. As mentioned in Sect. 1, T-cell costimulation is provided by ligation of CD28 on the CD4 + T cell with either CD80/CD86 on the APC, and it was hypothesized that treatment with a soluble form of CTLA4, i.e., CTLA4-Ig, would block this interaction (as shown in Fig. 1). Data show that CTLA4-Ig treatment at the time of disease induction for CIA inhibits disease induction and is associated with lack of lymphocyte expansion within the draining lymph nodes [68]. Similarly, treatment of mice at the time of disease induction of EAE with CTLA4-Ig is able to reduce the level of disease severity [69]. However, CD80 and CD86 have been shown to have differential roles in T-cell activation and differentiation [70]. To illustrate this point, conflicting results have been obtained using anti-CD80 and anti-CD86 mAbs to regulate autoimmune disease. Treatment with anti-CD80 mAb surrounding autoantigen priming has been shown to block EAE development induced with suboptimal concentrations of PLP_{139–151} or MBP_{84–104} in SJL mice, whereas anti-CD86 mAb treatment has been reported to either exacerbate disease [71] or have no effect [72]. In contrast, treatment with intact anti-CD86 mAb initiated during the remission following acute disease does not affect disease progression (relapses) in proteolipid protein (PLP)_{139–151}-induced relapse-remitting (R)-EAE [73]. Treatment with monovalent, non-cross-linking anti-CD80 Fab fragments during EAE remission blocked clinical relapses and epitope spreading to the PLP_{178–191} epitope [73], whereas treatment with intact anti-CD80 mAb led to a profound exacerbation of disease relapses concomitant with accelerated epitope spreading [74], and inflammatory CD4⁺ T cell cytokine production [75]. Likewise, treatment of mice with a small molecule inhibitor of CD28 during disease decreased disease severity and proliferation of myelin-specific CD4⁺ T cells upon ex vivo activation, and increased CD4⁺ T-cell apoptosis [76].

The presence of activated peripheral myelin-reactive T cells appears to distinguish MS patients from normal subjects, in whom circulating myelin-reactive T cells do not express activation markers. Two genetic alleles have been identified that best correlate with increased risk of MS development. The first is *HLA DR2*, and an increased percentage of MS patients also express an allelic variant of *CTLA4* [77]. In addition to the positive regulating CD28 costimulatory molecule, activated CD4⁺ T cells upregulate surface expression of CTLA4, a CD28 homolog, which also binds CD80/86 [78], and serves as a negative regulator of T-cell activation. CD80 is expressed by all cell types in the CNS-infiltrating mononuclear cells during EAE, including CD4⁺ T cells [79], and therefore blockade of this B7/CD28 interaction is a very attractive target therapeutically. Previously tested immunotherapeutic strategies have been shown to work at least in part through the alteration of signal 1, i.e., TCR signal, and/or inhibition of costimulatory molecule stimulation (signal 2). In this manner, CD4⁺ T-cell anergy is hypothesized to be induced in peptide-specific T cells undergoing activation at the time of treatment via short-term blockade of CD28-CD80/CD86 interactions. CD28-CD80/CD86 inhibitory reagents such as CTLA4-Ig are currently approved for the treatment of RA [68, 80, 81]. The goal for treatment of autoimmune diseases such as RA or MS is to re-establish tolerance to self-antigens. The difficulty in the development of these therapies lies in maintaining the ability of the patient to normally recognize and react to non-self-antigens following viral or bacterial infection.

