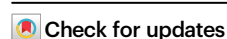


Brucella abortus impairs T lymphocyte responsiveness by mobilizing IL-1RA-secreting omental neutrophils

Received: 1 August 2023

Accepted: 30 December 2024

Published online: 20 January 2025



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Immune evasion strategies of *Brucella*, the etiologic agent of brucellosis, a global zoonosis, remain partially understood. The omentum, a tertiary lymphoid organ part of visceral adipose tissue, has never been explored as a *Brucella* reservoir. We report that *B. abortus* infects and replicates within murine omental macrophages. Throughout the chronic phase of infection, the omentum accumulates macrophages, monocytes and neutrophils. The maintenance of PD-L1⁺Sca-1⁺ macrophages, monocytes and neutrophils in the omentum depends on the *wadC*-encoded determinant of *Brucella* LPS. We demonstrate that PD-L1⁺Sca-1⁺ murine omental neutrophils produce high levels of IL-1RA leading to T cell hyporesponsiveness. These findings corroborate brucellosis patient analysis of whole blood displaying upregulation of *PDL1* and *Ly6E* genes, and of serum exhibiting high levels of IL-1RA. Overall, the omentum, a reservoir for *B. abortus*, promotes bacterial persistence and causes CD4⁺ and CD8⁺ T cell immunosuppression by IL-1RA secreted by PD-L1⁺Sca-1⁺ neutrophils.

To establish a chronic infection, intracellular bacteria need to survive, disseminate, and reach a favorable organ embedding their sheltered replicative niche¹. The anatomic environments they preferentially invade and colonize are diverse and adapted to their lifestyle. *Brucella* spp., Gram-negative facultative intracellular coccobacilli, are the causative agents of brucellosis, a worldwide re-emerging zoonosis. As organ reservoirs, *Brucellae* have a predilection for the lymph nodes,

spleen, lungs, bone marrow and the reproductive system², where their cellular niches, the professional phagocytes reside^{1–3}. Although *B. abortus* is able to replicate inside a murine fibroblastic derived adipocyte-like cell line⁴ and *B. canis* has been detected in adipocytes of the gastro-splenic ligament from naturally infected fetuses⁵, nothing more is known about *Brucella* and adipose tissue. The omentum, whose capability to fight bacteria has been appreciated clinically for

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years⁶, is part of the visceral adipose tissue and is recognized as a tertiary lymphoid organ. It contains leukocyte aggregates called milky spots (MS), strategic vascular and lymphatic connections, and constitutes a favored site for metastatic tumors⁷. The omentum rapidly accumulates antigens from the peritoneal cavity and plays a critical role in both innate and adaptive immunity in various models of inflammation or infection^{7–15}. Adipose tissue has been identified as a reservoir for intracellular pathogens that stay dormant for years after the primary infection, such as *Mycobacterium tuberculosis*¹⁶, *Rickettsia prowazekii*¹⁷, *Coxiella burnetii*¹⁸ or *Trypanosoma brucei*¹⁹; however, the mechanisms involved in the establishment of chronicity remain unexplored.

During the early stages of infection, *Brucella* has the peculiarity of minimally activating host innate immunity, due in part to the expression of an atypical non-endotoxic lipopolysaccharide (LPS) that tunes down dendritic cell (DC) and macrophage activation^{20–24}. *Brucellae* infect neutrophils without intracellular replication, resist their killing mechanisms, inhibit their degranulation, and marginally activate them²⁵. Premature cell death of neutrophils, elicited by *Brucella* without neutrophil extracellular trap (NET)osis, triggers efferocytosis, i.e., phagocytosis of dying neutrophils by macrophages²⁶. Infected neutrophils were hence proposed to act as Trojan horses for the dissemination of *B. abortus*²⁶. Neutrophils are essential for *B. abortus* persistence during the chronic phase of infection and dampen adaptive immunity especially T cell activation through an unidentified mechanism^{27,28}.

Here, we show that the omentum acts as a reservoir for *B. abortus* that sustains the mobilization of myeloid cells expressing the immunosuppressive markers Programmed cell Death-Ligand 1 (PD-L1)²⁹ and Stem Cell Antigen-1 (Sca-1)^{30,31}. The additional identification of biomarkers of brucellosis in humans led us to uncover a key role of IL-1RA secreted by PD-L1⁺Sca-1⁺ neutrophils in T-cell immunosuppression.

Results

B. abortus infects omental macrophages and persists during the chronic phase of infection

We first assessed the ability of *Brucella abortus* to infect the omentum in mice. The bacterial loads in the spleen, peritoneal fluid, and omentum were determined at different time points after intraperitoneal (i.p.) infection of C57BL/6 mice (Supplementary Fig. S1a). High CFU/g were observed in both the omentum and spleen from the onset (2 days post-infection (p.i.)) to the chronic phase (30 days p.i.), while lower values (CFU/mL) were detected in the peritoneal fluid (Fig. 1a). Given that omentum is located in the peritoneal cavity where the initial *Brucella* inoculum was injected, we next infected C57BL/6 mice by gavage (Supplementary Fig. S1a). Gavage infection also resulted in significant bacterial loads in the omentum at 30 days p.i. (Fig. 1b), formally demonstrating a colonization of this organ by *Brucella*. To determine which cell type was hosting *B. abortus* within the omentum, we performed confocal microscopy of omentum infected with DsRed *B. abortus*, harvested at 8 days p.i. and stained for F4/80⁺ macrophages, Ly6G⁺ neutrophils and B cells known *Brucella* target cells^{1,32,33}, as well as Ly6C⁺ monocytes and CD3⁺ T cells (Fig. 1c). A 3D reconstituted image of the infected macrophage shown in Fig. 1c confirms that omental F4/80⁺ macrophages were colonized intracellularly by replicating DsRed *B. abortus* (Fig. 1d). Although C57BL/6 and BALB/c mouse strains differ in their susceptibility to *B. abortus* infection, colonization of the omentum in BALB/c mice infected by gavage followed a similar trend as in C57BL/6 (Supplementary Fig. S1a, b). Mice were positive for *Brucella* in the omentum at 90 days p.i., the chronic phase, at CFU levels comparable to the classical *Brucella* reservoirs, the spleen, or lymph nodes. These findings demonstrate that *B. abortus* has the ability to reach the omentum, infect F4/80⁺ macrophages, and persist at the chronic stage of infection.

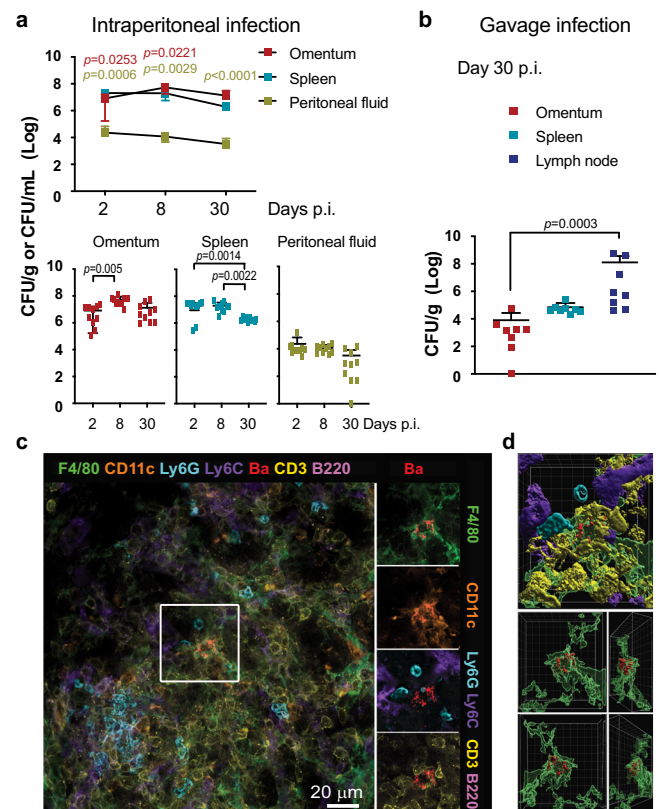


Fig. 1 | *Brucella abortus* infects macrophages in the omentum and persists during the chronic phase. **a** C57BL/6 mice were injected intraperitoneally (i.p.) with 1×10^6 *B. abortus* strain 2308 CFU. At the indicated time points after infection of C57BL/6 mice, omentum (magenta), spleen (dark cyan), and peritoneal fluid (olive green) were analyzed for bacterial loads (mean of CFU per g, or for the peritoneal lavage per mL, from pooled data $\pm$ SD). 5 mice per group (but omentum day 8, 5–6 mice per group), $n = 2$. Significant differences from the spleen are displayed for the kinetics (two-way ANOVA followed by Dunnett's multiple comparison test; $F = 13.56$; $DF = 82$) and between time points for organ individual points (Kruskal-Wallis followed by Dunn's multiple comparison test). Absence of p -value, non-significant. **b** C57BL/6 mice were infected by gavage with 1×10^9 *B. abortus* strain 2308 CFU. At 30 days post-infection (p.i.), omentum (magenta), spleen (dark cyan), and cervical lymph nodes (dark blue) were harvested, and CFU per g was determined. Each square represents one animal. Mean $\pm$ SD from pooled data. 4 mice per group, $n = 2$. Significant differences are shown (Kruskal-Wallis followed by Dunn's multiple comparison test). Absence of p -value, non-significant. **c** Confocal microscopy image of wholemount staining of the omentum at day 8 p.i. from C57BL/6 mice i.p. infected with a DsRed strain of *B. abortus*. DsRed (Ba), F4/80 (green, macrophages), Ly6G (magenta, neutrophils), Ly6C (violet, monocytes, and blood vessels), B220 (pink, B cells) and CD3 (cyan, T cells). Individual stainings of cells present in the selected square are on the right. 3 mice, $n = 4$. Scale bar, 20 μ m. **d** 3D reconstitution of cells in the square selected in (c), showing macrophage colonization by, and multiplication of, *B. abortus*. Top image, all labelings. Other images, Ba and F4/80 staining only. One square of the grid, 5 μ m. Source data are provided as a Source Data file.

