

## REVIEW

# A novel role for coinhibitory receptors/checkpoint proteins in the immunopathology of sepsis

Eleanor A. Fallon | Bethany M. Biron-Girard | Chun-Shiang Chung |  
Joanne Lomas-Neira | Daithi S. Heffernan | Sean F. Monaghan | Alfred Ayala

Division of Surgical Research, Department of Surgery, Rhode Island Hospital, Brown University, Providence, Rhode Island, USA

## Correspondence

Alfred Ayala, Rhode Island Hospital, 593 Eddy St, 227 Aldrich Bldg., Providence, RI 02903, USA.  
Email: aayala@lifespan.org

## Abstract

Coinhibitory 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 up-regulation 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 nonimmune cells during sepsis and/or shock contributes to functional alterations often with detrimental sequelae.

## KEYWORDS

immunomodulation, immunosuppression, infection, innate immunity, PD-1, PD-L1

## 1 | INTRODUCTION

### 1.1 | 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.<sup>1</sup> Sepsis is the leading cause of death in noncardiac Intensive Care Units, with 250,000 deaths each year in the United States.<sup>1</sup> Additionally, it ranks as one of the most expensive conditions treated in U.S. hospitals, with costs exceeding 20 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 with the 1970s.<sup>2–5</sup> 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.<sup>6</sup>

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 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.<sup>6–8</sup> All organs are susceptible, including the pulmonary, hepatic, renal, and gastrointestinal organ systems.

Abbreviations: Akt, protein kinase B; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; A<sub>0</sub>, apoptosis; ARDS, acute respiratory distress syndrome; AT, adoptive transfer; B7-H1, B7 Homolog 1; BTLA, B and T lymphocyte attenuator; CARS, compensatory anti-inflammatory response syndrome; CCL, C-C motif ligand; CD, cluster of differentiation; CLP, cecal ligation and puncture; CTLA, CTL-associated protein; CXCL12, C-X-C motif chemokine 12; DCs, dendritic cells; EC, endothelial cell; EpiCs, epithelial cells; Hem-CLP, hemorrhage-CLP; HLA-DR, HLA-D related; HVEM, herpes virus entry mediator; IEC, intestinal epithelial cells; iNKT cell, invariant NK T cell; IT, intratracheal; ITSM, immunoreceptor tyrosine-based switch motif; KC, keratinocyte chemoattractant; LSECs, liver sinusoidal endothelial cell; PD-1, programmed cell death receptor-1; PD-L1, programmed cell death receptor ligand-1; PD-L2, programmed cell ligand-2; 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; SLAMF4, signaling lymphocytic activation molecule 4; sPD-1, soluble programmed cell death receptor-1; Tie2, tyrosine kinase with Ig and epidermal growth factor 2; WT, wild-type; ZO-1, zona occludens-1.

## 1.2 | Systemic inflammatory response syndrome/compensatory anti-inflammatory response syndrome

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.<sup>6,9,10</sup> Unfortunately, however, although numerous treatments that were based on anti-inflammatory or anticoagulant concepts showed promise in the experimental setting, they have all failed to provide a survival benefit in randomized human clinical trials.<sup>11,12</sup> And although 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 toward 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.<sup>13</sup> 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 1960s. 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.<sup>14–20</sup> 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 complete blood count with differential, and persistence of or failure to resolve an infection. The mechanisms proposed include decreased HLA-D related (HLA-DR), lymphocyte reprogramming (movement from a Th1/M1 immune cell phenotype to a more Th2/M2 phenotype), the induction of programmed cell death/apoptosis ( $A_0$ ), increased expression of anti-inflammatory mediators (prostaglandin E, IL-10, steroid hormones), and finally increased expression of cell-associated coinhibitory receptors and ligands (such as programmed cell death receptor-1 [PD-1]/programmed cell death receptor ligand-1 [PD-L1], CTL-associated protein [CTLA-4]).<sup>19–23</sup>

## 2 | 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 coinhibitory/checkpoint protein signaling cascades play in this process of immunomod-

ulation, and how does this affect the balance of innate and/or adaptive immunity?

## 3 | OBJECTIVES

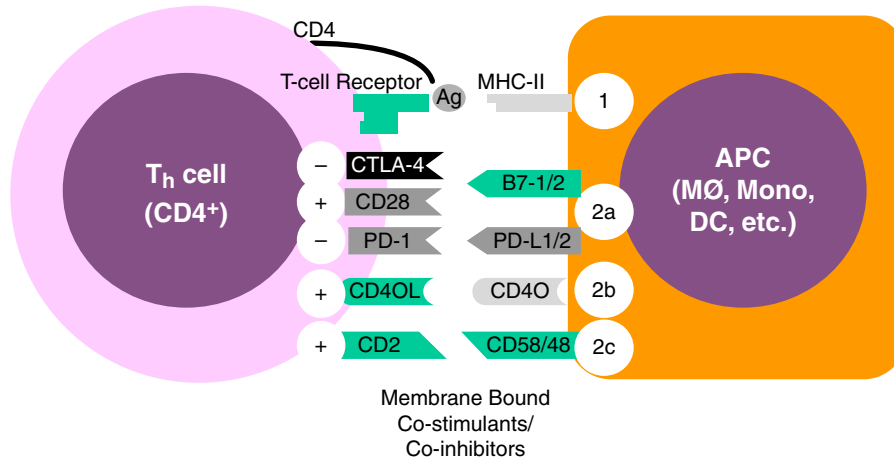
From our central question, we derived 4 objectives for this discussion, as follows:

