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A Novel Role for Co-Inhibitory Receptors/Checkpoint Proteins in the Immunopathology of Sepsis.

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Abstract

Co-inhibitory molecules, such as PD-1, CTLA-4, 2B4 and BTLA, are an important new family of mediators in the pathophysiology of severe bacterial and/or fungal infection, as well as the combined insults of shock and sepsis. Further, the expression of these molecules may serve as indicators of the immune status of the septic individual. Using PD-1:PD-L as an example, we discuss in this review how such checkpoint molecules may affect the host response to infection by mediating the balance between effective immune defense and immune-mediated tissue injury. Additionally, we explore how the upregulation of PD-1 and/or PD-L1 expression on not only adaptive immune cells (e.g. T cells) but also on innate immune cells (e.g. macrophages, monocytes and neutrophils) as well as non-immune cells during sepsis and/or shock contributes to functional alterations, often with detrimental sequelae.

Summary Sentence:

Review of checkpoint proteins' contributions to immunological signaling in shock and sepsis.

Keywords

Infection; Immunosuppression; Immunomodulation; PD-1; PD-L1; Innate Immunity

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E.A.F. wrote the initial review outline, edited the figures and wrote the manuscript. A.A. provided the framework for the review and substantial revisions to the manuscript, as well as preparing the figures. B.M.B contributed to the literature search and citation of references. C.S.C, S.F.M., J.L.N. and D.S.H. provided revisions and conceptual feedback. All authors reviewed and approved the final manuscript.

CONFLICT OF INTEREST DISCLOSURE:

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INTRODUCTION:

Sepsis

Sepsis remains a serious clinical problem worldwide and in the United States, with an annual incidence in the U.S. estimated at 1.5 million (1). Sepsis is the leading cause of death in non-cardiac Intensive Care Units, with 250,000 deaths each year in the United States (1). Additionally, it ranks as one of the most expensive conditions treated in U.S. hospitals, with costs exceeding twenty billion dollars annually. A concerning factor is that the incidence of sepsis is increasing, with current levels giving rise to more than triple the mortality compared to the 1970's (2–5). Despite its concerning prevalence within the healthcare system, sepsis and related terminology remain difficult to define due to the heterogeneity and complexity of sepsis pathobiology. In 2016, the definition for sepsis was updated by the Third International Consensus Definitions for Sepsis and Septic Shock. Today sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (6).

Sepsis is a complex clinical syndrome that is thought to develop when the initial host response against infection and/or injured tissue becomes inappropriately amplified, then dysregulated. Ultimately this results in a harmful host response. The homeostasis between eliminating invading pathogens and protecting tissue health is disrupted; multisystem organ failure (MSOF) ensues. Much of sepsis related mortality and long-term morbidity is driven by organ dysfunction and failure. On the cellular level, disruption of the immune system's balanced response to infectious challenge and/or tissue injury causes circulating immune cells to influx in abnormal proportions into distal organs in the absence of a clear nidus of infection and/or overt tissue damage (6–8). All organs are susceptible, including the pulmonary, hepatic, renal and gastrointestinal organ systems.

SIRS/CARS

The central hypothesis that has driven the search for novel therapeutics is that sepsis induces an overwhelming Systemic Inflammatory Response Syndrome (SIRS). Sequelae include significant bystander cell/tissue injury within the host. Although the term SIRS has been largely overtaken by the modified definition encompassing an expanded pathophysiologic perspective of sepsis, the underlying principle of SIRS has historically influenced the framework with which sepsis has been researched (6, 9, 10). Unfortunately, however, while numerous treatments that were based on anti-inflammatory or anti-coagulant concepts showed promise in the experimental setting, they have all failed to provide a survival benefit in randomized human clinical trials (11, 12). And while one can argue the nature and quality of the clinical trials designed to test these anti-inflammatory approaches, it remains critical to continue to work towards a better understanding of the complex pathology of the septic state in order to develop a truly effective therapy.

The term Compensatory Anti-inflammatory Response Syndrome (CARS) was coined by Roger Bone to describe the immune suppression that occurs after sepsis (13). It arose from the initial descriptions of the development of immune suppression associated with severe shock/trauma/sepsis and with reports dating back to as early as the 1960's. The syndrome is

characterized by the immune system undergoing reduced activation with limited efficacy; in its most severe form it has even been referred to as immunoparalysis (14–20). Historically, however, this facet of the severely injured or septic patient has not been considered as a significant therapeutic target.

Characteristics of septic immune suppression/CARS include cutaneous anergy as manifested by delayed type hypersensitivity, leukopenia as determined by decreased lymphocyte count on a patient's CBC (complete blood count) with differential, and persistence of or failure to resolve an infection. The mechanisms proposed include decreased human leukocyte antigen-D related (HLA-DR), lymphocyte reprogramming (movement from an Th1/M1 immune cell phenotype to a more Th2/M2 phenotype), the induction of programmed cell death/apoptosis, increased expression of anti-inflammatory mediators (prostaglandin E, IL-10, steroid hormones), and finally increased expression of cell-associated co-inhibitory receptors and ligands (such as PD-1/PD-L1, CTLA-4) (19–23).

CENTRAL QUESTION:

Out of this history grew the principal question which we undertake to review here: does modulating aspects of developing immune suppression improve the capacity of the critically-ill patient to ward off end-organ and tissue injury that thus far has been commensurate with the septic state? Specifically, what roles do such co-inhibitory/checkpoint protein signaling cascades play in this process of immunomodulation, and how does this affect the balance of innate and/or adaptive immunity?

OBJECTIVES:

From our central question, we derived four objectives for this discussion, as follows:

- To provide a succinct overview of membrane co-stimulatory/co-inhibitory molecules and their role in classic immunity;
- To discuss how sepsis and sepsis plus hypovolemic shock affect the expression of co-inhibitory molecules such as PD-1 and its ligands;
- To describe the data that speak to the patho-physiological contributions of PD-1:PD-Ls to the morbidity/mortality seen in models of sepsis and/or shock (hemorrhage); and
- To consider possible adaptive and innate immune mechanisms by which PD-1:PD-L1 interactions may drive morbidity.

DISCUSSION:

1. The role of checkpoint proteins in classic antigen-driven immunity.

Antigen presentation is mediated by the antigen presenting cell (APC), with examples including innate immune cells such as professional phagocytes, B cells, or dendritic cells, as well as non-classic immune cells in some cases, such as endothelial cells [EC]. The process is incited by a bacterial challenge or tissue injury, during which time components of the

pathogen or damaged cell are taken up by the APC. The products of these lytic processes are antigenic peptides that are associated with the HLA/MHC molecule (Figure 1, Signal 1). A $CD4^+$ or $CD8^+$ T cell receptor binds to the APC's HLA/MHC complex as well as the checkpoint molecule concomitant with the T cell receptor's binding of the APC's antigen-containing HLA/MHC molecule (Figure 1, Signal 2). If the predominant co-receptors on the APC are co-stimulatory molecules/receptors, such as CD28, ICOS, or CD40, this licenses the T cells that see presented antigen to respond to this connection by activation or differentiation. This transformation is a driving force behind what has been characterized as a Th1-driven response into an activated effector T cell (cell-mediated immunity) response. Alternatively, if the APC expresses a preponderance of co-inhibitory molecules (i.e. checkpoint proteins), the $CD4^+$ or $CD8^+$ T lymphocytes are not licensed to respond (i.e. becoming anergic and potentially apoptotic). It should be noted that many of these co-inhibitory cell surface receptor molecules *actively* signal --the simple over-expression of these receptors, or their cell surface ligands in some cases (e.g. the most overt example being the over-expression of some of these co-inhibitory ligands on tumor cells), is sufficient to directly (via check point protein cell signaling and/or the indirect stimulation immune suppressive mediators, like IL-4, IL-10 and IL-13) prompt an anergic state (Th1 to Th2 and/or M1 to M2 phenotypic shift) among cells within such an environment (24). That such shifting occurs in the setting of the experimental animal and septic patient has been documented by several labs (19, 23). Ultimately, these receptors and their ligands are often first regarded as toleragens (25, 26).

