

T Cell Co-inhibitory Receptors: Functions and Signalling Mechanisms

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Abstract

T cell activation is a central event in the adaptive immune response and essentially begins with the recognition of an antigenic peptide in the context of a major histocompatibility complex (MHC) on an antigen-presenting cell by the T cell receptor (TCR). The process of T cell activation consists of an orchestration of various functional modules such as actin polymerization, cell surface receptor patterning, calcium fluxing, immunological synapse formation, enhanced adhesion and gene transcription. These modules are mediated by a number of signalling proteins through inducible phosphorylation, enzyme activation and protein-protein & protein-lipid interactions. This complex and dynamic interplay of signalling events governs the decisions the T cell makes in terms of gene expression, proliferation, differentiation, survival and migration. These outcomes are influenced by the magnitude, duration and context of the activation signals. The activation signals have the potential to be modulated by a family of receptors termed, co-inhibitory receptors that include PD-1, LAG-3, TIM-3 and CTLA-4. Co-inhibitory receptors modulate signalling by utilising mechanisms such as ectodomain competition with counter receptors and by the use of intracellular mediators such as protein phosphatases. Co-inhibitory receptors can act as threshold-setters, modulators, check-points and feedback mechanisms that can potentially fine tune the quality and magnitude of the T cell immune response. Given the key roles of these receptors in modulating the immune response, they are increasingly being targeted for immune intervention in a variety of disease settings. This review discusses current understanding of the role of co-inhibitory receptors in influencing T cell signalling.

Keywords: Co-inhibitory receptor; CTLA-4; PD-1; BTLA; LAG-3; TIM-3; SHP-1; ITIM

Introduction

The two major types of lymphocytes, B cells and T cells, are the main mediators of the adaptive immune response. T lymphocytes differentiate in the thymus and migrate to secondary lymphoid organs such as the lymph nodes and spleen. Here, the T lymphocytes play a prominent role to sense, monitor and respond to the presence of foreign antigens that have been engulfed by highly phagocytic immature dendritic cells (DCs) at sites of infection and brought in to the secondary lymphoid organs. Mature DCs display these foreign antigens as proteasome-processed peptides in the context of major histocompatibility complex (MHC) glycoproteins. Intracellular peptides (e.g. derived from viral proteins) bind to MHC class I molecules and are recognized by the CD8⁺ T cell subtype, while extracellular peptides (e.g. derived from bacterial proteins) bind to MHC class II molecules and are recognized by CD4⁺ T cells. The T cell receptor complex (TCR) expressed by CD8⁺ and CD4⁺ T cell subsets serve as highly variable receptor molecules that enable these cells to recognize and respond to antigen [1].

The binding of the peptide-MHC complex to the TCR initiates the formation of microclusters at distinct contact areas between the T cell and the antigen presenting cell (APC). These microclusters represent essential membrane-associated platforms where TCR-mediated signalling is initiated and maintained, before the formation of the immunological synapse (IS) [2]. TCR engagement is translated into changes in the TCR-associated CD3 subunits that allow tyrosines within their immunoreceptor tyrosine-based activation motifs (ITAMs) to be phosphorylated by CD4- and CD8-p56 Lck complexes. This event represents the start of assembly of the TCR signalling machinery or TCR signalosome which consists of a complex modular architecture made up of three independent signalling modules whose connectivity and inter-regulation are increased upon TCR stimulation. These are the src-family protein tyrosine kinase (PTK) regulation module, the signal triggering module which consist of the ITAM motifs associated with the TCR complex and the PTK ZAP70, and the signal diversification

and regulation module that includes components such as ITK and Vav1 [3]. Following the phosphorylation of CD3 ITAMs by the src-family PTK Lck, ZAP 70 is recruited and activated which induces the assembly of the signal diversification and regulation module (these include the regulators of Ca²⁺ signalling and DAG production; PLCγ and ITK, actin polymerization; NCK and Vav1 and integrin activation; ADAP) by phosphorylating the adaptor protein LAT found in subsynaptic intracellular vesicles beneath the IS. This is followed by the consequent phosphorylation of multiple key downstream adaptor proteins such as SLP-76, Grb2 and GADS [4].

Since the TCR lacks endogenous kinase function, the phosphorylation of the paired tyrosine residues in the cytoplasmic tails of CD3 and ζ-chain accessory molecules relies on the Src-family kinase (SFK) Lck. SFK activity is pivotal for orchestrating the threshold sensitivity and strength of TCR signalling. To prevent aberrant cellular activation, Lck is regulated and kept in check by a dynamic equilibrium between the protein kinase and phosphatase activities of Csk and CD45, respectively [5]. Thus, the phosphorylation state and activity of Lck is continuously altered with a slight change in either CD45 or Csk leading to Lck activity and resulting in signal transduction.

The primary TCR signal provides the minimal scaffold for the recruitment of essential signalling molecules that then form nexus points

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to other major signalling pathways such as the MAPK/Erk pathways. Signalling molecules recruited by co-stimulatory receptors such as CD28 add to this central scaffold and act as amplifiers, enabling signalling thresholds to be overcome when TCR occupancy is low [6]. CD28 is expressed by T cells and binds to ligands B7.1 (CD80) and B7.2 (CD86) expressed by professional APCs such as DCs. Upon receptor triggering, signal transduction cascades are initiated when signalling proteins associate with the cytoplasmic tail of CD28. Within the cytoplasmic tail, the proximal YMMN motif binds to the p85 subunit of PI3K and Grb2 or GADS. The distal proline-rich motifs PRRP and PYAP bind to the proteins Itk and Grb2/filamin A/Lck, respectively [7]. The PI3K signal pathway ultimately leads to increased NF- κ B transcriptional activity and increased survival. Binding of Grb2 to the distal motif initiates the formation of two signalling complexes. The vav-Sos complex leads to cytoskeletal rearrangement and formation of the AP1 transcriptional complex via JNK activation, while the SLP-76-LAT complex leads to NF- κ B transcriptional activation and enhanced transcription of NFAT-dependent genes including IL-2 [7]. The efficiency of T cell signalling is increased by spatial organisation of receptors and signalling mediators. In particular, the triggered receptors and their downstream signalling proteins are spatially organised into cholesterol- and sphingolipid-rich plasma membrane microdomains termed lipid rafts [8]. Sustained T cell signalling requires prolonged T cell-APC interactions. Integrins such as lymphocyte function-associated antigen 1 (LFA1) constitute a family of proteins that are capable of inside-out signalling, mediate T cell-APC adhesion and are essential for IS formation [9].

In addition to the considerable array of stimulatory receptors found on T cells, there are also receptors that are inhibitory in function. These receptors are termed T cell co-inhibitory receptors (Figure 1 & Table 1). Inhibitory receptors attenuate and counterbalance activation signals initiated by stimulatory receptors. The subsequent outcomes on T cell function can range from temporary inhibition to permanent inactivation and cell death [10]. TCR signalling can be controlled by various mechanisms that differ in their time of action and/or target molecule. Negative regulatory mechanisms are in place to act before T cell activation to maintain its quiescent state. These range from the sequestration of ITAM motifs in the lipid bilayers [11] to the autoinhibitory autoinhibition of the phosphatase calcineurin, the action of I κ B and its transcriptional activators Foxj and Foxo3a [12]. Rapidly after activation of the TCR signalosome, immediate feedback is provided by the protein tyrosine phosphatase SHP-1, miR-181a and DOK adaptor proteins. Delayed feedback is conducted by the HPK1-SLP76-14-3-3 pathway and STS proteins. Hours after T cell activation, modulation of TCR signalling is conducted by inhibitory receptors such as CTLA-4, PD-1 and LAG-3 [3]. This review focuses on some of the key T cell co-inhibitory receptors and their influence on T cell signalling and functions.

