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1 # Lymphocyte subset expression and serum concentrations of

2 # PD-1/PD-L1 in sepsis – pilot study

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1 ## Abstract

2 **Background:** Sepsis remains a major cause of mortality in critical care, for which specific
3 treatments are lacking. The dysregulated response to infection seen in sepsis includes
4 features of lymphocyte dysfunction and exhaustion, suggesting that immune-stimulatory
5 therapy may improve outcomes in certain patient groups. Monoclonal antibodies targeting
6 checkpoint molecules, such as programmed-death 1 protein (PD-1) and its ligand PD-L1,
7 have shown success in stimulating the immune response in cancer patients, and are being
8 considered for future sepsis trials. The aims of this pilot study were to compare lymphocyte
9 subset expression of PD-1 and its ligands between sepsis patients and controls; to
10 characterize serum levels of PD-1 and PD-L1 in sepsis patients and controls, and determine if
11 serum concentrations correlated with cell surface expression.

12 **Methods:** Expression levels of PD-1, PD-L1 and PD-L2 on four lymphocyte subsets
13 (CD27+CD19+ B cells, CD27-CD19+ B cells, CD27+CD4+ T cells and CD27-CD4+ T cells) were
14 compared between 22 sepsis patients (including 11 survivors and 11 non-survivors) and 11
15 healthy controls using flow cytometry. Levels of soluble PD-1 and PD-L1 were also compared
16 using commercially available ELISA kits.

17 **Results:** Expression of PD-1 and PD-L1 was higher on all lymphocyte subsets in sepsis
18 patients compared to controls ($p<0.05$). PD-L2 expression on CD27+ B cells was also higher
19 in sepsis patients ($p=0.0317$). There was differential expression of PD-1 by CD27 status, with
20 expression being higher in the B and T cell subsets associated with memory status (CD27+
21 and CD27-, respectively; $p<0.001$). Higher PD-1 and PD-L1 expression was not associated
22 with mortality nor with a higher risk of nosocomial infections. There were no differences in
23 levels of soluble PD-1 or PD-L1 between sepsis patients and controls.

Conclusions: Higher expression of PD-1 by memory subpopulations of B cells and CD4+ T cells, with normal soluble PD-1 and PD-L1 in sepsis patients, are novel findings. This information may be useful to enrich sepsis populations for trials of PD-1/PD-L1 blockade.

Keywords: sepsis, septic shock, Programmed death-1, enrichment, outcomes

Background

Sepsis is a dysregulated host response to infection [1], with concomitant immune activation and suppression. Sepsis-related immunosuppression contributes to poor outcomes by increasing the risk of nosocomial infection and death [2-4]. A common feature of sepsis-related immunosuppression is impaired lymphocyte function, with increased expression of inhibitory checkpoint molecules, such as programmed-death 1 protein (PD-1) [2]. PD-1 serves to limit excessive immune responses by negatively regulating lymphocyte activation and function, and promoting immune cell apoptosis. It has two known ligands: programmed death ligand-1 (PD-L1), which is widely expressed by a variety of immune and non-immune cell types; and programmed death ligand-2 (PD-L2), which is expressed by antigen-presenting cells [2]. Increased expression of PD-1 and PD-L1 by T cells, monocytes and neutrophils has been demonstrated in sepsis, while upregulation of the PD-1 pathway is associated with higher mortality [2, 5-8]. As this dysfunction is potentially reversible with anti-PD-1 or anti-PD-L1 monoclonal antibody treatment [2, 5-8], manipulating the PD-1 pathway represents a potential target for sepsis trials.

Against this background, we hypothesized that lymphocyte surface PD-1, PD-L1 and PD-L2 expression by B and T cell subsets will vary by CD27 expression status. CD27 is a marker of lymphocyte activation; CD27 positive (CD27+) B cells correspond to memory B cells [9], while CD27 negative (CD27-) T cells represent a high antigen-recall subset of memory T cells [10]. Another rationale for assessing CD27 based memory lymphocyte subsets is the selective depletion of memory B cells in sepsis [8]; it is not known whether PD-1 expression varies by lymphocyte memory status. We therefore measured PD-1, PD-L1 and PD-L2 surface expression on CD27+ and CD27- subsets of CD4+ T and B lymphocytes using flow

1 cytometry in adult patients with sepsis on the intensive care unit (ICU). We compared
2 expression between sepsis and healthy controls, and between subgroups of sepsis patients
3 by nosocomial infection and survival status. PD-1 and PD-L1 also exist in a soluble form in
4 the serum, however the relevance of these soluble forms to sepsis pathogenesis is unclear.
5 We hypothesized that cell-surface PD-1, PD-L1 and PD-L2 expression would correlate with
6 serum concentrations, and so measured corresponding serum PD-1 and PD-L1 levels in the
7 same samples.

