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Check Point Inhibitors and Their Role In Immunosuppression In Sepsis

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Introduction:

Sepsis is a life-threatening organ dysfunction which results from a dysregulated host response to infection and it remains a common, deadly, and expensive problem. [1] Treatment advances ranging from measured resuscitation strategies to broad spectrum antibiotics have improved the morbidity and mortality associated with sepsis. Despite these advances, nearly 50,000 cases occur annually in the United States and sepsis remains the leading cause of death in the non-coronary ICUs, costing more than $24 billion as of 2014. [2,3] In response to septic insults, a profound immune activation occurs as the body attempts to corral the infectious source. The result of such extreme immune activation is a multifactorial disharmony of immune cells. This disruption of immune homeostasis results in circulating immune cell influx into distal organs, leading to multi-organ failure. [4] This necessary balance between activation and suppression is mediated by immune checkpoint regulators. These regulators are implicated in multiple instances of immune imbalance, including in sepsis.

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The immune system utilizes checkpoint regulators to balance immune activity throughout the body. Checkpoint regulators are membrane bound proteins which serve as a second signal to direct the immune response to a particular antigen. [5] An antigen bound by MHC class I or II receptors on an antigen presenting cell (APC) is presented to a T-cell Receptor (TCR), acting as the first signal. The second signal, from a checkpoint regulator, is necessary to instruct the T-cell on how to respond to this antigen. Without such signals the immune response is attenuated or absent. These proteins further allow for modification of the immune response over time, enabling different immune cells to respond to various environmental cues in unique ways. [6,7] Stimulatory signals from regulators can lead to activation, and subsequent cell and humoral mediated immunity, while inhibitory signals can lead to anergic T-cells unable to respond to further signals.[8]

In the setting of overwhelming infection checkpoint regulators serve as key mediators of the immune dysfunction that portends a risk of secondary infection. These regulators promote tolerance and can inhibit further immune reactivity to an infectious stimulus, traits that can be detrimental in the setting of overly profound infection.[7] It has been demonstrated that alteration in signaling through a variety of such regulators can alter outcomes after sepsis in animal models, and this has introduced new therapeutic targets that are beginning to be tested in clinical settings. [9] These regulatory systems are made up of a variety of proteins with non-redundant spatial and temporal functions, yet, their ultimate signaling outcomes demonstrate substantial redundancy. The purpose of such repetition is incompletely understood. With a more complete understanding of these immune policing proteins it is thought that their power might be harnessed to better treat patients enduring septic insults. [10] We will elaborate on the roles of diverse checkpoint regulators, such as Programmed Cell Death-1, V-domain Ig suppressor of T-cell activation, Cytotoxic T-Lymphocyte Associated Protein 4, and Herpes Virus Entry Mediator in immune regulation after sepsis and their contribution to post-septic immunosuppression.

Programmed Cell Death −1 (PD-1, *Pdcd1*)

PD-1 is an immune checkpoint protein first identified as a classical programmed cell death induced gene, *Pdcd1.* PD-1 bears a V-immunoglobulin domain and belongs to the B7-CD28 superfamily of checkpoint proteins. Both PD-1 and it's ligands are expressed on a variety of immune cell types, and the PD-1 signaling pathway uniquely allows for maintenance of both central and peripheral tolerance by limiting the activation of T-cells. [11]

PD-1 and its Ligands

Non-redundant signaling pathway

PD-1 signals as a receptor tyrosine kinase. Its cytoplasmic domain contains an immunoreceptor tyrosine-based switch motif (ITSM) domain and an immunoreceptor tyrosine-based inhibitory motif (ITIM) domain. [12] Following ligation to the PD-1 ligands, the ITSM and ITIM domains are phosphorylated and Src Homology region 2 domain-containing Phosphatases (SHPs) 1 and 2 dock at the ITSM domain. Upon activation by PD-1, SHP-2 dephosphorylates kinases, phosphoinositide 3-kinase (PI3K) and ZAP70 to

inhibit the Akt and Erk/MAPK pathways as shown in Figure 1. [13,14,15] Both TCR antigen stimulation and PD-1 ligation are requisite for PD-1 mediated suppression. [16]

Broad expression patterns promote tolerance

PD-1 is expressed on conventional T (Tconv) cells, regulatory T-cells (Tregs), Natural Killer T (NKT) cells, B-cells, dendritic cells (DCs), and macrophages. [17,18] Transcription factors such as NFAT2, STATs, Notch, and FoxO1 bind the *Pdcd1* promoter to transiently upregulate PD-1 expression following T-cell receptor (TCR)-antigen stimulation. [19–21] The expression of PD-1 during antigen stimulation sets a threshold for reactivation of lymphocytes. [14] As lymphocyte stimulation subsides, PD-1 expression is downregulated by Blimp-1 and T-bet transcription factors to prevent overt T-cell exhaustion. [22,23]

PD-L1 (B7-H1) and PD-L2 (B7-DC) are type 1 transmembrane proteins and CD28-B7 family members containing an extracellular region with IgV- and IgC- like domains. PD-L1 and PD-L2 compete for PD-1 binding with different affinities, and both bind additional ligand-specific partners B7-1 and repulsive guidance molecule b (RGMb), respectively. [24,25] The differential cell and tissue-specific expression patterns and competing molecular mechanisms of PD-L1 and PD-L2 promotes dynamic activity of PD-1 based on spatial and temporal context. [26]

PD-L1 is broadly expressed on both lymphoid and non-lymphoid cells in peripheral tissues, mediating PD-1 activation at sites of infection. PD-L1 is constitutively expressed at high levels by naïve $CD4^+$T-cells, $CD8^+$T-cells, B-cells, DCs, and macrophages. Upon activation of lymphoid cells PD-L1 surface expression is upregulated and induced on monocytes. [27] Non-lymphoid cells, including cardiac endothelium, lung, placenta, kidney, salivary gland, glial cells, muscle cells, epithelial cells, and liver non-parenchymal cells express PD-L1 constitutively as well. [28] These expression patterns ensure that activated T-cells are systemically regulated to maintain peripheral tolerance. [26] PD-L2 is more tightly regulated with weaker expression on a smaller subset of cells and high binding specificity to PD-1. PD-L2 expression is induced specifically on activated bone marrow-derived DCs and macrophages found in the liver, lung, and spleen. Despite the differences between these ligands, they do share some expression characteristics. Both PD-Ls are expressed on tumor cells, though PD-L1 expression extends to hematopoietic and non-hematopoietic tumors and PD-L2 is largely restricted to leukemias. In addition, PD-L1/2 expression on splenic DCs is upregulated by interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-4 (IL-4). [27] PD-1 and its ligands mediate interactions between both immune and non-immune cell types to prevent autoreactivity in the periphery. However, the expansive influence of the PD-1 pathway has been exploited to curtail immune surveillance in cancer and chronic viral infections. This pathway has also demonstrated detrimental activity in other diseases such as sepsis and acute lung injury.