Macrophages, monocytes and neutrophils are recruited to the omentum after *B. abortus* infection during the acute and chronic phases

The infection of omental macrophages by *B. abortus* raised the possibility of changes in this highly dynamic cell population upon infection. We examined by flow cytometry the peritoneal fluid and omentum at 8 and 30 days post-intraperitoneal *B. abortus* (Ba) infection of C57BL/6 mice (Fig. 2) as per the gating strategy in Supplementary Fig. S1c. We first looked at the F4/80^{hi} (large peritoneal macrophage, LPM) resident macrophages and F4/80^{int}MHCII^{hi} (small peritoneal macrophage, SPM) subsets, well-known macrophage cells populating the peritoneal cavity

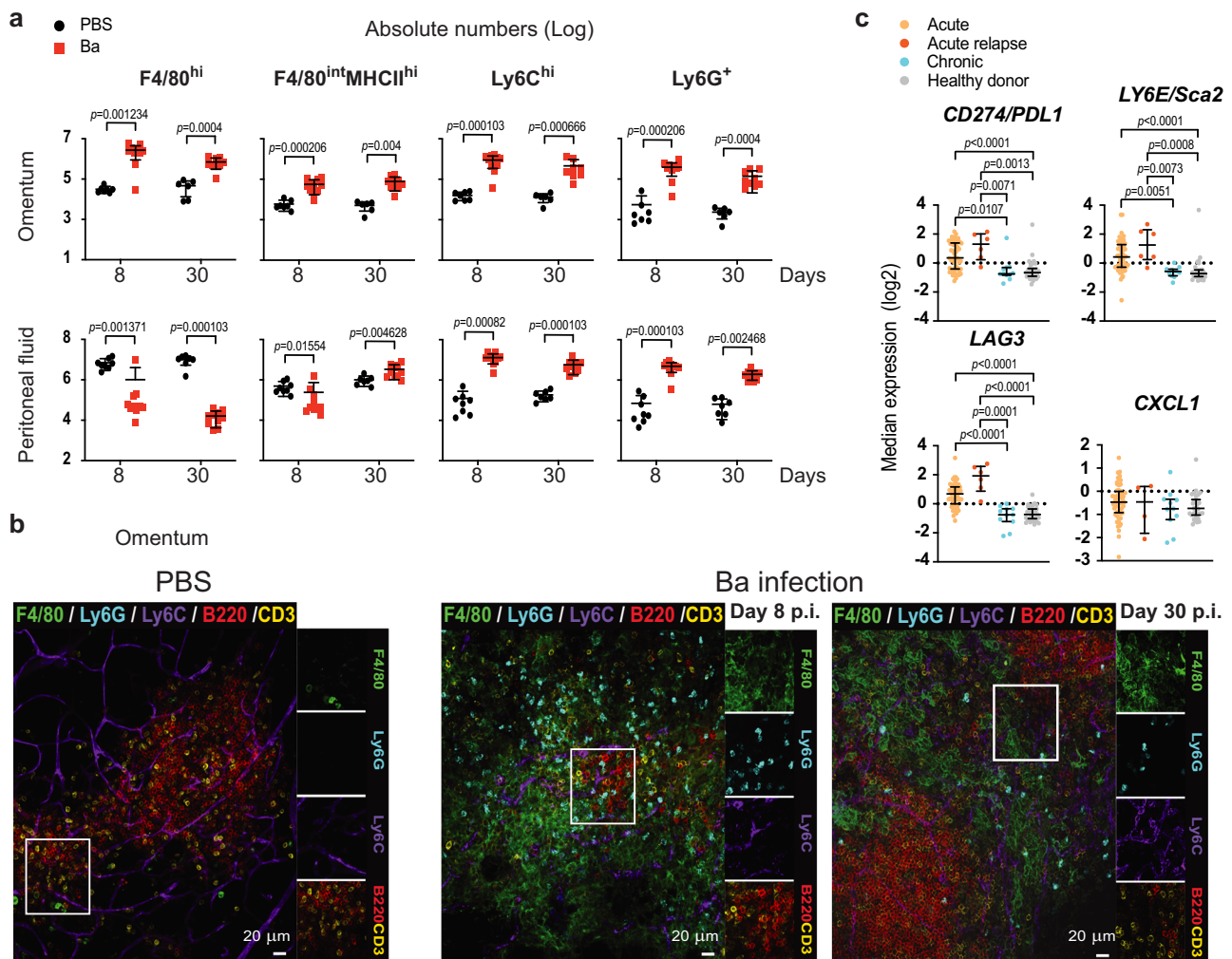


Fig. 2 | Macrophages, monocytes and neutrophils are recruited in the omentum after *B. abortus* infection from the acute to the chronic phase. **a** At 8 or 30 days post-infection (p.i.) of C57BL/6 mice i.p. infected with *B. abortus* (Ba) (red square) or mock-injected with PBS (black circle), omentum and peritoneal fluid were harvested, and individualized cells analyzed by flow cytometry. Normalized absolute numbers of omental and peritoneal F4/80^{hi} LPM, F4/80^{int}MHCII^{hi} SPM, Ly6C^{hi} monocytes, or Ly6G⁺ neutrophils at the indicated time points are shown. Each symbol represents one animal. (PBS, 2–3 mice; Ba, 3–4 mice), $n = 3$. Mean from pooled data $\pm$ SD. Significant differences are shown (Mann-Whitney test, two-sided). Absence of p -value, non-significant. **b** Confocal microscopy images of wholemount staining of omenta harvested at day 8 or 30 p.i. from i.p. infected C57BL/6 mice or mock-PBS treated. F4/80 (green, macrophages), Ly6G (magenta, neutrophils), Ly6C (violet, monocytes and endothelial cells of blood vessels), B220

(red, B cells), and CD3 (cyan, T cells). B and/or T cell localization defines the milky spot (MS). Individual stainings of cells present in the selected square are on the right. 3 mice, $n = 2$. Scale bar, 20 μ m. **c** Median expression of selected differentially expressed genes from published RNA seq transcriptomic profiling obtained from whole blood samples from healthy donor controls or primary brucellosis patients in acute, acute relapse, or chronic phase of infection^{37,38}. X-axis: Healthy donor controls $n = 32$ (Gray); Brucellosis patients: Acute $n = 56$ (Yellow), Acute with relapse $n = 6$ but for *CXCL1* $n = 4$ (Orange), Chronic $n = 11$ (Blue). Y-axis: log2 residual gene expression counts. Each symbol represents one patient or healthy donor. Mean $\pm$ SD. Significant differences are shown (Multiple comparison Kruskal Wallis test, followed by post-hoc Dunn's test). Absence of p -value, non-significant. Source data are provided as a Source Data file.

at resting state and upon inflammation, respectively³⁴. A significant decrease in the numbers of LPM and SPM subsets was observed in the peritoneal fluid at 8 days p.i. (Fig. 2a, bottom) that coincided with a significant increase in the omentum (Fig. 2a, top), inferring a possible migration of macrophages from the peritoneal fluid to the omentum during the acute phase of infection. The disappearance of resident macrophages in the peritoneal cavity is usually followed by a complete recovery mediated by monocyte-derived SPM³⁵. Here, in the peritoneal fluid at 30 days p.i., LPM rates remained low whereas SPM cell numbers slightly increased (Fig. 2a). However, both macrophage subset numbers augmented in the omentum at the chronic phase (day 30) upon *B. abortus* infection (Fig. 2a).

Since SPM, which are derived from Ly6C^{hi} blood monocytes in response to stimuli³⁶, accumulated in the omentum from day 8 p.i.

onward and in the peritoneal fluid at the chronic phase of infection, we analyzed the levels of Ly6C^{hi} monocytes (Fig. 2a). Ly6C^{hi} monocytes were readily mobilized to the peritoneal fluid and omentum at high numbers at days 8 and 30 p.i. In the spleen, their numbers were elevated at 8 days p.i. and decreased to values still higher than those from PBS controls at 30 days p.i. (Supplementary Fig. S1d).

Neutrophils playing an essential role in brucellosis²⁵, we also determined the kinetics of Ly6G⁺ neutrophil mobilization. At 8 days p.i., neutrophils significantly accumulated in the peritoneal fluid, omentum, and spleen (Fig. 2a and Supplementary Fig. S1c), and this recruitment remained in these three organs at 30 days p.i. By comparison, high blood neutrophil counts were found in infected mice at 8 days p.i., which diminished to levels marginally lower than those of mock-treated mice at 30 days p.i. (Supplementary Fig. S1e).

Altogether, *B. abortus* causes a decrease in LPM and SPM in the peritoneal fluid (at the acute phase only for the latter), and at the same time elicits an increase of both populations in the omentum at the acute and chronic phases of infection. An accumulation of monocytes and neutrophils also occurs in the peritoneal fluid and omentum that persists at the chronic phase of infection. Confocal microscopy images of *B. abortus*-infected omenta further illustrate the spatial localization of these cells in the omentum at day 8 and 30 p.i. (Fig. 2b) and shows their presence in the vicinity of the milky spots (MS) until the chronic phase.

PDL1 and LY6E genes are upregulated in blood samples from acute phase brucellosis patients

To get some clues about the immune responses associated with *Brucella* and disease stage in humans, RNA-seq transcriptional profiles obtained from whole blood samples of brucellosis patients in acute, acute relapse (i.e., patients who developed disease relapse while treated with antibiotics) or chronic phase of infection, or healthy donors were previously determined by an international consortium comprising our laboratory^{37,38}. Using this database, we looked for genes that might help *Brucella* persistence and immune escape, and analyzed the blood expression of genes encoding immune inhibitory molecules^{30,31,39}, like PD-L1, a potential ortholog of the mouse Sca-1/Lymphocyte antigen 6A-2/6E-1 in humans (where the LY6 gene family comprises 11 members (*PSCA*, *LY6K*, *SLURP1*, *LYPD2*, *LYNX1/SLURP2*, *LY6D*, *GML*, *LY6L*, *LY6H*, *GPIHBP1* and *LY6E*⁴⁰) and LAG3 (Fig. 2c). Overexpression of *CD274/PDL1* and *LAG3* was detected in the blood of acute and acute with relapse patients but not in chronic brucellosis patients. The *LY6E* gene followed the same pattern of expression. As a control, expression of the *CXCL1* chemokine gene remained stable in all groups.

In mice, mobilized macrophages, monocytes and neutrophils upregulate PD-L1 and Sca-1 during acute and chronic phases of *B. abortus* infection

We then assessed if *B. abortus* controls the expression of PD-L1 and Sca-1, two molecules linked to the acquisition of a negative regulatory profile in the recruited peritoneal macrophages, monocytes and neutrophils by quantifying the total number of positive cells at 8 and 30 days p.i. by flow cytometry (Fig. 3a). In the peritoneal fluid, similar reductions in the single or double positive PD-L1 Sca-1 F4/80^{hi} LPM cell subsets from *B. abortus*-infected mice were observed at both time points when compared to non-infected PBS-injected controls, whereas the number of F4/80^{int}MHCII^{hi} SPM co-expressing the PD-L1 and Sca-1 markers significantly augmented. Monocytes recruited to the peritoneal cavity displayed a consistent phenotype with enhanced numbers of PD-L1⁺Sca-1⁺Ly6C^{hi} cells at days 8 and 30 p.i. The numbers of peritoneal Ly6G⁺ neutrophils expressing PD-L1 with or without Sca-1 co-expression were also significantly increased at days 8 and 30 p.i. (Fig. 3a). When analyzing by spectral cytometry the number of peritoneal cells expressing LAG-3 at day 8 p.i. (undetected in our settings by flow cytometry and using TIM4 as an additional marker for LPM⁴¹), a pattern evoking that of the PD-L1⁺Sca-1⁺ cells was obtained (Fig. 3b and Supplementary Fig. S1f). We then looked at the expression of these three markers in the F4/80^{int}TIM4⁺ LPM, F4/80^{int}MHCII^{hi} SPM, Ly6C^{hi} and Ly6G⁺ cells recruited in the omentum at day 8 p.i. by spectral cytometry (Fig. 3c). We found that the total numbers of these four double positive PD-L1 and Sca-1 cell populations were significantly higher than those of non-infected PBS-injected controls. Moreover, a large fraction of these cells was also LAG-3 positive (Fig. 3c).

Together, these data reveal in the omentum a constant influx of macrophages/monocytes and neutrophils overexpressing PD-L1 and Sca-1 after *B. abortus* infection, inferring a possible role of these cells in the chronic stage of infection.