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

## 4 | DISCUSSION

### 4.1 | The role of checkpoint proteins in classic Ag-driven immunity

Ag presentation is mediated by the APC, with examples including innate immune cells such as professional phagocytes, B cells, or dendritic cells (DCs), as well as nonclassic 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 (Fig. 1, Signal 1). A cluster of differentiation (CD)4<sup>+</sup> or CD8<sup>+</sup> 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 Ag-containing HLA/MHC molecule (Fig. 1, Signal 2). If the predominant coreceptors on the APC are costimulatory molecules/receptors, such as CD28, ICOS, or CD40, this licenses the T cells that see presented Ag 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 coinhibitory molecules (i.e., checkpoint proteins), the CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes are not licensed to respond (i.e., becoming anergic and potentially apoptotic). It should be noted that many of these coinhibitory cell surface receptor molecules *actively* signal – the simple overexpression of these receptors, or their cell surface ligands in some cases (e.g., the most overt example being the overexpression of some of these coinhibitory ligands on tumor cells), is sufficient to directly (via checkpoint protein cell signaling and/or the indirect stimulation of 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



**FIGURE 1** Ag presentation is typically a 2-signal process, in which Ag derived from a foreign pathogenic source (and/or at times tissue components/debris) are processed (commonly in a lytic fashion) by an APC, that is, macrophage (Mφ), 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<sup>+</sup> T helper cell) (This is signal one; ①). However, for formal T cell activation/differentiation to proceed, the APC must not only provide a 2nd costimulatory (+) signal (Signal 2; ②) that licenses T cell differentiation, but this must overcome and/or suppress concomitant coinhibitory (–) signals that are often expressed by the APC (but not exclusively by them). Of note, there are 3 loosely termed families of these costimulatory/coinhibitory 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 and select integrins

environment.<sup>24</sup> That such shifting occurs in the setting of the experimental animal and septic patient has been documented by several labs.<sup>19,23</sup> Ultimately, these receptors and their ligands are often first regarded as toleragens.<sup>25,26</sup>

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

#### 4.1.1 | PD-1

PD-1, with pseudonyms including Pcdcl and CD279, is a Type I transmembrane glycoprotein-Ig (IgV) superfamily member, containing an 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.<sup>27–29</sup> 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/ITAM activation<sup>30–34</sup> (Fig. 3).

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 nonimmune tissues and organs.<sup>35–37</sup> Alternatively, PD-L2 is more restrictively expressed on APCs and immune cells.<sup>38</sup> 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 intracellular signaling may still be achieved via these PD-1 ligands themselves, perhaps from surrogate ligation of B7.1/CD80.<sup>26,39</sup>

#### 4.1.2 | B and T lymphocyte attenuator and CTLA-4

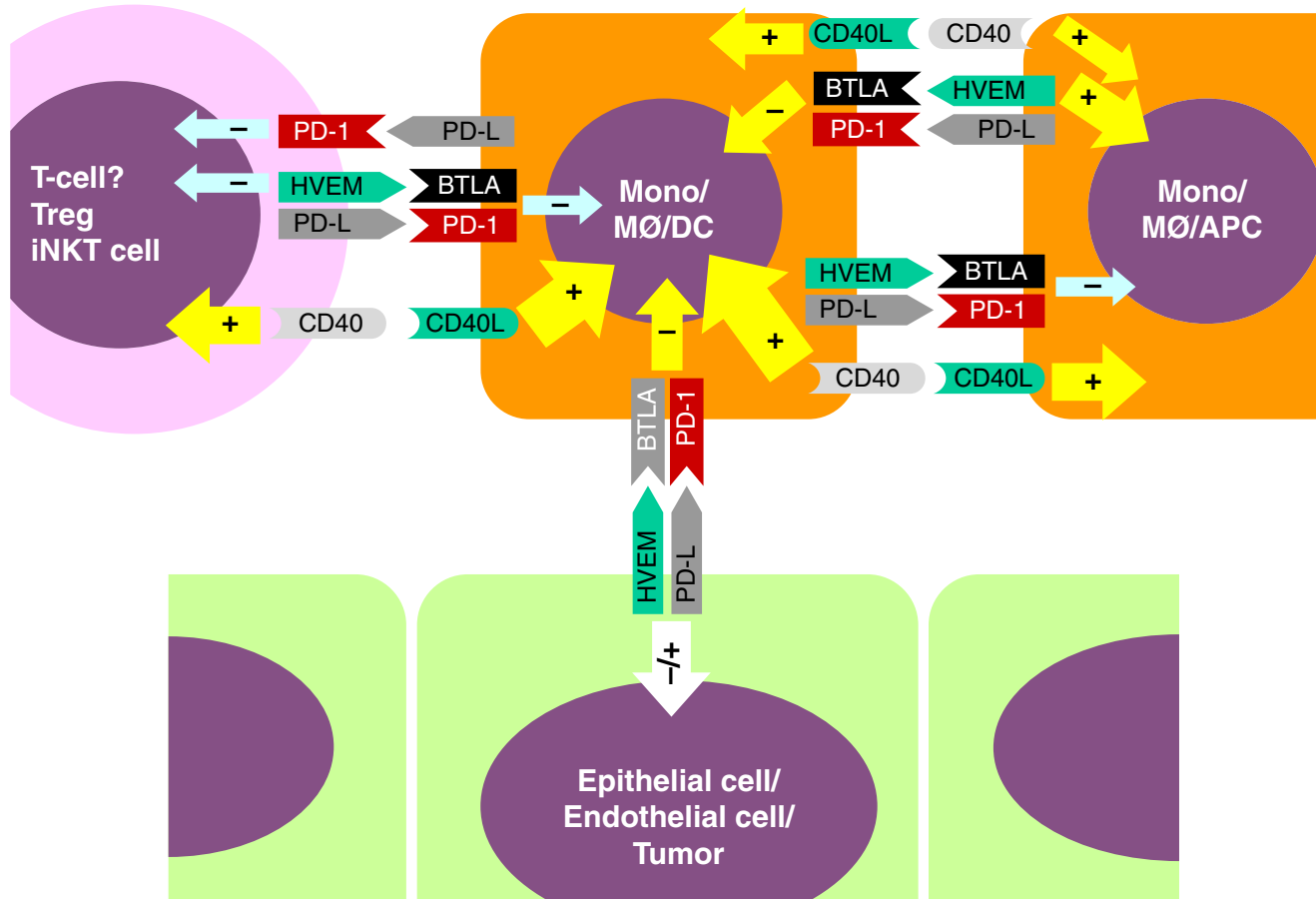
B and T lymphocyte attenuator (BTLA) is considered a coinhibitory receptor, related in molecular structure to PD-1; it acts as an inhibitor