The Role of PD-1 in Immunopathology

Sepsis

As aforementioned, sepsis is characterized by systemic immune dysfunction. As with other immune-related diseases such as cancer and chronic viral infection, T-cell anergy is implicated in sepsis development. The large impact that lymphocyte regulation and activity have in immune dysfunction prompted Huang *et al.* to investigate the role of PD-1 in sepsis progression. This group found that adult *PD-1*$^{-/-}$ mice have a survival advantage following intra-abdominal septic insult via the cecal ligation and puncture (CLP) procedure. The PD-1 deficient mice maintained macrophage function, demonstrating improved bacterial clearance and reduced inflammatory cytokine production[29]. The survival results were recapitulated in a neonatal murine sepsis model using the neonatal cecal slurry technique. PD-1 deficiency promoted neonatal survival compared to Wild Type (WT) controls, but did not alter bactericidal efficacy. [30]

Monaghan *et al.* expanded on the adult murine findings by analyzing the PD-1 expression patterns and cytokine profile of patients with septic shock. In septic patients, PD-1 was significantly upregulated upon circulating monocytes, granulocytes, and lymphocytes when compared to healthy controls. This upregulation positively correlated to IFN-γ, IL-4, and IL-2 levels which are associated with the T helper cell 1/2 (T_H1/T_H2) response and cytokine storm. The upregulation of PD-1 surface expression and cytokine production also correlated with the severity of illness as determined by the Acute Physiology And Chronic Health Evaluation II (APACHE II) score. [31,32] Thus, the increase in disease severity (APACHE II > 20) associated with PD-1 overexpression demonstrates the potential of PD-1 blockade as a therapeutic intervention for sepsis. [31]

Acute Lung Injury and Respiratory Distress Syndrome

Acute lung injury (ALI) can develop following an infectious insult ranging from sepsis, trauma, shock or pneumonia, and can progress into the more severe acute respiratory distress syndrome (ARDS). ALI/ARDS development is largely induced by neutrophils, lung epithelial, and lung endothelial cells. As PD-1 and its ligands exert suppressive function in the periphery, it was postulated that this pathway may play a role in ALI/ARDS [33]. In a murine model of indirect acute lung injury (iALI), PD-1 was upregulated on several immune populations, such as T-cells ($CD4^+$), tissue resident DCs ($CD11c^+$), and $Gr1^+$ cells in the lung. This result was also mirrored in ARDS patients with a significant increase in PD-1 expressing $CD3^+$ T-cells in the blood compared to healthy controls. In ARDS patients, the level of TNF-α found in the bronchoalveolar lavage fluid and immune cell apoptosis was significantly higher. The loss of lung barrier function due to apoptosis of epithelial and endothelial cells is another phenotype of ALI/ARDS patients, implicating TNF-α further in the development of this disease. In *PD-1−/−* mice, pathological indices of ARDS such as TNF-α levels, tissue congestion, neutrophil influx in the lungs, and immune cell apoptosis were significantly lower than WT mice. PD-1 deficient mice also demonstrated a survival advantage when compared to WT mice (70% v 31.25%). These murine and human results further support a role for PD-1 in the development of ALI/ARDS. [33]

Viral control

Evading immune surveillance is a phenomenon that is typically associated with cancer. Antitumoral immunity can be dampened by exploiting negative checkpoint pathways, allowing tumors to expand and thrive in the host. The PD-1/ PD-L1 pathway has been targeted pharmacologically as an anticancer treatment with great efficacy, resulting in the development of multiple Food and Drug Administration (FDA) approved PD-1 inhibitors. [34] This method of immune evasion is not limited to cancer. Human Rhinovirus (HRV) is among the most common infections and has evolved a mechanism to escape the immune response through the PD-1/PD-L1 pathway. Kirchberger *et al.* demonstrated that HRV induces PD-L1 expression on DCs, blunting the ability to stimulate T-cells and promoting T-cell tolerance. In line with the known PD-1/PD-L1 function, T-cells were also reduced to a hypo-proliferative state. [35] These results support a clear role for the PD-1/PD-L1 pathway in sepsis, ARDS, and chronic infections.

V-domain Ig Suppressor of T-cell Activation (VISTA, *Vsir,* PD-1H)

VISTA is a negative checkpoint regulator that belongs to the B7-CD28 family and contains an IgV domain, a transmembrane domain, a cytoplasmic tail, and an extracellular domain homologous to PD-L1. Despite belonging to the B7-CD28 family, VISTA has several unique characteristics that are not shared with other family members.[36] For instance, the cytoplasmic tail lacks both an ITIM and ITSM domain but contains proline residues, additional cysteines, and potential protein kinase c binding sites. It is theorized, based on these characteristics, that VISTA signals through a nonredundant pathway that remains undefined.[37] Another unique characteristic of VISTA is its receptor and ligand-like structure, expression, and function.[38] Acting as a receptor and ligand, VISTA inhibits T-cell proliferation, production of cytokines such as IL-2, and IFN-γ, and chemokine production following TCR activation.[39] Through this inhibition, VISTA promotes peripheral tolerance. [36]

VISTA as a receptor and a ligand

VISTA-VISTA interaction promotes context dependent regulation

VISTA is almost exclusively expressed on hematopoietic lineages such as neutrophils, monocytes, DCs, and macrophages at high levels. VISTA is also constitutively expressed at lower levels on naÏve $CD4^+$ T-cells, $CD8^+$ T-cells, Tregs, and tumor infiltrating lymphocytes. [40] When expressed on Tconv cells, such as $CD4^+$ T-cells, VISTA functions as a receptor and inhibits antigen-specific proliferation, cytokine, and chemokine production through cell intrinsic regulation [38]. VISTA can also act as a ligand when expressed on APCs and Tregs. The VISTA ligand exerts cell extrinsic regulation of Tconv cells by interacting with the uncharacterized VISTA receptor on T-cells to suppress activation.[41]

The proposed VISTA-VISTA interaction between Tconv cells and Tregs could also promote the differentiation and suppressive function of Tregs.[39] Interestingly, VISTA also appears to have a stimulatory role when expressed as a receptor on myeloid cells by enhancing inflammatory cytokine production and antigen presentation.[37] Thus, the VISTA receptor

inhibits Tconv cells whereas the VISTA ligand stimulates APC and Treg function. The ability to function as a ligand and a receptor depending on cellular context demonstrates the multi-faceted, dynamic regulatory role of VISTA.

VISTA-VSIG-3 interaction represents a novel checkpoint pathway

In addition to VISTA-VISTA interactions, a new VISTA ligand has been identified. V-set and Immunoglobulin domain containing 3 (VSIG-3, IGSF11, BT-IgSF) is a ligand in the immunoglobulin family and functionally interacts with VISTA expressed on T-cells.[36] VSIG-3 is expressed in the brain, kidney, skeletal muscles, and germinal centers. It is also overexpressed in hepatic, colorectal, and gastric cancers.[42] VSIG-3 is a homophilic adhesion molecule and shares structural homology with B7 family members. Association between the VSIG-3 and VISTA ectodomains suppress the T-cell response, cytokine, and chemokine production. [36] In parallel with other checkpoint proteins mentioned thus far, VISTA activity can also exacerbate immune related pathologies.