Macrophage, monocyte and neutrophil accretion in the omentum at late time-points following *B. abortus* LPS stimulation mimics that of infection and is core LPS-mediated

To gain insight into the mechanisms involved, we interrogated the behavior in the omentum upon infection of a mutant of *B. abortus* exhibiting a mannosyltransferase (*wadC*) gene deletion (Ba-*wadC*). This mutation causes a lack of a characteristic core lateral pentasaccharide in *Brucella* smooth LPS that eases its detection by TLR4 and, therefore, makes the mutant strain less virulent^{21–23}. Bacterial counts in the spleen and omentum at 15 days (the start of the chronic phase) post-intraperitoneal infection of C57BL/6 mice with the wild-type *B. abortus* (Ba-wt) or Ba-*wadC* strains showed a significant attenuation of the Ba-*wadC* mutant strain in the omentum, which was more marked than that in the spleen (Supplementary Fig. S1g). Noteworthy, while CFU/organ between the wt and the *wadC* strains varied in the spleen by a 1.17 Log fold, this variation was of 2 in the omentum, supporting a paramount role of this organ in brucellosis. This importance of an intact LPS core in the persistence of *Brucella* at the chronic phase in the omentum led us to explore the role of LPS in the mobilization of cells in the peritoneal fluid and omentum of mice i.p.-injected with the LPS purified from the *B. abortus* Ba-wt or Ba-*wadC* strains, or from the canonical LPS from *E. coli* (Ec), which differs by its overall structure from *Brucella* LPS²³ (Fig. 4 and Supplementary Fig. S2a, b). In the omentum, Ba-wt LPS injection triggered an early influx of F4/80^{hi}TIM4⁺ resident macrophages (LPM), which persisted until 72 h, recalling the pattern previously observed upon *B. abortus* mouse infection (Fig. 4a, left top). Similar recruitment of LPM was observed with Ba-*wadC* or Ec LPS at an early time-point (12 h) of stimulation, the omental LPM population declining promptly afterward and reaching almost steady-state levels in the case of the Ec LPS samples. In the peritoneal fluid, LPM decreased at 12 h after exposure to any of the three LPS and remained diminished at 72 h only after Ba-wt LPS injection, in accordance with the infection data (Fig. 3a, left top). Concerning F4/80^{hi}MHCII^{hi} SPM, Ba-wt LPS injection drove higher numbers than did the other LPS at 72 h post-LPS injection in both the peritoneal fluid and omentum (Supplementary Fig. S2c). Ly6C^{hi}CCR2⁺ circulating monocytes rapidly accumulated in the peritoneal fluid and omentum 4 h after Ba-wt LPS stimulation and stayed in both locations until 72 h, consistent with our observations in *Brucella*-infected mice, unlike what happened after Ba-*wadC* and Ec LPS administrations (Fig. 4a, left bottom). In contrast to Ec LPS, both Ba-wt and Ba-*wadC* LPS administration generated an early accumulation of Ly6G⁺ neutrophils in the peritoneal fluid and omentum of mice, which was sustained only for Ba-wt LPS, again in agreement with *Brucella* infection findings (Fig. 4a, right). Together, these data indicate that the influx of macrophages, monocytes, and neutrophils observed after *B. abortus* LPS stimulation in the omentum and peritoneal fluid was similar to that following infection and relies at late time points on the LPS core lateral determinant, added by the *wadC* mannosyl transferase.

PD-L1⁺Sca-1⁺ macrophage, monocyte and neutrophil maintenance in the omentum after *B. abortus* LPS stimulation is core LPS-mediated and independent of PD-L1

We further explored by flow cytometry the effect of the three types of LPS on the expression of the immunosuppressive PD-L1 and Sca-1 surface markers on the macrophages, monocytes, and neutrophils of the omentum (Fig. 4b and Supplementary Fig. S2d, e). From 4 h to 24 h post-LPS injection, high numbers of PD-L1⁺Sca-1⁺ F4/80^{hi}MHCII⁺TIM4⁺ LPM, Ly6C^{hi}CCR2⁺ monocytes, and Ly6G⁺ neutrophils were triggered at similar levels by all types of LPS (Supplementary Fig. S2d). However, at 48 h and 72 h, this accumulation of PD-L1⁺Sca-1⁺ macrophages (LPM and SPM), monocytes, and neutrophils was sustained for Ba-wt LPS only (Fig. 4b and Supplementary Fig. S2d, e). So, *B. abortus* LPS drives a fast mobilization to the omentum of macrophages, monocytes, and

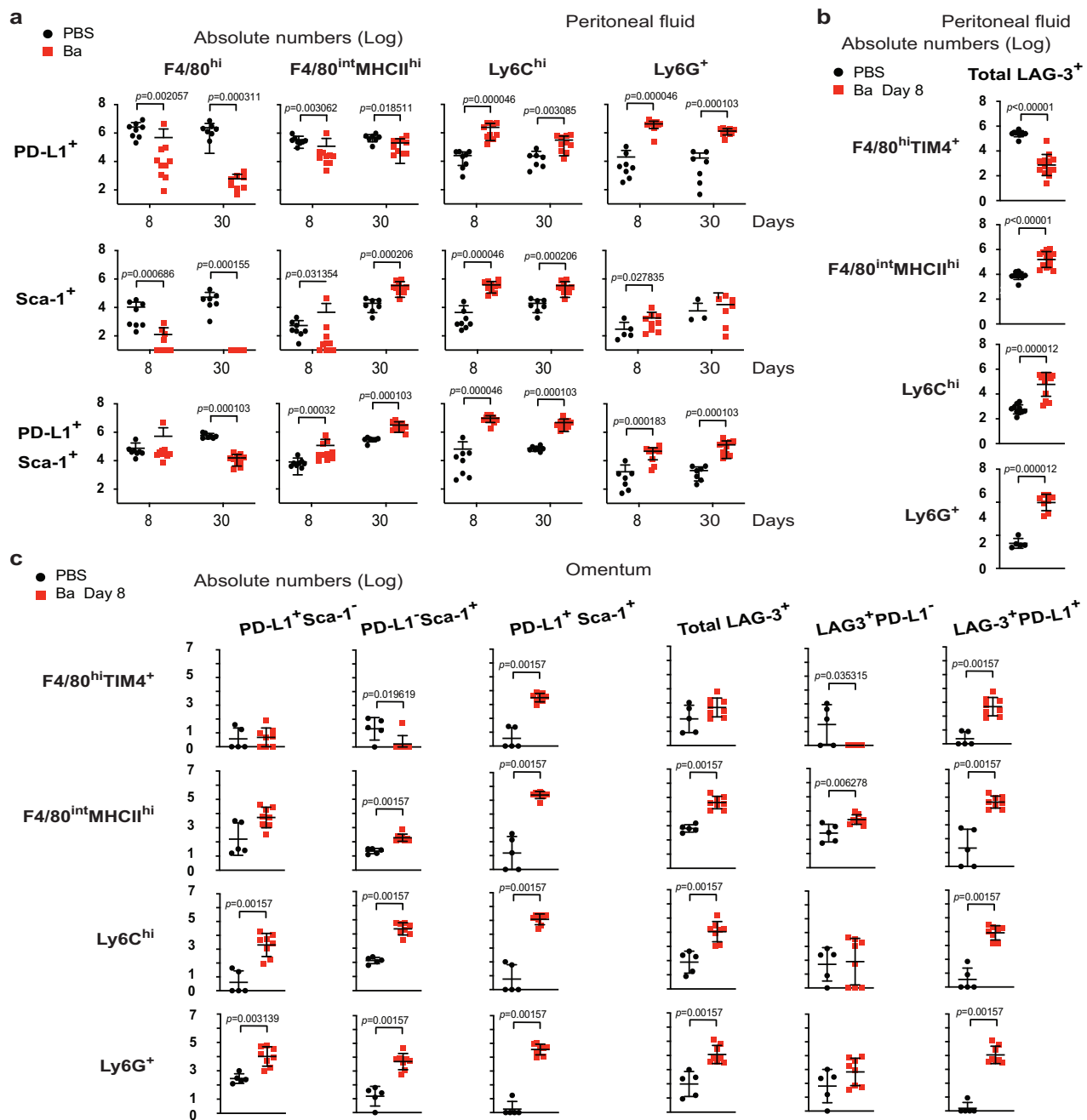


Fig. 3 | Macrophages, monocytes and neutrophils recruited in the omentum after *B. abortus* infection upregulate PD-L1 and Sca-1. **a** Normalized absolute numbers of peritoneal LPM, SPM, monocytes, and neutrophils expressing PD-L1, Sca-1, or both markers at 8 and 30 days post-infection, obtained by flow cytometry from mice treated as in Fig. 2a are shown. Each symbol represents one animal. (PBS (black circle), 2–3 mice; Ba (red square), 3–4 mice), $n = 3$. Mean from pooled data $\pm$ SD. Significant differences are shown (Mann-Whitney test, two-sided). Absence of p -value, non-significant. **b** Normalized absolute numbers of peritoneal LPM, SPM, monocytes, and neutrophils expressing total LAG-3 only at 8 days p.i. obtained by spectral cytometry from mice infected as in (a) are shown. Each symbol represents

one animal. (PBS (black circle), 3–4 mice; Ba (red square), 4–5 mice), $n = 3$. Mean $\pm$ SD. Significant differences are shown (Mann-Whitney test, two-sided). **c** Normalized absolute numbers of omental LPM, SPM, monocytes, and neutrophils expressing PD-L1, Sca-1, or both markers or expressing LAG-3 only or LAG-3 with or without PD-L1 at 8 days p.i. obtained by spectral cytometry from mice infected as in (a) are shown. Each symbol represents one animal. (PBS (black circle), 2–3 mice; Ba (red square), 4 mice), $n = 2$. Mean $\pm$ SD. Significant differences are shown (Mann-Whitney test, two-sided). Absence of p -value, non-significant. Source data are provided as a Source Data file.

neutrophils exhibiting a PD-L1⁺Sca-1⁺ phenotype, the persistence of which depends on the LPS core lateral pentasaccharide moiety.

Next, we asked if the blockade of PD-L1 impacts *Brucella* infection. C57BL/6 mice were i.p. infected with *B. abortus*, and neutralizing anti-PD-L1 or isotype control antibodies were administered at day 2, 4, and 6 p.i.; bacterial loads in the spleen and omentum, determined at day 8 p.i. revealed no difference in either case (Supplementary Fig. S3a).

