

Considering B7-CD28 as a family through sequence and structure

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Impact statement

Immunotherapy as a field has dramatically expanded in the last decade in the area of oncology with efficacy demonstrated by PD-1, PD-L1, and CTLA-4 blockade. With all three “checkpoint blockade” receptors being in the B7-CD28 family, there has been increased interest in targeting other members in this family due to redundancy in immune regulation, i.e., the combination of therapeutic agents targeting multiple co-inhibitory receptors may yield additional antitumor efficacy. Therefore significant resources are being dedicated to developing additional B7-CD28 treatment options.

Abstract

With the emergence of immuno-oncology, new therapeutic agents that modulate immune activation and regulation are being used to treat cancer patients with durable response. It is well known that following T-cell receptor (TCR) activation, many co-receptors can augment or suppress the TCR signal, and therapeutically targeting these co-receptors has proven effective. The B7-CD28 family is comprised of such immune-regulatory receptors, and antibodies against its members programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have revolutionized cancer treatment. These therapies promote an immune response against tumor cells, which demonstrated better long-term survival and tolerability compared to traditional cancer treatments. In this review we describe the history of the expanding B7-CD28 family, and by comparison of sequence and structure reveal that it is a non-

traditional family. The family has grown to include proteins that share low sequence identity, generally grouped by regulation of immune response, which utilize the common immunoglobulin fold. This low level of commonality has provided additional challenges to the drug discovery process as the mechanisms and therapeutic potency between family members can vary greatly.

Keywords: Protein–protein interactions, immunology/molecular, oncology, structural biology, immunotherapy, immuno-oncology

Experimental Biology and Medicine 2019; 244: 1577–1583. DOI: 10.1177/1535370219855970

Introduction

An effective, durable, and non-self-immune response is essential for maintaining human health. To achieve this balance and be adaptable, the immune system consists of various cell types that work in concert to evoke the appropriate response. For example, the B-cell response focuses predominantly on extracellular antigens, whereas the CD8 T-cell response is largely focused on antigens presented by major histocompatibility complexes. Both responses rely on initial antigen recognition, then subsequent activity tuning through co-stimulatory and inhibitory receptors, along with cytokine signaling. The discovery of these receptors, and subsequent targeting of inhibitory receptors for therapeutic benefits has been tremendously impactful and was highlighted by the 2018 Nobel Prize in Physiology or Medicine, which was awarded to James Allison and Tasuku Honjo. This new class of checkpoint inhibitors enhances the T-cell response to tumor antigens¹ and tends

to be more durable than traditional chemotherapy.² This pioneering work has generated much interest in the B7-CD28 family, and reviews published in recent years provide details about the functionality of the B7 and CD28 co-receptor pathways.^{3–5} In this review we discuss the chronology leading to the current definition of the B7-CD28 family, the sequence identity and structure characteristics of the family, and how its non-classical characteristics provide challenges to drug discovery.

Chronology of B7-CD28 discoveries led to an expanding family

The discovery of B7 resulted from efforts to find antibodies against antigens to allow researchers to classify distinct B lymphocytes. With its members loosely related by sequence and function, the B7-CD28 family has gradually expanded over decades. The initial discovery of B1 (CD20) in 1980⁶ was quickly followed by B2 (CD21),⁷ B3 (CD22),⁸

B4 (CD19),⁹ B5,¹⁰ and finally B7 (CD80).¹¹ CD28, previously identified as a T-cell activator,¹² was shown to bind CD80 by demonstrating CD80:CD28-mediated cell adhesion could be blocked by anti-CD80 and anti-CD28 antibodies.¹³

Not long after, a second receptor competing with CD28 for CD80, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), was identified.¹⁴ CTLA-4 had previously been described as homologous in sequence and structure to CD28 and both are located on chromosome 2,¹⁵ clearly showing familial relationship, but the biological role of CTLA-4 was unknown. The picture became more complicated in 1993 when four papers were published within a day of each other describing a second member of the B7 family, B7-2 (CD86), which also bound both CD28 and CTLA-4.¹⁶ Interestingly, while they have limited sequence similarity, CD80 and CD86 are located on chromosome 3.¹⁷ Whereas CD80 is expressed on activated antigen presenting cells (APC), CD86 is constitutively expressed on monocytes and dendritic cells, and on activated B-cells.¹⁸ Meanwhile, work progressed describing the biological role of CTLA-4, where it was proposed as a T-cell co-stimulatory molecule¹⁹ or a suppressing molecule.²⁰ It was definitively shown in mid-1995 that CTLA-4 outcompetes CD28 for CD80/CD86 binding and inhibits T-cell activation.^{21,22} In early 1999, inducible T-cell co-stimulator (ICOS), a third CD28-related molecule was identified and shown to be inducible on activated T-cells; ICOS induces interleukin (IL)-10 but not IL-2 as seen with CD28 activation.²³ With three related co-stimulatory molecules that modulate T-cell response (CD28, CTLA-4, and ICOS) and two identified ligands (CD80 and CD86), characterization of the B7-CD28 family seemed relatively clear.