The Role of VISTA in Immunopathology

Sepsis

Though VISTA has mostly been discussed in the context of cancer and autoimmune disease, some recent work by Bharaj *et al.* demonstrated that monocytes upregulate VISTA following CLP in humanized BTL mice. BLT mice have severe immune-deficient non-obese diabetic phenotypes and produce inflammatory monocytes. Following CLP in BLT mice VISTA expression was significantly upregulated on monocytes[43]. These results support a role for VISTA in activating monocytes and contributing to the inflammatory response during sepsis progression.

Chronic Viral Infection (HIV)

As previously discussed, PD-1 plays an important role in chronic viral and bacterial infections. VISTA is no exception. *In vitro* treatment with Toll-like receptor (TLR) 3 and TLR5 correlated with significant VISTA upregulation in circulating $CD14^+$ monocytes and macrophages. TLR3 and TLR5 are virus and bacteria-associated agonists respectively. Based on these preliminary data, Bharaj *et al* also investigated VISTA in the context of HIV-infected patients. This research group found significant upregulation of VISTA on monocytes associated with enhanced cytokine secretion in these individuals.[37] It appears that the VISTA ligand plays a stimulatory role when expressed on monocytes during chronic viral infection. The differential role of VISTA as a ligand and receptor based on cell-type and disease context, further highlight the complex regulatory function of this checkpoint protein.

Cytotoxic T lymphocyte Ag-4 (CTLA-4)

CTLA-4 is a negative checkpoint regulator that was first identified as a homolog to costimulatory protein CD28 and belongs to the Ig superfamily. [44] CD28 and CTLA-4 are founding members of the CD28-B7 family and serve as the paradigm for immune checkpoint pathways. Both regulators bind B-cell activation antigens (B7-1/CD80 and B7-2/

CD86) and are expressed on the surface of T-cells. B7-1 and B7-2 expression is restricted to lymphoid tissues. B7-1 expression is induced in T-cells, B-cells, monocytes, and DCs. B7-2 is constitutively expressed on B-cells, DCs, and monocytes. Following activation B7-2 is also upregulated on B-cells, DCs, and monocytes and induced on T-cells. [45] Despite structural similarity and shared ligands, CTLA-4 is a co-inhibitory protein whereas CD28 is co-stimulatory. CTLA-4 has higher affinity and avidity to B7s so it competes against CD28 to suppress activated T-cells. [46]

Complex molecular mechanisms of CTLA-4-mediated suppression

CTLA-4 and CD28 target the same signaling pathway

Both CTLA-4 and CD28 associate with serine/threonine phosphatase PP2A to regulate T-cell activation. However, CTLA-4 is able to regulate T-cells through cell intrinsic and extrinsic inhibition as seen in Figure 2. When the TCR is stimulated, CTLA-4 recruits PP2A [47], inhibiting the Akt tyrosine kinase cascade responsible for potentiating T-cell activation. [48,47] This T-cell intrinsic mechanism targets Akt pathway kinases that are distal to the membrane whereas PD-1 targets this pathway proximal to the TCR (Fig 1 & 2a). In addition to AKT pathway inhibition, CTLA-4 competes for B7 binding to inhibit CD28 stimulatory activity and further suppress T-cell activation. [46]

CTLA-4 also suppresses $CD4^+$ and $CD8^+$ T-cell activity through cell extrinsic mechanisms. Interaction of CTLA-4 with B7-1/ B7-2 promotes trans endocytosis to sequester these ligands from antigen presenting cells (APCs), preventing CD28 binding, thereby, inhibiting T-cell activation. Constitutively expressed CTLA-4 also enhances the suppressive function of Tregs to further inhibit Tconv activation. [49] Phosphorylated CTLA-4 also enhances the production of signaling molecules by T-cells. This downregulates B7 expression in the APC and reduces the ability of APCs to stimulate T-cells (Fig 2b). [50] The multiple molecular mechanisms by which CTLA-4 suppresses effector T-cells demonstrates the importance of this regulatory pathway in maintaining immune tolerance.

CTLA-4 expression is not restricted to lymphoid cells

Unlike CD28 which is constitutively expressed on T-cells, CTLA-4 expression is induced in naÏve T-cells upon TCR stimulation and constitutively expressed on Tregs. CTLA-4 is also expressed on B-cells, Natural Killer (NK) cells, NKT cells, and DCs. [51,52] This restricted expression can be attributed to strict regulation through multiple mechanisms including transcriptional control, post transcriptional modifications, and intracellular trafficking of CTLA-4. NFAT and Foxp3 are transcription factors that induce CTLA-4 transcription in Tconv cells and Tregs, respectively.[53,54] MicroRNA activity and the post-transcriptional modifications of the CTLA-4 3′ UTR regulate CTLA-4 mRNA stability. These transcriptional and post-transcriptional mechanisms provide temporal control of CTLA-4 surface expression. [55,56]

Intracellular trafficking regulates CTLA-4 surface expression via endocytic and secretory pathways, polarizing CTLA-4 surface expression to the immune synapse, and maintaining spatial control of CTLA-4 activity. [57,58] In $CD4^+$ T-cells, CTLA-4 is localized within

secretory lysosomes. Within these lysosomes, CTLA-4 is rapidly degraded when it is not being actively transcribed. When the TCR is stimulated, CTLA-4 gene expression is induced, intracellular CTLA-4 accumulates, and lysosomes secrete CTLA-4 to the cell surface.[57] In $CD8^+$ T-cells, intracellular trafficking of CTLA-4 is mediated through endocytic pathways. [58] In activated T-cells, the LRBA protein promotes migration of CTLA-4 containing endosomes to the plasma membrane.[59] Once expressed proximal to the TCR, CTLA-4 effectively suppresses T-cell function. [47] To limit its activity and overall abundance in the absence of TCR stimulation, CTLA-4 is negatively regulated by clathrin-associated adaptor proteins AP-1 and AP-2. In resting T-cells, AP-1 and AP-2 interact with the unphosphorylated cytoplasmic tail of CTLA-4 to promote internalization of this receptor into the lysosomal network for degradation.[59] This phosphorylation dependent trafficking allows for rapid and dynamic regulation of CTLA-4 at the cell surface.

CTLA-4 In Immunopathology:

CTLA-4 has been implicated in multiple roles in human disease processes varying from autoimmune phenomena to oncologic pathogenesis to infectious immunosuppression. Similar to other checkpoint regulators it often mediates overly robust tolerance of abnormal signals in the setting of profound disease, preventing necessary immune responsiveness to threat.