Mechanisms of Regulation by Co-inhibitory Receptors

The majority of T cell co-inhibitory receptors belong to the immunoglobulin (Ig) superfamily [13]. There are three major mechanisms that are utilized by membrane bound inhibitory receptors

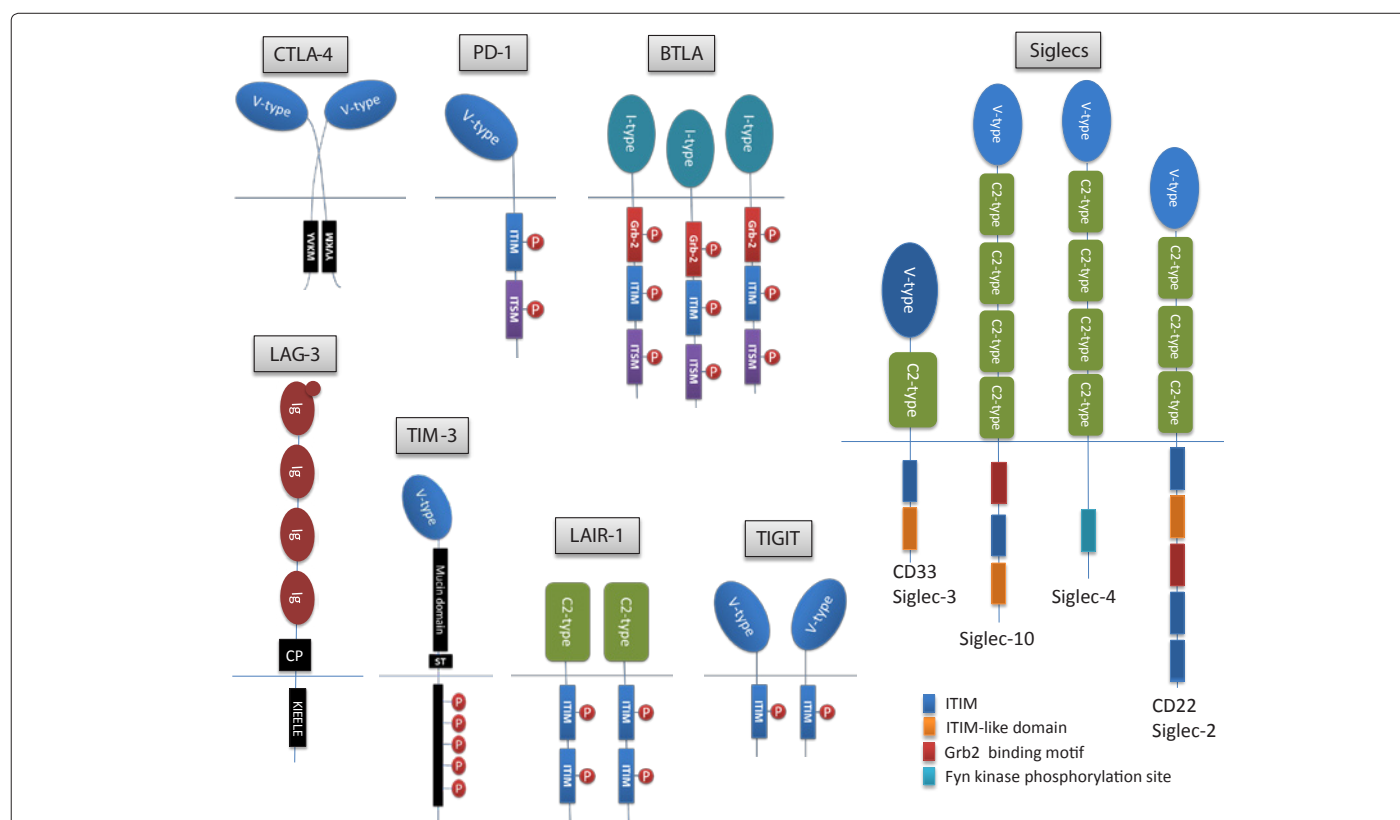


Figure 1: Schematic structure of T cell co-inhibitory receptors

The domain structures of the following co-inhibitory receptors are schematically depicted: B- and T-lymphocyte attenuator (BTLA), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Lymphocyte-activation gene 3 (LAG-3), Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1), Programmed Death 1 (PD-1) sialic acid binding Ig-like lectins (Siglecs), T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), T cell immunoglobulin mucin-3 (TIM-3). Extracellular immunoglobulin domains of co-inhibitory receptors include Ig-like (Ig), V-type, C2-type and I-type. ITIM- immunoreceptor tyrosine-based inhibition motif, ITSM- immunoreceptor tyrosine-based switch motif.

Receptor (Ligand)	Intracellular motif	Mechanism of Action	Receptor distribution	Ligand distribution	Disease	Biologic (Target)	Refs.
CTLA-4 (CD80, CD86)	YxxM	¹ Use of intracellular mediators—SHP-2, PI3K ³ Ectodomain competition—with counter receptor (CD28); interference of lipid-raft and microcluster formation	Activated T cells	B cells, Mo, DCs, T cells, inducible in some somatic tissues	RA, MS, LN, Melanoma	abatacept (B7) ipilimumab (CTLA-4) tremelimumab (CTLA-4)	[31,89] [13,90] [34,91]
PD-1 (PD-L1, PD-L2)	ITIM, ITSM	¹ Use of intracellular mediators—recruitment of SHP-1, SHP-2; suppression of transcription factor SKP2 ² Inducing genes that negatively regulate T cell signalling—BATF	Activated T cells, B cells, DCs, NKT cells, Mo	B cells, T cells, inducible in Mo, DCs, some somatic tissues	Melanoma, RCC, HIV	CT-011(PD-1) MDX1106 (PD-1) MPDL3280A (PD-L1)	[41,92] [93,94] [95]
BTLA (HVEM)	ITIM, ITSM	¹ Use of intracellular mediators—SHP-1, SHP-2 ³ Ectodomain competition with counter receptor (LIGHT)	T cells, B cells, DCs, Myeloid cells	naive T cells, B cells, DCs, NK cells, myeloid cells, inducible in somatic tissues	GVHD, Autoimmune diabetes	N/A	[51,52]
LAG-3 (MHC II)	KIEELE	³ Ectodomain competition with counter receptor (CD4)	T cells, B cells, NK cells	DCs, MΦ, B cells, Mo, thymic epithelial cells	Melanoma, RCC	IMP321 (MHC II)	[96,97] [58]
TIM-3 (Galectin-9, PS)	Y235, Y242	¹ Use of intracellular mediators—Lck, Fyn, p85 PI3K, Bat3 (repressor of TIM-3 signalling)	T cells, B cells, NK cells, NKT cells, DCs, MΦ	T cells, eosinophils, endothelial cells, DCs, MΦ	MS, colitis, HIV, HCV	N/A	[66,69] [64,70]
LAIR-1 (Collagen)	2×ITIM	¹ Use of intracellular mediators—SHP-1, SHP-2, Csk	T cells, B cells, NK cells, DCs, Mo, eosinophils, Basophils, Mast cells	Extracellular matrix components	RA	N/A	[71]
TIGIT (CD155)	2×ITIM	³ Ectodomain competition with the counter receptor (CD266)	T cells, NK cells	T cells, B cells, DCs	MS	N/A	[77,78]
Siglecs (sialylated glycoconjugates)	variable number of ITIM, Grb2 and ITIM-like domains	¹ Use of intracellular mediators—SHP-1, SHP-2, PI3K ³ Ectodomain competition with the counter receptor (cis/trans-ligand binding)	T cells, B cells, DCs, pDCs, MΦ, Mo, Neutrophils, NK cells, eosinophils	widespread on cell surfaces, proteins, pathogens	Leukaemia, SLE, B cell-NHL	gemtuzumab (Siglec-3), epratuzumab (Siglec-2)	[98,99]