8

9 # Methods

10 ## Conceptual approach

11 Immune responses in sepsis differ between patients [3, 4]. The ability to identify who would
12 – or would not – benefit from a therapy based on specific biological mechanisms, will offer a
13 crucial step forward in patient management, especially when that mechanism is dominant,
14 linked to an outcome of interest, and present at the time of assessment of trial eligibility
15 [11]. These principles informed our study design. Our conceptual approach was that sepsis
16 trial eligibility criteria assessments are often done on the day of ICU admission and that
17 patients with increased expression of PD-1 and PD-L1 have a greater risk of nosocomial
18 infections and/or death, as this would be a dominant mechanism contributing to these
19 outcomes.

20 ## Study design and setting

21 This was an analysis of a subpopulation of patients enrolled into a previous prospective
22 observational cohort study performed in a general medical-surgical tertiary ICU (HRA

1 Research Ethics Committee approval reference: 12/LO/0326). Details of the study design
2 have been published previously [8, 12]. From this cohort we randomly selected 22 adult
3 patients with sepsis, with an ICU length of stay ≥48 hours, and included equal numbers of
4 survivors and non-survivors [8, 12]. As our original study was designed prior to the Sepsis-3
5 definitions, sepsis was identified using the previous definition requiring proven or suspected
6 infection, two or more systemic inflammatory response system (SIRS) criteria, and at least
7 one organ system dysfunction (cardiovascular, respiratory, renal, hematologic, hepatic,
8 neurologic or metabolic) [13]. We highlighted in a recent cohort study that the prevalence
9 of SIRS negative sepsis in ICU patients in England was approximately 3% [14], with a 92%
10 overlap in sepsis cases identified by Sepsis-2 and Sepsis-3 [15]. We excluded patients less
11 than 18 years old, and those with known immune dysfunction, including; those with
12 congenital hypogammaglobulinemia, known protein-losing enteropathies, nephrotic
13 syndrome, and neoplastic or proliferative hematologic diseases; those having received
14 intravenous immunoglobulin therapy in the last 3 months; those receiving high-dose
15 corticosteroid therapy; those with ongoing blood loss (defined by blood transfusion
16 requirement > 2 units in a 24 hour period); those with retroviral disease; and those with
17 immune dysfunction as defined by Acute Physiology and Chronic Health Evaluation
18 (APACHE) II score comorbidities [16].

19 **Blood sampling, flow cytometry, ELISA and healthy controls**

20 Peripheral blood mononuclear cells were isolated by density centrifugation from blood
21 samples collected within 12 hours of ICU admission, and stored in liquid nitrogen. Serum
22 samples from the same patients were stored at -80°C. Anti-human fluorochromes were used
23 to identify lymphocyte subsets: anti-CD19 (PerCP Cy5.5; HB19); anti-CD3 (APC-H7; SK7)

(both BD Biosciences, Wokingham, Berkshire, UK); anti-CD4 (Pacific Blue; SK3); anti-CD27 (FITC; 0323); anti-PD-1 (APC; EH12.2H7); anti-PD-L1 (PeCy7; 29E.2A3); and anti-PD-L2 (PE; 24F.101C12) (all Biolegend, London, UK). Amcyan (L34957; Invitrogen, Carlsbad, CA) was used to identify live cells. All flow cytometry experiments were carried out by the same investigator. Flow cytometer set up, calibration and compensation were carried out prior to each experiment using BD CompBeads (BD Biosciences). Reagents remained the same during the course of the study. Gating to identify cell subsets was achieved using isotype controls and fluorescence minus-one (FMO) controls (eFigure-1). FlowJo software (https://www.flowjo.com) was used for analysis of flow cytometry data. Percentage positivity for PD-1, PD-L1 and PD-L2 was defined as the percentage of cells above the gate set using the above controls, with the proportion of positive cells and the corresponding geometric mean fluorescence intensity (MFI) used as indicators of expression. Quantitative detection of serum levels of PD-1 and PD-L1 was done in duplicate using commercial ELISA kits (Proteintech, Manchester, UK), according to the manufacturer's instructions. Detection ranges for PD-1 and PD-L1 were 125-8000 pg/ml and 0.156-10 ng/ml, respectively. All flow cytometry and ELISA experiments used anonymised healthy controls who were consented prior to sampling as per the King's College London Infectious Diseases Biobank protocol [8].