Checkpoint proteins are not limited to solely the APC to T cell interaction. Communication among monocytes/macrophages/dendritic cells with epithelial/endothelial/tumor cells works via this mechanism (Figure 2).

(a) Programmed cell death receptor-1 (PD-1): Programmed Cell Death Receptor (PD)-1, with pseudonyms including Pcdc1 and CD279, is a Type I transmembrane glycoprotein-Ig (IgV) superfamily member, containing an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) for intracellular signaling. PD-1 participates across a spectrum of immune responses relative to many other B7:CD28 superfamily members (27–29). Most observations indicate that ligation of PD-1 recruits phosphatases Src homology region 2 domain-containing phosphatase (SHP)-1 and/or SHP-2, prompting an inhibition of PI3K pathway signaling resulting typically from CD28/CD3/immunoreceptor tyrosine-based activation motif (ITAM) activation (30–34) (Figure 3).

Programmed Cell Death Receptor Ligand-1 (PD-L1), also known as B7-H1 or CD274, is considered the primary ligand of PD-1. Importantly, it is ubiquitously expressed on not only immune, but also a wide variety of non-immune tissues and organs (35–37). Alternatively, PDL2 is more restrictively expressed on APCs and immune cells (38). Like PD-1, these ligands are both type I transmembrane glycoprotein receptors (IgC in addition to IgV). Unlike PD-1, they have neither an ITIM nor ITSM motifs. However, there are reports that intra-cellular signaling may still be achieved via these PD-1 ligands themselves, perhaps from surrogate ligation of B7.1/CD80 (26, 39).

(b) B and T Lymphocyte Attenuator (BTLA) and Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4): B and T Lymphocyte Attenuator (BTLA) is considered a co-inhibitory receptor, related in molecular structure to PD-1; it acts as an inhibitor of B cell and $CD4^+$ T cell function, as its name describes (40). It is induced on anergic $CD4^+$ T cells and is associated with attenuated pro-survival signaling (41, 42).

Cytotoxic T Lymphocyte-Antigen-4 (CTLA-4) is another inhibitory regulator of T cell activation and proliferation. CTLA-4 competes with CD28 (a potent co-stimulatory molecule/receptor) for binding to CD80 and CD86 on APCs, thus, preventing T cell activation (43, 44). CTLA-4 plays a prominent role in maintaining T cell responses which is evident in the phenotype of CTLA-$4^{-/-}$ mouse strain where CTLA-4 deficiency results in death from lymphoproliferative disorders (45, 46)

2. Checkpoint proteins and their ligands during sepsis and shock.

Studies from several laboratories initially utilizing experimental models of sepsis, and subsequently models complexed with aspects of severe shock or secondary infectious challenge, have shown that several of these families of cell-surface molecules have an impact on survival. This points to potential roles in pathological processes driving sepsis. Studies by Huang *et al.* (47) showed that mice in which the gene for PD-1 was deleted exhibited a marked reduction in mortality in response to CLP-induced polymicrobial septic challenge. From an experimental perspective, the role of PD-1:PD-L ligation in driving these morbid effects was further corroborated by two independent investigations utilizing antibodies against PD-1 (33), and its primary ligand PD-L1 (48), where both demonstrated that CLP morbidity and mortality could be significantly reduced by such antibody post-treatment. Importantly, observational clinical studies have shown that critically ill patients who developed severe septic shock plus secondary nosocomial infections expressed markedly levels of PD-1 and PD-L on various leukocyte subsets (49). A similar observation was made on severely injured patients, where the expression of high PD-1 blood leukocyte levels was correlated with worsening physiological dysfunction (50). Furthermore, to the extent that these are simply the aberrations of the CLP model as applied in *adult* male mice, the importance of PD-1 on throughout the age spectrum has been demonstrated (51, 52). Work by Young *et al.* (51), using a cecal slurry model, documented that PD-1 gene deficiency also confers a survival advantage in this model of polymicrobial sepsis in *neonatal* mice. Several studies have also shown a clear role for PD-1 in modulating the *geriatric* immune response to sepsis (53, 54).

Using a similar experimental approach, Shubin *et al.* (55) subsequently demonstrated that $CD4^+$ T cells' BTLA expression among ICU patients was associated with sepsis and development of subsequent nosocomial infections. When the interaction between BTLA and its primary ligand herpes virus entry mediator (HVEM) is limited by genetic deletion of BTLA, murine survival improves in the setting of septic challenge (56). Among adult septic patients and septic adult mice, BTLA and HVEM are both elevated on myeloid and lymphoid cell populations (55, 56).

Unsinger *et al.* (57) have also shown that in response to septic challenge, expression of CTLA-4 on CD4, CD8, and regulatory T cells is increased in the murine CLP model.

Furthermore, antibody neutralization of CTLA signaling capacity with anti-CTLA-4 antibody treatment after the onset of CLP led to a decrease in sepsis-induced apoptosis, better antigen recall responsiveness, and improved survival (58). In studies looking at the role of CTLA-4 in sepsis-induced immunosuppression, blockade of CTLA-4 in conjunction with PD-1 also improved survival and reversed sepsis-induced immunosuppression as measured by reduced lymphocyte apoptosis in a sequential sepsis challenge model where CLP was followed by a secondary fungal insult (59). Recent studies by Chen *et al.* (60) investigating the co-inhibitory molecule 2B4 indicate it may also markedly suppress CLP-induced complications. Together these results point to pathologically important roles for these co-inhibitory/checkpoint proteins and their signaling as underpinning the development of septic morbidity.

(a) From PD-1 to its Ligands: Similar to the survival analysis undertaken for PD-$1^{-/-}$ mice, a survival analysis of PD-L1 gene deficient mice after sepsis demonstrated comparable results(61). In this respect, elevated PD-L1 and PD-L2 expression has been documented in pulmonary epithelia from patients who died of sepsis (62). These investigators found that not only the expression of PD-1, but also the expression of its two ligands PD-L1 and PD-L2, are associated with poor outcome in sepsis and shock. Other groups have also reported the expression of the ligands for PD-1, namely PD-L1 and PD-L2, as being elevated on a variety of non-professional immune cells/tissue beds/tumor cells (63–65). As such, the over-expression of these toleragenic checkpoint protein ligands represents a novel, oft-overlooked, interface between non-professional immune cells/tissue beds/organs that may speak not only to how local immune, but organ function might be regulated directly orindirectly through their ligation (Figure 2 and Figure 4). So, what evidence is there for these ligands of co-inhibitors having an effect on immune dysfunction in sepsis and shock?

3. Immune Dysfunction in Sepsis.

(a) Adaptive Immunity

(i) Classic T cell Immune Dysfunction: Studies of sepsis with PD-1 gene-deficient mice and anti-PD-1 antibody treatments point to PD-1's impact on adaptive immune responsiveness. These changes include the restoration of the delayed type hypersensitivity response and a reduction of sepsis-induced $CD4^+$/$CD8^+$ T as well as B lymphocyte cell death (47, 57, 61). In this regard, Chen *et al.* (60) have recently reported that the expression of the co-inhibitory molecule 2B4 (CD244, SLAM4), which was initially reported to be upregulated on activated natural killer cells and $CD8^+$ T cells (but not in experimental models of sepsis), is markedly upregulated on the splenic $CD4^+$ T cells of not only CLP mice, but also on human patients with severe sepsis. Further, these $CD4^+2B4^+$ cells appear to upregulate PD-1 and CTLA-4, but inhibition of 2B4 activity is non-redundant to either PD-1 or CTLA-4. Importantly, gene deficiency of 2B4 preserves CD4 cell Th1 functions and cell survival (reduced septic CD4 T cell loss), while providing a survival benefit to CLP mice (59).