of B cell and CD4<sup>+</sup> T cell function, as its name describes.<sup>40</sup> It is induced on anergic CD4<sup>+</sup> T cells and is associated with attenuated prosurvival signaling.<sup>41,42</sup>

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

## 4.2 | 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 pathologic processes driving sepsis. Studies by Huang et al.<sup>47</sup> showed that mice in which the gene for PD-1 was deleted exhibited a marked reduction in mortality in response to cecal ligation and puncture (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 2 independent investigations utilizing antibodies against PD-1,<sup>33</sup> and its primary ligand PD-L1,<sup>48</sup> where both demonstrated that CLP morbidity and mortality could be significantly reduced by such Ab 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.<sup>49</sup> A similar observation was made on severely injured patients, where the expression



**FIGURE 2** While coinhibitors (a.k.a., checkpoint proteins)/costimulants are best appreciated for their role in stimulating or inhibiting the activation/differentiation of the CD4<sup>+</sup> T helper cell, these same cell-surface coinhibitors/costimulants appear to have potentially unique roles in cell:cell interactions between not only various leukocyte sub-sets, but with nonimmune cells within tissue. Positive (+), stimulatory activity reported; negative (–), inhibitory activity reported

of high PD-1 blood leukocyte levels was correlated with worsening physiologic dysfunction.<sup>50</sup> 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 throughout the age spectrum has been demonstrated.<sup>51,52</sup> Work by Young et al.,<sup>51</sup> 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.<sup>53,54</sup>

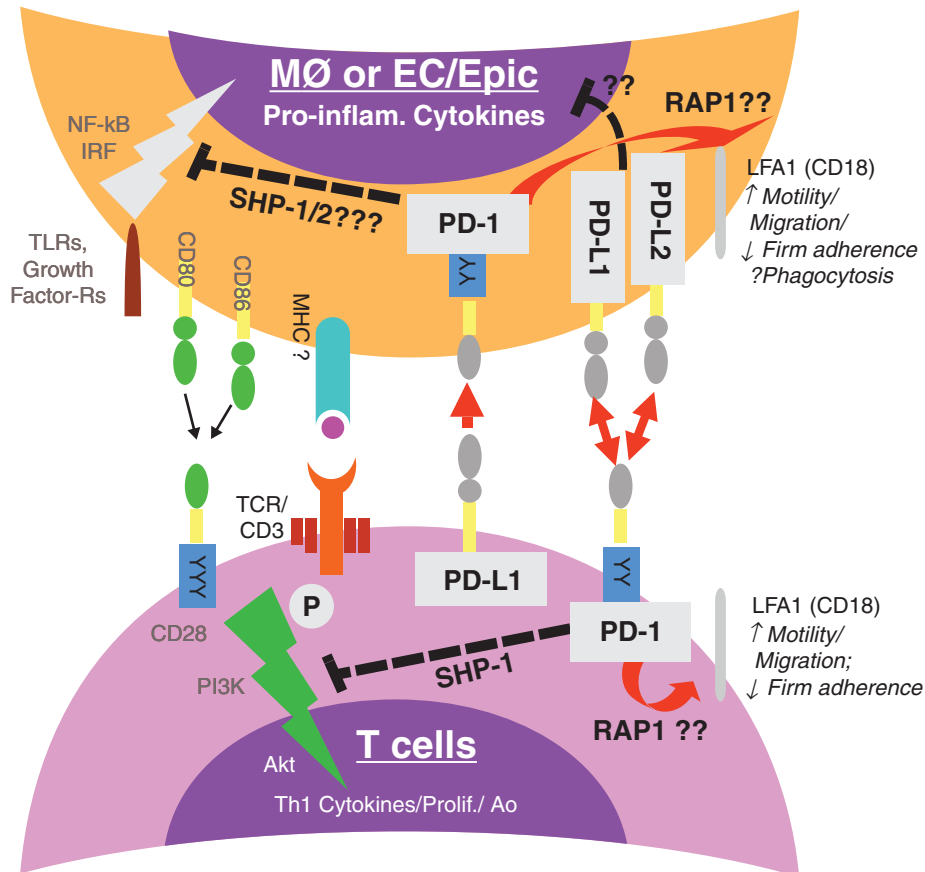
Using a similar experimental approach, Shubin et al.<sup>55</sup> subsequently demonstrated that CD4<sup>+</sup> T cells' BTLA expression among ICU patients was associated with sepsis and development of subsequent nosocomial infections. When the interaction between BTLA and its primary ligand herpes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