Statistics

Mann-Whitney U tests were used to compare PD-1, PD-L1 and PD-L2 expression on B and T cells between septic patients and controls, and between subgroups of septic patients based on mortality and nosocomial infection status, with nosocomial infection defined as a new antibiotic start for suspected new infection, after an antibiotic-free period of ≥24 hours. Within sepsis patients, PD-1, PD-L1 and PD-L2 expression on B and T cells was compared by

1 CD27 status using the paired Wilcoxon test. All statistical analyses were performed with
2 GraphPad Prism software (GraphPad Software, La Jolla, CA). Significance levels were set as p
3 values <0.05.

4

5 Results

6 Study cohort

7 The median (IQR) age of the septic patients was 68.5 (54.8 – 84.3) years, with 73% being
8 male. The respiratory tract was the most common infection site (73%), followed by intra-
9 abdominal (18%) and wound/soft tissue (9%). The mean (SD) total white cell and
10 lymphocyte counts were 15.9 (7.8) x 10^9 cells/litre and 1.1 (0.7) x 10^9 cells/litre, respectively.
11 The median (IQR) ICU length of stay was 10.5 (7 – 20) days. Nosocomial infection occurred in
12 11 patients and was more common in patients with an ICU length of stay ≥4 days (61% vs
13 0%). Patient characteristics are summarized in eTables 1 and 2.

14 Memory B cells in sepsis patients had more PD-1, PD-L1 and PD-L2 positive cells with
15 higher expression

16 The proportion of B cells positive for PD-1, and the corresponding MFI, were significantly
17 higher in sepsis patients than in controls (26.5% vs 8.8%, p=0.0002; 483 vs 348, p=0.0003)
18 (eTable 3; Figure 1). This was true of both CD27+ and CD27- subsets (eTable 4; eFigure 2;
19 eFigure 3). The proportion of PD-L1 positive B cells was also higher in sepsis patients
20 compared to healthy controls (2.4% vs 1.2%, p=0.0244). The percentage positivity of PD-L2
21 was higher in sepsis than controls in the CD27+ subset only (3.59% vs 0.41%, p=0.0317)
22 (eFigure 3). Within B cells in septic patients, the CD27+ subset had significantly higher PD-1

1 and PD-L2 MFI (574.5 vs 471.5, p<0.0001; 1189 vs 744, p<0.0001) and percentage positivity
2 (34.05% vs 24.80%, p<0.0007; 3.59% vs 0.86%, p<0.0001) (Figure 1).

3 **Memory CD4+ T cells in sepsis patients had more PD-1, PD-L1 and PD-L2 positive cells with**
4 **higher expression**

5 The proportions of PD-1 and PD-L1 positive T cells were significantly higher in sepsis than in
6 controls (38.90% vs 21.25%, p=0.0023; 1.9% vs 0.2%, p=0.0083), as were the corresponding
7 MFI values (780 vs 627, p=0.0013; 1314 vs 1007, p=0.0276) (Figure 2). This was true of
8 CD27+ and CD27- subsets (p<0.05 for all comparisons) (eTable 4; eTable 5; eFigure 4;
9 eFigure 5). There were no significant differences in CD4+ T cell PD-L2 expression between
10 sepsis and healthy controls. In sepsis patients, expression of PD-1 was higher on CD27- T
11 cells than CD27+ T cells, by both percentage positivity and MFI (70.45% vs 35.15%,
12 p<0.0001; 972 vs 716, p<0.0001) (Figure 2).