However, the addition of a third B7 member, B7-H1 (programmed cell death protein 1 [PD-L1]), complicated the definition of the family,²⁴ and expanded possibilities of what might be included. PD-L1 was initially identified as a co-stimulatory ligand for T-cells, but was later shown to be a suppressive when its receptor programmed cell death protein 1 (PD-1) was discovered.²⁵ PD-1 is located on chromosome 2, but not in the locus shared by CD28, CTLA-4, and ICOS. Furthermore, PD-L1 is located on chromosome 9, whereas CD80 and CD86 are on chromosome 3. This series of additions broadened the family, with PD-L1 and PD-1 situated at new chromosomal locations and not interacting with any previously identified members. Additions to the family continued as more immunoglobulin (Ig) domain-containing molecules were found, usually involving APC:T-cell interactions, but sometimes only broadly related by structure and function. The B7 family grew to include B7-H2 (ICOSL),²⁶ B7-H3 (CD276),²⁷ B7-DC (PD-L2),²⁸ B7-H4 (B7x),²⁹ B7-H5 (VISTA),³⁰ B7-H6 (NCRLG1),³¹ and B7-H7 (HHLA2).³² Concurrently, NKp30 and CD28 homolog (CD28H; TMIGD2) were identified as receptors for B7-H6 and B7-H7, respectively, thus enlarging the CD28 family. Recently, VSIG-3 has been proposed as a receptor for B7-H5.³³ Receptors for B7-H3 and B7-H4, and if they are CD28-like (i.e. Ig domains), have yet to be determined. Through this chronology, we see that the family has its origins in B-cell characterization, where a series of discoveries and additions led to the creation of

a family without distinctive features to separate itself from other Ig-containing receptors that also affect the immune system.

Sequence homology

A defining characteristic of the B7-CD28 family members is their ability to modulate the immune response. However, traditional characteristics that often define a family such as conserved signaling mechanisms, sequence homology, structural-similarity, or chromosome location do not apply across members of this family, except an Ig domain that is broadly utilized in immune regulation. Lacking these distinguishing protein features makes it difficult not only to clearly define the family, but also difficult to predict potential undiscovered members. Interestingly, B7-H1 (PD-L1) was initially identified as an IgV and IgC containing protein with low sequence identity to CD80, broadly expressed and affecting T-cells,²⁴ though with more limited protein expression.³⁴ We suppose that this classification may have led to the growth in the B7-CD28 family as other Ig-containing "family members" were discovered. Indeed, by initially naming PD-L1 as B7-H1, PD-1 entered a group containing CD28, CTLA-4, and ICOS, which are clearly related molecules that interact with CD80, CD86, and ICOSL, and share a conserved interface,³⁵ but PD-1 and PD-L1 do not interact with these members.

Most families of homologous proteins have significant sequence identity, as they potentially derive from a common ancestor, gaining diversity through gene duplication and subsequent mutations. Proteins with more than 30% sequence identity have a higher likelihood of being structurally homologous (90%), and below 25% identity the chance of being a homolog falls to 10%.³⁶ With high sequence identity comes more likelihood of being structurally similar, thus potentially conserving any structure-function relationships. However, B7-CD28 members have low sequence identity when quantified by globally aligning their full-length sequences (Figure 1(a)). The overall average sequence identities among human B7 and CD28 members from this alignment is 20% and 18%, respectively. This is comparable to the 17% sequence identity shared by all Ig domain sequences (as annotated in PFAM),³⁷ indicative of a level of identity resulting from structural conservation alone.