Unsinger et al.<sup>57</sup> have also shown that in response to septic challenge, expression of CTLA-4 on CD4, CD8, and regulatory T cells (Tregs) is increased in the murine CLP model. Furthermore, Ab neutralization of CTLA signaling capacity with anti-CTLA-4 Ab treatment after the onset of CLP led to a decrease in sepsis-induced A<sub>0</sub>, better Ag

recall responsiveness, and improved survival.<sup>58</sup> 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 A<sub>0</sub> in a sequential sepsis challenge model where CLP was followed by a secondary fungal insult.<sup>59</sup> Recent studies by Chen et al.<sup>60</sup> investigating the coinhibitory molecule 2B4 indicate it may also markedly suppress CLP-induced complications. Together these results point to pathologically important roles for these coinhibitory/checkpoint proteins and their signaling as underpinning the development of septic morbidity.

#### 4.2.1 | From PD-1 to its ligands

Similar to the survival analysis undertaken for PD-1<sup>–/–</sup> mice, a survival analysis of PD-L1 gene-deficient mice after sepsis demonstrated comparable results.<sup>61</sup> In this respect, elevated PD-L1 and PD-L2 expression has been documented in pulmonary epithelia from patients who died of sepsis.<sup>62</sup> These investigators found that not only the expression of PD-1, but also the expression of its 2 ligands PD-L1 and PD-L2, re associated with poor outcome in sepsis and shock. Other groups have also reported the expression of the ligands for



**FIGURE 3** Overview of proposed PD-1 and PD-L1/L2 intracellular 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, whereas suppressive effects are denoted with a dashed line

PD-1, namely PD-L1 and PD-L2, as being elevated on a variety of nonprofessional immune cells/tissue beds/tumor cells.<sup>63–65</sup> As such, the overexpression of these toleragenic checkpoint protein ligands represents a novel, oft-overlooked, interface between nonprofessional immune cells/tissue beds/organs that may speak not only to how local immune, but organ function might be regulated directly or indirectly through their ligation (Figs. 2 and 4). So, what evidence is there for these ligands of coinhibitors having an effect on immune dysfunction in sepsis and shock?