13 **Admission day PD-1, PD-L1 and PD-L2 expression did not differ by nosocomial infection**
14 **and hospital mortality**

15 There were no differences in PD-1, PD-L1 or PD-L2 expression between patients who
16 subsequently developed a nosocomial infection and those who did not (eFigure 6). When
17 patients with an ICU length of stay ≤7 days were excluded, PD-L1 expression on lymphocytes
18 was significantly higher in those who subsequently developed an infection (p=0.0068)
19 (eFigure 7), however this did not reach significance for any B or T cell subset (eFigure 8).
20 There were no differences between survivors and non-survivors in PD-1, PD-L1 or PD-L2
21 expression for any lymphocyte subset (eFigure 9).

22 **Soluble PD-1 and PD-L1 expression did not correlate with cell surface expression**

There were no differences in serum sPD-1 and sPD-L1 concentrations between sepsis patients and healthy controls (eFigure 10). Serum sPD-1 and sPD-L1 concentrations in sepsis patients showed no correlation to lymphocyte surface expression (eFigure 11).

Discussion

The novel findings from this pilot study include the first report of higher B cell expression of PD-1, PD-L1 and PD-L2 in sepsis, and a differential expression of PD-1 by CD27 status in both B and CD4+ T cells. We also report results that are consistent with the published literature such as higher PD-1 and PD-L1 expression in CD4+ T cells in sepsis compared with controls [7, 17], which gives external validity to our report. The overexpression of these checkpoint inhibitors in most sepsis patients is consistent with the published literature suggesting this is a feature of sepsis-related immunosuppression.

PD-L2 has been less well-studied in sepsis than PD-L1, as PD-L1 is the more important binding partner for PD-1. The contribution of PD-L2 to the pathophysiology of sepsis remains unknown although increased PD-L2 expression by monocytes was reported in one observational study of patients with septic shock [7]. A key finding of our present study in critically ill adult patients with sepsis was a higher expression of PD-1/PD-L in the lymphocyte subsets associated with memory status, i.e. CD27+ B cells and CD27- CD4+ T cells. Memory lymphocytes are formed after encountering a specific pathogen, and are vital for generating rapid and effective immune responses upon future encounters with the same pathogen [18]. We chose CD27 as a marker of memory status. In circulating B cells, CD27 expression is associated with activation [9]. CD27+ B cells are larger in size and exhibit

1 greater and more rapid proliferation and immunoglobulin production in response to
2 antigenic stimulation [9]. In CD4+ T cells, loss of CD27 expression is seen in memory cells at
3 a late stage in differentiation, and is associated with an increased capacity for IL-4
4 production [19]. Functional studies of CD4+ T cells report that CD27 expression distinguishes
5 two distinct subpopulations, of which the CD27- subset shows a stronger antigen-recall
6 response and increased cytokine secretion [10]. A relatively higher PD-1 expression on
7 CD27- T cells may therefore have a greater negative effect on antigen-specific responses in
8 both B cells and T cells, as there is T cell-dependent B cell development within the germinal
9 centres of secondary lymphoid organs [20].

10 We measured soluble PD-1 and PD-L1 levels as high levels of sPD-1 or sPD-L1 in sepsis could
11 reduce the efficacy of anti-PD-1 or anti-PD-L1 antibody therapy by neutralisation.
12 Furthermore, should serum levels correlate with cell surface expression, this may offer a
13 potential point-of-care biomarker to identify patients who could benefit from early PD-1
14 pathway blockade. We did not find any significant differences in sPD-1 or sPD-L1 levels
15 between sepsis patients and controls; of note, levels were towards the lower limit of
16 detection in the majority of subjects. Previous studies measuring serum sPD-1/L levels in
17 sepsis have yielded inconsistent results [21-23] (eTable-6). Importantly, none of these
18 studies measured concurrent cell surface expression. Timing of measurement may
19 contribute to the differences; we measured sPD-1/PD-L1 within 12 hours of ICU admission
20 whereas the others varied from time of presentation to the emergency department [21] to
21 within 24 hours of ICU admission [22, 23]. Our pilot study results suggest that sPD-1 or sPD-
22 L1 levels within 12 hours of ICU admission do not identify patients with high cell surface PD-
23 1/L expression [11]. This needs confirmation in larger cohorts, ideally using the same
24 inclusion criteria to those planned for interventional trials.