The anti-PD-L1 neutralizing antibodies, whose efficacy was attested by the negligible numbers of all PD-L1⁺ cell types, had no effect either on the numbers of macrophages, monocytes, and neutrophils accumulated in the omentum at 48 h post-Ba-wt LPS injection (Supplementary Fig. S3b). However, they drove a significant increase in all Sca1⁺ myeloid cell populations. Altogether, the interactions between PD-L1 and PD-1 or CD80, both impaired by the neutralizing antibody⁴²,

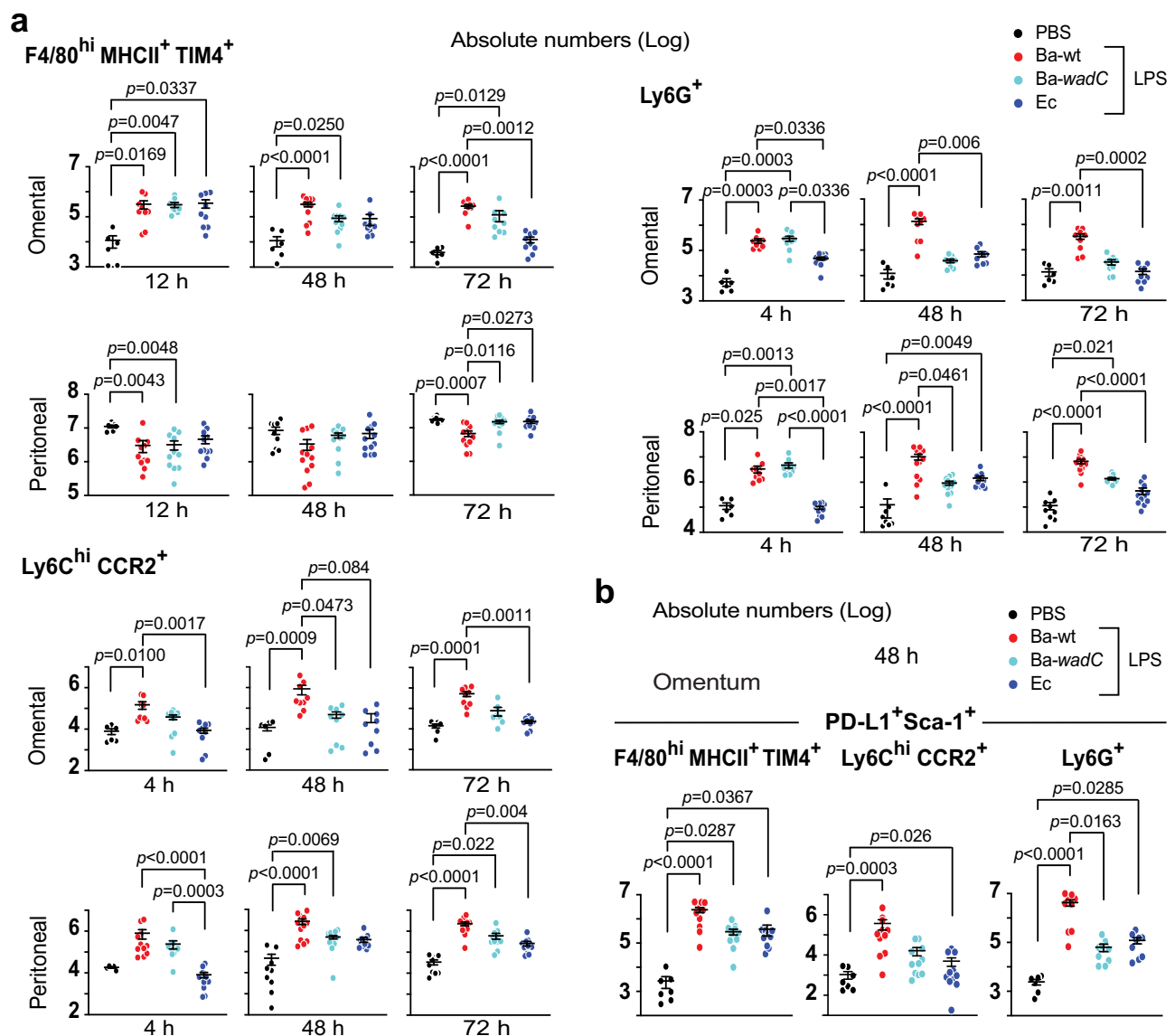


Fig. 4 | Core Ba-wt LPS-dependent sustained recruitment of PD-L1⁺Sca-1⁺ macrophages, monocytes and neutrophils in the omentum. **a** Omentum and peritoneal fluid were harvested at indicated time-points after i.p. challenge with Ba-wt (red circles), Ba-wadC (cyan circles) or Ec LPS (blue circles), or mock-PBS injection (black circles), and individualized cells analyzed by flow cytometry. Kinetics of omental and peritoneal F4/80^{hi}MHCII⁺TIM4⁺ macrophages, Ly6C^{hi}CCR2⁺ monocytes, and Ly6G⁺ neutrophils are shown at indicated time points. Each symbol represents one animal. Mean $\pm$ SD from pooled data of normalized absolute

numbers. (PBS, 2-3 mice; each LPS, 3-4 mice), $n = 3$. Significant differences are shown. Kruskal-Wallis followed by Dunn's multiple comparison test. Absence of p -value, non-significant. **b** Absolute numbers of omental PD-L1⁺Sca-1⁺ macrophages, monocytes and neutrophils obtained as in (a), with each symbol representing one animal at 48 h post-challenge. (PBS, 2-3 mice; each LPS, 3-4 mice), $n = 3$. Significant differences are shown (Kruskal-Wallis, followed by Dunn's multiple comparison test). Absence of p -value, non-significant. Source data are provided as a Source Data file.

are dispensable or insufficient for recruiting cells in the omentum at 48 h after Ba-wt LPS stimulation and for *B. abortus* infection. The significant accretion of Sca1⁺ cells suggests that PD-L1 and Sca-1 expression may converge on common downstream signaling events and/or that Sca-1 can compensate for the lack of PD-L1 availability.

CXCL1 secretion parallels the late omental recruitment of neutrophils elicited by Ba-wt LPS

CXCL1 is one of the major chemokines involved in neutrophil mobilization from the bone marrow to the blood and tissues, and in neutrophil aggregation in the omentum by mesothelial cells upon zymozan exposure¹². To investigate if this molecule contributes to the persistent cell recruitment observed in our settings, we first analyzed

by ELISA the levels of CXCL1 in the serum, peritoneal fluid and omentum harvested at 48 h or 72 h after injection of mice with PBS or Ba-wt LPS (Fig. 5a). CXCL1 levels were upregulated in the serum at 48 h post-LPS stimulation only, unchanged in the peritoneal fluid independently of the time point, and elevated in the omentum at both 48 h and 72 h post-LPS administration. Although total CXCL1 concentration at 48 h post-LPS injection was higher in the serum, the fold induction at 48 h or 72 h versus the PBS condition was 3.1 and 3.2, whereas in the omentum it reached 5.6 and 6.6, respectively (Fig. 5a). This indicates a higher and stable CXCL1 secretion in the omentum from 48 h onward. When comparing by confocal microscopy CXCL1 secretion in the omentum at 4 h and 72 h after Ba-wt LPS or PBS injection, CXCL1⁺ cells were detected in Ba LPS challenged omentum only at 72 h, with no

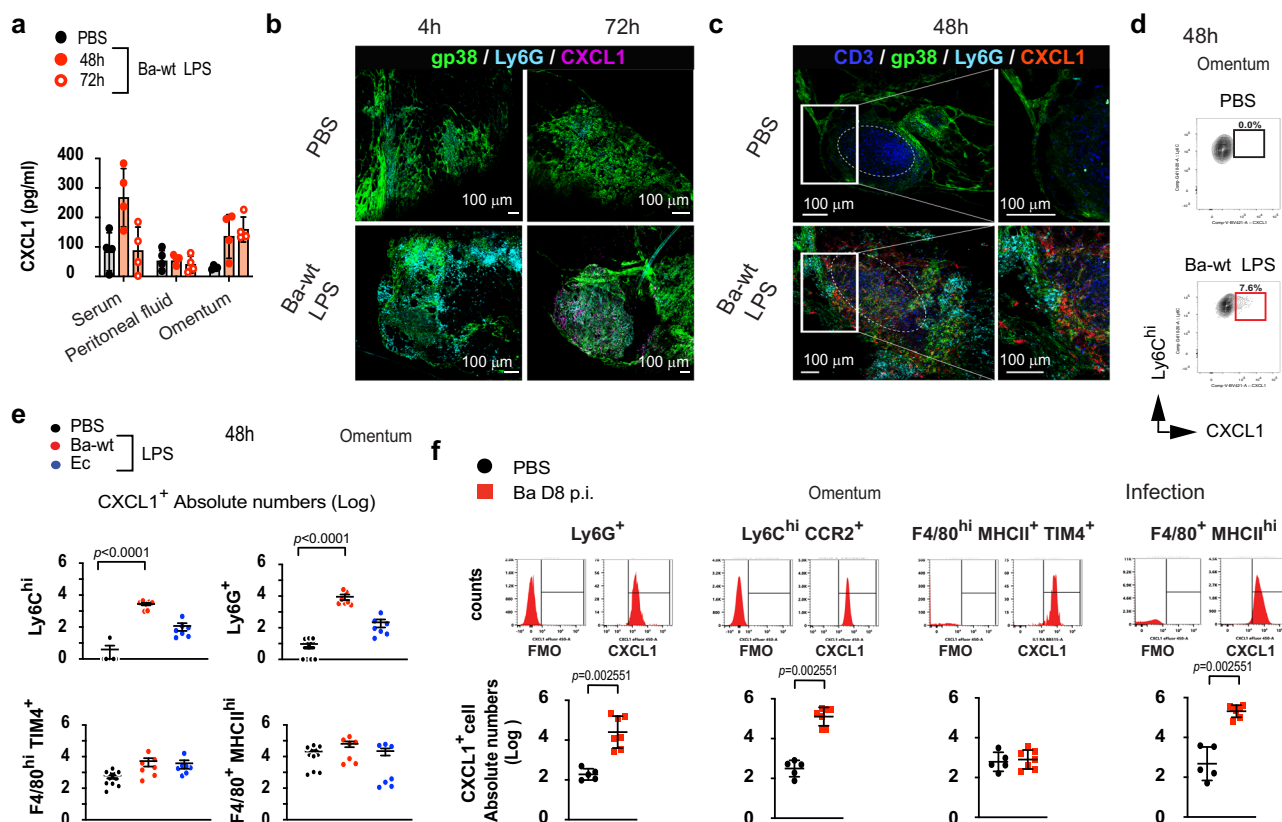


Fig. 5 | CXCL1 secretion in omentum coincides with the late Ba-wt LPS-driven omental accretion of neutrophils and occurs during *B. abortus* infection.

a Serum, peritoneal fluid, and omentum were harvested from C57BL/6 mice at 48 h (filled circles) or 72 h (empty circles) after i.p. injection with Ba-wt LPS (red circles) or PBS (black circles). CXCL1 was measured by ELISA. Mean of pooled data from normalized absolute number concentrations $\pm$ SD. 2 mice per group, $n = 2$.

b Confocal microscopy images of whole mount staining of omentum harvested at 4 h and 72 h after Ba-wt LPS or PBS injection of C57BL/6 mice. CXCL1 (magenta), gp38 (green), Ly6G (cyan). Scale bar 100 μ m. 3 mice per group, $n = 3$. **c** Confocal microscopy images of wholemount staining of omentum from Ba-wt LPS- or PBS-injected C57BL/6 mice at 48 h post-stimulation. CD3 (blue), gp38 (green), CXCL1 (red), Ly6G (cyan). Dotted circles indicate the contour of the milky spot (MS). Scale bar 100 μ m. 2–3 mice per group, $n = 3$. **d** Flow cytometry plots of Ly6Chi monocytes secreting CXCL1+ from omentum harvested at 48 h post-administration of C57BL/6 mice with

Ba-wt LPS or PBS (PBS, pooled cells from 6 mice). **e** Flow cytometry of indicated cells, analyzed as in (d) for CXCL1 positivity, from omentum harvested at 48 h post-injection of C57BL/6 mice with Ba-wt or Ec LPS, or PBS. Each symbol represents one animal. Mean from pooled data of normalized absolute numbers of CXCL1+ cells $\pm$ SD. 3–4 mice per group, $n = 2$. Significant differences are shown (Multiple comparison Kruskal-Wallis test, followed by post-hoc Dunn's test). Absence of p -value, non-significant. **f** Spectral flow analysis of Ba-infected (red) omentum at Day 8 p.i. showing representative CXCL1 expression profiles of the myeloid cells indicated, together with their fluorescence minus one (FMO) control (top), and normalized absolute numbers of these omental CXCL1+ Ba-infected and PBS control (black) cells (bottom). Each symbol represents one animal. 1–2 mice per group, $n = 4$. Mean $\pm$ SD. Significant differences are shown (Mann-Whitney test, two-sided). Absence of p -value, non-significant. Source data are provided as a Source Data file.

colocalization with mesothelial cells (gp38), while Ly6G+ neutrophils were visible at both time-points (Fig. 5b). The high influx of neutrophils triggered by Ba-wt LPS at 48 h post-Ba-wt LPS stimulation (Fig. 5c) occurred in the vicinity of the enlarged milky spot (MS) defined by CD3+ T cells with a nearby production of CXCL1.