Since many co-inhibitory receptors, e.g., PD-1, BTLA, CD47 and CTLA-4, express an ITIM and/or ITSM, it is thought that the suppression of classic $CD4^+$/ $CD8^+$ T cell function is a result of the recruitment of the phosphatase, SHP-1 and/or SHP-2 (27, 30, 66). These SHPs

then antagonize not only T cell receptor driven, but other pathways that drive the activation of the Akt/ PI3K pathway, which underpin the immune-suppressed'exhausted' T cell phenotype seen with PD-1 in conditions such as cancer and viral infection (27, 30, 66). In this respect, studies by Bandyopadhyay *et al.* (67) have documented that, at least in T lymphocytes isolated from severely injured patients, which they have previously reported as expressing a number of co-inhibitory molecules as well as exhibiting T cell anergy/ 'exhaustion' (21, 68), indicate that these cells appeared hyper-responsive to expressing the activated form of the phosphatase SHP-1 (Figure 3). Importantly, Huang *et al.* (47, 61) also showed that macrophages derived from septic mice had a markedly increased capacity to produce the immune suppressive/ anti-inflammatory cytokine IL-10 and this, more so than many of the mediators examined, could be attenuated by the loss of either PD-1 or PD-L1 gene expression. This implies that these cells shifted from an M1 to an M2 macrophage phenotype, and this change also has the capacity to support an antigen-independent induction of immune suppressive/Th2/anergic T cell state.

Intriguingly, an alternative signaling mechanism has been put forward for PD-1 related co-inhibitors, such as CTLA-4, involving interactions with integrins and various components of the cell cytoskeletal machinery (69–71) or the recruitment of alternative receptors like CD80, CD24 versus TLR4 by Siglec10 (72, 73) (Figure 3). Much, however, remains to be understood about sepsis-induced changes in T cell co-inhibitor molecule signaling and how it shapes the septic cellular phenotype and function.

(ii) Regulatory Lymphocytes:

invariant Natural Killer T-cell (iNKT-cell): The innate regulatory lymphocyte populations are emerging as key regulators of the immune and inflammatory response a variety of infectious insults. Among these sub-populations, the *i*NKT-cell has been shown to play some of the most central roles in immune response to a wide variety of infections and septic events. *i*NKT-cells are capable of both a Th1 and a Th2 response and interact with a diverse array of immune cells serving to both positively and negatively regulate the immune and inflammatory response *i*NKT-cells are capable of trafficking both within the liver in response to a sterile injury (74), in and out the lung in response to an infection (75). As well as distally to a septic source in the abdomen (76). *i*NKT-cells are themselves regulated by co-inhibitory/checkpoint proteins, most notably PD-1. Initial activation of *i*NKT-cells is associated with increased PD-1 expression upon *i*NKT-cells (77) (Figure 4, Component [2]). Young *et al.* (76) showed that direct ligation of PD-1 with its ligand PD-L1 is essential to trafficking and migration of *i*NKT-cells and PD-1 is essential to regulation of chemokine receptor expression upon *i*NKT-cells. Conversely, repeated stimulation of PD-1 upon *i*NKT-cells has been shown to induce anergy (78) a mechanism that is believed to have developed to protect against an over-exuberant *i*NKT-cell driven immune response. In similar fashion, PD-1:PD-L blockade has been shown to prevent the induction of *i*NKT-cell anergy (79). Although PD-1 has been the most explored co-inhibitory pathway in *i*NKT-cell responses to stimuli, several other co-inhibitory molecules have been described. CD28 and OX40 have been described as indispensable for full activation of *i*NKT-cells, and glucocorticoid-induced TNF receptor (GITR) plays a key negative regulatory role in antigen-induced activation of *i*NKT-cells (80). Akin to $CD8^+$ T-cells, the negative checkpoint regulator 2B4 has also been

shown to play a key role in suppressing an excessive *i*NKT-cells response. Ahmad *et al* demonstrated that among patient with HIV infection, levels of 2B4 expression was significantly upregulated upon both $CD3^+$ and HIV-specific $CD8^+$ T-cells as well upon and *i*NKT-cells. Levels of 2B4 expression correlated with degree of suppression of intra-cellular production of IFN-gamma within *i*NKT-cells. Importantly, treatment with anti-retroviral therapy among these HIV patients was noted to lead to a decline of 2B4 levels upon $CD8^+$ T-cells, but not among *i*NKT-cells (81).

$CD4^+CD25^+$ Regulatory T Cells (Tregs): A regulatory/anti-inflammatory role for T regulatory cells (Tregs) has been identified in several models of lung diseases: allergy/asthma, *pneumocystis* and tuberculosis (82–84). However, lymphocytes, especially the small population of Tregs that reside in the lung, have garnered little attention relative to the role of these cells in the pathophysiology of ARDS, let alone indirect (i)ARDS. Using a well-established model of direct lung injury (IT instillation of lipopolysaccharide [LPS]), D'Alessio *et al.* (85) were the first to demonstrate that Tregs accumulate in the bronchoalveolar lavage fluid (BALF) of mice. In addition, they identified the presence of Tregs in patients with ARDS suggesting a role for Tregs in the resolution of ARDS. Based on these results, Aggarwal *et al.* (86) documented that adoptive transfer of Tregs to lymphocyte-deficient recombinase-activating gene-1-deficient ($Rag\text{-}1^{-/-}$) mice restored them to a normal pattern of resolution after IT LPS-induced direct ALI. Furthermore, a recent study demonstrated that transplantation of human umbilical cord mesenchymal stem cells ameliorated ARDS by restoring the diminished levels of alveolar $CD4^+CD25^+Foxp3^+$Tregs via a re-balancing of levels of anti- and pro-inflammatory factors in a direct ARDS mouse model (87). More recently, a study by Ehrentraut *et al.* has indicated that CD73-dependent adenosine generating Tregs could promote the resolution of LPS-induced direct ARDS (88). However, none of these studies illustrated a specific role of Tregs in iARDS. Using a two-hit model of Hem-CLP, Venet *et al.* observed that $CD4^+CD25^+Foxp3^+$ cells appeared to be recruited to the lung in iARDS and that the Foxp3 as well as IL-10 gene expression in these cells were increased during this process (89). Most importantly, Venet *et al.* also showed that down-regulation of the Tregs' function using Foxp3 targeting siRNA, administered 12 hours before the induction of experimental iARDS, echoed lung injury obtained in CD4-gene deficient as well as anti-CD4 antibody depleted mice; this was consistently associated with a reduction in lung IL-10 levels (89). However, how the activation of this small sub-population of lymphocytes regulated iARDS in this study was only partially elucidated.

With respect to the role of the checkpoint protein PD-1, Tang *et al.* (90) demonstrated a significant role for PD-1 in regulating the Tregs response to iARDS. Adoptive transfer of Tregs derived from WT naïve mice were capable of suppressing iARDS, including decreased pulmonary neutrophil influx. However, these beneficial effects upon the severity of lung injury were not evident when $PD\text{-}1^{-/-}$ derived Tregs were transferred into recipient WT mice during the resuscitation period of Hem or 24 hours before CLP via tail vein injection. Furthermore, the expression of CTLA-4, another co-inhibitory regulator, was also affected by the presence of PD-1, wherein the expression of CTLA-4 upon $CD4^+Foxp3^+$ T-cells was increased following transfer of Tregs from WT, whereas no increase in CTLA-4

expression was noted in mice receiving PD-$1^{-/-}$ Tregs. (Figure 4, Component [3]). Subsequently, Tang *et al.* expanded the significance of PD-1:PD-L1 ligation on Tregs on mediating lung-protective effects by documenting that AT transfer of WT donor Tregs into PD-L1 gene deficient recipient mice did not confer protection against lung injury/inflammation. Further, the ligation PD-1:PD-L1 in this system also appears to preferentially signal through SHP-1 as opposed to SHP-2, similar to that reported above for septic patient T cells (91, 92).