### 4.3 | Immune dysfunction in sepsis

#### 4.3.1 | Adaptive immunity

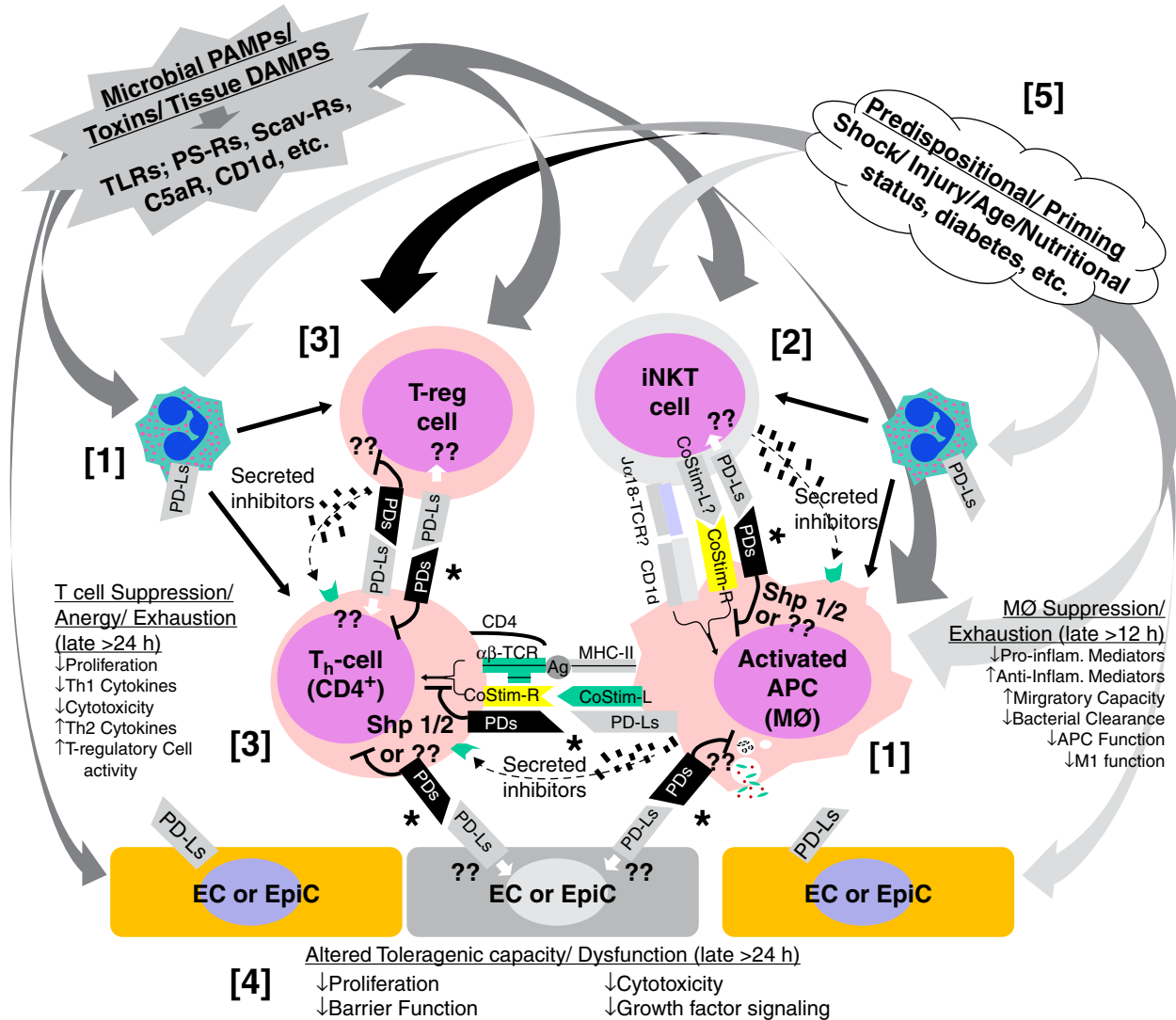
##### (i) Classic T cell immune dysfunction

Studies of sepsis with PD-1 gene-deficient mice and anti-PD-1 Ab 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<sup>+</sup>/CD8<sup>+</sup> T as well as B lymphocyte cell death.<sup>47,57,61</sup> In this regard, Chen et al.<sup>60</sup> have recently reported that the expression of the coinhibitory molecule 2B4 (CD244, signaling lymphocytic activation molecule 4 [SLAMF4]), which was initially reported to be up-regulated

on activated natural killer cells and CD8<sup>+</sup> T cells (but not in experimental models of sepsis), is markedly up-regulated on the splenic CD4<sup>+</sup> T cells of not only CLP mice, but also on human patients with severe sepsis. Further, these CD4<sup>+</sup>2B4<sup>+</sup> cells appear to up-regulate PD-1 and CTLA-4, but inhibition of 2B4 activity is nonredundant 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.<sup>59</sup>

Since many coinhibitory receptors, for example, PD-1, BTLA, CD47, and CTLA-4, express an ITIM and/or ITSM, it is thought that the suppression of classic CD4<sup>+</sup>/CD8<sup>+</sup> T cell function is a result of the recruitment of the phosphatase, SHP-1 and/or SHP-2.<sup>27,30,66</sup> 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.<sup>27,30,66</sup> In this respect, studies by Bandyopadhyay et al.<sup>67</sup> have documented that, at least in T lymphocytes isolated from severely injured patients, which they have previously reported as expressing a number of coinhibitory molecules as well as exhibiting T cell anergy/“exhaustion,”<sup>21,68</sup> indicate that these cells appeared hyperresponsive to expressing the activated form of the phosphatase SHP-1 (Fig. 3). Importantly, Huang et al.<sup>47,61</sup> also showed that macrophages





**FIGURE 4** Hypothetical points at which Programmed Cell Death Receptor (PD) family members, that is, PD-1, BTLA, and their ligands (PD-Ls, HVEM) interact to alter iNKT 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 iNKT cell activation through CD1d-Ag stimulation; [3] immune suppression of classic CD4 Th1 cell actions via anergy/chronic stimulation by Treg cells and/or iNKT 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, and so on, may serve to alter PD-family member expression, contributing to a state of priming/predisposition/innate immune memory.

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 Ag-independent induction of immune suppressive/Th2/anergic T cell state.

Intriguingly, an alternative signaling mechanism has been put forward for PD-1 related coinhibitors, such as CTLA-4, involving interactions with integrins and various components of the cell cytoskeletal machinery<sup>69–71</sup> or the recruitment of alternative receptors like CD80, CD24 versus TLR4 by sialic acid-binding Ig-like lectin 10<sup>72,73</sup>

(Fig. 3). Much, however, remains to be understood about sepsis-induced changes in T cell coinhibitor molecule signaling and how it shapes the septic cellular phenotype and function.