To define which omental myeloid cells might account for the secretion of CXCL1, omentum harvested at 48 h post-injection with Ba-wt LPS, Ec LPS, or PBS was analyzed by flow cytometry. Intracellular staining for CXCL1 disclosed a significant production of CXCL1 by Ly6Chi monocytes and Ly6G+ neutrophils, with a negligible contribution of omental F4/80hiTIM4+ or F4/80+MHCIIhi macrophages (Fig. 5d, e). Omental cells from Ec LPS-treated mice barely produced CXCL1, explaining the low influx of neutrophils observed at 48 h after Ec LPS exposure (Fig. 4a). Upon *B. abortus* infection, CXCL1 was also secreted by Ly6G+ neutrophils, Ly6ChiCCR2+ monocytes as well as by F4/80+MHCIIhi SPM, in contrast to Ba-wt LPS stimulation for the latter, as shown by spectral flow analysis of infected omentum (Fig. 5f). Overall, the delayed CXCL1 production by omental monocytes and neutrophils correlates with the late omental retention of neutrophils upon Ba-wt LPS challenge, while upon infection beside neutrophils and monocytes CXCL1 is also produced by SPM.

PD-L1+Sca-1+ neutrophils restrain T cell lymphocyte responsiveness

To verify that the PD-L1+Sca-1+ cells generated by *B. abortus* infection were immunosuppressive, neutrophils were isolated from the peritoneal fluid of Ba-wt LPS injected mice or from the bone marrow of a mock PBS-injected mouse. Neutrophils were then cocultured with PMA/ionomycin-stimulated fluorescently labeled T cells for 72 h before flow cytometry analysis for cell proliferation and activation marker expression, IL-7-treated T cells serving as a negative control of proliferation of surviving T cells (Fig. 6 and Supplementary Fig. S4a). Compared to the PMA/ionomycin-stimulated T cells alone, decreased numbers of proliferative T cells were observed in a dose-dependent manner in T cell/neutrophil cocultures, when T cells were in the presence of neutrophils isolated from the peritoneal fluid of Ba-wt LPS challenged mice only (Fig. 6a, b and Supplementary S4b, c). These neutrophils expressed the suppressive markers PD-L1 and Sca-1 (Fig. 6a, left bottom). Moreover, inhibition of proliferation was accompanied by a significant reduction of the MFI of the activation markers, CD69 and CD25 at the surface of CD4+ and CD8+ T cells (Fig. 6b and Supplementary S4c). This ability to suppress T cell responses is shared by both Ba-wt and Ba-wadC

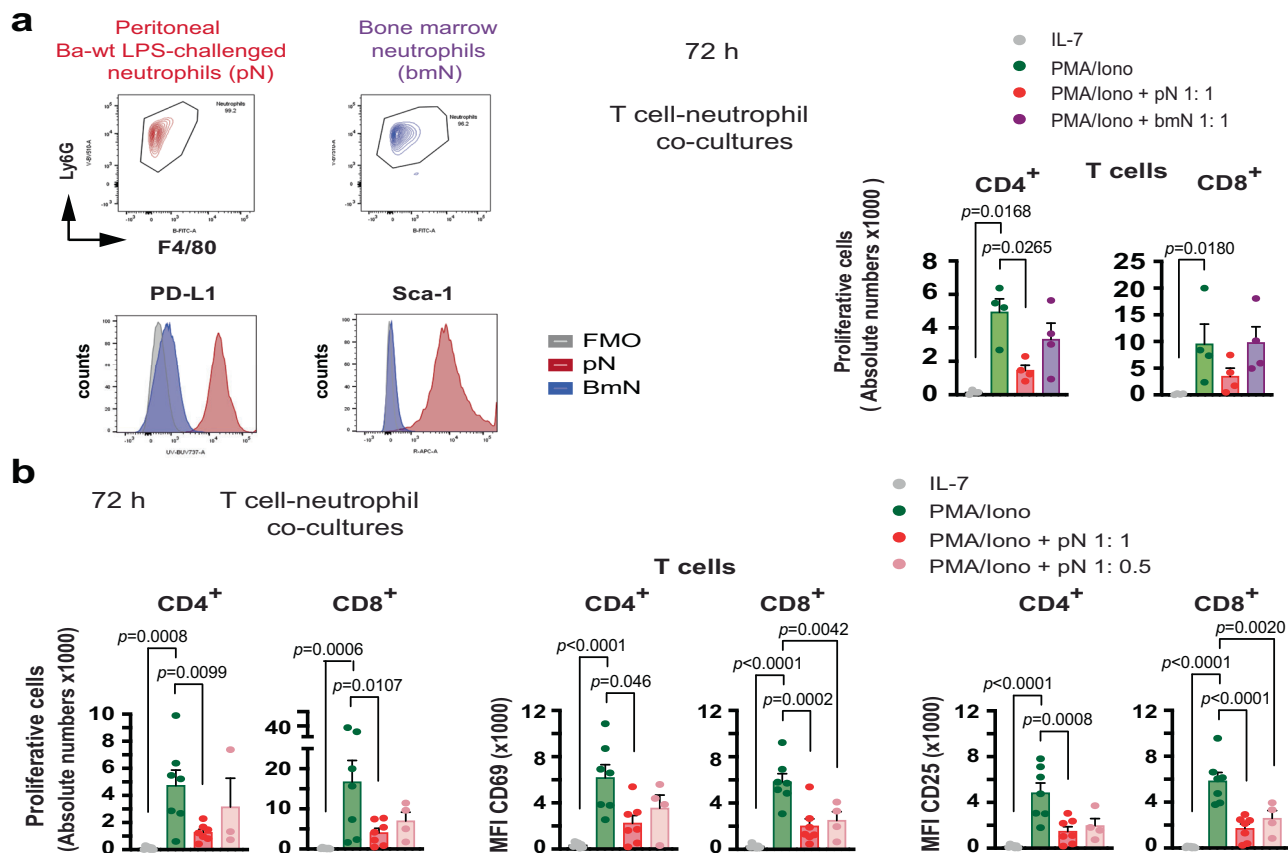


Fig. 6 | Ba-wt LPS-elicited PD-L1⁺Sca-1⁺ peritoneal neutrophils are immuno-suppressive. **a** Dot plots showing the high purity of neutrophils (left top) and histograms showing expression of PD-L1 and Sca-1 in neutrophils purified from the bone marrow of naïve C57BL/6 mice (bmN, violet) or from the peritoneal fluid exudate of 48 h Ba-wt LPS-challenged mice (pN, magenta) (left bottom), and absolute numbers of proliferative cells in PMA/ionomycin-stimulated CTV-fluorescently labeled naïve T cells (green) cocultured at a 1:1 ratio for 72 h with Ba-wt LPS-challenged peritoneal neutrophils (pN, red) or with bone marrow neutrophils from naïve C57BL/6 mice (bmN, parma violet) (right). IL-7 stimulation ensures T cell viability in culture in the absence of a proliferative signal. Each symbol represents the mean of two independent culture wells, $n = 4$. Mean $\pm$ SD. Significant differences from PMA/ionomycin-stimulated conditions are shown.

One-way ANOVA followed by Dunnett's multiple comparison test ($F = 15.80$, $DF = 15$ CD4⁺; $F = 10.48$, $DF = 15$ CD8⁺). Absence of p -value, non-significant. **b** Absolute numbers of proliferative cells (left) and MFI of CD69 (middle) and CD25 (right) in PMA/ionomycin-stimulated CTV-fluorescently labeled naïve T cells (green) cocultured at 1:1 (red) or 1:0.5 (pink) ratios for 72 h with Ba-wt LPS-challenged neutrophils (pN). Each symbol represents the mean of three independent culture wells, $n = 7$, but ratio 1:0.5, $n = 4$. Mean $\pm$ SD. Significant differences from PMA/ionomycin are shown (One-way ANOVA followed by Dunnett's multiple comparison test; Proliferation: $F = 7.628$ CD4⁺, $F = 7.150$ CD8⁺ cells; MFI CD69: $F = 9.966$ CD4⁺, $F = 17.28$ CD8⁺ cells; MFI CD25: $F = 13.03$ CD4⁺, $F = 23.89$ CD8⁺ cells; for all $DF = 21$ but CD4⁺ T cell proliferation $DF = 20$). Absence of p -value, non-significant. Source data are provided as a Source Data file.

challenged omental neutrophils (Fig. 7a). Hindrance of T cell responsiveness operated also at the T cell receptor (TCR) engagement level (Supplementary Fig. S4d–f). We next asked whether the interaction of PD-L1 at the surface of neutrophils with PD-1 on T cells⁴³ was involved in this blockade. When Ba-wt LPS-challenged peritoneal neutrophils were incubated with neutralizing anti-PD-L1 antibodies for 15 min prior to their addition to stimulated T cells, CD4⁺ and CD8⁺ T responses triggered by PMA/ionomycin or anti-CD3/anti-CD28 antibodies remained similar to levels observed with isotype control-treated cells (Supplementary Fig. S3c, d). Reduced PD-L1 and higher Sca-1 levels were found in neutrophils after 24 h of culture (Supplementary Fig. S3e), confirming the efficacy of PD-L1 neutralization and, again a compensatory overexpression of Sca-1 when PD-L1 is missing. Thus, the PD-L1/PD-1 axis is insufficient to generate T-cell suppression, suggesting that another mechanism operates. Indeed, the inhibition of CD4⁺ and CD8⁺ T cell proliferation and activation does not require direct contact between T cells and Ba-wt LPS-challenged neutrophils, as revealed by the persistence of the inhibitory effect in transwell assays (Fig. 7b and Supplementary Fig. S4g). This points out the possible involvement of a soluble factor.