La *et al.* also noted the suppressive effect of PD-1 upon Tregs in a model of hydatidosis, a parasitic infection (93). They noted that the percentage of $CD4^+CD25^+PD\text{-}1^+$ cells correlated with duration of infection and size of abscess. The authors speculated that the suppressive effect of PD-1^+ expression upon Tregs was essential for infection growth and avoidance of the immune surveillance. These findings have been correlated clinically, wherein Liu *et al.* noted PD-1 expression was increased on Tregs in patients with sepsis when compared with healthy controls. Most significantly, levels of PD-1 expression upon Tregs were lowest among patients who subsequently survived the septic event, again implying the negative regulatory effect of PD-1 upon Tregs may significantly contribute to sepsis related mortality (94).

(b) Innate Immunity

(i) Macrophages: One of the more interesting observations made by Huang *et al.* is that early following septic challenge/CLP (as defined as 12-hours post-CLP or less), changes in PD-1 expression on $CD4^+$ T cells, $CD8^+$ T cells or B cells are not yet evident (typically these are not seen until at 24 hours post CLP) (61). Further, dendritic cells did not express PD-1 at this time point. However, Huang *et al.* observed that peritoneal leukocytes, as defined by $F4/80^+$ cells harvested from peritoneal lavage, demonstrated increased PD-1 and PD-L1 expression (61), at both protein and message levels, after as little as 12-hour post-CLP. This effect was sustained at the 24-hour and 48-hour time points. Mouse blood monocytes and Kupffer cells have also been, subsequently, demonstrated to exhibit increased PD-1 expression in response to CLP (47, 95). This upregulation of PD-1, from a naïve state of nearly undetectable levels, was also a little surprising as a number of groups had indicated that tissue macrophage did not express elevated levels of PD-1 typically. However, as most of this work was conducted with naïve cells' response to LPS as a stimulant, unsurprisingly this was not detected. To elaborate, LPS, unlike TNF-α or interferon-γ, was not reported to be a strong inducer of PD-1 expression (49). In fact, Guignant *et al.* (49) noted that monocytes derived from patients with severe sepsis express both PD-1 and PD-L1 out to 3–5 days post diagnosis of sepsis.

These initial studies by Huang *et al.* also showed that following CLP, there was a decline in both phagocytic capacity (which occurs with both opsonized and non-opsonized targets) and stimulant-induced cytokine production among macrophages (47). Additionally, random migratory capacity (measured both in rate and direction) and cell spreading (following surface adherence) increased markedly after sepsis among WT mice. Importantly, these phenomena were equivalent to findings among sham control samples in the setting of PD-1 gene deficiency (96). These latter changes in migratory capacity, cell spreading and motility,

appear to be partly due to PD-1-mediated changes in cytoskeletal alpha-actinin and F-actin aggregation capabilities. Proposed mechanisms include PD-1 association with CD11b and changes in PD-1-mediated upstream suppression of phosphatase and Rap-1-dependent migration. Ultimately, septic PD-$1^{-/-}$ macrophages exhibit less dysfunctional migration compared to their septic WT counterparts.

With this stated, it is unknown whether macrophages and monocytes expressing PD-1 under the stress of sepsis and/or severe shock/injury are the same cells that express PD-L1 or whether they are unique sub-populations. Does it affect macrophage M1 to M2 polarization? Does paracrine or even autocrine signaling occur for PD-1: PD-L1 expressed on these macrophages? Further, while the study by Ayala *et al.* suggests that phosphatase signaling, as reported in classical T helper cells and Tregs, appears to be involved in some of the changes seen in these septic macrophages, the exactnature of this signaling remains to be elucidated (Figure 3).

(ii) Neutrophils: Neutrophils (PMNs) are proposed to have an important role in inducing iARDS. When recruited to a site of infection, they exert a variety of beneficial functions critical to clearance of the invading pathogen (97). However, it is also thought that the recruitment of activated PMNs may be harmful when these functions are directed at otherwise normal host tissue (bystander injury) (97, 98). In this regard, Ayala *et al.* (99) and Lomas *et al.* (100) reported on PMNs that were isolated from donor animals after hemorrhage (a form of PMN priming) (Figure 4, Component [5]), and were then adoptively transferred into naïve animals. They found that when these naïve animals were then subjected to a septic challenge, the recipient lungs became inflamed. In this setting, PMN *in vivo* 'priming' was not only associated with an increase in respiratory burst capacity *in vitro*, but also with a decrease in PMN apoptosis (Ao) (99, 100); a finding confirmed by others in experimental iARDS (101). Jimenez *et al.* (98) found a significant decline in PMN AO in patients with SIRS and the plasma from these individuals suppressed A_O of PMNs derived from healthy controls (98). These findings and others (102–104) support the hypothesis that while delayed A_O in an infectious environment aids PMN mediated pathogen killing, in a pathogen-free setting delay in A_O contributes to inflammation/SIRS and organ failure/injury (98). With that stated, the mechanisms underpinning these effects in PMN have not been fully clarified.

As mentioned earlier, while it has been documented that neutrophils can express the co-inhibitory ligand PD-L1 (105), until recently little was known about how disease states may alter its expression, and what might be the pathological significance of such changes. In this regard, using the CLP model of sepsis Huang *et al.* (105) initially documented a trend toward increased frequency of PD-L1 $(B7\text{-}H1)^+$ cells in the mouse blood PMN population following CLP, though this was not statistically significant due to wide variance. Upon closer inspection of the data, however, expression of PD-$L1^+$ staining appeared to segregate into distinct PD-$L1^{high}$ and $PDL1^{low}$ sub-populations. Further it was found that if mice exhibited PD-$L1^{high}$ expressing PMNs in their peripheral blood at 24 hours post-CLP this correlated not only with higher levels of systemic pro- and anti-inflammatory cytokine/chemokine levels (CCL2, IL-6, CXCL2, KC, TNF-α, and IL-10), but with lethal outcome as well. Subsequently, Wang *et al.* (106) showed that PMNs from CLP mice and patients

diagnosed with severe sepsis exhibited a substantial rise in their PD-L1 expression, correlating with increased morbidity in the septic patients. Additionally, these cells, via direct contact, were capable of inducing a marked increase in lymphocytic cell death/dysfunction (another common feature of the septic animal/patient) as well as altering the migrational functionality of the cell (a dysfunction also noted by Young *et al.* (107) in septic mouse *i*NKT cells) (22). More recently, Patera *et al.* (53) have further found that peripheral blood PMNs from septic patients that expressed high levels of PD-L1 exhibited a decline in their capacity to phagocytize bacteria, a reduction of CD168 expression and a poor production of TNF-α in response to stimuli. Utilizing neutralizing antibodies directed against either PD-1 or PD-L1, to treat these septic patients' PMNs *ex vivo*, the investigators also found that the decline in PMN bacterial phagocytic function could be markedly reversed. Together this illustrates that via direct ligation of PD-L1, or via indirect ligation of PD-1 and its downstream signaling, it appears possible that sepsis can drive altered PMN function as well as set up the PMNs to serve as a potent toleragenic cell population through PD-1:PD-L1 ligation.

3. End-organ sequelae of cellular immune disharmony

(a) End-organ Injury – the Gut—In the intestine, the epithelial lining serves as an important barrier to prevent the absorption of toxins, antigens, and microorganisms across the intestinal wall. Intestinal barrier dysfunction and increased intestinal permeability are morbid states that often accompany developing sepsis. This developing epithelial cell barrier dysfunction has been thought to contribute to the development of the multiple organ dysfunction syndrome (MODS) during sepsis (108–110).