#### (ii) Regulatory lymphocytes

**Invariant NK T cell (iNKT cell):** The innate regulatory lymphocyte populations are emerging as key regulators of the immune and inflammatory response to a variety of infectious insults. Among these subpopulations, the iNKT cell has been shown to play some of the most central roles in immune response to a wide variety of infections and septic events. iNKT 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. iNKT cells are capable of trafficking both within the liver in response to a sterile injury,<sup>74</sup> in and out the lung in response to an infection, as well as distally to a septic source in the abdomen.<sup>75,76</sup> iNKT cells are themselves regulated by coinhibitory/checkpoint proteins, most notably PD-1. Initial activation of iNKT cells is associated with increased PD-1 expression upon iNKT cells<sup>77</sup> (Fig. 4, Component [2]). Young et al.<sup>76</sup> showed that direct ligation of PD-1 with its ligand PD-L1 is essential to trafficking and migration of iNKT cells and PD-1 is essential to regulation of chemokine receptor expression upon iNKT cells. Conversely, repeated stimulation of PD-1 upon iNKT cells has been shown to induce anergy<sup>78</sup> a mechanism that is believed to have developed to protect against an overexuberant iNKT cell driven immune response. In similar fashion, PD-1:PD-L blockade has been shown to prevent the induction of iNKT cell anergy.<sup>79</sup> Although PD-1 has been the most explored coinhibitory pathway in iNKT cell responses to stimuli, several other coinhibitory molecules have been described. CD28 and OX40 have been described as indispensable for full activation of iNKT cells, and glucocorticoid-induced TNF receptor plays a key negative regulatory role in Ag-induced activation of iNKT cells.<sup>80</sup> Akin to CD8<sup>+</sup> T cells, the negative checkpoint regulator 2B4 has also been shown to play a key role in suppressing an excessive iNKT cells response. Ahmad et al.<sup>81</sup> demonstrated that among patient with HIV infection, levels of 2B4 expression was significantly up-regulated upon both CD3<sup>+</sup> and HIV-specific CD8<sup>+</sup> T cells as well as iNKT cells. Levels of 2B4 expression correlated with degree of suppression of intracellular production of IFN-gamma within iNKT cells. Importantly, treatment with antiretroviral therapy among these HIV patients was noted to lead to a decline of 2B4 levels upon CD8<sup>+</sup> T cells, but not among iNKT cells.<sup>81</sup>

**CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells:** A regulatory/anti-inflammatory role for Tregs has been identified in several models of lung diseases: allergy/asthma, *pneumocystis*, and tuberculosis.<sup>82-84</sup> 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 acute respiratory distress syndrome (ARDS), let alone indirect (i)ARDS. Using a well-established model of direct lung injury (intratracheal [IT] instillation of LPS), D'Alessio et al.<sup>85</sup> were the first to demonstrate that Tregs accumulate in the bronchoalveolar lavage fluid 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.<sup>86</sup> documented that adoptive transfer (AT) of Tregs to lymphocyte-deficient recombinate-activating gene-1-deficient (Rag-1<sup>-/-</sup>) 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<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs via a rebalancing of levels of anti- and proinflammatory factors in a direct ARDS mouse model.<sup>87</sup> More recently, a study by Ehrentauf et al.<sup>88</sup> has indicated that CD73-dependent adenosine generating Tregs could promote the resolution of LPS-induced direct ARDS. However, none of these studies illustrated a specific role of Tregs in iARDS. Using a 2-hit model of hemorrhage-

CLP (Hem-CLP), Venet et al.<sup>89</sup> observed that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> 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. Most importantly, Venet et al.<sup>89</sup> also showed that down-regulation of the Tregs' function using Foxp3 targeting siRNA, administered 12 h before the induction of experimental iARDS, echoed lung injury obtained in CD4-gene deficient as well as anti-CD4 Ab depleted mice; this was consistently associated with a reduction in lung IL-10 levels.<sup>89</sup> However, how the activation of this small subpopulation 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.<sup>90</sup> demonstrated a significant role for PD-1 in regulating the Tregs response to iARDS. AT of Tregs derived from wild-type (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-1<sup>-/-</sup>-derived Tregs were transferred into recipient WT mice during the resuscitation period of Hem or 24 h before CLP via tail vein injection. Furthermore, the expression of CTLA-4, another coinhibitory regulator, was also affected by the presence of PD-1, wherein the expression of CTLA-4 upon CD4<sup>+</sup>Foxp3<sup>+</sup> T cells was increased following transfer of Tregs from WT, whereas no increase in CTLA-4 expression was noted in mice receiving PD-1<sup>-/-</sup> Tregs (Fig. 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.<sup>91,92</sup>

La et al.<sup>93</sup> also noted the suppressive effect of PD-1 upon Tregs in a model of hydatidosis, a parasitic infection. They noted that the percentage of CD4<sup>+</sup>CD25<sup>+</sup>PD-1<sup>+</sup> cells correlated with duration of infection and size of abscess. The authors speculated that the suppressive effect of PD-1<sup>+</sup> expression upon Tregs was essential for infection growth and avoidance of the immune surveillance. These findings have been correlated clinically, wherein Liu et al.<sup>94</sup> 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.<sup>94</sup>