As mentioned previously, cross-linking CD80 on the surface of a CD4⁺ T cell with anti-CD80 mAb has been shown to promote increased production of IFN- γ by T_H1 cells [82], as well as an increase in the level of T_H17 cell-produced IL-17. Available data also shows that anti-CD80 mAb treatment cross-links CD80, and binds to both APCs and to peptide-specific

CD4⁺ T cells within the CNS following treatment. Published studies also show that cross-linking CD80 on the surface of APCs increases the level of inflammatory cytokine produced. Taken together, these findings provide a secondary mechanism by which cross-linking CD80 may increase the level of disease severity in EAE. During the activation of CD4⁺ T cells within the CNS, the dendritic cell would provide signal 1 and signal 2, while secreting increased levels of inflammatory cytokines, and providing the required stimulatory signals to spread epitope-specific CD4⁺ T-cell activation within the CNS [83]. While previous studies have concentrated on the effect of CD80 cross-linking on T_H1 cell function, the effect of CD80 cross-linking on T_H17 cell and iT_{reg} cell function has yet to be adequately addressed. As with most of the B7/CD28 family members, the effect on the immune response achieved following treatment with a specific mAb is dependent upon the cell type being studied, the manner in which the cells were activated, and the specific clone of mAb being used [84]. This later point will be discussed below. As an example of alternative functional outcomes ensuing depending upon the cell type studied, in contrast to the increase in CD4⁺ T-cell survival and cytokine production following anti-CD80 mAb treatment [75]; if B cells are activated with IFN- γ and lipopolysaccharide in the presence of anti-CD80 mAb, decreased production of IgG2a and enhanced apoptosis is observed [85]. Therefore, the potential outcome following costimulatory molecule blockade/cross-linking must be considered from multiple vantage points.

The aforementioned genetic analyses of MS patients support the hypothesis that CD80 expressed by autoreactive CD4⁺ T cells may contribute to the disease exacerbation. There is a positive correlation between the expression of allelic variants of CTLA4 that have an increased affinity for binding to CD80 in MS patients [77]. In this scenario, the expression of CTLA4 might have the opposite effect of the ‘classic’ negative regulatory role CTLA4 has on CD4⁺ T-cell activation [86–88]. Instead, CTLA4, with its increased affinity for CD80, might act as a cross-linker for CD80 sending a positive signal to autore-active T cells during T cell–T cell interactions. When taking the in vitro and in vivo data into consideration, a putative mechanism is suggested to explain why the timing of anti-CD80 mAb treatment has differing effects on disease outcome in R-EAE. Alternatively, anti-CD80 mAb has been approved for the treatment of CD80⁺ non-Hodgkin’s B-cell lymphoma [89–91]. The presumed mechanism of action was the elimination of CD80⁺ non-Hodgkin’s B-cell lymphoma cells via antibody-directed cellular cytotoxicity (ADCC); however, based on the clinical data it was concluded that ADCC could not account for one-hundred percent of the anti-CD80 mAb activity seen in patients [92–95]. Based upon our previously published data, it is very tempting to hypothesize that this increase in anti-CD80 mAb treatment efficacy is due to an increase in T_H1- [82, 96] and CTL-specific [97] anti-B-cell lymphoma activity. However, this remains to be formally tested.

In contrast to the hypothesis detailed above, recent data suggest that CTLA4 can perform a non-redundant effector function for T_{reg} cells, requiring a cell-extrinsic mechanism of action [98, 99]. Thus, one functional mechanism of CTLA4 activity is by the removal of co-stimulatory ligands from APCs via trans-endocytosis [100]. Moreover, this mechanism is specific for CTLA4/CD28 ligands and operates in an antigen-dependent manner in vivo. This finding correlates with several studies that demonstrate a reduced level of costimulatory ligand expression in the presence of CTLA4-expressing T_{reg} cells [98]. T_{reg} cells have been shown to play contrasting roles in both auto-immunity and chronic viral infection. T_{reg} cells are protective against autoimmune disease [101–106]; most notably, the balance of T_{reg} cells and effector T cells in the CNS has been reported to switch in favor of T_{reg} cells allowing for recovery from acute disease [45, 107]. In contrast, T_{reg} cell function has been shown to impede virus clearance during chronic viral infections [108–115].