Abbreviations: ¹⁻³three general types of distinct mechanisms of action; B cell-NHL: B cell non Hodgkin's Lymphomas; Bat3- Human Leukocyte Antigen B (HLA-B)-associated Transcript 3; BATF: Basic Leucine Zipper Transcription Factor, ATF-like; BTLA: B- and T-lymphocyte Attenuator; Csk: COOH-terminal; CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; DC: Dendritic Cells; Fyn: a Src family tyrosine-protein kinase; Grb2: Growth factor receptor-bound protein 2; GVDH: Graft Versus Host Disease; HCV: Hepatitis C Virus; HIV: Human Immunodeficiency Virus; HVEM: Herpes Virus Entry Mediator; ITIM- Immunoreceptor Tyrosine-based Inhibition Motif; ITSM: Immunoreceptor Tyrosine-based Switch Motif; LAG-3: Lymphocyte-Activation Gene 3; LAIR-1: Leukocyte-Associated Immunoglobulin-like Receptor 1; Lck: Lymphocyte-specific protein tyrosine kinase; LIGHT: Lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes; LN: Lupus Nephritis; MHC II: Major Histocompatibility Complex II; Mo: Monocyte; MS: Multiple Sclerosis; MΦ: Macrophage; N/A: Not Applicable; NK: Natural Killer cells; NKT: Natural Killer T cells; PD-1: Programmed cell death protein 1; pDC: plasmacytoid dendritic cell; PD-L1: Programmed cell death 1 ligand 1; PI3K: Phosphoinositide 3-kinase; PS: Phosphatidyl Serine; RA: Rheumatoid Arthritis; RCC: Renal Cell Carcinoma; SHP-1/2: Src homology 2-containing tyrosine phosphatase 1/2; Siglecs: Sialic acid binding Ig-like lectins; SLE: Systemic Lupus erythematosus, Srk kinase; SKP2: S-phase Kinase-associated Protein 2; TIGIT: T-cell immunoreceptor with immunoglobulin and ITIM domains; TIM-3: T cell Immunoglobulin Mucin-3

Table 1: T cell co-inhibitory receptors.

in T cells (Figure 2). The first mechanism involves the sequestration of the ligands for co-stimulatory receptors, depriving the T cell from receiving activation signals necessary for complete activation. The second mechanism involves the recruitment of intracellular phosphatases by an immunoreceptor tyrosine-based inhibition motif (ITIM) and/or an immunoreceptor tyrosine-based switch motif (ITSM) that make up the cytoplasmic tail of certain inhibitory receptors, which dephosphorylate signalling molecules downstream of the TCR and co-stimulatory pathways, leading to a quantitative reduction in activation-induced gene expression. The third mechanism involves the upregulation (or downregulation) of genes that code for proteins involved in the inhibition of immune functions [13]. A co-inhibitory receptor could use a combination of the above and possibly other yet to be discovered mechanisms to regulate T cell signalling.

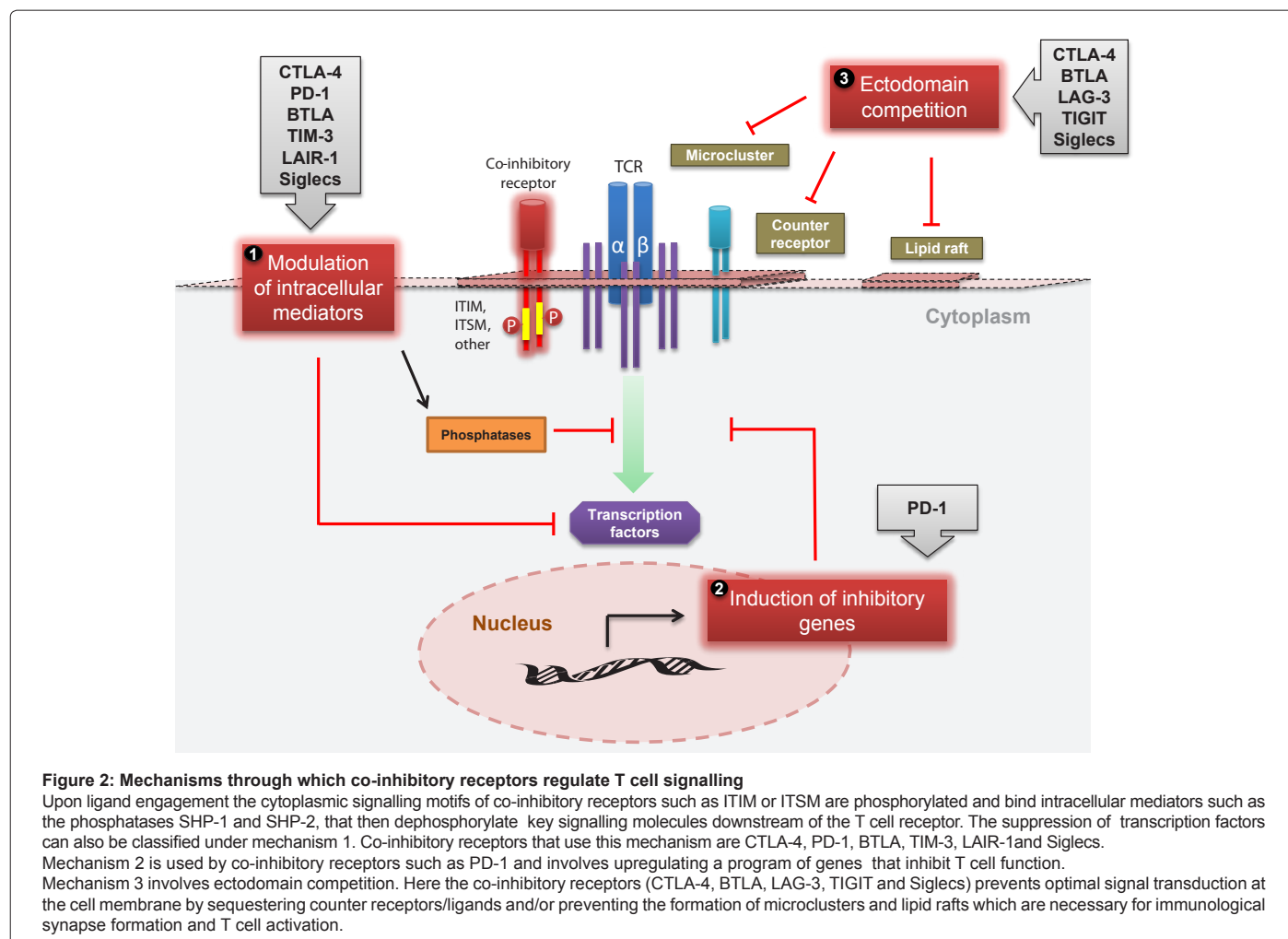
Protein Tyrosine Phosphatases as Mediators of Regulation by Co-inhibitory Receptors

A large number of co-inhibitory receptors recruit intracellular phosphatases to their cytoplasmic domains in order to dephosphorylate signalling molecules downstream of the TCR and co-stimulatory pathways. Most of these co-inhibitory receptors contain one or several ITIMs within the cytoplasmic domain which recruit Src homology 2 (SH2) domain-containing phosphatases. ITIMs are structurally defined as a six-amino acid consensus sequence (I/V/L)xYxx(L/V),

containing a single tyrosine (Y), a hydrophobic residue (I, V or L) at the C-terminal position Y+3 and a less conserved hydrophobic residue at the N-terminal position Y-2 [14,15]. The motif is highly conserved except for the amino acids surrounding the single tyrosine residue, where x denotes any amino acid.