Sepsis

CTLA-4 has been implicated as a key mediator in septic immunosuppression. Early work by Inoue *et al.* demonstrated that CTLA-4 upregulation on CD4, CD8 and Tregs increased after experimental sepsis using a CLP model.[60] Administration of intraperitoneal anti-CTLA-4 antibody generated a dose dependent survival benefit, with low doses producing profoundly improved survival associated decreased septic induced splenic apoptosis. Interestingly, high dose CTLA-4 was less protective inducing increased mortality.[60]

CTLA-4 has additionally demonstrated a significant role in the pathophysiology of both primary and secondary *Candida albicans* fungal sepsis [61]. Survival was improved in mice treated with anti-CTLA-4 antibody both after primary fungal sepsis, induced through tail vein injection, or secondary fungal sepsis, modeled by tail vein injection 72 hours after CLP. This benefit was associated with an increase in splenocyte derived IFN-γ production suggesting that in vivo CTLA-4 expression inhibits this necessary protective IFN-γ phenotype.

CTLA-4's role in septic immunosuppression extends to viral pathogens, with significant roles in the pathogenesis of two contemporary and highly morbid viruses, Human Immunodeficiency Virus (HIV) and Hepatitis C. While current anti-retroviral therapy has dramatically improved long-term outcomes in HIV by suppressing viral replication, latent stores of HIV have proven a major barrier to ultimate disease cure. CTLA-4 $CD4^+$ T-cells have been identified as a major reservoir of latent viral particles, and therefore a target for future therapeutics.[62] Further, specific genetic variants of CTLA-4 have been associated with chronic Hepatitis C infection, suggesting that some variants may predispose individuals to risk of chronic conversion upon viral exposure. [63]

CTLA-4 in Critical Illness

Patients who are critically ill from non-septic sources are known to be similarly at risk of secondary infections. For example, patients with acute liver failure frequently overexpress CTLA-4 on circulating T-cells compared to healthy controls. [64] Further, T-cells isolated from patients with acute liver failure were hypo proliferative when challenged with CD3 or antigen stimulation. Serum from these patients possessed increased amounts of soluble B7 which was shown to induce CTLA-4 upregulation in healthy control T-cells.[64] This implicates CTLA-4 as a prime mediator of decreased innate immune responses to infectious challenges in critical illness.

Herpes Virus Entry Mediator (HVEM)

Herpes Virus Entry Mediator (HVEM) is a type I transmembrane receptor member of the tumor necrosis factor receptor superfamily (TNFRSF) first discovered by Montgomery *et al.* as the necessary cell surface receptor for Herpes Simplex Virus-1 (HSV-1) entry. [65,66] It was separately identified in an expressed sequence tag survey seeking additional members of the TNFRSF. [67] The TNFRSF consists of 10 cell-surface proteins that regulate immune development and homeostasis. HVEM contains 4 cysteine rich domains (CRDs) in its extracellular region, a characteristic feature of TNFRSF utilized for ligand engagement. The cytoplasmic region of HVEM associates with TRAF1, TRAF2, TRAF3, TRAF5, and Stat3,and when transfected, cells demonstrate significant activation of NF-κB, Jun N-terminal kinase, and AP-1 [67,68]

Broad expression enables tissue specific behaviors:

Unlike many other checkpoint regulators whose expression is confined to immune cells, HVEM is expressed diffusely on multiple tissue types as well as many immune cell subsets. Northern blot survey of human tissue types demonstrated HVEM expression in most tissues, with the highest levels in adult spleen, peripheral blood leukocytes, fetal lung and kidney. [67] Immune cell characterization demonstrated monomeric HVEM expression on T-cells, B-cells, NK cells, DCs, and myeloid cells. [7] Specifically, human naÏve and memory B-cells express high levels of HVEM while germinal center B-cells lack HVEM expression, and T-cells constitutively express HVEM, unique from other TNFRs. [7]

HVEM and its Ligands:

HVEM behaves as both a receptor and a ligand, with variable downstream effects:

HVEM has a total of 5 described ligands including members both within, and outside of, the TNFSF, a unique trait not possessed by other TNFRSF members.[7] Immunoglobulin family ligands include B and T lymphocyte attenuator (BTLA), and CD160, while TNF superfamily ligands include Lymphotoxin Alpha (LTα), and LIGHT.[69] Additionally, HVEM binds to Herpes Virus glycoprotein-D (HSV gD).[65] The net function of HVEM, illustrated by *HVEM*$^{-/-}$ murine modeling, is inhibitory, with HVEM deficient T-cells exhibiting enhanced concanavalin (ConA) stimulation.[70] HVEM deficient mice similarly demonstrate increased mortality in T-cell dependent autoimmune hepatitis models with increased T-cell proliferation and cytokine production. [70]

HVEM signaling is variable, dependent on the ligand interacted with, the orientation of HVEM within the membrane, and the surrounding environment.[69] HVEM binding TNFSF members generally results in immune stimulation via an NFκB dependent mechanism, while binding of immunoglobulin family members results in immune inhibition. [7,71] Interestingly, in addition to its role as a receptor, HVEM behaves as a ligand for BTLA and CD160, inducing inhibitory signaling within the immunoglobulin expressing cells. [72]

LIGHT and Lymphotoxin Alpha (LTα):

LIGHT, which stands for "homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes," is also known as TNFSF14 or CD258. It is a 29kD, type II transmembrane protein, and member of the TNF family which formulates a homotrimer and exhibits highest expression in spleen, immature DC, granulocytes, and activated T-cells. [7,66,73] LIGHT acts as a ligand for both Lymphotoxin-β receptor (LTβR) and HVEM, which it binds in a 3:3 complex of trimeric LIGHT attached to 3 HVEM CRD2/3 regions.[7,73] When binding LTβR, LIGHT activates a cell death pathway within T-lymphocytes resulting in chemokine production, TRAF2 degradation, and caspase 8 activation.[71] However, when binding HVEM, LIGHT stimulates a robust stimulatory signal which, after TRAF2 recruitment results in NFκB activation, and promoting cell survival. [71]

LTα is a compact trimer which is assembled from subunits expressed by B-cells, T-cells and NK cells. [7,66] Lacking a transmembrane domain, LTα is secreted in its homotrimeric form and binds to HVEM in a stimulatory manner similar to LIGHT resulting in NFκB recruitment and cell survival. [66] Like LIGHT, LTα binds to the CRD2/3 of the inner surface of HVEM forming a 3:3 complex on the membrane. [7]

B and T Lymphocyte Attenuator (BTLA) and CD160:

A member of the immunoglobulin superfamily, BTLA possesses an intermediate type immunoglobulin fold in its ectodomain and two cytosolic ITIM inhibitory signaling domains.[72,74] BTLA expression is highest in spleen, lymph nodes, activated T-cells, and resting B-cells. [7] BTLA engagement by HVEM results ITIM activation, inducing SHP-1/2 phosphatase recruitment, and subsequent attenuation of IL-2 within the BTLA expressing cell. [72,74] Within the HVEM expressing cell, BTLA engagement mirrors the stimulatory LIGHT and LTa pathway, resulting in NFκB activation. [69] This is supported by evidence that BTLA binding to HVEM promotes survival and memory generation in $CD8^+$ T-cells.[72] Given that the net function of HVEM signaling is inhibitory, the ultimate signal generated from BTLA and CD160 ligation remains unclear. Evidence points to inhibition, yet no mechanism connecting the known stimulatory activation of NFκB to an inhibitory end result has been discovered.[7] It is this behavior, with inhibition when acting as a ligand, but stimulation while acting as receptor, that leads to the description of HVEM as a bidirectional switch.