Studies have shown that human gastric epithelial cells constitutively express basal levels of PD-L1, but when exposed to *Helicobacter pylori* this expression significantly increases (111). PD-L1 mRNA was also detected in colonic epithelial cells from healthy individuals and PD-L1 protein expression has been reported on the surface of colonic epithelial cells from patients with inflammatory bowel disease (IBD). Interestingly, blockade of PD-L1/B7-H1 ligation has been shown to suppress the development of experimental chronic intestinal inflammation (63–65), which implies that this ligand could be involved in mediating aspects of gastrointestinal dysfunction seen in septic or traumatic pathologies. In this vein, Huang et al. (61, 112) showed that jejunal villus injury was evident on histologic analysis as well splenic apoptosis in CLP mice – two findings which were ameliorated upon deletion of either PD-1 or its primary ligand PD-L1 (B7-H1) gene. However, a patho-mechanistic basis for these findings in the gut were not further explored in those studies.

In this respect, Wu *et al.* have recently shown that PD-L1 protein expression increases in small intestine and colon after CLP (113). This corresponds to changes in PD-L1 expression on colonic tissue sections of septic patients (113). Additionally, a time course demonstrated that intestinal epithelial cells maximally expressed PD-L1 at 24 hours after septic challenge – a finding also markedly increased across all sham time-points (113). Intestinal epithelial cell barrier integrity was markedly attenuated after sepsis in those mice with PD-L1 deficiency (113). But what is the possible basis for the improvement of gut barrier function? Wu *et al.* further demonstrated that an absence of PD-L1 leads to decreased expression of

cytokines/chemokines, such as TNF-α, MCP-1, IL-6 and IL-10, in the ileum following CLP (113). Absence of PD-L1 also allows for a preservation of the levels of tight junction proteins as measured by western blot for ZO-1, Occludin and Claudin-1. To the extent that these findings are a result of PD-L1 ligation, it was observed by Wu *et al.* that antibody blockade of PD-L1 ligation produced a partial, yet significant, restoration of tight junction protein expression on the epithelial cell line that had lost such expression when subjected to inflammatory stimuli, as encountered in septic animals and septic patients (113). Taken together, these data support the concept that blockade of PD-1's primary ligand PD-L1, and not just PD-1 itself, has a restorative effect on gut histology and function in the face of experimental septic challenge (Figure 4, Component [4]).

(b) End-organ Injury – the Liver—Liver Sinusoidal Endothelial Cells (LSECs) are located within the hepatic sinusoid. They have a characteristically discontinuous basement membrane; this fenestration facilitates exchange of material with adjacent hepatocytes. A well-known phenotypic marker of the LSEC population is CD146. The LSECs can also act as antigen presenting cells (as alluded to earlier) as marked by their expression of CD80, CD86, MHC I and MHC II. They are also considered to have roles in protecting hepatocytes from infection and maintaining the liver's immunotolerant state.

These characteristics are in contrast to the *vascular* endothelial cells which have a continuous basement membrane, express CD31, secrete inflammatory mediators such as IL-6, and directly interact with the intra-luminal leukocytes during an inflammatory state by expressing selectins (114–116).

Hutchins *et al.* (117) explored the LSEC population's response to experimental septic challenge in relation to PD-L1 expression and found that indeed its increased expression appears to contribute to LSEC dysfunction and liver injury in response to CLP (Figure 4, Component [4]). Indices of dysfunction include: increased apoptosis, decreased VEGF-R2 expression, decreased barrier function, and decreased proliferative capacity – all of these parameters of injury were diminished in the setting of PD-L1 gene deletion/loss (116). Additionally, depletion of Kupffer cells (on which increased levels of PD-1 were expressed) reversed septic mouse LSEC rise in PD-L1 (95, 117). Zhu *et al.* (118) also investigated liver damage after CLP, finding that *in vivo* anti-PD-L1 antibody treatment reduced the onset of transaminitis otherwise experienced in the sham-treated wildtype mice.

(c) End-organ Injury – the Lung—Using an experimental model of sequential hemorrhagic shock (Hem) followed by CLP, which is reported to more consistently produce a condition clinically resembling moderate acute respiratory distress syndrome (ARDS), pulmonary pathophysiology has been studied. This was chosen over the model of standalone CLP because the combination of insults consistently produced moderate or worse acute pulmonary injury in the setting of a low lethality rate at 24 hours (99, 119).

Among wildtype mice after Hem-CLP, the P:F ratio (arterial P02:Fi02) decreases, suggesting loss of pulmonary compliance (99, 120). Among mice who were either PD-$1^{-/-}$ or PD-$L1^{-/-}$, indices of lung inflammation (e.g. IL-6, MIP-2, MPO) and injury (pulmonary edema, protein leak) were mitigated following the same Hem-CLP challenge (99, 121).

Histologic analysis has also revealed increased leukocyte presence and cellular apoptosis in lung tissue following Hem-CLP, a finding attenuated by PD-1 gene deletion (122). As a clinical correlate, PD-1 expression on circulating T cells of ICU patients corresponded with ARDS and poor survival (122).

Returning to the membrane-bound checkpoint proteins: by what mechanism are PD-1 and PD-L1 protecting the lung against septic injury? The answer may lie in endothelial cell regulation through the Angiopoietin/Tie2 pathway. Angiopoietin (Ang)-1 is constitutively released by surrounding pericytes surrounding vascular ECs (123). The tyrosine receptor Tie2, which is expressed on vascular ECs, is stimulated by the binding of Ang-1. This interaction results in the synthesis of a cascade of anti-inflammatory/pro-survival proteins, as well as the down-regulation of ICAM-1 expression, thus leading to diminished leukocyte recruitment.

Ang-2 has the opposite effects, causing downstream Rho kinase expression with subsequent de-stabilization of cell-cell junctions and exaggerated vascular permeability (124, 125). Ang-2 is elevated in the experimental model for indirect ARDS mentioned earlier, as well in the blood of critically ill patients with ARDS (124). Direct EC:neutrophil interactions contribute significantly to EC Ang-2 release (124). Suppression of Ang-2 (using either *in vivo* siRNA or blocking Ab) significantly decreases inflammatory lung injury, neutrophil influx, and lung as well as plasma IL-6 and TNF-α (124, 126, 127). Does this mean that alteration of the expression of PD-1 and/or PD-L1 affects the Ang-1:Ang-2 regulatory axis during the development of iARDS in mice? Yes, as gleaned from the experiments of Lomas-Neira *et al.* (Lomas-Neira, J.L., Monaghan, S.F., Huang, X., Ayala A. 2017. Novel role for PD-1:PD-L1 as mediator of neutrophil:endothelial interactions in pathogenesis of indirect ARDS in mice. *Front Immunol. [Submitted]*). Gene deletion of either PD-1 or PD-L1 suppresses the rise in lung Ang-2, but not Ang-1 following Hem-CLP. Similarly, phosphorylated Tie-2 in the lung is diminished after lung injury, but PD-1 or PD-L1 gene deletion restores the Tie-2 to baseline levels (122). Synthesis of this data suggests a role for PD-1:PD-L1 in endothelial cell interaction and septic dysfunction.

Of note, soluble PD-1 (sPD-1), a solubilized form of the cell-surface receptor PD-1 that appears to be synthesized and released as a product of alternative splicing, is increased in patients with ARDS and in Hem-CLP mice compared to sham controls (128). Furthermore, Monaghan *et al.* documented that the sPD-1 that was released also appears to be biologically functional (128). This was demonstrated by the reduced capacity of splenocytes to respond to stimuli following *ex vivo* murine serum co-culture with sPD-1 following Hem-CLP (an experimental model for ARDS, as discussed) (128). Questions regarding soluble PD-1 remain, specifically: what is the overall significance of sPD-1, not only as a potential biomarker, but also as a contributor to sepsis/ARDS pathophysiology? The larger question is: what is the significance of the changes in alternative splicing itself that not only lead to sPD-1, but many other soluble protein isoforms seen in response to stress? Further investigations are underway to delineate these unknowns.