Despite the large number of different ITIM-bearing receptors on a single cell type, each co-inhibitory receptor has a crucial and non-redundant role in immunoregulation. The ITIM-bearing co-inhibitory receptors vary in their expression patterns, level of inhibition and the type of signal delivered. Upon T cell activation ITIM-bearing receptors translocate to the immunological synapse with activating receptors that brings them in close proximity to a kinase, which in the majority of cases is a Src family kinase (e.g. Lck). The kinase phosphorylates the tyrosine residue within the ITIM motif and leads to the recruitment of SH2 domain-containing phosphatases SHP-1 and/or SHP-2, SHIP, which then dephosphorylate several key molecules that are involved in the initial steps of the T cell signalling pathway. SHIP1 and SHIP2 are lipid phosphatases while SHP-1 and SHP-2 are tyrosine phosphatases [16].

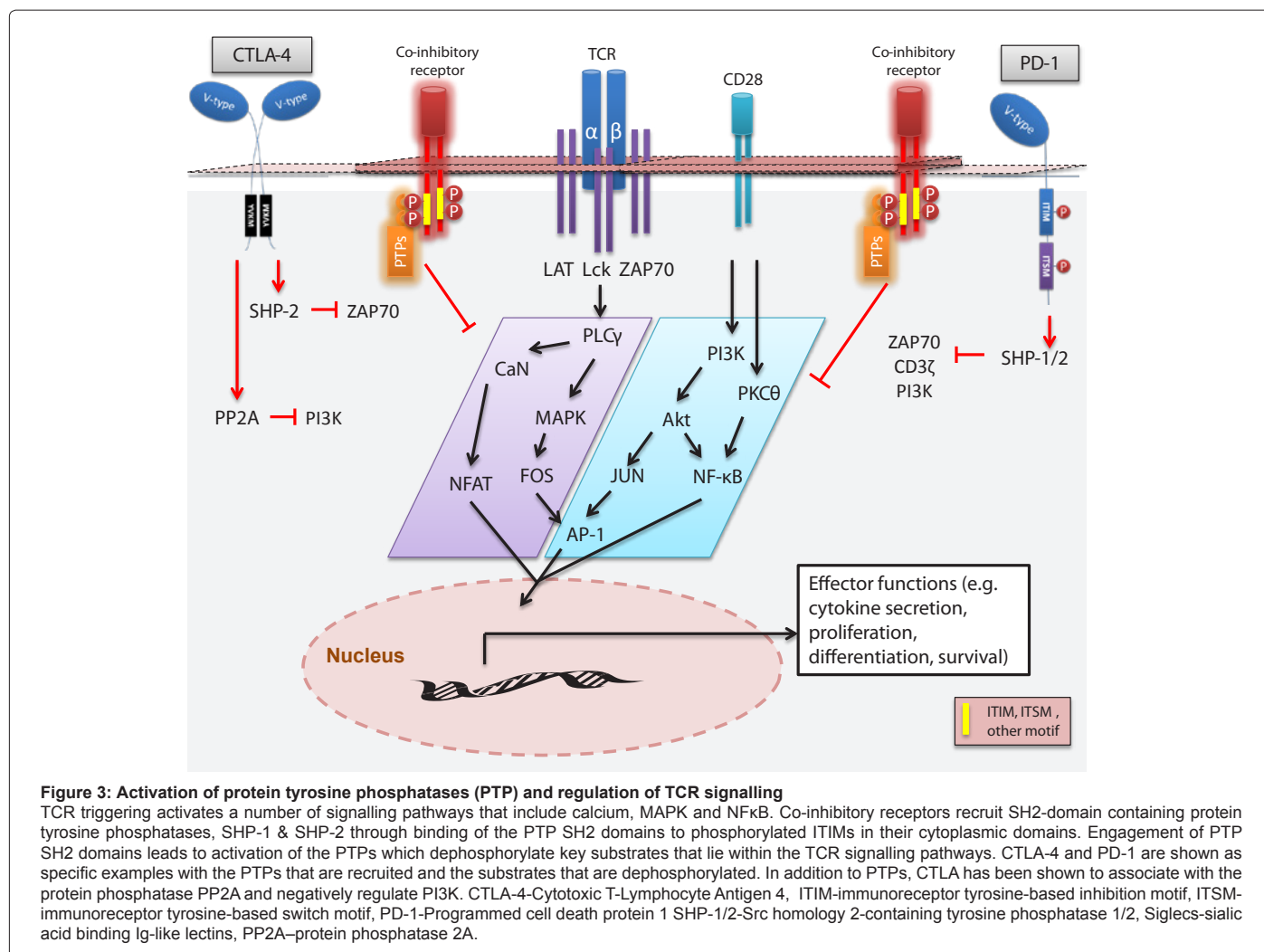
Programmed cell death protein 1 (PD-1), B- and T-lymphocyte attenuator (BTLA) and Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) belong to the group of ITIM-bearing co-inhibitory receptors which recruit SHP-1 and SHP-2; structurally related protein



tyrosine phosphatases that differ in expression patterns and biological function. Both phosphatases consist of two N-terminal SH2 domains, a central catalytic domain with the characteristic PTP signature motif VHCSAGIGRTG, and a C-terminal tail with two tyrosine phosphorylation sites [17]. SHP-1 but not SHP-2 contains a novel six amino acid motif (SKHKED) within the C-terminus that mediates constitutive lipid raft localization, a functional NLS and phosphatidic acid binding activity [16,18]. Endogenous SHP-1 is targeted into rafts following TCR engagement and is thought to associate with LAT [19]. Disruption of LAT activation impairs subsequent downstream signalling events without influencing proximal signalling such as TCR ζ and ZAP-70 tyrosine phosphorylation. The lipid raft-targeted SHP-1 is considered to increase the TCR activation threshold and modify the strength of the transmitted TCR signal [20]. The N-terminal SH2 keeps the phosphatase in an inactive state through an auto-inhibitory mechanism. The two SH2 domains of SHP-1 are required to bind cooperatively to two adjacent phospho-ITIMs. Once the N-terminal SH2 domain binds to a phosphorylated phospho-ITIM, a resulting conformational change alleviates the steric occlusion induced auto-inhibition of the catalytic PTP domain and enables the interaction of the enzymatic active site with substrates such as members of the Syk family (ZAP-70 and Syk), and also adaptor proteins (SLP-76 and LAT) (Figure 3). The allosteric switch between the two mutually exclusive conformational states is thought to exist in a dynamic equilibrium

and regulated by the C-terminal SH2 domain [21]. Furthermore, the catalytic activity of SHP-1 can be enhanced when a C-terminal tyrosine residue is phosphorylated by Lck [22,23]. In addition to the signalling molecules involved in the initial phase of activation, SHP-1 may also dephosphorylate downstream effectors such Vav1, a regulator involved in the re-organization of the actin cytoskeleton [24].