The first B7 member discovered was CD80, which shares 24% identity with CD86, its closest human homolog (Figure 1(a)). The rest of the human members have an average of 20% sequence identity compared to CD80. Comparing each member to all others, human B7-H3 has the highest average sequence identity of 23%, and B7-H5 has the lowest at 17%. Overall, the two most similar human proteins are B7-H1 and B7-DC (37%), and the two least similar are B7-H4 and B7-H5 (14%). Globally, B7 members have five pairs with sequence identity above 25%, suggesting little homology *a priori* beyond that of the Ig superfamily (IgSF). Intracellularly, the cytoplasmic tails vary in length from 1 (B7-H4) to 171 (B7-H6) residues, there is little evidence a common motif in the tail is widely utilized, and understanding their role in biology is ongoing.³⁸

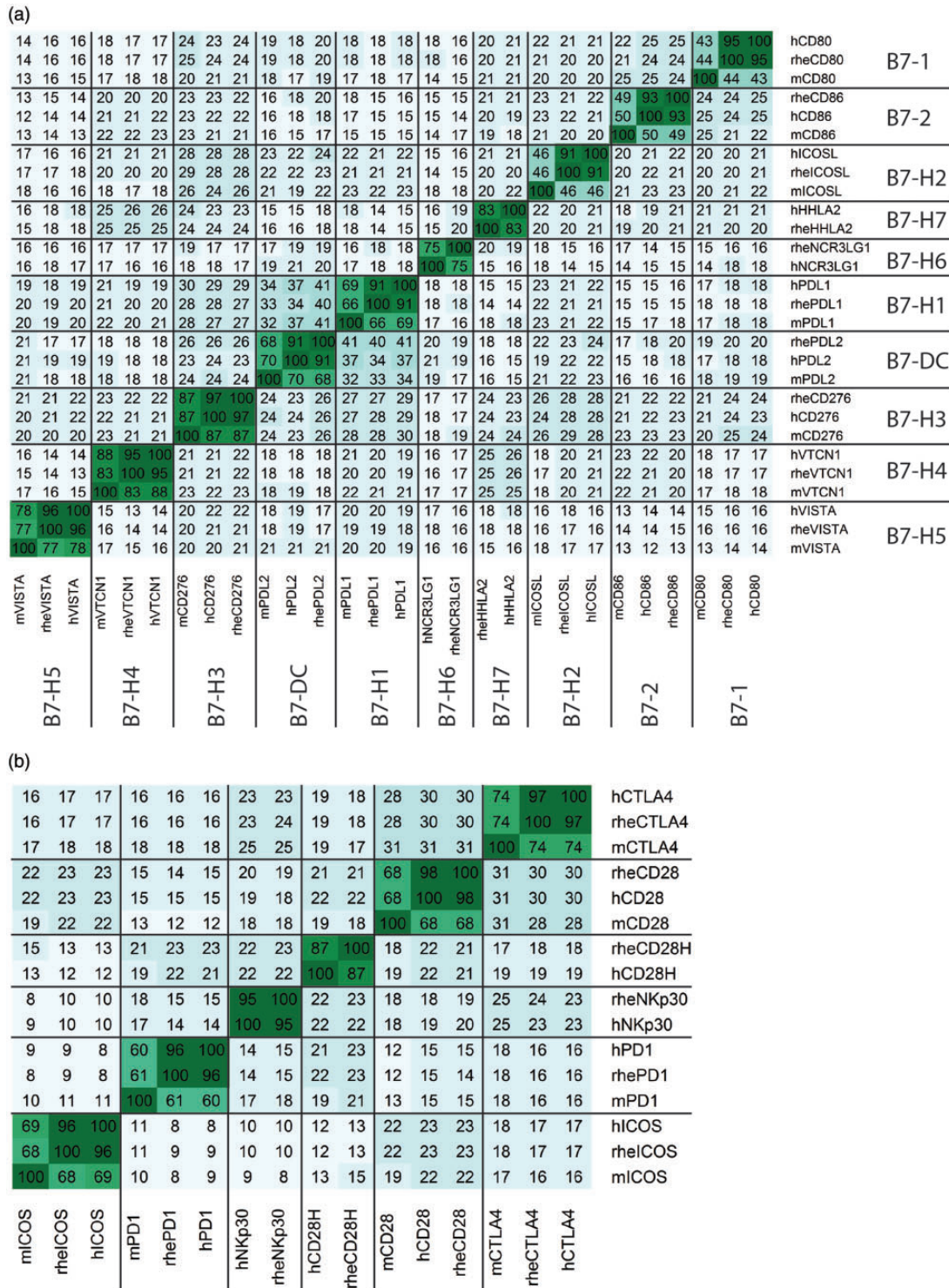


Figure 1. Full sequence alignment of available B7 and CD28 members. (a) Global alignment of human (h), *Rhesus macaque* (rh), and *Mus musculus* (m) B7 members. (b) Global alignment of human, rhesus, and mouse CD28 members. Number represents percent identity. Alignment performed using ClustalOmega.