IL-1RA secreted by PD-L1⁺Sca-1⁺ neutrophils lessens the response of T cell lymphocytes

The levels of cytokines and chemokines were quantified in the sera from the patients and healthy donors analyzed in Fig. 2c and submitted to sparse Partial Least Squares analysis. Heat map analysis revealed that each group could be differentiated by its serum chemokine/cytokine profile (Fig. 8a). In particular, acute patients, who subsequently responded well to treatment, were differentiated by comparatively superior levels of IL-18, BDNF, CXCL1, FGF-2, IL-2, CXCL8, TNF- β , HGF, CCL3, IL-4, VEGF-A, and IL-7. Acute patients who experienced relapse showed comparatively higher CXCL10, IFN- γ , IL-10, TNF- α , VEGF-D, IL-1RA, IL-31, SCF, PDGF-BB, IL-9, PIGF-1, IL-15, CCL4, IL-6, β -NGF, IL-5, IL-22, IL-13 and CXCL12. By contrast, chronic patients were characterized by comparatively greater levels of GM-CSF, IL-1 β , IL-23, IL-1 α , IL-27, IL-17A, CCL5, IFN- α , and IL-21. Interestingly, moderate levels of CXCL1, BDNF, IL-31, FGF-2, IL-2, IL-9, CXCL8, PIGF-1, IL-5, IL-13, and CXCL12 were maintained in the sera of patients in chronic phase. IL-1RA levels were always higher in the brucellosis-infected groups than in healthy donor controls.

Since IL-1RA acts as a marker of brucellosis in humans and IL-1RA is able to inhibit IL-1-enhanced T cell proliferation elicited by PHA⁴⁴, we

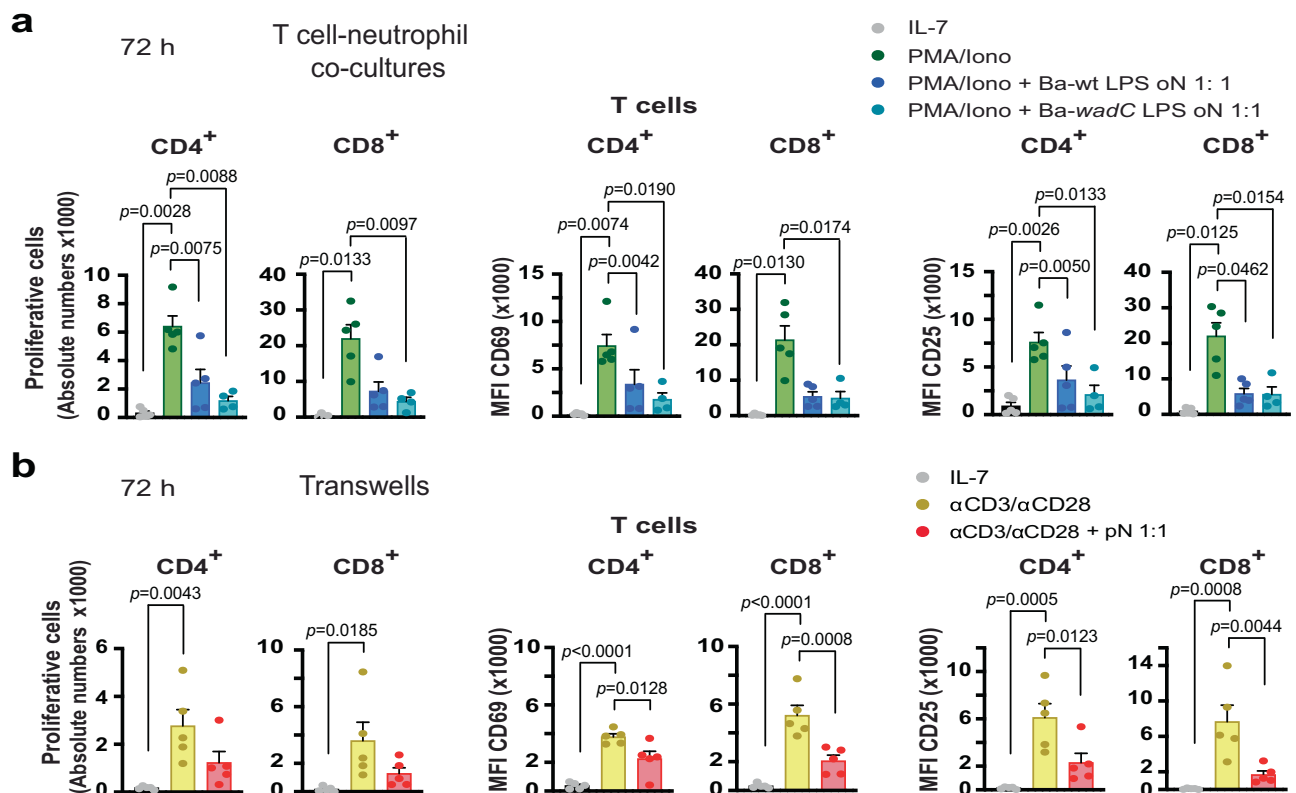


Fig. 7 | Ba LPS-elicited PD-L1⁺Sca-1⁺ omental neutrophils are immunosuppressive. **a** Absolute numbers of proliferative cells (left) and MFI of CD69 (middle) and CD25 (right) in PMA/ionomycin-stimulated CTV-fluorescently labeled naïve T cells (green) cocultured at a 1:1 ratio for 72 h with Ba-wt (blue) or Ba-wadC (dark cyan) LPS-challenged omental neutrophils (oN). Each symbol represents the mean of two independent culture wells, $n = 5$ but Ba-wadC, $n = 4$. Mean $\pm$ SD. Significant differences from PMA/ionomycin condition are shown (Mixed-effects analysis followed by Dunnett's multiple comparison test). Absence of p -value, non-significant. **b** Absolute numbers of proliferative cells (left) and MFI of CD69 (middle) and CD25

(right) in anti-CD3/CD28-stimulated CTV-fluorescently labeled proliferative T cells (yellow) cultured for 72 h in a two-chamber system separately from the peritoneal 48 h Ba-wt LPS-challenged neutrophils (pN) (red) at a 1:1 ratio. Each symbol represents 1 culture well. Mean of absolute numbers $\pm$ SD. 1 well/condition, $n = 5$. Significant differences are shown (One-way ANOVA followed by Dunnett's multiple comparison test; Proliferation: $F = 7.488$ CD4⁺, $F = 4.847$ CD8⁺ cells; MFI CD69: $F = 26.44$ CD4⁺, $F = 28.28$ CD8⁺ cells; MFI CD25: $F = 13.41$ CD4⁺, $F = 13.16$ CD8⁺ cells; for all, DF = 12). Absence of p -value, non-significant. Source data are provided as a Source Data file.

postulated that IL-1RA may be the soluble factor accounting for the T cell inhibition elicited by neutrophils upon Ba LPS stimulation. We examined if IL-1RA was produced in vivo in the omentum after LPS injection (Fig. 8b) and in vitro in T cell-neutrophil co-cultures (Fig. 8c). In all cases, IL-1RA was detected at physiological levels⁴⁵. We then investigated if Ba LPS was the driver of IL-1RA expression and analyzed the numbers of peritoneal neutrophils producing IL-1RA in Ba-wt LPS-, Ec LPS- or mock-treated cells isolated at 48 h post-in vivo challenge (Fig. 8d). Significantly higher IL-1RA-producing cells were triggered by Ba-wt LPS compared to the numbers present in the mock or Ec LPS conditions (Fig. 8d, left). When focusing on the IL-1RA⁺Ly6G⁺ neutrophils, similar percentages of PD-L1⁺Sca-1⁺ neutrophils were generated by Ba-wt and Ec LPS (Fig. 8d, right). To ultimately prove the immunosuppressive role of IL-1RA on T cell responsiveness, we analyzed the proliferation and activation marker expression of fluorescently labeled T cells 72 h after TCR engagement by anti-CD3/CD28 antibody stimulation in the presence or absence of 10 ng/ml of recombinant IL-1RA (Fig. 8e, f). Proliferation and MFI levels of CD69 and CD25 of both CD4⁺ and CD8⁺ T cells were significantly impaired in the presence of the cytokine. Most importantly, during infection in the mouse, IL-1RA was detected in the omentum both at the acute and chronic stages (Fig. 9a) and produced by omental neutrophils, as well as monocytes and SPM at day 8 p.i. (Fig. 9b). The IL-1RA⁺Ly6G⁺ neutrophils were mostly PD-L1⁺Sca-1⁺ (Fig. 9c). Analysis of the correlation between each of the IL-1RA⁺ myeloid cell type with the total, PD-1⁺, LAG-3⁺ or LAG-3⁺PD-1⁺ CD4⁺ or CD8⁺ or CD4⁺CD62L⁺CD44⁺CD25⁺GITR⁺ T

regulatory (Treg)⁴⁶ T cells during *B. abortus* infection revealed that IL-1RA⁺Ly6G⁺ neutrophils positively associated with CD4⁺ and CD8⁺ expressing both PD-1 and LAG-3 suppressive markers (Fig. 9d and Supplementary Figs. S1f, S4h). IL-1RA⁺Ly6C⁺ monocytes were preferentially linked to PD-1⁺LAG-3⁺ CD4⁺ T cells and Treg, while no significant association was seen with IL-1RA⁺ macrophages.

Together, these results reveal that Ba-wt LPS governs a long-lasting omental accumulation of PD-L1⁺Sca-1⁺ neutrophils, stimulating them to produce IL-1RA, which in turn suppresses T cell expansion and activation during infection.

Discussion

The role of the omentum in establishing chronic infection and the underlying mechanisms remain unclear. Using *Brucella* as a stealthy intracellular bacterial paradigm and the mouse model, we demonstrate that *B. abortus* not only replicates in omental macrophages but remains in the omentum for protracted periods of time (up to 90 days), similarly to what happens in the spleen or lymph nodes, the main known reservoirs for chronic brucellosis. This colonization occurs even when mice are inoculated via gavage, a peritoneal distant site of inoculation and a more natural route⁴⁷, in two different genetic backgrounds, considered as somewhat resistant (C57BL/6) or sensitive (BALB/c) to *Brucella* infection, hence establishing a preferential tropism of *Brucella* for this organ. The ability of *B. abortus* to infect and persist in the omentum and its associated macrophages has never previously been described as a bacterial pathogen, even though other