Our laboratory has had a long-standing interest in studying the pathogenesis of Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), a mouse model of chronic MS, which provides an ideal system to study the interplay of genes, infection and immunoregulation in the induction and progression of a virus-induced CNS autoimmune demyelinating disease [116, 117]. Susceptibility to TMEV-IDD is controlled by multiple genes, predominantly *MHC class I*, wherein resistant strains of mice [e.g., C57BL/6 (H-2^b)] mount an efficient CTL response and rapidly clear the infection, while susceptible strains (H-2^{s,q,r,p}, and ^f) of mice [e.g., SJL/J (H-2^s)] fail to mount an effective CTL response [118–127] resulting in a life-long persistent CNS TMEV infection leading to induction of CNS autoimmunity via epitope spreading [83, 128, 129]. Our findings demonstrate that inactivation of T_{reg} cells via anti-CD25 mAb treatment prior to infection of susceptible SJL/J results in delayed disease onset and progression, increased viral clearance, and increased adaptive anti-viral CD4, CD8, and antibody immune responses [115]. Clinically, the monitoring of patients on immune modulatory therapy such as CTLA4-Ig has been completed. Recently published data show that in patients with a combination of RA and chronic hepatitis B infection, if antiviral therapy was begun in combination with CTLA4-Ig, then there was a decrease in RA disease score and no reactivation of hepatitis B. However, if RA patients with chronic hepatitis B infection did not receive antiviral therapy, then there was not a decrease in RA disease score following CTLA4-Ig treatment and the patients experienced a reactivation of hepatitis B [130]. Therefore, consideration of latent and chronic viral infections within a patient must be accounted for before the beginning of CTLA4-Ig treatment.

4 Therapy Using CTLA4-FasL

Besides the use of extracellular domains of cell surface receptors fused with the constant region of Ig heavy chain, there is also a growing interest in the use of bi-functional fusion proteins. One such example is the CTLA4-FasL fusion protein that is being developed. The Fas-Fas ligand receptor pair triggers activation-induced cell death (AICD) within CD4⁺ T cells. A fusion protein composed of CTLA4-FasL is intended to target the fusion protein to the surface of activated APCs [131–133]. In this manner, CD80/CD86 expressed by the APC acts as a scaffold to bind CTLA4-FasL, while the FasL portion of the fusion protein presumably will induce apoptosis in Fas-expressing activated CD4⁺ T cells. Fas is a member of the TNF superfamily of proteins and is expressed by monocytes, NK cells, T cells, and B cells [134–136]. A potential functional benefit of a fusion protein like CTLA4-FasL as a therapy in comparison with soluble FasL treatment is that soluble FasL tends to aggregate in vivo causing toxicity within the liver [137]. This toxicity of FasL can be alleviated via the generation of a membrane-bound form of the protein. One of the original ideas proposed was to link Fas to a glycosyl-phosphatidylinositol (GPI)-modification, which would allow for the FasL-GPI to be incorporated into cellular membranes following in vivo treatment [138]. However, since FasL is a type-II transmembrane protein, the C-terminal requirement for linking a GPI-modification to a protein was not feasible. Therefore, the use of CTLA4 as an anchoring protein has been completed [139]. Beside the direct effect of FasL on Fas expressing cells, the use of the present fusion may also inhibit the activation of CD4⁺ T cells via the blockade of CD80/CD86-CD28 interaction, as detailed above in Sect. 3. The utilization of CTLA4-FasL treatment has shown promise in transplantation models. An example of this is the adenovirus generation of CTLA4-FasL, which has been successful in animal models of pancreatic β -islet cell [140] and cardiac allograft transplantation [141].