A similar trend can be seen when comparing CD28 members (Figure 1(b)), which have an overall average sequence identity of 18%. CD28 and CTLA-4, the two initial members, share 30% identity (Figure 1(b)). Homology falls to 15–23% comparing other human members to CD28. ICOS has the lowest average sequence identity compared to other CD28 members at 14%, and CD28 has the highest at

22%. Pairwise, CD28 and CTLA-4 share the highest identity as mentioned, while PD-1 and ICOS only have 8% identity, the lowest across all comparisons. Besides the initial members, CD28 and CTLA-4, no other receptor pair has identity greater than 25%. Intracellularly, the cytoplasmic tail range from 38 to 111 residues, with many members having tyrosine signaling motifs.⁵

The low sequence homology of B7-CD28 members is in contrast to a more traditional family like butyrophilin. Butyrophilin members have been described as B7-like,³⁹ as they modulate the immune response and contain Ig domains. The family is clustered on chromosome 6, has an overall 42% sequence identity (unpublished analysis), and typically contains the B30.2 intracellular domain.⁴⁰ The lack of similar sequence features among B7-CD28 family members results in difficulties predicting additional members, potential binding partners, and therapeutic potency.

Structure homology

Protein structure is another feature by which a family can be classified. Both B7 and CD28 members utilize the common Ig fold for their extracellular domains (Figure 2 (a)), and belong to the IgSF. The Ig fold is widely utilized from antibodies to receptors, and is not associated with any

specific biological function. Ig fold can be described as roughly 100 amino acids, consisting of anti-parallel beta strands stabilized through a hydrophobic core and a disulfide bond. The Ig fold is further classified as IgV, IgC1, or IgC2 based on strand number and connectivity,⁴¹ which are utilized in B7 and CD28 members, with IgV domains being involved in receptor and counter-receptor binding (Figure 2(a)). It should be noted that strand swapping was observed in some structures of CTLA-4, B7-H4, and murine B7-H3. This strand swap is a potential artifact of the protein refolding process or crystallization conditions and has been observed in other Ig structures, but may also have a physiological role.^{42,43} With the exception of B7-H5,⁴⁴ B7 members contain at least two Ig domains, whereas all identified CD28 members consist of a single Ig domain. Overall the B7 members share a consistent structural fold and align well (2.4Å RMSD), with similar number and positioning of beta sheets. We do see