The importance of SHP-1 in T cell biology is displayed by the phenotype of the *motheaten* (*me/me*) and the *viable motheaten* (*me^v/me^v*) mice [25]. Mice with *me/me* phenotype have a mutation that leads to a frameshift near the 5'-end of the SHP-1 coding sequence and results in no detectable protein. These mice display severe immunodeficiency and autoimmunity (autoantibodies and glomerulonephritis) due to impaired development of virtually all hematopoietic lineages and eventually die due to hyperactivation of cells from the myeloid lineage. Mice with *me^v/me^v* phenotype are caused by a different splicing mutation in the SHP-1 locus where a few amino acids are inserted or deleted within the phosphatase domain resulting in diminished PTP activity and thus, have a similar but less severe phenotype than *me/me* mice [18]. SHP-1 is expressed in hematopoietic cells and plays a major role as a negative regulator of signalling pathways downstream of activated cytokine, integrin, growth factor and antigen receptors. In contrast, SHP-2 is ubiquitously expressed and is thought to act as a positive regulator of cytokine and growth factor receptor signalling. However, there is some evidence portraying SHP-2 as a negative regulator of



cell activation when recruited to ITIMs-bearing receptors. The way in which activating signals are modified depends on the phosphatase recruited to the ITIM motif. In addition to the SH2 domain and C-terminal phosphorylation mediated regulation, the activity of SHP-1 can be regulated by two other possible mechanisms. SHP-1 can be translocated to different subcellular locations upon cell activation which could alter its rate of dephosphorylation as most of its substrates are membrane receptors. Finally, the expression of the *SHP-1* gene is regulated at a developmental and tissue specific level, and is expressed as multiple transcript isoforms which themselves could have novel regulatory properties at the splicing/transcription level [16].

A variant of the ITIM termed immune receptor tyrosine-based switch motif (ITSM) is found in some co-inhibitory receptors. PD-1 and BTLA contain both an ITIM and an ITSM within their cytoplasmic domain (Figure 1). The ITSM motif has a consensus sequence (T) xYxx(V/I) and binds to an adaptor protein called SH2 domain-containing molecule 1A, which influences the activating signals by regulating the recruitment of SHP-1 and SHP-2 [26]. In addition to ITIMs and ITSMs, it is thought that under specific configurations the ITAM motif can propagate an inhibitory signal. The so called inhibitory ITAM (ITAMi) confers a new immunoregulatory function to ITAMs as dual function modules. Unlike the ITIMs which represent an instant activation-dependent regulatory step, the ITAMi operates continuously

as a control mechanism of immune cell activation. The ability of an ITAM motif to mediate inhibitory signals was originally shown in an experiment where altered peptide ligands under certain conditions lead the TCR complex to produce differential signals that could even be inhibitory in nature. It is possible that partial phosphorylation of ITAM tyrosines can be inhibitory in function and lead to altered signalling output [27].

Inhibition can also be executed by a co-inhibitory receptor in the absence of an intracellular ITIM motif. For example, CTLA-4 does not have ITIMs but does contain two tyrosine residues that could act as potential SH2-domain binding sites. Although SHP-2 might have a debatable role in mediating the inhibitory effect of CTLA-4, its presence is not always required for optimal CTLA-4 function [16,28,29]. The role of SHP-1 in CTLA-4 function is yet to be determined.

Key Co-inhibitory Receptors Involved in Regulating T cell Signalling

CTLA-4

CTLA-4 has an essential role in negative regulation and maintenance of T cell homeostasis. The expression of CTLA-4 on T cells controls the thresholds of T cell activation following TCR ligation [30]. CTLA-4 is a type I transmembrane glycoprotein of the Ig superfamily (Figure

1) found at low levels as a non-functional covalent homodimer on the surface of T cells 24-48 hours following TCR stimulation and binds to the homodimeric ligand CD80 (B7-1) and CD86 (B7-2) expressed by APCs (Table 1). These ligands also bind to the more abundant CD28 receptor, although with a lower affinity. The structural topology of the CTLA-4 homodimer enables bivalent binding to B7, resulting in a higher avidity compared to CD28 which can only bind B7 monovalently. The CD28 and CTLA-4 receptors use the same MYPPPY motif in their extracellular domain to bind to both B7-1 and B7-2. The cytoplasmic domain of CTLA-4 lacks any intrinsic enzymatic activities and relies on its protein-protein interaction motifs to associate with a variety of signalling molecules [31]. CTLA-4 is not present on naive T cells but is constitutively expressed in the CD4⁺ CD25⁺ regulatory T cell subset. Naive and memory T cells have a large pool of CTLA-4 localized to intracellular vesicles (TGN, endosomes and lysosomes) which can be mobilised to accumulate at the IS and partitioned to lipid rafts upon T cell activation. The amount of CTLA-4 transported depends on the strength of the TCR signal and calcium influx, and is facilitated by the chaperone adaptor TCR-interacting molecule (TRIM) [32]. CTLA-4 uses a mechanism of signal initiation that does not fit with the current paradigm for receptor-mediated signalling where ligand-induced conformational changes or ligand-induced dimerization take place [32].

The tyrosine-containing motif YVKM present in the cytoplasmic domain of CTLA-4 interacts specifically in its dephosphorylated state with the $\mu 2$ subunit of the clathrin-associated adapter protein AP-2 which mediates receptor internalisation. CD28 on the other hand also contains several tyrosines in its cytoplasmic domain but does not interact with $\mu 2$ and thus, is constitutively expressed on the T cell surface compared to the transient expression of CTLA-4 [33]. Phosphorylation of the Y-165 residue which requires Lck-dependent and ZAP-70-dependent TCR signals leads to the retention of the CTLA-4 receptor on the cell surface and thus regulates both signal transduction and surface expression. The phosphorylated YVKM motif is recognised by the SH2 domains of signal transducers such as the tyrosine phosphatase Syp and the p85 subunit of PI3K [34].

Several models have been proposed to explain how CTLA-4 can influence TCR signalling. These include competitive antagonism of CD28, direct engagement of negative signalling molecules and the inhibition of lipid-raft and microcluster formation (Figure 2 & 3). The higher affinity and avidity of CTLA-4 for CD80/CD86 molecules outcompetes CD28 binding and thus impairs co-stimulation leading to a dampened T cell response. Studies have shown that CTLA-4 can bind negative signalling molecules such as SHP-2 and PP2A (a serine/threonine phosphatase) which can then dissociate upon CTLA-4 ligation and dephosphorylate certain proximal TCR signalling proteins such as TCR- ζ and LAT [35]. This may lead to inhibition of the PKB/Akt signalling pathway and/or enhance the activity of Cbl-b and Rap1. Cbl-b is a key molecule that tunes the threshold for T cell activation and the activation of Rap1 would lead to the inhibition of ERK-1/-2 in a Ras-independent manner [36]. Following PP2A dissociation, CTLA-4 is able to sequester PI3K and thus limit its availability to mediate CD28-dependent co-stimulation. CTLA-4-PI3K-PKB/Akt-induced phosphorylation of the pro-apoptotic protein Bcl-2 antagonist of cell death (BAD). This leads to the release of Bcl-X_L and Bcl-2 which mediate mitochondrial-dependent T cell survival that may result in T cell energy and potentially ensuring T cell tolerance. Furthermore, CTLA-4 can also activate JNK which is thought to contribute to T-helper cell type 1 (T_H1) versus T_H2 cell differentiation [36].

CTLA-4 disrupts the formation of lipid-rafts and the availability of key signalling components within this platform required for TCR signal propagation. The formation of ZAP70-containing microclusters as well as calcium mobilization can also be impaired by CTLA-4, resulting in reduced phosphorylation of TCR substrates [37]. Upon TCR ligation, there is a reduction in T cell motility in order to form an extended contact with the APC (termed conjugate formation) and a stable IS for the induction of signalling cascades. This reduction in motility termed the 'stop signal' is induced by an increase in avidity and affinity of LFA-1 for its ligand upon TCR activation. LFA-1 is an adhesion molecule on T cells which binds to the intercellular adhesion molecule 1 (ICAM1) expressed on APCs. CTLA-4 reverses the 'stop signal' and essentially acts as a gatekeeper of conjugation by interfering with the formation of stable ISs and limiting the dwell time between the T cell and the APC. In addition to preventing the stop signal, CTLA-4 engagement results in reduction in ZAP70 microcluster formation, calcium mobilization and IL-2 production [38]. CTLA-4 can also act in a cell-extrinsic manner to regulate T cell responses through altering APC function. It has been demonstrated that CD80 and CD86 on APCs can be captured and degraded by CTLA-4 via a process of trans-endocytosis thereby resulting in impaired T cell responses [39]. During CD8⁺ T cell proliferation replicating cells come in contact with each other and upon engagement of the CTLA-4 with CD80/CD86 between activated CD8⁺ T cells trigger the contact-dependent Hippo pathway which activates a serine/threonine kinase cascade. This induces the phosphorylation and proteosomal degradation of the transcription cofactor, Yap and the expression of the transcriptional repressor Blimp-1 which regulates the differentiation of effector CD8⁺ T cells [40].