In contrast to one previous study [7], we did not observe significant differences in PD-1/PD-L1 expression by either survival or nosocomial infection status. Aside from our small sample size, there are several alternative explanations. The kinetics of these checkpoint inhibitors in critically ill sepsis patients are unknown. There is also a variable degree of immunosuppression even at the time of ICU admission though we specifically excluded any patients with previously documented immunosuppression. However, our timing of sampling within 12 hours of ICU admission may be too early for differentiating survival status. This inference is supported by a recent study examining PD-1 expression by CD4+ T cells in a sepsis cohort using serial measurements on days 1, 3 and 7 of ICU admission, which found that while all septic patients had raised PD-1 at days 1 and 3, only survivors normalised PD-1 expression by day 7 [17]. This highlights the need for further work to characterize how PD-1/L expression changes over the course of sepsis, how this relates to outcome, and the optimal recruitment window for any future trial of anti-PD-1 therapy. With regard to nosocomial infection, we found that lymphocyte PD-L1 expression was significantly higher in those who subsequently developed an infection, but only when the ICU length of stay exceeded 7 days. This may be explained by the competing risk of nosocomial infection with early ICU discharge or death. The additional risk provided by over-expression of these checkpoint inhibitors may be overwhelmed by stronger risk factors for mortality such as age, comorbidity and illness severity [14].

When interpreting our results, key limitations to consider include the small sample size, the use of healthy controls instead of non-sepsis critical illness controls, and that this was a post-hoc sub-study designed to test a hypothesis to inform future trials. We chose healthy controls as critically ill patients exhibit a range of immune deficits similar to those seen in sepsis patients [24]; the use of non-infected critically ill controls could confound the

association between PD-1 expression and outcomes. The key strengths of the study include the hypothesis-driven set of experiments that highlight the need for further research to define PD-1, PD-L1 and PD-L2 expression in sepsis, and its relationship to two competing events: nosocomial infection and death [11].

Conclusions

In conclusion, our pilot study contributes to the further understanding of sepsis immunology by highlighting increased expression of these checkpoint regulators in B cells, and their differential expression by memory subset status in both B and T cells. The utility of CD27 status in lymphocytes as a putative biomarker for patient enrichment in anti PD-1 or anti PD-L1 trials warrants further study.

List of abbreviations

ELISA: enzyme-linked immunosorbent assay; FCS: flow cytometry standard; FMO: fluorescence minus one; ICU: intensive care unit; MFI: mean fluorescence intensity; PD-1: programmed death protein 1; PD-L1: programmed death-ligand 1; PD-L2: programmed death-ligand 2; SD: standard deviation; sPD-1: soluble programmed death protein 1; sPD-L1: soluble programmed death-ligand 1

Declarations

Ethics approval and consent to participate

All patients who met the eligibility criteria were consented prior to blood sampling. Ethics approval for the study was given by HRA Research Ethics Committee London (Camberwell St Giles). Address: East of England REC Centre Victoria House, Capital Park Fulbourn, Cambridge CB21 5XB. Approval reference: 12/LO/0326.

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

MS is on the steering committee for a clinical trial of anti PD-L1 therapy in sepsis. The other authors have no competing interests to declare.

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Authors' contributions

JW performed the flow cytometry and ELISA experiments and analysed the data. MSH designed the study, and obtained the samples. MSH and JW wrote the first version of the

1 manuscript. JS and YZ supervised the laboratory experiments, including ELISA and flow

2 cytometry. All authors read, and approved the final manuscript.

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Additional files

eTable 1. Sepsis patient characteristics

eTable_1.docx

Table showing characteristics of sepsis patients included in the study. Figures are shown for all patients, and for survivors and non-survivors

eTable 2. Infection site/microbiology of patients with nosocomial infections

eTable_2.docx

Table showing the survival status, microbiology results and nosocomial infection site of those patients who developed nosocomial infection.

eTable 3. Proportion of positive B and T cells

eTable_3.docx

Table showing the proportion of B and CD4+ T cells which express PD-1, PD-L1 and PD-L2, compared between sepsis patients and healthy controls.

eTable 4. Proportion of positive cells by CD27 expression status

eTable_4.docx

Table showing the proportion of CD27+ B cells, CD27- B cells, CD27+ CD4+ T cells and CD27- CD4+ T cells which express PD-1, PD-L1 and PD-L2, compared between sepsis patients and healthy controls.