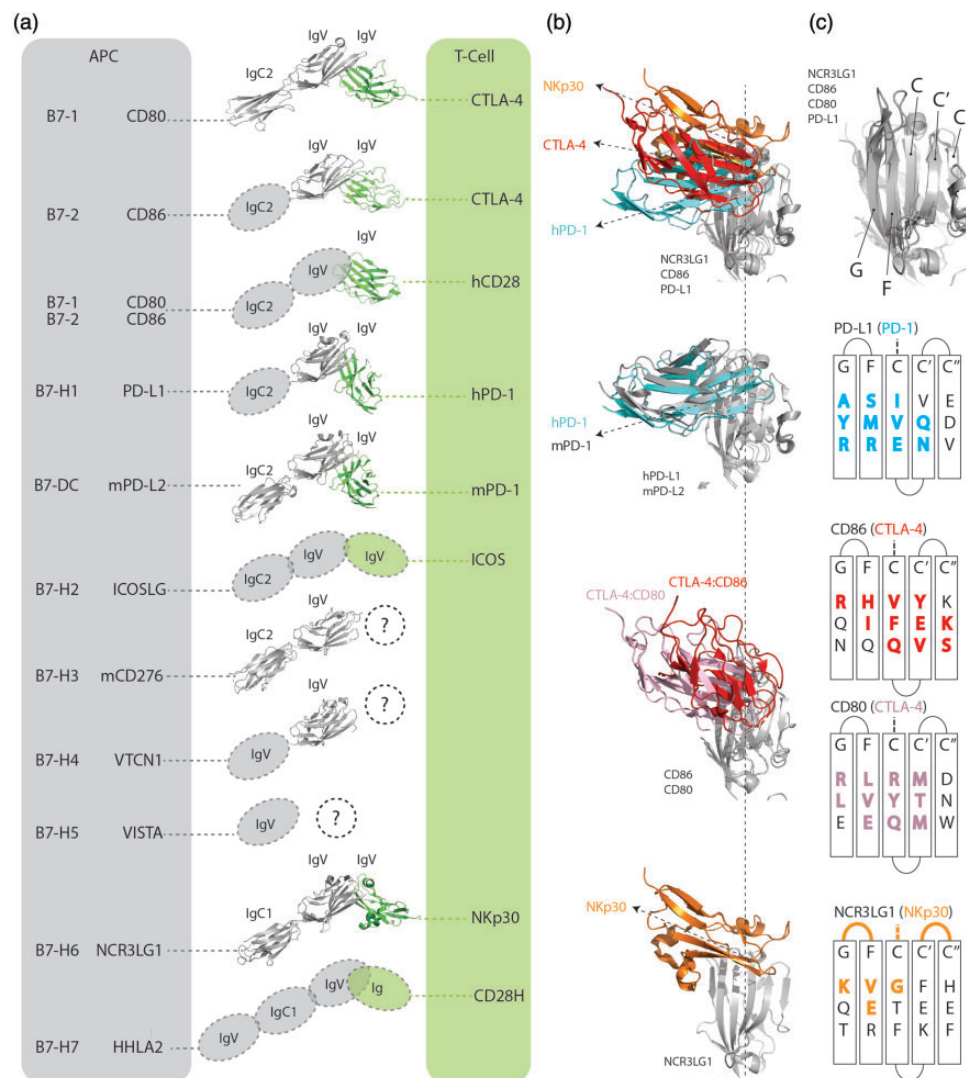


Figure 2. Structural analysis of B7 and CD28 members. (a) Structures of select B7 (gray) and CD28 (green) members. From top to bottom PDB codes: 118L (hCD80:hCTLA4), 1185 (hCD86:hCTLA4), 1YJD (hCD28), 4ZQK (hPD-1:hPD-L1), 3BP5 (mPD-1:mPD-L2), 4I0K (mCD276 has two Ig domains; hCD276 has four), 4GOS (hVTCN1), 3PV6 (hNCR3LG1:NKp30). (b) Comparing IgV domains interaction angle for 4ZQK (hPD-1:hPD-L1), 3BP5 (mPD-1:mPD-L2), 118L (hCD80:hCTLA4), 1185 (hCD86:hCTLA4), and 3PV6 (hNCR3LG1:NKp30). (c) Beta sheets of aligned B7 members at the counter receptor interface, and specific residues that structurally align in 3D space. Residues and loops that are in contact with the CD28 members are colored and in bold.

variability in loops that connect the beta sheets, and bend angle between the two Ig domains. The variability in loop position is not unexpected as loops are structurally flexible and could vary from structure to structure. The length of these loops can also vary, and this is related to sequence differences. The subtle bend angle differences between the Ig domains can also be attributed to experimental effects of structural determination, and may not reflect actual differences.

This Ig structural fold however is not a distinguishing feature for B7 members, as there are other receptors with similar extracellular folds including the butyrophilin family. The butyrophilin family members contain two Ig domains, a membrane distal IgV, and overall look similar to B7 members.⁴⁰ Members of CD28 consist of a single Ig fold, and the structural layout is shared with many other immune receptors such as the T-cell Ig and mucin domain (TIM) family also having immunological functions.⁴⁵ Likewise, the two Ig domain CD200 ligand is broadly expressed, while its inhibitory receptor CD200R, also composed of two Ig domains, is expressed on myeloid, NK, B-, and T-cells.⁴⁶ CD200:CD200R have an interface similar to the B7-CD28 family.⁴⁷ These examples demonstrate the versatility of the Ig fold and its prevalence in immunology, therefore not unique to B7-CD28 members.