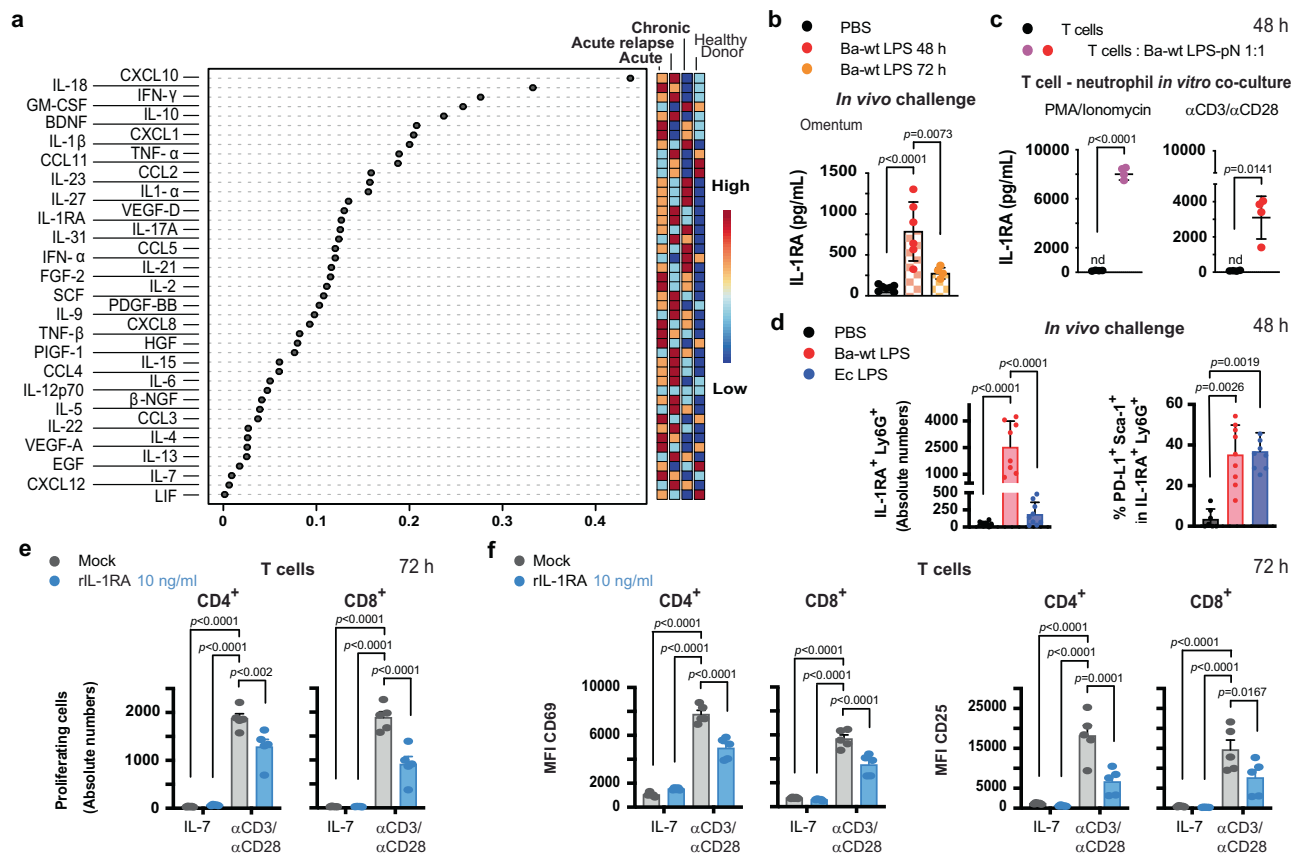


Fig. 8 | IL-IRA produced by Ba LPS-challenged PD-L1 $^{+}$ Sca-1 $^{+}$ neutrophils inhibits T cell responsiveness. **a** SPS-DA loading plots of cytokines/chemokines measured at admission in serum of acute, acute with relapse, and chronically infected brucellosis patients and healthy donors. Healthy donors $n = 20$; Brucellosis patients: Acute $n = 20$, Acute with relapse $n = 6$, Chronic $n = 5$. **b** IL-IRA ELISA values in omentum from C57BL/6 mice at 48 h (red) or 72 h (orange) after Ba-wt LPS or PBS (black) administration. Mean of pooled data $\pm$ SD. 1-3 mice/group, $n = 3$. Significant differences are shown (one-way ANOVA followed by Tukey's multiple comparison test; $F = 17.41$, $DF = 14$). No p -value, non-significant. **c** IL-IRA ELISA values, normalized to omentum, from PMA/ionomycin-stimulated (left, magenta) or anti-CD3/ α CD28-stimulated (right, red) T cells cocultured or not (black) with Ba-wt LPS-challenged peritoneal neutrophils (pN) at 1:1 ratio for 48 h. 1 well/condition, $n = 4$. nd, non-detected. Mean $\pm$ SD. Significant differences are shown (two-sided unpaired Welch's t test; PMA/ionomycin, $t = 33.86$, $DF = 3.012$; anti-CD3/anti-CD28, $t = 5.164$, $DF = 3.001$). **d** Absolute numbers of Ly6G $^{+}$ neutrophils secreting IL-IRA $^{+}$ (left) and % of PD-L1 $^{+}$ Sca-1 $^{+}$ among those (right), from peritoneal fluid exudates at

48 h post-challenge of C57BL/6 mice with Ba-wt (red) or Ec (blue) LPS, or PBS (black), and analyzed by flow cytometry. Each symbol represents one animal. Mean from pooled data $\pm$ SD. 2-3 mice/group, $n = 3$. Significant differences are shown (left, one-way ANOVA with Tukey's multiple comparison test, $F = 22.42$, $DF = 21$; right, multiple comparison Kruskal-Wallis test, followed by post-hoc Dunn's test). No p -value, non-significant. **e** Absolute numbers of proliferative cells in anti-CD3/ α CD28-stimulated CTV-fluorescently labeled T cells cultured for 72 h with (blue) or without (black) recombinant IL-1RA. Mean $\pm$ SD. 1-2 wells/condition, $n = 3$. Significant differences from mock-treated anti-CD3/ α CD28-stimulated cells are shown (One-way ANOVA followed by Dunnett's multiple comparison test; $CD4^{+}$ $F = 95.07$, $CD8^{+}$ $F = 76.77$; all $DF = 16$). **f** MFI of CD69 (left) and CD25 (right) in proliferative cells in (e). Mean $\pm$ SD. 1-2 wells/condition, $n = 3$. Significant differences from mock-treated anti-CD3/ α CD28-stimulated T cells are shown (One-way ANOVA followed by Dunnett's multiple comparison test; $CD69$: $CD4^{+}$ $F = 142.8$, $CD8^{+}$ $F = 91.25$; $CD25$: $CD4^{+}$ $F = 30.02$, $CD8^{+}$ $F = 18.83$; all $DF = 16$). Source data are provided as a Source Data file.

intracellular bacteria (like *M. tuberculosis*, *R. prowazekii*, *C. burnetii*), parasites (such as *P. berghei*, *T. cruzi* and *T. brucei*) or viruses (e.g., HIV, SIV) have been detected in adipose tissue or adipocytes^{16–19,48,49}.

As reported in different infectious contexts^{7,8,50}, peritoneal exposure to *B. abortus* triggers a series of events, including the recruitment and homing of myeloid cells to the omentum that increases the cellularity of milky spots (MS). The early omental influx of myeloid cells that we observed is coherent with the increased differentiation of hematopoietic stem cells toward the myeloid lineage triggered by the interaction of SLAMF1/CD150 with *B. abortus* outer membrane protein Omp25 during the acute phase of infection⁵¹. Its maintenance fits also perfectly with the augmented levels of GM-CSF, a major regulator of myeloid cell development and function⁵², that we detected in the serum of patients with chronic brucellosis. Moreover, the late persistence of CXCL1 that is specifically produced in the omentum upon *B. abortus* LPS exposure and upon infection suggests that as described after *E. coli* infection¹¹, neutrophils are directly mobilized from the blood through the high endothelial venules. Our identification of

CXCL1 as a serum marker of brucellosis further infers that an analogous omental accretion of neutrophils is likely to take place in humans, which would explain the neutropenia observed in one-third of patients⁵³. However, the early influx of neutrophils is CXCL1-independent, in contrast to the response to zymosan¹². This initial neutrophil mobilization mechanism does not involve the peculiar LPS core of *Brucella* and is partially shared with the Ec LPS, possibly involving eicosanoid leukotrienes⁵⁴, whose synthesis pathway is stimulated in brucellosis^{55,56}. Considering that infected macrophages/monocytes or neutrophils may undergo efferocytosis^{25,26,57}, and are spatially co-located around the MS with its high blood and lymphatic vessel density, we propose that the omentum functions as a reservoir and cell dissemination platform for intracellular pathogens like *B. abortus*, forming a central hub for the egress of bacteria engulfed cells from the omentum to the circulation, and vice versa.

Remarkably, myeloid cells accumulating in the peritoneal cavity during *B. abortus* infection are characterized by a suppressive PD-L1 $^{+}$ Sca-1 $^{+}$ phenotype. This accretion in the peritoneal fluid and

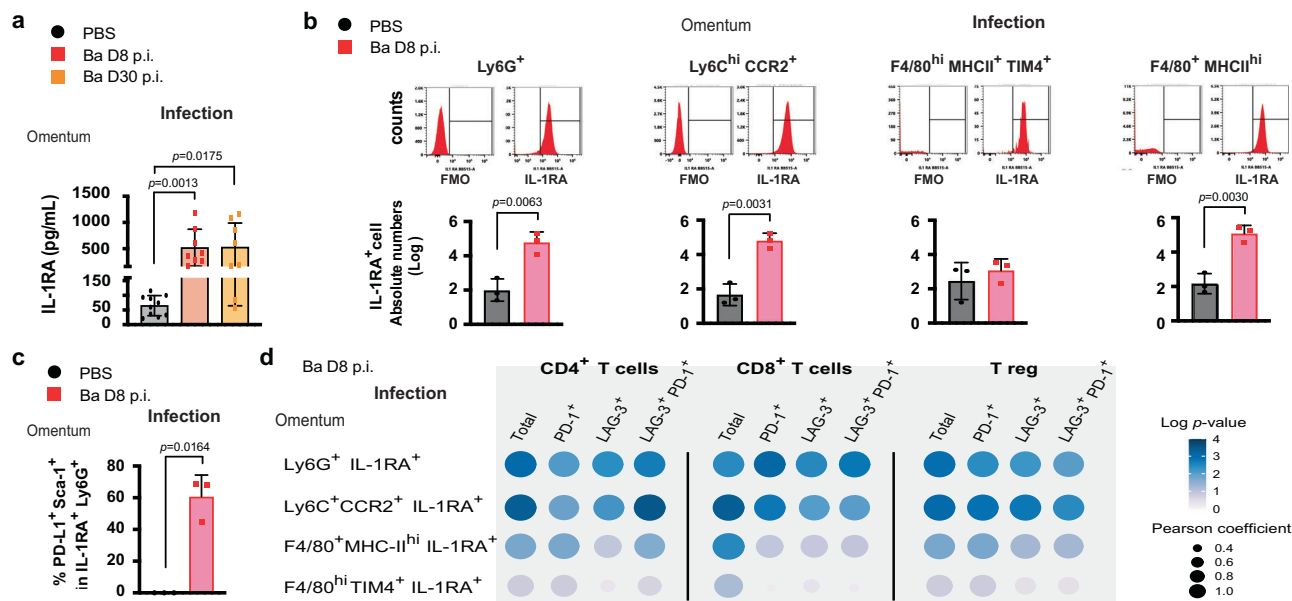


Fig. 9 | IL-1RA is produced in omentum until the chronic phase of infection and omental IL-1RA production by Ba-infected neutrophils correlates with a general T cell suppressive phenotype. **a** Omenta from C57BL/6 mice infected i.p. by *B. abortus* (Ba) were harvested at Day 8 (red squares) and 30 p.i. (orange squares) or mock-injected with PBS (black circles). IL-1RA was measured by ELISA. Each symbol represents one animal. Mean of pooled data $\pm$ SD. 2 mice per group, $n = 4$. Significant differences are shown (Kruskal-Wallis followed by Dunn's multiple comparison test). Absence of p -value, non-significant. **b** Spectral flow analysis of Ba-infected omenta showing IL-1RA expression profiles of the myeloid cells indicated at Day 8 p.i., together with their fluorescence minus one (FMO) control (top). Normalized absolute numbers of these omental IL-1RA⁺ Ba-infected (red) or PBS-

treated (black) cells at Day 8 p.i. (bottom). Each symbol represents one animal. 1 mouse, $n = 3$. Mean $\pm$ SD. Significant differences are shown (two-sided unpaired Welch's t test; neutrophils, $t = 5.287$, $df = 3.972$; monocytes, $t = 7.081$, $df = 3.589$; LPM, $t = 0.8302$, $df = 3.369$; SPM, $t = 6.668$, $df = 3.853$). **c** Percentages of PD-L1⁺Sca-1⁺ cells among the IL-1RA⁺Ly6G⁺ Ba-infected cells in (b). Each symbol represents one animal. Mean $\pm$ SD. 1 mouse per group, $n = 3$. Significant differences are shown (Welch's t test; $t = 7.721$, $df = 2$). **d** Dot plots represent the correlation between each of the IL-1RA⁺ myeloid cell types (p -value, color) with the absolute numbers of total, PD-1⁺, LAG-3⁺ or LAG-3⁺PD-1⁺ CD4⁺ or CD8⁺ or Treg T cells (Pearson correlation coefficient, size). Two-sided Pearson correlation test, no adjustment. Color and size scales are indicated on the right. Source data are provided as a Source Data file.