CD160 was the last of HVEM's ligands to be discovered, identified through an attempt to isolate NK cell-specific receptors. [7] It is a member of the immunoglobulin superfamily of receptors and contains a glycosylphosphatidylinositol (GPI)-anchor and single IgV-like

domain.[75] It is expressed highly in spleen, small intestine, as well as peripheral blood leukocytes, NK cells, and γδT-cells. [7] BTLA and CD160 both associate with the CRD1 domain of HVEM, demonstrating competitive binding. However, mutagenesis studies have demonstrated that the two possess overlapping but not identical binding domains within this CRD1 region. [76]

Ligand binding can occur simultaneously:

HVEM's binding complexity is increased by the discovery that its orientation within the membrane alters its binding site availability, affecting the manner in which it interacts with available ligands.[77] Like other members of the TNF family, HVEM orients into a trimer within the membrane meaning it forms 3:3 complexes with many of its ligands.[7] With its multiple CRDs located on alternate faces of the trimer complex this allows HVEM to bind both at its CRD1 and CRD2/3 regions simultaneously, forming complexes with 3 LIGHT or LTα molecules bound to one face and 3 BTLA or CD160 to the other when HVEM is expressed in its *trans* confirmation.[7] In addition to these complexes, HVEM and BTLA can be co-expressed on single cells, forming a stable complex, with both proteins expressed in the *cis* confirmation on the membrane.[77] This is seen almost exclusively in naïve T-cells, where HVEM appears to associate with LIGHT, but no down-stream signaling, neither stimulatory nor inhibitory, is noted within the cells. [77] It is felt that this cis-complex competitively inhibits HVEM activation with the surrounding environmental ligands, maintaining T-cell naiveite.

HVEM's Role in Septic Immunosuppression:

HVEM's role in septic immunosuppression is best characterized by its behavior at mucosal barriers, but it has also been implicated in more systemic roles in indirect lung injury, viral illness and non-septic systemic critical illnesses, such as liver failure[78–80]. Unlike other checkpoint regulators, HVEM has been implicated both in septic immunosuppressive roles, mediating inappropriate levels of tolerance, and as mechanism of excessive immune activation, responsible for tissue injury. This represents a physical manifestation of its unique bidirectional behavior, making it an interesting therapeutic target allowing more context specificity.

HVEM is essential to mucosal immunity:

The mucosal surfaces serve as a primary entry site for many infectious threats, and the immune presence within these tissues is extensive. An investigation of innate lymphoid cell (ILC) checkpoint regulation demonstrated that HVEM signaling within the ILC3 subset was both necessary and sufficient to generate an appropriate IFN-γ response to protect against *Yersinia enterocolitica* infection.[81] Improved survival, via IFN-γ production, was mediated through the HVEM-LIGHT axis, with no affect from BTLA or CD160.

Shui *et al.* echoed HVEM's invaluable role in mucosal barrier signaling utilizing intestinal *Citrobacter rodentium* and *Streptococcus pneumoniae* pulmonary infection models. [68] HVEM stimulation, by either BTLA or CD160, in colonic epithelial cells induced STAT3 phosphorylation and innate inflammatory responses such as IL-6, CXCL1, and CCL20

production. *HVEM*$^{-/-}$ mice survived significantly worse than WT when subjected to *Citrobacter rodentium* infection, a surrogate for enteropathogenic *Escherichia coli* infection. These mice also had higher bacterial burden and lower STAT3 activation. They established this effect was mediated exclusively through CD160 interaction using *BTLA*$^{-/-}$, *LIGHT*$^{-/-}$and CD160 antibody administration.[68] Their results were confirmed in mice subjected to a *Streptococcus pneumoniae* pulmonary infection model where again the HVEM-CD160 axis was essential to survival and bacterial clearance. In both examples the HVEM axis defends mucosal barriers against infection, with blockade of deletion resulting in decreased mucosal barrier defense, such as is common after sepsis.

HVEM in respiratory immunity:

The role of HVEM in immune dysfunction in respiratory tissues has been similarly well established. In a murine model of *Chlamydia psittaci* respiratory infection *LIGHT*$^{-/-}$mice demonstrated a profound survival deficit with increased weight loss, higher bacterial burden, and heightened severity of lung tissue injury. [82] Lung tissue from these mice demonstrated decreased IFN-γ, TNF-α, and IL-12 mRNA levels, with elevated Treg abundance. Mice subjected to iALI using a double hit model of hemorrhage followed by CLP upregulate HVEM expression in lung tissue. [78] Administration of intratracheal HVEM siRNA attenuated this increased HVEM expression and conveyed a transient early survival benefit. [78] Similar to *LIGHT*$^{-/-}$ mice, HVEM siRNA treated mice had reduced cytokine and chemokine levels in respiratory mucosa, suggesting that HVEM signaling was necessary for this local inflammatory response, an example of inappropriate immune activation following sepsis. Together this demonstrates that the HVEM pathway, while necessary for response to respiratory infectious threats, can be inappropriately activated by distant infectious challenges resulting in inappropriate tissue injury.

HVEM and Herpes Simplex Virus-1:

In addition to allowing cell entry for Herpes Simplex Virus-1 (HSV-1), HVEM mediates HSV-1's ability to chronically infect individuals by influencing the Treg population. [80] After HSV infection there is marked expansion of regulatory T-cell populations, HVEM is upregulated on $CD4^+FoxP3^+$ Tregs after HSV infection. *HVEM*$^{-/-}$ are more susceptible to HSV ocular disease and these mice had reduced T-cell expansion compared to wild type mice[80]. This suggests that HVEM regulation of Treg expansion initially aids in control of the infection and direct tissue injury but, may ultimately enable a definitive reservoir for chronic HSV infection.

HVEM expression in critically ill patients:

Expression of HVEM and its major ligand BTLA was explored in critically ill surgical patients by Shubin *et al.* demonstrating BTLA up regulation on $CD4^+$ T-cells, monocytes and granulocytes and similar HVEM upregulation on granulocytes and monocytes in septic patients.[83,84] Non-septic critically ill patients with >80% BTLA expression on $CD4^+$ T-cells were at increased risk of developing secondary infection.[84] Also, *BTLA*$^{-/-}$ mice demonstrate a survival benefit over WT controls with improved bacterial clearance after CLP. [83] Finally, critically ill patients with Hepatitis B-induced acute on chronic liver failure were shown to co-express HVEM, BTLA and fibrinogen-like protein (a virus induced

molecule) on liver macrophages, implying absence of HVEM signaling by HVEM-BTLA complexing may play a role in Hepatitis pathogenesis and acute reactivation.[79]

Conclusion

Checkpoint regulators are crucial in producing an appropriate and controlled immune response to insults. Their expansive roles in immune modulation described above highlights their essential function. However, their powerful role is often mistakenly used to reduce immune reactions when true non-self-threats exist or to activate immune responses in the absence of infectious pathogens resulting in tissue injury. The immense role of checkpoint regulators in immune dysfunction, especially during sepsis progression, makes them an attractive target for therapeutic interventions.