The surfaces utilized by B7-CD28 members to interact, critical for antagonizing therapies blocking the interaction, are comprised of sidechain interactions from beta strands and loops at the interface. This is similar to the classic IgV domain pairing of the variable heavy and light chains of antibodies that can vary by their packing angle,⁴⁸ which dictates the orientation of the two variable domains. In antibodies, this angle impacts how the binding loops are presented. By aligning and orienting the B7 IgV domains of complex structures (hPD-L1:hPD-1, mPD-L2:mPD1, CD80:CTLA-4, CD86:CTLA-4 and NCR3LG1:NKp30), differences in interface angles are apparent (Figure 2(b)) as previously described.⁴⁹ The change in interface angle indicates different residues of the IgV domain can be utilized for protein interactions. The angles observed exhibit how CTLA-4 interacts differently with two closely related molecules, CD80 and CD86, whereas PD-1 interacts similarly with both of its ligands. A closer look at the IgV:IgV interface reveals that certain residues on beta strands C, C', C'', F, and G share spatial positioning, but differ in charge and size of the sidechains, suggesting little is shared at this interface within the family (Figure 2(c)). This positional conservation at the interface is only apparent with structural alignment; IgV domain sequence alignment shows an average percent identity of 22% for B7 and 18% for CD28 members, with different residues aligned at some interface positions due to low conservation. Taken together, the available human complex structures show while they share similar structure, residues at the IgV interface differ in composition and contacts. This further highlights that though members are related through use of the Ig fold, and have beta strand interactions similar to other IgV:IgV complexes, the family has few consistencies in its interactions.

Therapeutic considerations

Since the FDA approval of cancer therapies targeting B7-CD28 family members, anti-CTLA-4 in 2011 and subsequent approvals for anti-PD-1 and PD-L1, there has been tremendous discovery and clinical efforts in reversing immune suppression. B7-CD28 members are amenable targets for antibody therapy, as they rely on extracellular domains to initiate contact and subsequent signaling. However, dissimilarities within B7-CD28 family members have made it difficult to utilize insights gained from research and clinical development targeting the potent PD-1 and CTLA-4 pathways.

Another important aspect to drug discovery is utilizing mouse models. As there is low sequence conservation from human to mouse (Figure 1(a,b)), obtaining cross-species reactive antibodies can be difficult, but more importantly this limits the functional relevance of mouse models. For example there is 50% or less sequence identity between mouse and human for CD80, CD86 and ICOSL, whereas human and rhesus share over 90% sequence identity for these receptors. To further highlight the immunological differences of human and mouse, there are no clear functional mouse orthologues for B7-H6,³¹ NKp30,⁵⁰ or B7-H7.⁵¹ These differences between mouse and man, and the dynamics of the immune system, make interpreting and translating efficacy difficult for checkpoint inhibitors. An example of this is targeting CTLA-4, where tumor clearance in mouse has been linked to depletion of intratumoral regulatory T-cells,⁵² but this mechanism may not be reflected in the clinic.⁵³

Discussion

While the core B7-CD28 family can be designated as a trio of related molecules on APC (CD80, CD86, ICOSL) and their counterparts on T-cells (CD28, CTLA-4, ICOS), the broad family criteria of regulating an immune response and containing an Ig fold have led to ever-increasing growth of the family. To illustrate this, NKp30 was initially identified on NK cells, without any notable relationship to the B7-CD28 family.⁵⁴ A decade later, a previously unannotated gene with a sequence identity "comparable" to other B7 members, therefore named B7-H6 (NCR3LG1), was identified as its receptor.³¹ Consequently, NKp30 is now considered a CD28 member, and understanding its role in T-cells continues.⁵⁵ Perhaps a more dramatic example that resulted from lack of similarity among family members is B- and T-lymphocyte attenuator (BTLA), which was initially proposed to be related to CTLA-4 and PD-1 with binding to B7-H4.⁵⁶ However, it was later revealed that it did not bind B7-H4 and subsequently BTLA is no longer considered part of the B7-CD28 family.⁵⁷ As two B7 members have not been de-orphanized, there may yet be additional CD28 members. An example of this is the developing story of B7-H5:VSIG-3, which raises the question of VSIG-3 being "CD28-like". With these lax criteria, there may be more inclusions in the family. This fluidity of B7-CD28 members, with contractions and expansions, limits biological understanding and therapeutic development.

The overwhelming success of targeting the initial B7-CD28 members with therapeutic antibodies altered how cancer is treated. These advances have thrust the complex and dynamic interactions of immune cells into the spotlight, and discovery and classification of these diverse receptors and ligands have created a non-traditional family. Since members are only related in a broad sense, additional members have been included based on Ig domain similarities with little specific functionality in common. Ultimately, family classifications provide value in organizing proteins phylogenetically and evaluating function, but when a family expands without consistency, this value is diminished.

Authors' contributions: SMW and XAD contributed equally to writing the review and have read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank our colleagues Arvind Rajpal, Pavel Strop, John Engelhardt, and Cathy Bolger for their thoughtful comments and insights during the preparation of this manuscript.



DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: SMW and XAD are employed by Bristol-Myers Squibb.

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