eTable 5. MFI results

eTable_5.docx

Table showing the MFI of PD-1, PD-L1 and PD-L2 on CD27+ B cells, CD27- B cells, CD27+ CD4+ T cells and CD27- CD4+ T cells, compared between sepsis patients and healthy controls.

eTable 6. sPD-1/sPD-L1 studies in sepsis

eTable_6.docx

Summary of previous studies measuring soluble serum PD-1 and PD-L1 (sPD-1; sPD-L1) levels in sepsis.

eFigure 1. Example of gating used in data analysis

eFigure_1.docx

The isotype (and FMO) was used to set the negative gate, and then the percentage of positive cells was taken as the percentage above this gate. Separate healthy and sepsis isotypes were used (eFigure 1a and 1b respectively). 1c shows an example of MFI signalling in healthy and sepsis isotypes, and healthy, sepsis survivor and sepsis non-survivor samples.

eFigure 2. B cell subset MFI

eFigure_2.docx

Box and whisker plots comparing expression by MFI of PD-1, PD-L1 and PD-L2 between sepsis patients and healthy controls on B cell subsets (CD27+ and CD27-).

eFigure 3. B cell percentage positivity

eFigure_3.docx

Dot plots comparing the expression levels by percentage positivity of PD-1, PD-L1 and PD-L2 between sepsis patients and healthy controls on B cell subsets (CD27+ and CD27-).

eFigure 4. CD4+ T cell subset MFI

eFigure_4.docx

Box and whisker plots comparing expression by MFI of PD-1, PD-L1 and PD-L2 between sepsis patients and healthy controls on CD4+ T cell subsets (CD27+ and CD27-).

eFigure 5. CD4+ T cell percentage positivity

eFigure_5.docx

Dot plots comparing the expression levels by percentage positivity of PD-1, PD-L1 and PD-L2 between sepsis patients and healthy controls on CD4+ T cell subsets (CD27+ and CD27-).

eFigure 6. Comparison by nosocomial infection status

eFigure_6.docx

Box and whisker plots comparing expression of PD-1, PD-L1 and PD-L2 between patients who developed a nosocomial infection and those who did not.

eFigure 7. PD-L1 comparison by nosocomial infection status in patients with ICU length of stay ≥7 days

eFigure_7.docx

Box and whisker plot showing PD-L1 expression by lymphocytes compared between patients who developed a nosocomial infection and those who did not, when patients with an ICU length of stay less than 7 days are excluded.

eFigure 8. Comparison by nosocomial infection status in patients with ICU length of stay ≥7 days

eFigure_8.docx

Box and whisker plots comparing PD-1, PD-L1 and PD-L2 expression by B and CD4+ T cells between patients who developed a nosocomial infection and those who did not, when patients with an ICU length of stay less than 7 days are excluded.

eFigure 9. Comparison by survival status

eFigure_9.docx

Box and whisker plots comparing PD-1, PD-L1 and PD-L2 expression by B and CD4+ T cells between sepsis survivors and non-survivors.

eFigure 10. Serum level comparison

eFigure_10.docx

Dot plots comparing levels of serum PD-1 and PD-L1 between sepsis patients and healthy controls.

eFigure 11. Serum vs cell surface expression

eFigure_11.docx

Scatter graphs plotting serum levels of PD-1 and PD-L1 against cell surface expression levels on B cells and CD4+ T cells.

Figure titles/legends

Figure 1. PD-1/L expression on B cells.

Box and whisker plots (Figure 1a-c) comparing the MFI of PD-1, PD-L1 and PD-L2 on B cells in sepsis patients compared with healthy controls. Figure 1d-f compares the MFI of PD-1, PD-L1 and PD-L2 between CD27+ B cells and CD27- B cells within sepsis patients. The boxes below show the corresponding proportion of positive cells for each comparison, with corresponding p-values. Significant p-values are marked with an asterisk.

Figure 2. PD-1/L expression on CD4+ T cells.

Box and whisker plots (Figure 2a-c) comparing the MFI of PD-1, PD-L1 and PD-L2 on T cells in sepsis patients compared with healthy controls. Figure 2d-f compares the MFI of PD-1, PD-L1 and PD-L2 between CD27+ T cells and CD27- T cells within sepsis patients. The boxes below show the corresponding proportion of positive cells for each comparison, with corresponding p-values. Significant p-values are marked with an asterisk.

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