Multiple clinical trials have been undertaken to investigate blockade of various checkpoint regulators during sepsis. Unfortunately, all reported clinical trials have demonstrated lackluster results thus far. The most recent trial of immunotherapy in sepsis, utilizing an anti-PD-L1 antibody in septic patients, demonstrated no change in mortality or cytokine levels. A modest increase in monocyte human leukocyte antigen-DR expression was obtained with anti-PD-L1, but only at higher doses. [85] Earlier immunotherapy trials demonstrated similarly disappointing results, as treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) provided no survival benefit and only a modest reduction in ventilatory days.[86] However, treatment with IFN-γ correlates with decreased TNFα response to lipopolysaccharide stimulation and is FDA approved for treatment of fungal sepsis in patients with chronic granulomatous disease. Despite this small success no broadly applicable immunomodulatory agent is approved for use in sepsis treatment at this time.[10]

These underwhelming results may be due in part to the reliance on animal modeling to study these complex molecular mechanisms with limited confirmation from human sampling. Further, the lack of specific septic patient criteria make it difficult to select ideal patient cohorts for treatment as has successfully been done in cancer clinical trials. Finally, failures in sepsis clinical trials may stem from targeting single regulators in isolation, ignoring how molecules endogenously act in concert. The family of checkpoint regulators is extensive, diverse, and important. However, many of these regulators have overlapping roles without an obvious indication for such redundancy. Thus, a more thorough understanding of the behavior and hierarchy of checkpoint regulators in sepsis may afford a more effective combinatorial therapeutic approach.

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Key Points:

1. Checkpoint regulators are a diverse group of membrane bound proteins, with varied expression and notable redundancy, which dictate immune cell response to antigen presentation.
2. Septic Immunosuppression predisposing patients to secondary infection after a primary infectious insult is mediated, at least in part, by checkpoint regulators.
3. Checkpoint regulators have been manipulated in animal models improving outcomes after septic insult, but, have not been successfully harnessed as therapeutic targets in humans.

Synopsis:

Checkpoint regulators are a varied group of membrane bound receptors or ligands expressed on a variety of immune cells to regulate the immune cell response to antigen presentation and other immune stimuli such as cytokines, chemokines, and complement. In the context of profound immune activation such as sepsis, the immune system can be rendered anergic by these receptors to prevent excessive inflammation and tissue damage. However, if this septic immunosuppression is prolonged, the host is unable to mount the appropriate immune response to a secondary insult or infection. Here we describe the manner in which major regulators belonging to the B7-CD28 family, PD-1, VISTA, CTLA-4, HVEM, and their various ligands, mediate immunosuppression in sepsis.

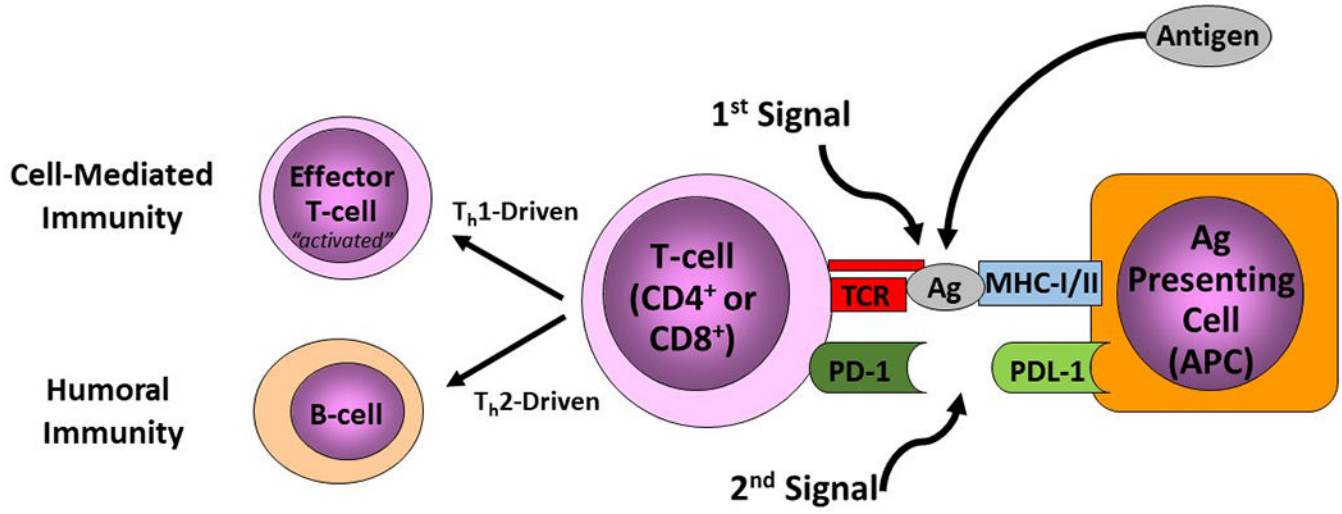

Figure 1. Checkpoint regulators serve as a necessary second signal for immune responses:
Checkpoint regulators are membrane bound proteins which serve as a second signal to direct the immune response to a particular antigen. When an antigen is present it is bound by MHC class I or II receptors on an antigen presenting cell (APC) and presented to a T-cell Receptor (TCR). Following this a second signal, from a checkpoint regulator, is necessary to instruct the T-cell on how to respond to this antigen, shown here as the PD-1/PDL-1 interaction. Stimulatory signals from regulators can lead to activation, and subsequent cell and humorally mediated immunity, while inhibitory signals can lead to anergic t-cells unable to respond to further signals.