PD-1

PD-1 belongs to the Ig superfamily (IgSF) of surface proteins (Figure 1 & Table 1). PD-1 is made up of one Ig domain, a ~20 amino acid stalk region, a transmembrane domain and an intracellular domain containing both an ITIM and an ITSM (Figure 1). PD-1 is inducibly expressed on the surface of T cells within 24 hours of activation, although its functional effects on for e.g. IFN- γ , TNF- α and IL-2 production are observable within a few hours [41]. PD-1 normally exhibits a uniform cell surface expression but redistributes itself close to the site of TCR engagement within microclusters for successful interaction with its ligands PD-L1 (constitutively expressed on T cells, B cells, DCs, macrophages, mesenchymal stem cells and bone marrow derived mast cells) and PD-L2 (inducibly expressed on DCs, macrophages and bone marrow derived mast cells) [42].

Upon PD-1 ligation, both of the cytoplasmic ITIM and ITSM tyrosine motifs are phosphorylated possibly by Lck and/or C-terminal Src kinase with a consequent recruitment of SHP-2. This reduces the TCR-triggered phosphorylation of CD3 ζ , ZAP70 and PKC θ , while also blocking the CD28-mediated activation of PI3K and Akt [43]. The inhibitory signal mediated by PD-1 depends on the strength of the TCR signal with much stronger phosphorylation of PD-1 and association with SHP-2 observed at high levels of TCR stimulation. Even in the absence of TCR engagement, a basal level of PD-1 phosphorylation and SHP-2 recruitment is detectable [44].

PD-1 ligation interferes with the induction of the cell survival factor Bcl-xL [26] and the expression transcription factors associated with effector cell function such as GATA-3, T-bet and Eomes [41]. PD-1 ligation can also block cell cycle progression and proliferation of T cells by interfering with multiple regulators of the cell cycle via a mechanism that utilises the suppression of transcription factors in order to downregulate genes that code for proteins involved in cell

cycle control. The standard cell cycle is traditionally subdivided into the G₁, S, G₂ and M phases with a G₀ resting state. Cyclin-dependent kinases (CDK) are key regulatory proteins that are activated at specific checkpoints during the cell cycle to influence downstream processes by phosphorylating specific proteins. There are five active CDKs during the cell cycle; during G₁ (CDK4, CDK6 and CDK2), S (CDK2), G₂ and M (CDK1), and different cyclins are needed at different phases to provide enzymatic activity to the cyclin-Cdk holoenzyme complexes which can be inhibited by Cdk inhibitors (CKI) of the Cip/Kip family such as p21 (Waf1, Cip1), p27 (kip1) and p57 (Kip2). Cdc25 are dual-specificity phosphatases that target CDKs and control the entry and progression through the cell cycle phases [45].

A recent study demonstrated that PD-1 ligation blocks cell cycle progression through the G₁ phase by suppressing the transcription of SKP2, a gene that encodes components of a p27^{kip1} degrading ubiquitin ligase called SCF^{Skp2}, via inhibition of PI3K-Akt and Ras-MEK-ERK signalling pathway. These result in the accumulation of p27^{kip1} and suppression of cyclin E-Cdk2 activity. Consequent impairment in phosphorylation of the transcription factor retinoblastoma (Rb) and the anti-proliferative signal transducer Smad3, increase Smad3 transactivation due to the inhibition of E2F-regulated gene expression. This results in the suppression of the gene encoding Cdc25A with concomitant increase in ubiquitin-dependent proteosomal degradation of the Cdc25A protein, increased p15 abundance and finally more suppression of SKP2 expression [46]. In addition to influencing cell cycle genes, PD-1 can cause T cell exhaustion by upregulating a program of genes including that of the basic leucine transcription factor, ATF-like (BATF) which functions as a negative regulator of AP-1 activity [47].

Continued interaction between PD-1 and its ligand PD-L1 suppress TCR-driven stop signals and prevent recruitment of key signalling molecules to initiate T cell activation. The inability to form stable immunological synapses with allogeneic APCs causes a long-term maintenance of the anergic state that seems to depend on PD-1-PD-L1 interaction [48]. The expression of PD-1 also modulates the threshold of antigen density required to activate cognate T cells which has an impact on antigen recognition in peripheral tissues especially in the context of tolerance [49]. Finally, PD-1 is being considered as a viable target for therapeutic immunomodulation. CD8⁺ T cell dysfunction brought about by chronic antigen stimulation (eg. during lymphocytic choriomeningitis virus LCMV infection) has been shown in animal models to be partially reversed by blockage of the PD-1/PD-L1 pathway [50].

BTLA

B and T lymphocyte associated (BTLA) belongs to the IgSF of co-inhibitory receptors and binds to the herpes virus entry mediator (HVEM) which belongs to the TNF superfamily of co-stimulatory molecules (Figure 1 & Table 1). In addition to BTLA, HVEM binds to four other ligands, the conventional TNF ligand LIGHT and Lymphotoxin α , the herpes simplex virus glycoprotein D and the glycosylphosphatidylinositol-linked Ig domain protein CD160. The interaction of HVEM with BTLA and multiple other ligands represents a functionally diverse set of intrinsic and bidirectional signalling pathways that balance activation and inhibition to regulate the outcome of T cell activation [51]. *Trans* interaction between two cells leads to the transmission of an inhibitory signal from the HVEM expressing cell to the BTLA expressing cell. *Cis* interactions between BTLA and HVEM co-expressed on the same cell are also possible and may function by affecting signalling capacity or quality of the *trans* interaction. For instance, *cis* interactions could prevent HVEM binding to LIGHT

expressed on other cells and thus, deprive the T cell of an additional co-stimulatory signal [52].

Surface expression levels of BTLA is low on naive CD4⁺ T cells, but is rapidly upregulated following TCR activation with peak surface expression after 48 hours [53]. In contrast, naive T cells display a high level of surface HVEM expression which decreases following T cell activation but is restored to high levels when cells reach a quiescent state. There is a widespread and dynamically regulated expression of BTLA and HVEM amongst the cells of the immune system. In addition to T cells, both surface proteins are also expressed on DCs, B cells, NK cells, NKT cells and $\gamma\delta$ T cells [51]. BTLA contains an ITIM, an ITSM and two Grb2-binding motifs in its cytoplasmic domain (Figure 1) [54-56]. BTLA functions via the recruitment of tyrosine phosphatases (SHP-1 and SHP-2) and interaction with lipid raft components in order to attenuate TCR downstream signalling pathways [51].

LAG-3

Another co-inhibitory molecule that plays an important role in regulating T cell activation is the lymphocyte activation gene-3 (LAG-3; CD223), a CD4-related transmembrane protein (Figure 1). LAG-3 binds to MHC class II molecules found on the surface of APCs but with a much higher affinity than CD4 and at a different site from CD4 endowing it with a possible role as a negative competitor for CD4 (Table 1).