While CTLA4 may potentially block the CD28-induced survival signaling pathway within activated CD4⁺ T cells and allow for Fas-FasL-induced apoptosis, one potentially restrictive issue to utilizing a fusion protein such as CTLA4-FasL is the blockade and/or the deletion of T_{reg} cells that are required for the maintenance of long-lived tolerance. Besides the intended

functional activity of the fusion protein, possible functional consequences must also be considered. For example, in the case of CTLA4-FasL fusion protein, the intended goal is to kill activated CD4⁺ T cells via Fas-FasL-mediated apoptosis. However, the use of such a molecule over time may also inhibit the development of T_{reg} cells and potentially eliminate them as well. The first portion of this concern is supported by published data showing that CD80/CD86 signaling is required for the development of T_{reg} cells within the thymus [142], and the latter may be supported as follows. One mechanism that regulates the CD4⁺ T-cell pool is AICD. This is mimicked in vitro by TCR restimulation-induced expression of the death ligand CD95L (FasL/APO-1L/CD178) in expanded T cells. These cells express the death receptor CD95 (Fas/ APO-1), and binding of CD95L to CD95 results in AICD. In contrast, T_{reg} cells do not undergo AICD upon TCR restimulation in vitro despite a functional CD95 cell death pathway. Data show that both human and murine T_{reg} cells express low levels of CD95L upon stimulation, and that knockdown of Foxp3 expression partially rescues CD95L expression and AICD in human T_{reg} cells [143]. Therefore, Foxp3 negatively regulates CD95L expression in T_{reg} cells and demonstrates that T_{reg} cells are susceptible to homeostatic control by CD95 stimulation.

5 B7/CD28 Family Member Proteins as Alternative Therapeutic Targets

The ability of the immune system to control peripheral activation of self-reactive T cells is dependent on the level and type of costimulatory molecules expressed on the surface of APCs, and/or the type of costimulatory molecule receptors expressed by CD4⁺ T cells [144]. Alternatively, ligation of inhibitory molecules associated with CD4⁺ T cells during and/or following T cell activation can effectively suppress T cell responses. Over the past several years, multiple B7/CD28 family members have been identified (Fig. 2). Prediction of the potential biologic effects of reagents directed towards agonism or antagonism of particular B7 family members is complicated by the fact that several of the B7/CD28 family members interact with multiple receptors (reviewed in [145] and [146]). For example, while CD80 is classically believed to interact with both CD28 and CTLA4, more recent data show that CD80 also interacts with PD-L1/B7-H1 [147]. To further confound the study of B7/CD28 family member proteins, the resultant effect of mAb treatment is dependent upon the clone of mAb used. For example, the 10F.9G2 clone of anti-PD-L1 mAb blocks both the PD-1:PD-L1 interaction and the CD80:PD-L1 interaction, while the 10F.2H11 clone only blocks the PD-1:PD-L1 interaction and not the CD80:PD-L1 interaction [148]. Likewise for anti-CD80 mAbs, the 1G10 clone blocks both the CD28, CTLA4:CD80 interaction and the CD80:PD-1 interaction, while the 16-10A1 clone only blocks the CD28, CTLA4:CD80 interaction [148] and induces direct intracellular signaling within the CD80+ target cell [149] and does not inhibit the CD80:PD-1 interaction [148]. Additionally, several of the B7/CD28 family members interact with unidentified receptors, and still other B7 family member proteins, like B7-H6, interact with non-classical CD28-like receptors, i.e., NKp30 expressed on the surface of NK cells [150–153].

Besides the typical costimulatory function of B7/CD28 family members, recent data show that some B7 family member proteins function via a skewing of the CD4⁺ T cell response toward a specific effector phenotype. An example of an immune regulatory receptor/ligand pair is PD-1/PD-L1. Data show that mice deficient in PD-L1 expression show an increase in the level of myelin oligodendrocyte glycoprotein (MOG)_{35–55}-induced chronic (C)-EAE as compared with wildtype C57BL/6 mice, and treatment of mice with anti-PD-L1 mAb (clone J43) induces an increase in the number of MOG_{35–55}-specific CD4⁺ T cells producing IFN- γ and IL-17 [154]. Also, activation of naïve CD4⁺ T cells in the presence of beads coated with anti-CD3/28 plus PD-L1 Ig in the presence of T_h1 cell- or T_h17 cell-promoting conditions has been shown to decrease the number of resultant IFN- γ and IL-17 producing cells, respectively, while increasing the number of CD25⁺/FoxP3⁺ cells when activated in