(d) The Interface of Endothelium with Epithelium—The lung, similar to the gut (with its epithelial digestive system interacting with endothelium) and the liver (with

sinusoidal tissue interacting with robust trees of vascular endothelium), has an interface of these two cellular lining entities, namely the endothelium and epithelium. To localize the PD-L1 expression within the lung, expression of CD31 as an endothelial marker and EpCAM as an epithelial marker were utilized – shock/sepsis conditions caused increased PD-L1 expressions on both of these cell types (Xu, S. *et al.* Blockade of PD-L1 attenuates shock/sepsis-induced iARDS in mice through targeting endothelial cells but not epithelial cells. *[Manuscript in preparation]*). Intra-tracheal (IT) delivery of naked siRNA to target pulmonary *epithelial* cells and intravenous (IV) delivery of liposomal-encapsulated siRNA to target vascular *endothelial* cells were undertaken (129, 130). The results support the concept that PD-L1 expression on the endothelial cell, but not on the pulmonary epithelial cell, contributes to shock/sepsis induced iARDS as produced in mice (129–131). In *ex vivo* studies, PD-L1 gene deficiency also renders the lung endothelial cell monolayer more resilient against tight junction loss following IFN-γ/TNF-α stimulation (Figure 4, Component [4]).

CONCLUSION:

The Future of Checkpoint Proteins in Sepsis

Expression of PD-1 and its ligands (as seen with gene deficient mice) appears to contribute to the morbidity and mortality seen in not only acute models of lethal septic challenge and hemorrhage/sepsis, but also in patients with septic shock. Additionally, inhibition of PD-1:PD-L signaling, when provided in a delayed (post-treatment) fashion in a low mortality model of chronic sepsis and/or fungal challenge, also appears protective. The effects of these co-inhibitors are not restricted to APC:lymphocyte communication associated with the antigen presentation process, but also phagocyte:phagocyte, phagocyte:lymphocyte (non-APC) and non-immune:immune cell interactions. These co-inhibitors (alone or together) and/or the downstream signaling process may represent novel diagnostic markers and/or potential therapeutic targets.

Finally, these investigative endeavors have potential to be highly clinically relevant and impactful. As evidenced by the body of work in cancer research, immunomodulatory therapy has been effective against several types of malignancies in clinical trials (132–135). Anti-PD-1 blocking antibody (Keytruda [pembrolizumab] by Merck and Opdivo [Nivolumab] by Bristol-Myers Squibb) are FDA-approved for treatment of a variety of metastatic cancers (136). Other anti-PD-1 and anti-PD-L1 antibodies (alone or with anti-CTLA-4) are in Phase I, II & III clinical trials for cancer patients. From malignancy back to sepsis, a phase I clinical trial has been initiated for anti-PD-1 monoclonal antibody in patients with severe sepsis and/or septic shock (137).

In conclusion, checkpoint proteins such as PD-1 and PD-L1, extensively considered here, appear to be important mediators in the pathophysiology of severe infection as well as the combined insults of shock and sepsis. PD-1 expression, either in membrane-bound or soluble form, may serve as and indicator of the immune status of the individual afflicted with sepsis or shock. PD-1 may influence the outcome of bacterial infection by influencing the balance between effective antimicrobial immune defense and immune-mediated tissue damage. Upregulated PD-1 expression on innate immune cells as well as on T cells during

sepsis and/or shock appears to play a role in their functional decline. The future of immune signaling pathways as regulated by checkpoint proteins may lie in clinical therapy targeted towards unleashing the potential not only of the adaptive, but of the innate immune system.

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ABBREVIATIONS:

Ao	Apoptosis
Ab	Antibody
Ag	Antigen
Akt	Protein kinase B
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
AT	Adoptive Transfer
BALF	Bronchoavleolar Lavage Fluid
B7-H1	B7 Homolog 1
BTLA	B and T Lymphocyte Attenuator
CARS	Compensatory
CBC	Complete Blood Count
CCL	C-C motif Ligand
CD	Cluster of Differentiation
CLP	Cecal Ligation and Puncture
CS	Cecal Slurry
CTLA	Cytotoxic T Lymphocyte-Associated protein
CXCL12	C-X-C motif chemokine 12
DCs	Dendritic Cells

EC	Endothelial Cell
EpiCs	Epithelial Cells
Foxp3	Forkhead Boxp3
Hem-CLP	Hemorrhage-CLP
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen - D Related
HVEM	Herpesvirus Entry Mediator
iALI	indirect Acute Lung Injury
IBD	Inflammatory Bowel Disease
ICAM-1	Intercellular Adhesion Molecule 1
IEC	Intestinal Epithelial Cells
IFN-□	Interferon-gamma
IgC	Immunoglobulin-Constant
IgV	Immunoglobulin-Variable
IL	Interleukin
IT	Intra-tracheal
IV	Intravenous
***i*NKT**	cell invariant Natural Killer T cell
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif
KC	Keratinocyte Chemoattractant
LPS	Lipopolysaccharide
LSECs	Liver Sinusoidal Endothelial Cell
MHC	Major Histocompatibility Complex
MIP-2	Macrophage Inflammatory Protein-2
MØ	Macrophage
MODS	Multiple Organ Dysfunction Syndrome
MOF	Multiple Organ Failure
MPO	Myeloperoxidase

NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PD-1	Programmed cell death receptor-1
PD-L1	Programmed cell death receptor Ligand-1
PD-L2	Programmed cell ligand-2
PI3K	Phosphoinositide 3-kinase
PMN	Polymorphonuclear leukocytes
Rap-1	Repressor Activator Protein 1
SHP	Src Homology region 2 domain-containing Phosphatase
Siglec10	Sialic acid-binding Ig-like lectin 10
SIRS	Systemic Inflammatory Response Syndrome
SLAM4	Signaling Lymphocytic Activation Molecule 4
sPD-1	soluble Programmed cell death receptor-1
Tie2	Tyrosine kinase with Ig and Epidermal growth factor 2
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor-alpha
TNFR	Tumor Necrosis Factor Receptor
WT	Wildtype
ZO-1	Zona Occludens-1

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Cover Letter Statement:

All authors herein affirm that we concur with contents of this manuscript submitted for publication. The materials being submitted are not under consideration for publication anywhere else.