LAG-3 is mainly retained intracellularly near the microtubule-organising centre (MTOC) and is found co-localised with Rab11b, a monomeric G-protein that regulates endosomal recycling of transferrin (Tfn) receptors to the plasma membrane [57]. Upon T cell activation LAG-3 is rapidly translocated to the plasma membrane. LAG-3 is first detectable 24hrs following activation and peak expression is observed at around day 2 followed by a gradual decline in expression by day 8 [58]. LAG-3 expressed on the surface can be cleaved within the transmembrane domain at the connecting peptide (CP) by two members of the TNF α converting enzyme (TACE) family of metalloproteases known as ADAM 10 and ADAM 17 [59], to release soluble LAG-3 (sLAG-3) which may contribute to the regulatory function of LAG-3. Although the mechanism of how LAG-3 associates with the TCR-CD3 complex and negatively regulates TCR-induced signal transduction is not completely understood, a single lysine residue 468 within a conserved KIEELE motif in the cytoplasmic domain of LAG-3 has been shown to be indispensable in executing the inhibitory effect on CD4-dependent T cell function (Figure 1) [60,61]. Similar to the protein kinase C binding site in the CD4 molecule, the cytoplasmic tail of LAG-3 contains a region with a potential serine phosphorylation site. Another motif that has been identified in the intracytoplasmic region of LAG-3 is an unusual 'EP' (glutamic acid-proline) repeat that binds a protein termed LAP (LAG-3-associated protein) and is predicted to be important in the anchorage of the IS to the microtubule network following TCR engagement [62]. These properties suggest that the LAG-3 cytoplasmic domain mediates intracellular signal transduction and/or molecular aggregation [61].

LAG-3 inhibits T cell expansion by blocking entry of activated T cells into the growth phase of the cell cycle and results in the accumulation of cells in the S-phase. LAG-3 is involved in limiting the expansion of activated T cells and controlling the size of the memory T cell pool [63]. LAG-3 is also thought to play a role in modulating DC function and also in enhancing the suppressive activity of CD4⁺CD25⁺ regulatory T cells. LAG-3 can negatively regulate T cell homeostasis by Treg-dependent and independent mechanisms.

TIM-3

T cell immunoglobulin-3 (Tim-3) is a type I glycoprotein receptor with a membrane distal immunoglobulin variable (IgV) domain and a membrane proximal mucin domain and is mainly expressed on IFN- γ -producing CD4⁺ T helper 1 (Th1) and CD8⁺ T cytotoxic 1 (Tc1) cells (Figure 1). Within the TIM-3 IgV domain there are two noncanonical disulfide bonds that reorient a CC' loop towards a FG loop forming a "cleft" structure that is thought to be involved in ligand binding and is not found in other IgSF members. Although protein sequence analysis of TIM-3 shows that the cytoplasmic domain is devoid of ITIMs or ITSMs, it does contain a conserved region of five tyrosine residues which are phosphorylated upon ligand binding (Figure 1) [64]. TIM-3 is also expressed by macrophages and DCs, where it plays a role in the clearance of apoptotic bodies possibly through binding phosphatidyl serine by the CC'-FG cleft of TIM-3 (phosphatidyl serine is exposed on the surface of apoptotic cells) [65].

TIM-3 is recognized by galectin-9, a member of carbohydrate-binding proteins that belong to a group of S-type lectins (Table 1) [66]. Galectin-9 recognizes the oligosaccharide chains on the TIM-3 IgV domain via its carbohydrate recognition domain. TIM-3 receptor triggering induces intracellular calcium flux, cell aggregation and the death of Th1 but not Th2 cells which provides a negative feedback loop to prevent uncontrolled harmful Th1 responses. TIM-3 expression gradually rises during Th1 polarization until a stable but high level of expression is reached on terminally differentiated Th1 cells. The expression of TIM-3 in T cells is partly regulated by the Th1-specific transcription factor T-bet which binds directly to the TIM-3 promoter and promotes TIM-3 expression independent of IFN- γ [67]. TIM-3 expression is dysregulated on CD4⁺ Th1 cells in certain autoimmune disease states such as multiple sclerosis (MS). Low level TIM-3 expression disrupts galectin-9-mediated negative regulation of IFN- γ -secreting CD4⁺ Th1 cells. Elevated IFN- γ levels in turn upregulates galectin-9, which allows autoreactive proinflammatory cells to escape galectin-9-induced cell death and expand in numbers. On the other hand, TIM-3 expression is found to be elevated on CD8⁺ T cells that experience repeated antigen stimulation for example, as in viral infection and certain cancers [68]. This resembles the pattern of PD-1 overexpression associated with T cell exhaustion. T cell effector function has been shown to be restored when the TIM-3/galectin-9 pathway is blocked either alone or in combination with the blockade of other inhibitory receptors [66]. Furthermore, blockade of the TIM-3 pathway using anti-TIM-3 mAbs in Th2-associated murine experimental allergic conjunctivitis augments antigen-specific IFN- γ production and reduces eosinophilic infiltration. Thus, targeting the TIM-3 receptor may also be a viable therapeutic option for a wide array of atopic diseases such as asthma [69].

A recent study has demonstrated that in the absence of any exogenous ligands, TIM-3 can positively contribute to the T cell signalling pathway following an acute stimulation through the TCR/CD3 and CD28 receptors. This early enhancement in activation might accelerate the shift to an exhausted T cell phenotype, while the extracellular domain of TIM-3 modulates its co-inhibitory function upon binding to galectin-9 [64]. Although devoid of any obvious signalling motifs, the two membrane-proximal tyrosines were shown to be required for TIM-3 signalling. Lck and Fyn were shown to mediate TIM-3 phosphorylation and NFAT activation [64]. TIM-3 mediated acute enhancement of T cell activation requires components of the TCR proximal signalling machinery, notably Src kinases, ZAP-70 and SLP-76. Thus, the downstream signalling pathway of TIM-3

might intersect closely with the TCR signalling pathway [64]. More recently the human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) was identified as a proline-rich adaptor protein that binds the intracellular tail of TIM-3 at residues 252-270. Bat3 then acts as a molecular scaffold and recruits catalytically active TCR-associated Lck to reduce TIM-3-dependent T helper type 1 (T_H1) cell exhaustion and galectin-9-mediated cell death, ultimately promoting both proliferation and proinflammatory cytokine production [70].

LAIR-1

Unlike the inhibitory receptors which are expressed after T cell activation, LAIR-1 is expressed in very high and relatively homogenous levels in naive T cells but in lower and more heterogeneous levels in memory T cells [71]. LAIR-1 consists of a type I transmembrane glycoprotein of 287 amino acids with a single extracellular C2-type Ig-like domain and a cytoplasmic domain with two ITIM motifs (Figure 1). LAIR-1 can inhibit TCR mediated signals possibly through the recruitment of C-terminal Csk, one or more of the phosphatases SHIP, SHP-1 or SHP-2, and to a certain extent on signalling through p38 MAP kinase and ERK signalling [72]. LAIR-1 has been shown to exist in a basally tyrosine phosphorylated state that constitutively recruits and activates SHP-1, which can then dephosphorylate downstream TCR signalling components and influence the basal threshold of T cell activation [73,74]. LAIR-1 expression levels might contribute to the higher threshold for TCR stimulation observed in naive cells compared to effector and memory cells. Moreover, cell surface expression of LAIR-1 is thought to be upregulated during incomplete CD28-deficient T cell activation but downregulated in the presence of full CD28-mediated co-stimulation [75]. The expression of LAIR-1 thus provides a mechanism to prevent the initiation of immune responses and also for the downregulation of ongoing immune responses [72]. LAIR-1 was recently identified to recognize and interact with high affinity to hydroxyproline containing collagen motifs. The interaction between collagen and LAIR-1 may set a threshold for the activation of T cells that have migrated into damaged tissues [71].