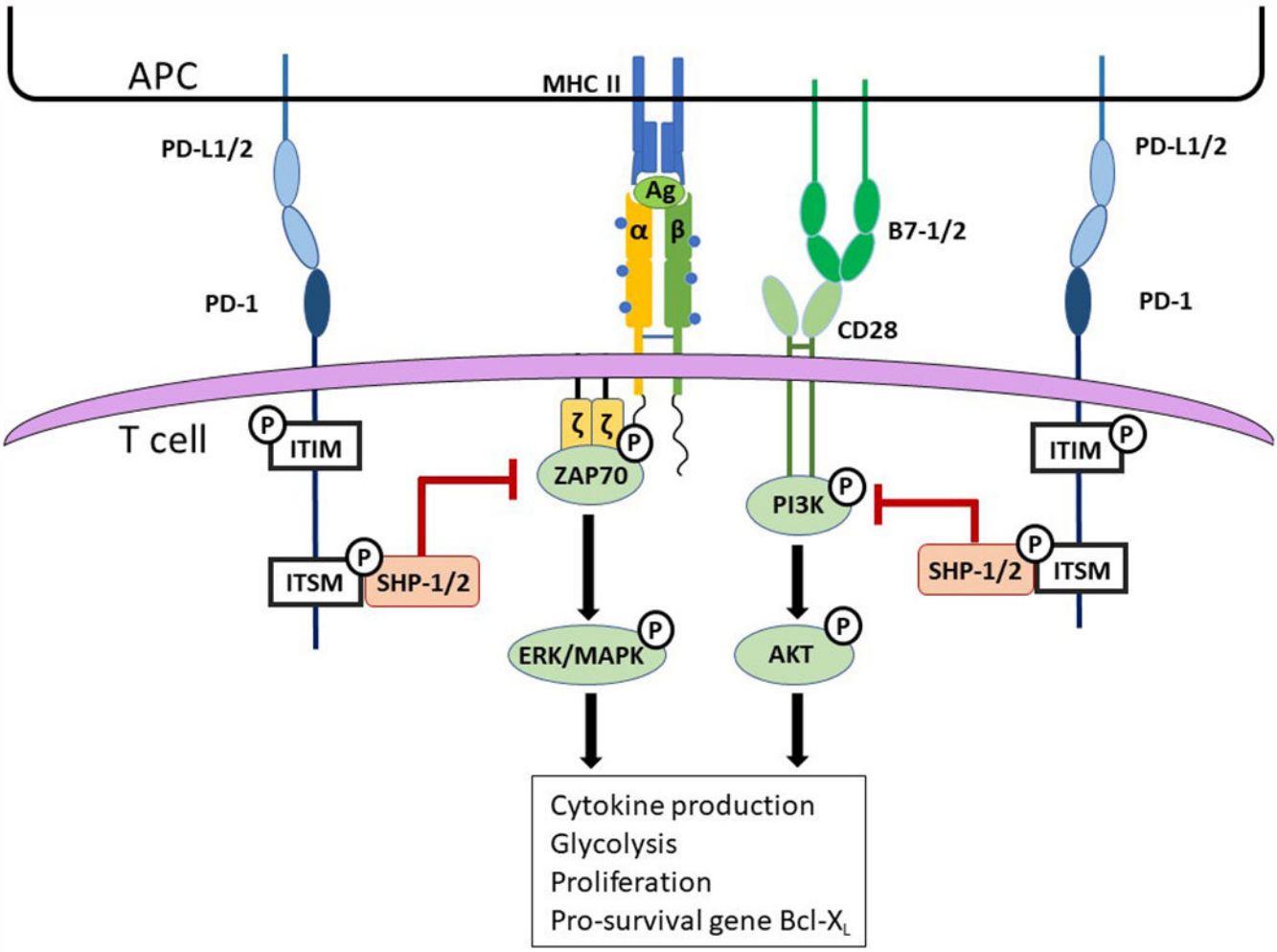

Figure 2. PD-1 exerts intrinsic suppression of T cell activity proximal to the TCR:

Ligation of PD-1 with its ligands results in recruitment of SHPs to the phosphorylated ITSM domain where they become functionally active. ZAP70 associates with the CD3-zeta chain and PI3K associates with the CD28 cytoplasmic tail upon TCR stimulation and CD28 ligation. ZAP70 is an adaptor protein that recruits and stabilizes a kinase complex to initiate the ERK/MAPK pathway. PI3K serves as the initial kinase in the AKT pathway. Active SHP-1/2 dephosphorylate ZAP70 and PI3K; thus, inhibiting these kinase pathways and preventing T cell activity, proper metabolic activity, T cell survival, and proliferation.