the presence of iT_{reg} cell-promoting conditions [155]. Along these same lines of immune modulatory B7 family proteins, the ICOS/ICOS-L interaction has been shown to favor T_h2 cell differentiation and IL-4 production [156]. In contrast to this finding, the induction of local expression of ICOS Ig within a xenograft induced an increase in graft survival, and potentially increased the number of CD4⁺CD25⁺Foxp3⁺ T cells at the periphery of the graft [157]. Besides PD-L1, PD-L2 is also a ligand for PD-1 [158]. Therapeutically, PD-L2-Ig has been shown to have a two to sixfold higher affinity for PD-1 than PD-L1 [159]. In comparison with the potential induction by PD-L1-Ig of iT_{reg} cells and decrease in CD4⁺ T cell effector function [155], PD-L2 knockout mice exhibit reduced levels of IFN- γ , reduced IgG2a responses, and a decreased capacity to clear hepatic tumors [160]. Therefore, while PD-L1 and PD-L2 have the same receptor, i.e., PD-1, there is a dramatic difference in function when comparing the two different ligands. This difference may be due to the higher affinity of PD-L2 for PD-1 as compared with PD-L1, and PD-L2 also appears to have an additional as yet unidentified receptor [159, 160]. This data supports the hypothesis that some of the more novel B7 family members regulate CD4⁺ T-cell differentiation towards specific effector cell phenotypes, which is in contrast to the function of CD28 expressed on CD4⁺ T cells.

The generation of a B7-H4-Ig fusion protein has been the focus of research by multiple research groups over the past several years. Besides the critical balance between T_h1/T_h17 cells and T_{reg} cells during MS/EAE, published data show that alternatively activated macrophages (M2 cells) are able to suppress EAE following transfer [161]. Furthermore, M2 cells have been suggested to have neuroprotective activity compared with the inflammatory M1 cells [162]. As a potential mechanism, co-culture of CD4⁺ T cells with IL-10/TGF- β -treated macrophages that also express B7-H4 decreases CD4⁺ T-cell proliferation and subsequently induces an increase in the number of CD4⁺ T cells that express Foxp3 [163]. B7-H4-Ig treatment directly modulates the level of inflammatory CD4⁺ T-cell function [164, 165], and is currently in clinical development. Furthermore, B7-H4 expression by cancer cells has been suggested to be a putative mechanism by which these cells evade anti-tumor immune responses [166–169]. The ability of B7-H4-Ig to be used therapeutically has been tested in both the NOD model of type I diabetes and in β -islet cell transplantation. Firstly, B7-H4-Ig treatment of pre-diabetic NOD mice reduced the disease incidence as compared with control Ig-treated mice [170]. Additionally, initial attempts to determine a putative mechanism of action for the B7-H4-Ig-induced decrease in the level of disease severity was found to be correlated with a decrease in the level of CD4⁺ T-cell proliferation in response to GAD34 stimulation *ex vivo*, and a transient increase in the number of T_{reg} cells *in vivo*. B7-H4 protects allografts and generates donor-specific tolerance. It also prevents the development of autoimmune diabetes. More importantly, B7-H4 plays an indispensable role in alloimmunity in the absence of the classic CD28/CTLA4:B7 pathway, suggesting a synergistic/additive effect with other agents such as CTLA4 on inhibition of unwanted immune responses [171].

As shown in Fig. 2, there are multiple members of the B7/CD28 family of receptors/ligands. Besides the present ones listed, there are also newer potential family members that have been identified *via in silico* screening over the last few years. For example, V-domain Ig suppressor of T-cell activation (VISTA) is primarily expressed on hematopoietic cells, and VISTA expression is highly regulated on APCs and CD4⁺ T cells. Data show that a soluble VISTA-Ig fusion protein or VISTA expression on APCs inhibits T-cell proliferation and cytokine production, and that VISTA overexpression on tumor cells interferes with protective antitumor immunity *in vivo* in mice, thereby illustrating that VISTA may play a role in the development of autoimmunity and immune surveillance in cancer [172].