TIGIT

T cell Ig and ITIM domain (TIGIT) is a more recently identified inhibitory receptor that is expressed on activated T cells. TIGIT is an immunoglobulin protein of the CD28 family with a structure that consists of an extracellular immunoglobulin domain, a type I transmembrane domain and two ITIMs in its cytoplasmic domain (Figure 1). The two ITIMs have recently been predicted to mediate T cell-intrinsic inhibitory effects, predominantly through the activation and phosphorylation of Erk [76]. The crystal structure of TIGIT in complex with its ligand, CD155 revealed a heterotetrameric assembly of two TIGIT molecules flanked by two CD155 molecules. The lateral TIGIT homodimers and the *trans*-TIGIT/CD155 heterodimers can oligomerize in a zipper-like fashion to facilitate the interactions between adjacent T cells and DCs in order for stable clustering of receptors required for effective I [76]. TIGIT binds to surface CD155 (poliovirus receptor) molecules on DCs (Table 1). CD155 is a prototypical Nectin/Nectin family member and in addition to binding IgSF molecules on lymphocytes such as TIGIT and CD226, it provides heterophilic interaction with other nectin family members such as nectin-3 on juxtaposed cells via bidirectional signalling [76].

Through the use of TIGIT^{-/-} knockout mice it has been demonstrated that the loss of TIGIT results in hyperproliferative T cell responses, proinflammatory cytokine production and increased susceptibility to autoimmunity [77]. Stimulation of the TIGIT receptor on T cells

with agonistic mAbs demonstrated a direct inhibitory effect on cell cycle entry, a decrease in the expression of transcription factors such as T-bet, GATA3, IFN regulatory factor 4 (IRF4) and retinoic acid-related orphan receptor γ (ROR γ), with a consequent inhibition of proinflammatory (IFN- γ) cytokine production [78]. TIGIT was also found to act upstream by directly interfering with molecules that comprise the TCR complex and may alter antigen density thresholds required for productive T cell activation [79].

TIGIT-CD155 interaction can indirectly inhibit T cell responses by driving DCs to a more IL-10 producing-tolerogenic phenotype [80]. Similar to the ligand competition exhibited by CTLA-4 with CD28 for the CD80/86 receptor on DCs, TIGIT competes with the CD226 co-stimulatory molecule for the CD155 receptor on DCs. CD226 is a leukocyte adhesion molecule also known as DNAM-1 and is a member of the V-set and has the ability to invoke a potent cytotoxic response from T cells. CD226 is constitutively expressed on CD8⁺ T cells and is upregulated on CD4⁺ T cells following activation [81]. Thus, TIGIT exerts immunosuppressive effects by competing with CD226 for the same ligand CD155 and thereby represents an alternative pathway for regulating T cell responses that can be targeted by immunomodulatory antibodies for autoimmune conditions. Certain tumours such as colorectal carcinomas and neuroblastomas over-express CD155 molecules; these can inhibit T cell activation when bound to TIGIT receptors and enable the tumour to escape immune attack. Similar to the antibody-mediated PD-1/PD-L1 blockade prevalent in current clinical trials for cancer therapy, disruption of TIGIT/CD155 interaction using blocking antibodies may also hold therapeutic benefits [78,81].

Siglecs

Sialic acid-binding Ig-like lectins (Siglecs) are type I transmembrane proteins of the IgSF that are made up of a sialic acid-binding N-terminal V-set domain, variable numbers of C2-set Ig domains, a transmembrane region and a cytoplasmic domain (Figure 1). Siglecs have the unique ability to transmit signals into immune cells upon binding to sialylated glycans [82]. Several Siglecs contain inhibitory signalling motifs within their cytoplasmic domains and are thought to be important contributors of negative signals that dampen the immune response [83]. Siglecs can be categorised into two broad groups based on sequence similarity and evolutionary conservation. The first group represents Siglecs that are structurally conserved among mammals and include Sialoadhesin (siglec-1), CD22 (siglec-2), myelin-associated glycoprotein (siglec-4) and siglec-15. The second group represents siglecs that are rapidly evolving by gene duplication events and display high homology (50-90% identity) to CD33 in their extracellular domain; in humans these CD33-related siglecs include siglec-3, 5-12, 14 and 16 [84]. B cells, conventional DCs and pDCs are the major cell types that express relatively high levels of siglecs, and may indirectly influence T cell biology. Although most siglecs are found at minute levels on T cells, gene expression data has recently revealed a striking elevation of siglec-9 in central memory T cells [85].

Siglecs can also be classified according to the transmembrane and cytoplasmic domains involved in the downstream signalling events. Siglec-1 and siglecs-4 are found in the first group and represent lectins that lack inhibitory signalling cytosolic motifs and possess neutral transmembrane domains. The majority of siglecs fall into the second group which represents siglecs with a single conserved membrane-proximal ITIM and sometimes also an additional membrane-distal ITIM-like motif with the consensus sequence D/ExYxEV/IK/R [85]. Engagement of these siglecs with their ligands exposes the cytosolic

ITIM tyrosine and the ITIM-like tyrosine for phosphorylation by Src family kinases such as Lyn, which then recruit SHP-1 or SHP-2. Siglecs that are categorised into the third group including the CD33-related siglecs, have a positively charged residue within their transmembrane anchor region and associate with a complementary negatively charged transmembrane residue found in signalling chains of molecules such as DAP12 and DAP10 [86]. DAP12 contains a cytosolic ITAM motif while DAP10 contains cytosolic YxxM motifs, which become accessible to nonreceptor tyrosine kinases following a conformational change upon ligation. The subsequent phosphorylation of paired tyrosine residues in the ITAM of DAP12 facilitates the recruitment of Syk family tyrosine kinases, the phosphorylation of the tyrosine in the YxxM motif of DAP10 and the activation of PI3K. The CD33-related siglecs are thought to play a more substantial role as negative immunoregulators of T cells influencing functional outcomes such as proliferation and cytokine secretion. The proximal ITIM motif of CD33-like siglecs in the phosphorylated state is a target of at least two E3 ubiquitin ligase families, Cbl and suppressor of cytokine signalling (SOCS). Ubiquitylation is a signal for endocytosis and may represent an alternative mechanism of terminating signalling through a tyrosine phosphorylated ITIM-bearing membrane-bound surface receptors [87]. Phosphorylated ITIMs can be prevented from binding to an endocytic adaptor when masked or dephosphorylated by SHP-1/SHP-2 [88].

Summary

Co-inhibitory receptors are transmembrane glycoproteins that transmit dominant negative signals mainly via intracellular phosphatases that bind to phosphorylated tyrosine residues in the cytoplasmic domain. Co-inhibitory receptors can act as safety mechanisms and threshold setters to prevent uncontrolled detrimental extremes of reactivity by counteracting the stimulatory signals. A considerable body of work has been carried out demonstrating the key roles played by co-inhibitory receptors in T cell function. However, the hierarchy of inhibition provided by each co-inhibitory receptor in different immune environments and cell differentiation states needs to be defined more thoroughly. Defining such a hierarchy of inhibition would allow a more targeted therapeutic strategy to be devised for different disease settings. The severity of diseases associated with knockouts for these co-inhibitory receptors in mouse models uncovers the vital role of negative inhibition in T cell responses and highlights the therapeutic potential of targeting the various components that comprise different inhibitory mechanisms used by these receptors. The susceptibility to several autoimmune diseases such as diabetes, multiple sclerosis and rheumatoid arthritis that can arise as a result of polymorphisms in co-inhibitory receptor genes further identifies these receptors as beneficial targets for immunomodulation. An increasing number of co-inhibitory receptor-targeting drugs (biologics) are being developed or are undergoing clinical trials for a variety of diseases (Table 1). A clear grasp of whether co-inhibitory receptors act individually or synergistically in regulating T cell effector responses, together with a mechanistic understanding of the downstream signalling cascades and their interactions during synergism would help in the development of more effective and safer immunomodulatory biologics.

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