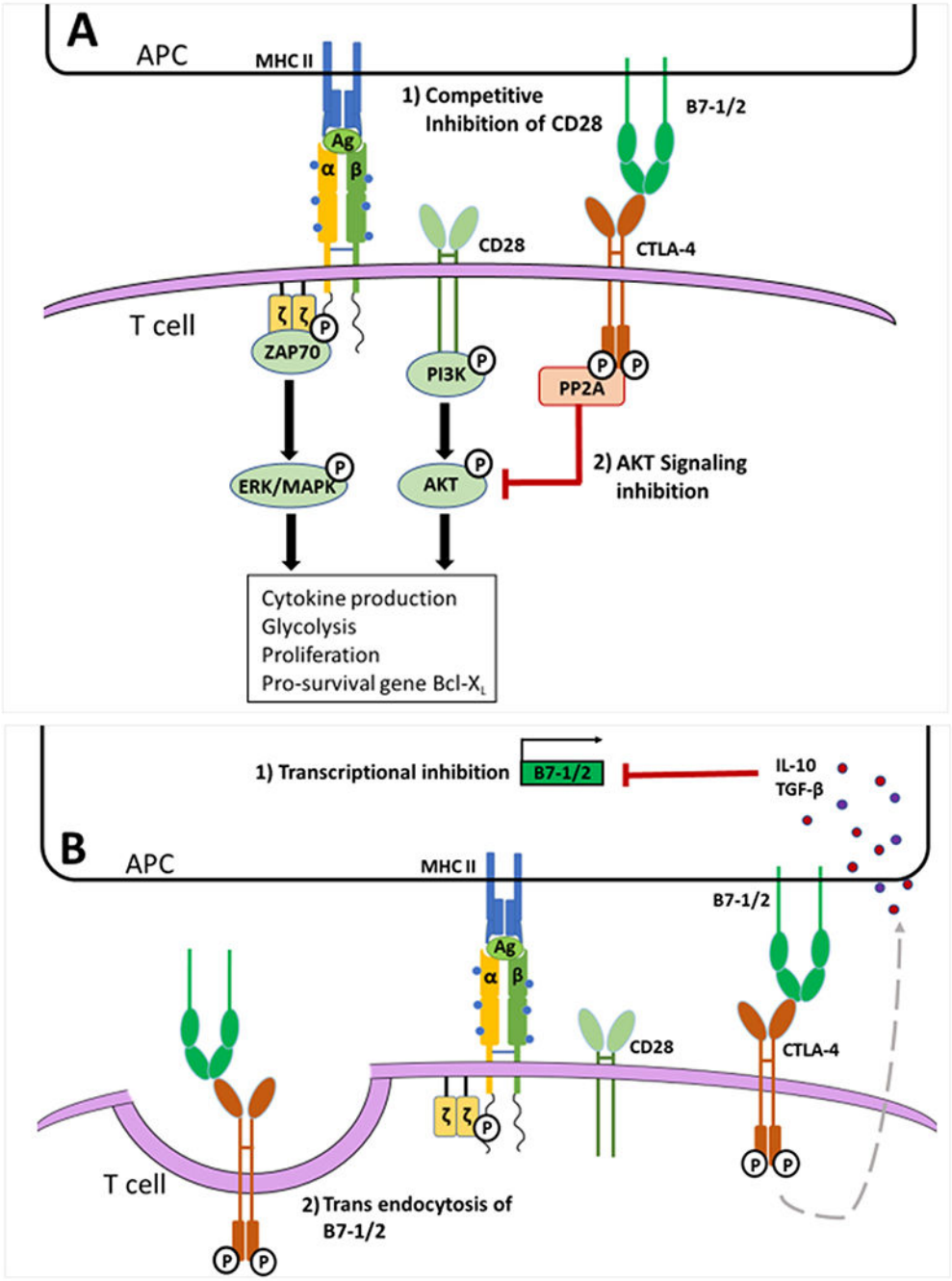

Figure 3. CTLA-4 suppresses T cells through both T cell intrinsic and extrinsic mechanisms:
A. Upon TCR activation CTLA-4 is expressed on the T cell surface and exerts cell intrinsic inhibition. CTLA-4 outcompetes CD28 for the B7-1/2 ligands resulting in reduced CD28 stimulatory activity. CTLA-4 also recruits the phosphatase PP2A to its phosphorylated cytoplasmic tail. Active PP2A dephosphorylates AKT preventing downstream signaling; thus, inhibiting T cell activity, proliferation, and survival. **B.** Following CTLA-4 phosphorylation and ligation, a signal cascade is initiated by which IL-10, TGF-β, and soluble-CTLA-4 (not shown here) are upregulated. These signaling molecules are

endocytosed by the APC and inhibit the transcription of B7-1 and B7-2, reducing the amount of B7-1/2 surface expression by APCs and preventing CD28 stimulation. When CTLA-4 binds to B7-1/2 it can also trigger *trans* endocytosis of these ligands. This reduces the surface expression of B7-1/2 on the APC and further hinders the ability of CD28 to be stimulatory.

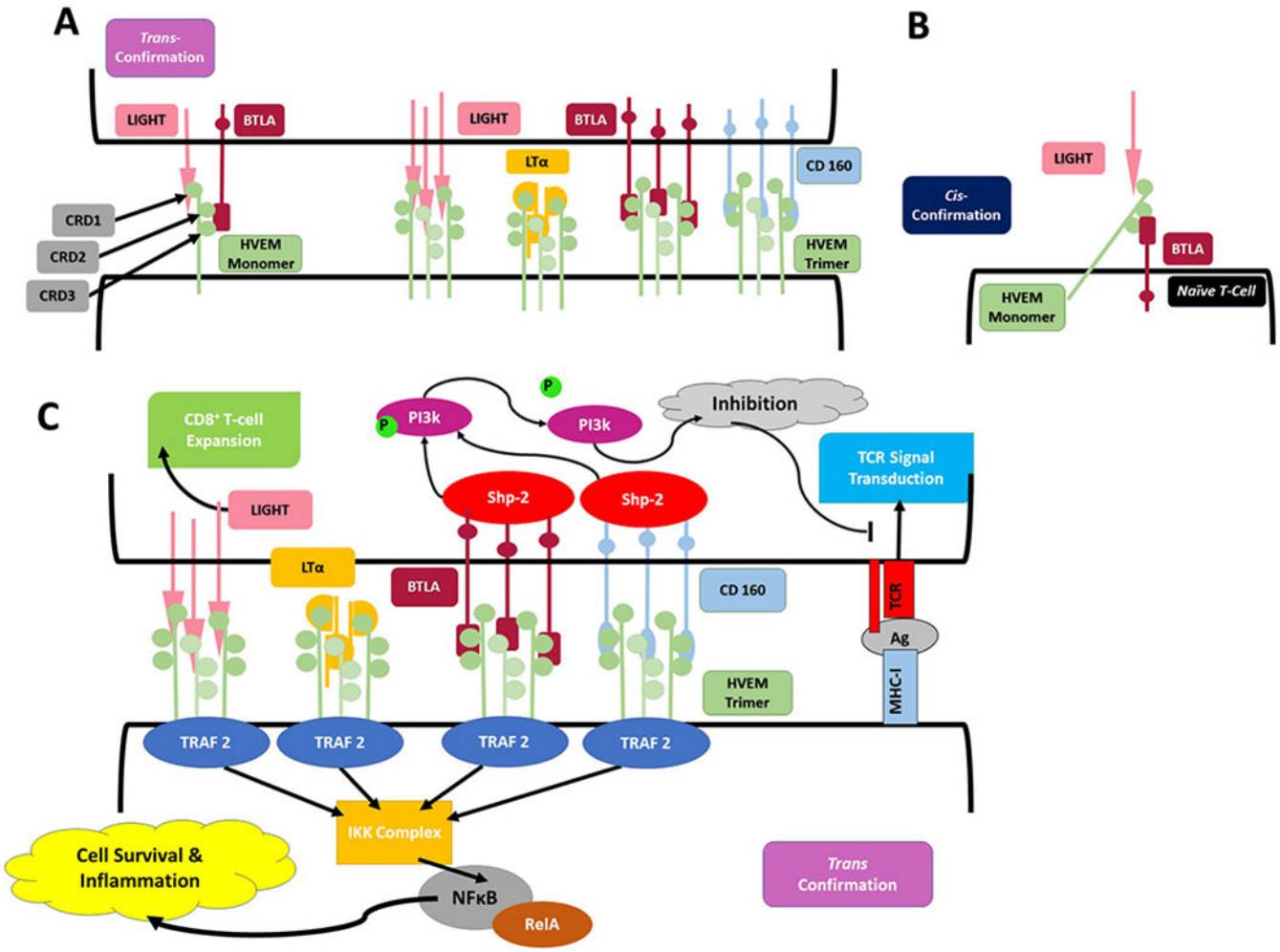

Figure 4. HVEM behaves as bidirectional switch based on environmental signals:

A. HVEM associates with LIGHT, LTα, BTLA, and CD160 in trimeric confirmations when expressed in *trans* confirmation. LIGHT and LTα associated with CRD1 binding domain, while BTLA and CD160 associate with CRD2/3. All ligands associate with HVEM in a trimeric confirmation, generating a 3:3:3 complex of 3 HVEM molecules with 3 BTLA or CD160 molecules and 3 LIGHT or LTα molecules. **B.** HVEM interacts with coexpressed BTLA to from an inert complex when both are expressed in their *cis* confirmation, most commonly on naÏve T-cells. In this formation HVEM can still associate with soluble LIGHT or LTα at its exposed CRD1 binding domain, yet no downstream signal is generated from the interaction. **C.** When HVEM is expressed in the *trans* confirmation on the membrane, ligation of LIGHT, LTα, BTLA or CD160 results in TRAF2 recruitment within the HVEM expressing cell. TRAF2 activates an IKK complex which in turn activates the RelA form of NFκB to promote cell survival. Within BTLA and CD160 expressing cells, ligation of HVEM results ITIM phosphorylation, recruiting SHP1 and SHP2, ultimately resulting in inhibitory signaling interrupting TCR signal transduction via dephosphorylation of PI3k - PKB pathway. Despite apparent absence of intracellular signaling motifs, LIGHT ligation of HVEM stimulates $CD8^+$ T-cell expansion.