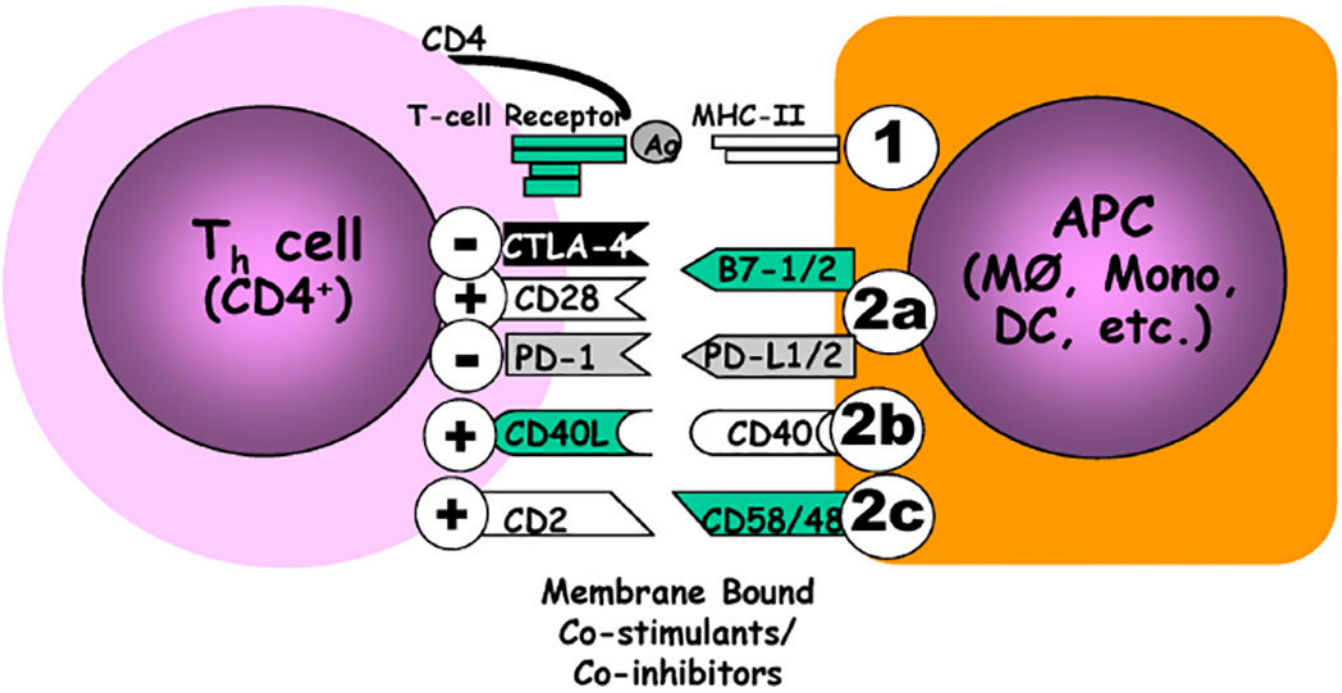

Figure 1.

Antigen presentation is typically a two-signal process, in which antigens derived from a foreign pathogenic source (and/or at times tissue components/debris) are processed (commonly in a lytic fashion) by an APC, i.e., macrophage (MØ), dendritic cell (DC), monocyte (Mono), for formal association with the an HLA/mouse MHC II receptor and presentation/exposure to the appropriate T cell receptor expressing lymphocyte ($CD4^+$ T helper cell)(This is signal one; ①.).However, for formal T cell activation/differentiation to proceed, the APC must not only provide a 2^{nd} co-stimulatory (+) signal (Signal 2; ②) that licenses T cell differentiation, but this must overcome and/or suppress concomitant co-inhibitory (−) signals that are often expressed by the APC (but not exclusively by them). Of note, there are three loosely-termed families of these costimulatory/co-inhibitory molecules, as broken down by protein structure: (2a) the B7:CD28 superfamily, (2b) the TNF:TNFRs that lack death receptor domain, and (2c) the CD2 superfamily & select integrins.

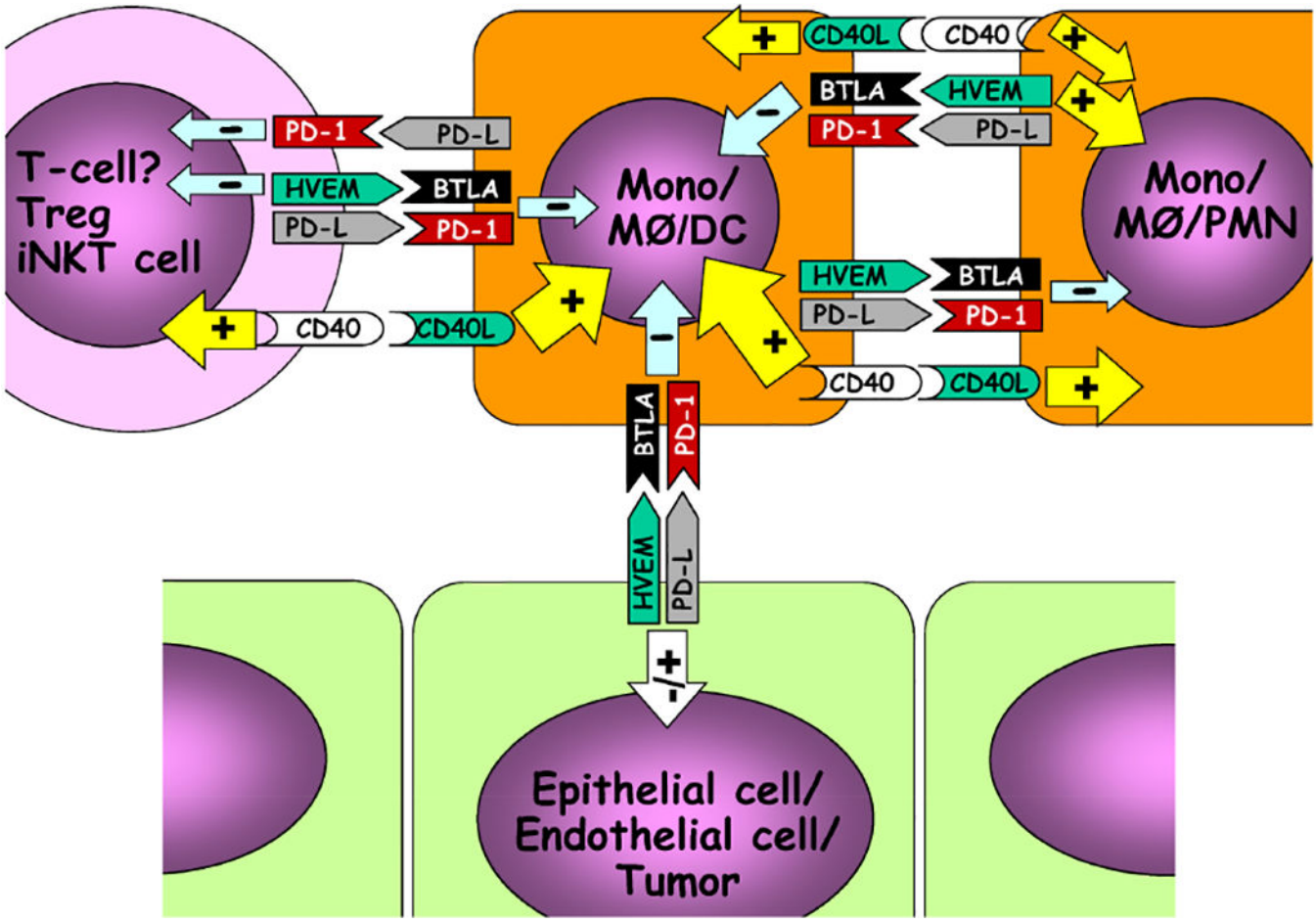

Figure 2.

While co-inhibitors (a.k.a., checkpoint proteins)/co-stimulants are best appreciated for their role in stimulating or inhibiting the activation/differentiation of the $CD4^+$ T helper cell, these same cell-surface co-inhibitors/co-stimulants appear to have potentially unique roles in cell:cell interactions between not only various leukocyte sub-sets, but with non-immune cells within tissue. Positive (+), stimulatory activity reported; negative (−), inhibitory activity reported.