6 Conclusion

Based on the success of CTLA4-Ig fusion protein for the treatment of RA, there has been a renewed interest in the further development of additional fusion proteins that may prove to be more efficacious than CTLA4-Ig and/or may be developed for additional indications. While the mechanisms of action for most of these drugs remain to be determined, there is considerable promise therapeutically. With regard to the question as to the mechanism of action for these later fusion proteins, this must be considered on a case-by-case basis for each specific fusion protein. For example, the literature shows that CTLA4-Ig treatment appears to block B7/CD28 interactions by binding to CD80 and CD86 on APCs [131]. This is in contrast to ICOS-Ig, PD-L1-Ig and B7-H4-Ig, which, as detailed above, appear to function by cross-linking their respective receptors and thereby modulating immune cell function at the T-cell level. Besides the overall immune consequence induced by the respective fusion protein, consideration must also be given to whether the desired functional outcome may be increased by the utilization of monomeric, dimeric, or multimeric forms of the respective fusion proteins. Consideration must also be given to whether or not the functional activity of the fusion protein is inhibited, unaffected, or enhanced by the binding of the fusion protein to host Fc receptors. For example, if the mechanism of action for the fusion protein is via cross-linking of a target receptor in vivo, then omission of the Fc portion of the fusion protein would cause a loss of function. Lastly, the serum half-life of the respective protein and the longevity of the treatment effect must be considered to optimize its desired efficacy while limiting undesired side effects. Much like currently used FDA-approved therapeutics, the discussed Ig fusion proteins are not antigen-specific therapeutics; however, the ideal Ig fusion protein therapeutic will gain its selectivity by only having to be administered for a limited time frame, while maintaining long-lived efficacy.

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Fig. 1.

CD4⁺ T cell activation in the presence of CTLA4-Ig. Naïve CD4⁺ T cell receives antigen-specific signals (signal 1) through its clonally derived T-cell receptor (*TCR*), which interacts with antigenic peptides presented by major histocompatibility complex (*MHC*) class II molecules expressed on the surface of antigen-presenting cells (*APCs*). A second set of signals are delivered by co-stimulatory molecules, such as CD80/CD86 (B7) expressed by activated APCs, which interact with the CD28 co-receptor that is constitutively expressed by CD4⁺ T cells. The overall effect of CD28 ligation is to increase the level of proliferation and cytokine production and to promote CD4⁺ T-cell survival. The costimulatory molecule pairs CD28–CD80/CD86 and CD40–CD40L and cellular adhesion molecules represent putative therapeutic targets for blockade of autoreactive CD4⁺ T-cell activation and trafficking to inflammatory sites. For example, CTLA4-Ig is hypothesized to block CD28 interaction with CD80/CD86 on the APC, thereby inducing anergy and/or activation-induced cell death of the CD4⁺ T cell

Fig. 2. B7/CD28 family of costimulatory molecules. B7/CD28 family member proteins provide costimulatory and co-inhibitory signals to both CD4⁺ T cells and antigen-presenting cells (APCs). This is classically illustrated by the CD80/CD86:CD28 interaction in comparison with the CD80/CD86:CTLA4 interaction, respectively, with regard to CD4⁺ T-cell function. However, over the past several years more B7/CD28 family member proteins have been identified, and can be subdivided into novel B7s like PD-L1, PD-L2, ICOSL, B7-H3 and B7-H4 that can both decrease CD4⁺ T-cell function and skew the CD4⁺ T-cell response toward a T_H2 cell or T_{reg} cell phenotype. Besides these B7 family members, there are also newly described potential family members like V-domain Ig suppressor of T-cell activation

(VISTA) and Cgen-15001T, and B7-H6 that is expressed by tumor cells and interacts with NKp30 expressed by NK cells