### 4.3.2 | Innate immunity

#### (i) Macrophages

One of the more interesting observations made by Huang et al.<sup>61</sup> is that early following septic challenge/CLP (as defined as 12-h post-CLP or less), changes in PD-1 expression on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells or B cells are not yet evident (typically these are not seen until at 24 h post CLP). Further, DCs did not express PD-1 at this time point. However, Huang et al.<sup>61</sup> observed that peritoneal leukocytes, as defined by F4/80<sup>+</sup> cells harvested from peritoneal lavage,

demonstrated increased PD-1 and PD-L1 expression, at both protein and message levels, after as little as 12-h post-CLP. This effect was sustained at the 24-h and 48-h time points. Mouse blood monocytes and Kupffer cells have also been subsequently demonstrated to exhibit increased PD-1 expression in response to CLP.<sup>47,95</sup> This up-regulation 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 macrophages 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- $\alpha$  or interferon- $\gamma$ , was not reported to be a strong inducer of PD-1 expression.<sup>49</sup> In fact, Guignat et al.<sup>49</sup> 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.<sup>47</sup> also showed that following CLP, there was a decline in both phagocytic capacity (which occurs with both opsonized and nonopsonized targets) and stimulant-induced cytokine production among macrophages. 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.<sup>96</sup> 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<sup>-/-</sup> macrophages exhibit less dysfunctional migration compared with 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, although 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 exact nature of this signaling remains to be elucidated (Fig. 3).

## (ii) Neutrophils

Neutrophils (polymorphonuclear leukocytes [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.<sup>97</sup> 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).<sup>97,98</sup> In this regard, Ayala et al.<sup>99</sup> and Lomas et al.<sup>100</sup> reported on PMNs that were isolated from donor animals after hemorrhage (a form of PMN priming) (Fig. 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 A<sub>0</sub><sup>99,100</sup>; a finding confirmed by others in experimental iARDS.<sup>101</sup> Jimenez et al.<sup>98</sup> found a significant decline in PMN A<sub>0</sub> in patients with SIRS and the plasma from these individuals suppressed A<sub>0</sub> of PMNs derived from healthy controls.<sup>98</sup> These findings and others<sup>102–104</sup> support the hypothesis that while delayed A<sub>0</sub> in an infectious environment aids PMN-mediated pathogen killing, in a pathogen-free setting, delay in A<sub>0</sub> contributes to inflammation/SIRS and organ failure/injury.<sup>98</sup> With that stated, the mechanisms underpinning these effects in PMN have not been fully clarified.

As mentioned earlier, although it has been documented that neutrophils can express the coinhibitory ligand PD-L1,<sup>61</sup> until recently little was known about how disease states alter its expression, and what might be the pathologic significance of such changes. In this regard, using the CLP model of sepsis Huang et al.<sup>61</sup> initially documented a trend toward increased frequency of PD-L1 (B7-H1)<sup>+</sup> 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<sup>+</sup> staining appeared to segregate into distinct PD-L1<sup>high</sup> and PD-L1<sup>low</sup> subpopulations. Further it was found that if mice exhibited PD-L1<sup>high</sup> expressing PMNs in their peripheral blood at 24 h post-CLP this correlated not only with higher levels of systemic pro- and anti-inflammatory cytokine/chemokine levels (CCL2, IL-6, CXCL2, keratinocyte chemoattractant [KC], TNF- $\alpha$ , and IL-10), but with lethal outcome as well. Subsequently, Wang et al.<sup>105</sup> 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.<sup>106</sup> in septic mouse iNKT cells).<sup>22</sup> More recently, Patera et al.<sup>53</sup> 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- $\alpha$  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.

## 4.4 | End-organ sequelae of cellular immune disharmony

### 4.4.1 | End-organ injury — the gut

In the intestine, the epithelial lining serves as an important barrier to prevent the absorption of toxins, A<sub>g</sub>s, 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 (EpiC) barrier dysfunction has been thought to contribute to the development of the multiple organ dysfunction syndrome during sepsis.<sup>107–109</sup>

Studies have shown that human gastric EpiCs constitutively express basal levels of PD-L1, but when exposed to *Helicobacter pylori*, this expression significantly increases.<sup>65</sup> PD-L1 mRNA was also detected in colonic EpiCs from healthy individuals and PD-L1 protein expression has been reported on the surface of colonic EpiCs from patients with inflammatory bowel disease. Interestingly, blockade of PD-L1/B7-H1 ligation has been shown to suppress the development of experimental chronic intestinal inflammation,<sup>63–65</sup> 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.<sup>61,110</sup> showed that jejunal villus injury was evident on histologic analysis as well splenic A<sub>0</sub> in CLP mice—2 findings, which were ameliorated upon deletion of either PD-1 or its primary ligand PD-L1 (B7-H1) gene. However, a pathomechanistic basis for these findings in the gut were not further explored in those studies.

In this respect, Wu et al.<sup>111</sup> have recently shown that PD-L1 protein expression increases in small intestine and colon after CLP. This corresponds to changes in PD-L1 expression on colonic tissue sections of septic patients.<sup>111</sup> Additionally, a time course demonstrated that intestinal epithelial cells (IECs) maximally expressed PD-L1 at 24 h after septic challenge—a finding also markedly increased across all sham time-points.<sup>111</sup> IEC barrier integrity was markedly attenuated after sepsis in those mice with PD-L1 deficiency.<sup>111</sup> But what is the possible basis for the improvement of gut barrier function? Wu et al.<sup>111</sup> further demonstrated that an absence of PD-L1 leads to decreased expression of cytokines/chemokines, such as TNF- $\alpha$ , MCP-1, IL-6, and IL-10, in the ileum following CLP. Absence of PD-L1 also allows for a preservation of the levels of tight junction proteins as measured by Western blot for zona occludens-1 (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 Ab blockade of PD-L1 ligation produced a partial, yet significant, restoration of tight junction protein expression on an EpiC line that had lost such expression when subjected to inflammatory stimuli, as encountered in septic animals and septic patients.<sup>111</sup> 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 (Fig. 4, Component 4).