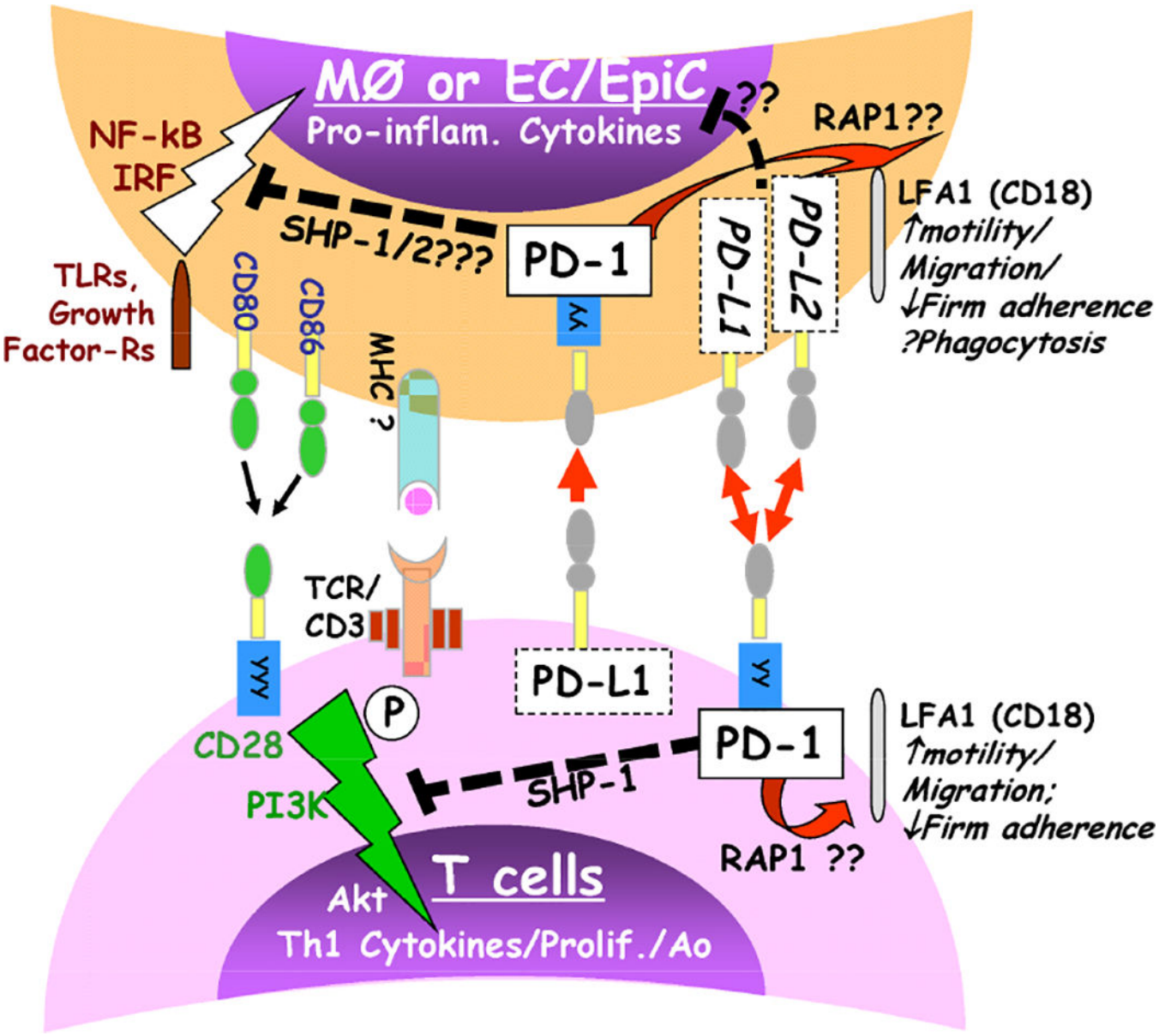

Figure 3.

Overview of proposed PD-1 & PD-L1/L2 intra-cellular signaling between T cells and/or macrophage/monocytes, among others (e.g. PMN, DC and/or EC/EpiCs, which may express PD-1 and/or PD-Ls). The activation pathway is indicated by a ‘thunder bolt’ symbol, while suppressive effects are denoted with a dashed line.

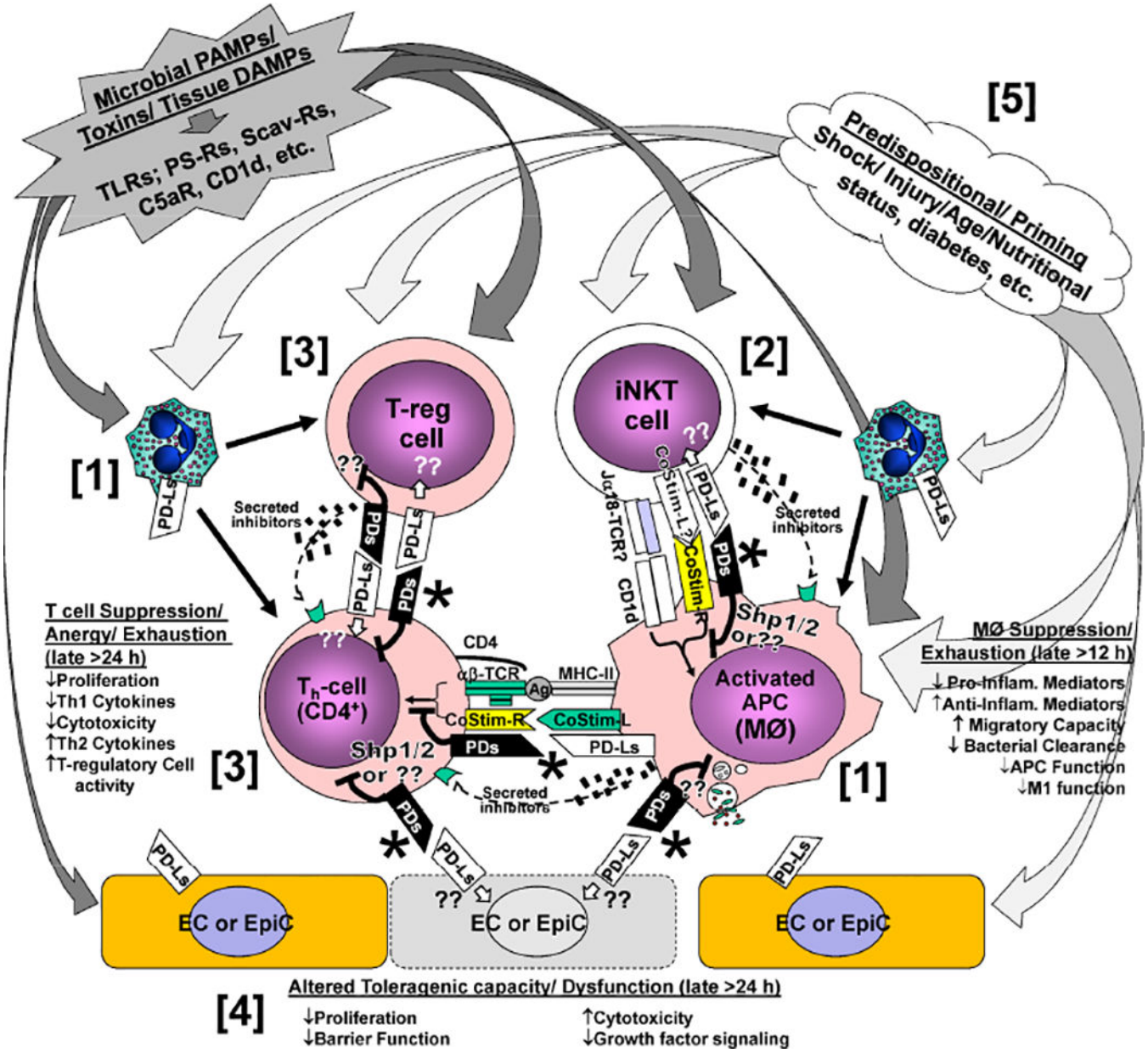

Figure 4.

Hypothetical points at which Programmed Cell Death Receptor (PD) family members, i.e., PD-1, BTLA and their ligands (PD-Ls, HVEM) interact to alter *i*NKT cell activation in response to the diverse signal(s) derived from tissue injury and infectious microbial challenge. This leads to: **[1]** suppression of phagocyte clearance of the septic challenge; PMN and macrophage dysfunction (via direct/indirect effects of PD-1/PD-L1 ligation [*]); **[2]** overzealous activation of innate immune cells via overt *i*NKT cell activation through CD1d-Ag stimulation; **[3]** immune suppression of classic CD4 Th1 cell actions via anergy/ chronic stimulation by Treg cells and/or *i*NKT-cells; and/or **[4]** through the induction of altered ECs/EpiCs capacity or dysfunction (hence a link to organ injury and dysfunction). Finally, **[5]** factors such as prior injury/shock and subjects age, background, nutritional

status, prior health, etc., may serve to alter PD-family member expression, contributing to a state of priming/ pre-disposition/ innate immune memory.