#### 4.4.2 | 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 APCs (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 ECs, which have a continuous basement membrane, express CD31, secrete inflammatory mediators such as IL-6, and directly interact with the intraluminal leukocytes during an inflammatory state by expressing selectins.<sup>112–114</sup>

Hutchins et al.<sup>115</sup> 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 (Fig. 4, Component [4]). Indices of dysfunction include: increased A<sub>0</sub>, 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.<sup>114</sup> Additionally, depletion of Kupffer cells (on which increased levels of PD-1 were expressed) reversed septic mouse LSEC rise in PD-L1.<sup>95,115</sup> Zhu et al.<sup>116</sup> also investigated liver damage after CLP, finding that *in vivo* anti-PD-L1 Ab treatment reduced the onset of transaminitis otherwise experienced in the sham-treated WT mice.

#### 4.4.3 | 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 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 h.<sup>99,117</sup>

Among WT mice after Hem-CLP, the P:F ratio (arterial P02:FiO2) decreases, suggesting loss of pulmonary compliance.<sup>99,118</sup> Among mice who were either PD-1<sup>-/-</sup> or PD-L1<sup>-/-</sup>, indices of lung inflammation (e.g., IL-6, MIP-2, myeloperoxidase) and injury (pulmonary edema, protein leak) were mitigated following the same Hem-CLP challenge.<sup>99,119</sup> Histologic analysis has also revealed increased leukocyte presence and cellular A<sub>0</sub> in lung tissue following Hem-CLP, a finding attenuated by PD-1 gene deletion.<sup>119</sup> As a clinical correlate, PD-1 expression on circulating T cells of ICU patients corresponded with ARDS and poor survival.<sup>119</sup>

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 EC regulation through the angiopoietin (Ang)/tyrosine kinase with Ig and epidermal growth factor 2 (Tie2) pathway. Ang-1 is constitutively released by pericytes surrounding vascular ECs.<sup>120</sup> 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/prosurvival 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.<sup>121,122</sup> Ang-2 is elevated in the experimental model for indirect ARDS mentioned earlier, as well in the blood of critically ill patients with ARDS.<sup>121</sup> Direct EC:neutrophil interactions contribute significantly to EC Ang-2 release.<sup>121</sup>

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- $\alpha$ .<sup>121,123,124</sup> 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.<sup>119</sup> Synthesis of this data suggests a role for PD-1:PD-L1 in EC 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 with sham controls.<sup>125</sup> Furthermore, Monaghan et al.<sup>125</sup> documented that the sPD-1 that was released also appears to be biologically functional. This was demonstrated by the reduced capacity of splenocytes to respond to stimuli following ex vivo murine serum coculture with sPD-1 following Hem-CLP (an experimental model for ARDS, as discussed).<sup>125</sup> 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.

#### 4.4.4 | The interface of endothelium with epithelium

The lung, similar to the gut (with its epithelial-lined digestive system interacting with villous endothelium) and the liver (with sinusoidal tissue interacting with robust trees of vascular endothelium), has an interface of these 2 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 ECs but not EpiCs. [Manuscript in preparation]). IT delivery of naked siRNA to target pulmonary epithelial cells and intravenous delivery of liposomal-encapsulated siRNA to target vascular endothelial cells were undertaken.<sup>126,127</sup> The results support the concept that PD-L1 expression on the EC, but not on the pulmonary EpiC, contributes to shock/sepsis-induced iARDS as produced in mice.<sup>126–128</sup> In ex vivo studies, PD-L1 gene deficiency also renders the lung EC monolayer more resilient against tight junction loss following IFN- $\gamma$ /TNF- $\alpha$  stimulation (Fig. 4, Component 4).

## 5 | CONCLUSIONS

### 5.1 | 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 coinhibitors are not restricted to APC:lymphocyte communication associated with the Ag presentation process, but also phagocyte:phagocyte, phagocyte:lymphocyte (non-APC) and nonimmune:immune cell interactions. These coinhibitors (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.<sup>129–132</sup> Anti-PD-1 blocking Ab (Keytruda [pembrolizumab] by Merck and Opdivo [Nivolumab] by Bristol-Myers Squibb) are FDA-approved for treatment of a variety of metastatic cancers.<sup>133</sup> Other anti-PD-1 and anti-PD-L1 antibodies (alone or with anti-CTLA-4) are in Phase I, II, and III clinical trials for cancer patients. From malignancy back to sepsis, a phase I clinical trial has been initiated for anti-PD-1 monoclonal Ab in patients with severe sepsis and/or septic shock.<sup>134</sup>

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 an 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. Up-regulated 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.

## AUTHORSHIP

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.

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## DISCLOSURES

The authors declare no conflicts of interest.

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