omentum at late time points after challenge is mediated by a *Brucella* wt LPS core moiety absent in the LPS purified from the *wadC* mutant, which presents a congruous attenuation in omentum upon infection. PD-L1 or Sca-1 upregulation has been implicated in CD4⁺ T cell immune suppression by *Burkholderia pseudomallei*-infected neutrophils⁵⁸ for the former and in acquisition of a suppressive regulatory phenotype by *T. gondii*³⁰ or gammaherpesvirus-infected³¹ monocytes for the latter. We now show that PD-L1⁺Sca-1⁺ macrophages, monocytes, and neutrophils accumulate in the omentum and peritoneal fluid in a Ba-wt LPS-dependent fashion. During *B. abortus* infection in the mouse, most of the PD-L1⁺Sca-1⁺ myeloid cells were positive for LAG-3. LAG-3 is a well-known T cell inhibitory receptor, which in myeloid cells has been so far found expressed only on human plasmacytoid dendritic cells from melanoma patients³⁹. We bring here direct evidence for an upregulation of LAG-3 during *B. abortus* infection in multiple myeloid cells and neutrophils in particular. Future work will bring clues as to the physiological relevance and function of LAG-3 expression in these cells. In accordance with these murine data, we found that the expression of *CD274/PDL1*, *LAG3*, and *Ly6E*, a putative human homolog of Sca-1, is upregulated in whole blood from acute phase brucellosis patients. This suggests that both the PD-1/PD-L1 axis and Sca-1, as well as LAG-3 play a role during natural *Brucella* infection in humans. PD-1 upregulation has been indeed detected at the surface of blood CD4⁺ and CD8⁺ T cells from acute and chronic phase brucellosis patients⁵⁹. Blocking PD-L1-PD-1 interaction did not increase *B. abortus* clearance or modify the influx of myeloid cells to the omentum, indicating that compensatory mechanisms e.g., a putative LPS-driven overexpression of Sca-1 may operate. In the mouse, during chronic *B. melitensis* infection, IFN- γ -blunted CD8⁺ T cells overexpressing PD-1 and LAG-3 have been described⁶⁰. *Brucella* LPS perdures at the surface of peritoneal macrophages for months, long after its disappearance in the

peritoneal fluid, remaining associated in macrodomains with MHC class II molecules⁶¹, thus reducing their antigen presentation ability to CD4⁺ T cells, which are consequently less activated²⁴. PD-L1 expression at the surface of macrophages may further provide a constitutive negative signal as shown in tumor-associated macrophages⁶² or during GHV infection⁶³.

Our data establish PD-L1⁺Sca-1⁺ neutrophils as critical determinants of T cell immunosuppression, impairing both the proliferation and activation of CD4⁺ and CD8⁺ T cells stimulated at the TCR level or downstream. These findings are supported by the enhanced *B. abortus* clearance and T cell activation at late time points of infection in murine experimental models devoid of neutrophils²⁷. Of note, PD-L1⁺ neutrophils with the ability to suppress CD8⁺ T cell functions have been described hitherto in viral or parasitic infectious contexts only^{64–66}. In the case of bacterial infections, CD11b⁺GRI⁺Sca-1⁺ myeloid cells produce more IFN- γ in the peritoneal fluid during infection by *S. aureus* and increase mortality upon transfer⁶⁷. The balance between Sca-1 and PD-L1 expression may be a way to instruct regulatory neutrophils toward a suppressive phenotype. IL-1RA is upregulated in the serum of brucellosis patients (this study) and genetic polymorphism in its gene, *IL1RN*, has been linked to human susceptibility to brucellosis⁶⁸. Upon Ba LPS exposure and during both the acute and chronic stages of infection in the mouse, omentum produces IL-1RA at comparable levels, in the range of those secreted by in vivo LPS-challenged peritoneal neutrophils cultured in vitro for two days. Consistently, recombinant IL-1RA leads to CD4⁺ and CD8⁺ T cell hypo-responsiveness in vitro. During *B. abortus* infection, the positive association of IL-1RA-producing neutrophils with CD4⁺ and CD8⁺ T cells expressing both PD-1 and LAG-3 suggests that this T cell immunosuppressive mechanism is definitely acting. Yet, IL-1RA is additionally produced by monocytes; the privileged link of IL-1RA-producing monocytes with PD-1⁺LAG-3⁺

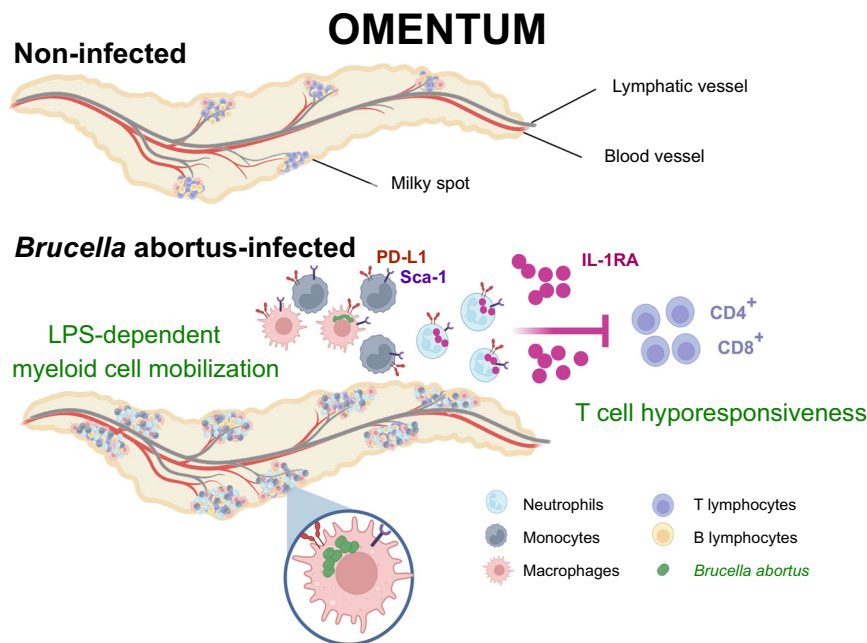


Fig. 10 | Model for a new function of omental neutrophil-secreted IL-1RA in limiting T cell responsiveness during the chronic stage of *B. abortus* infection. Upon *B. abortus* infection, the bacteria replicate in omental macrophages and persist until the chronic stage in the omentum, identified as a pathogenic reservoir. *B. abortus* mobilizes macrophages, monocytes, and neutrophils to the peritoneal cavity and the omentum, nearby milky spots. These cells harbor an immunosuppressive phenotype, characterized by the co-expression of PD-L1 and Sca-1 that

remains from the acute to the chronic phase of infection and is driven by *B. abortus* LPS. Maintenance of these immunosuppressive cells in the omentum depends on the *wadC*-encoded determinant of *Brucella* LPS. IL-1RA, which is notably secreted by PD-L1⁺Sca-1⁺ omental neutrophils and impairs CD4⁺ and CD8⁺ T cell responsiveness, is produced until the chronic phase of infection in the omentum, most likely favoring bacterial persistence. The illustration was created by Biorender.com.

CD4⁺ T cells and Treg during *B. abortus* infection opens up a complementary and specific role for these cells in immunosuppression, which needs future investigations. It remains that herein we provide the first demonstration of a T cell suppressive role of IL-1RA produced by neutrophils. Human neutrophils secrete IL-1RA in response to GM-CSF or TNF⁶⁹ or upon efferocytosis when infected by *Yersinia pestis*⁷⁰. This suggests that this inhibitory mechanism may represent a general subversion strategy in humans for intracellular bacterial pathogens that can stay dormant and relapse for long periods of time, like *Brucella*, *M. tuberculosis*, *R. prowazekii*, *C. burnetii*, or *Francisella tularensis*.

In conclusion, we have unraveled an unexpected evasion strategy employed by *B. abortus*, which through triggering a sustained local recruitment of PD-L1⁺Sca-1⁺ suppressive neutrophils secreting IL-1RA impairs T cell responsiveness and most likely favors bacterial persistence in host reservoirs such as the omentum during chronic stages of infection. A similar *modus operandi*, presented in Fig. 10, may exist for brucellosis in humans and natural hosts as well as for other intracellular bacterial infections using phagocytes as a replicative niche and/or the omentum as a reservoir. Targeting PD-L1 and Sca-1, and/or IL-1RA and/or omentum may provide promising therapeutic avenues for the treatment of chronic bacterial infections and eradicate the pathogens hidden in permissive niches.

Methods

Ethics

This study complies with all relevant ethical regulations. Animal experimentation (authorization APAFIS 32624) was conducted in strict compliance with good animal practice as defined by the French animal welfare bodies (Law 87-848, Decree 2001-464 and Decree 2001-131 relative to European Convention, EEC Directive 86/609/EC). Authorization of *Brucella* experimentation in the Center d'Immunophénomique (CIPHE) BSL3 facility, Marseille, was given under the numbers: AMO-076712016-5, AMO076712016-6, and AMO-

076712016-7, at the Universidad de Navarra BSL3, Pamplona, under the number CEEA-R077-20, by the Gobierno de Navarra following the Spanish (RD 53/2013) legislations, and at the Université de Namur BSL3, Namur, under the number UNLE-23/399 by The Animal Welfare Committee of the Université de Namur. Animal experimentations are supervised by the Ethical and Animal Welfare Committee of the institutions. All efforts were made to minimize suffering during animal handling and experimentation. The study in humans was approved by the Ethics Committee of the Medical Faculty in Skopje, Republic of North Macedonia (authorization 03-7670/2). Laboratory data were acquired during routine diagnostic procedures, and written consent was obtained from all the patients and healthy donors enrolled.

Mice

Female mice aged 7–10 weeks were obtained from Janvier Laboratories (C57BL/6J and BALB/c). Animals were housed in the CIML animal house facility (France) and in the animal facility of the Gosselies campus of the Université Libre de Bruxelles (Belgium) in ventilated cages with water and food ad libitum under specific pathogen-free conditions at an ambient temperature of 22 °C with a 12 h light/dark cycle and 30–70% (55% average) humidity. Infection experiments were mostly carried out in the BSL3 at the CIPHE animal house facility in Marseille. Two weeks before the start of experiments, mice were transferred to the BSL3 and kept under strict biosafety containment conditions all along with infection with live bacteria.

Bacterial strains

Brucella abortus smooth virulent 2308 strain and a derived transgenic DsRed-expressing strain were used⁴⁷. All *Brucellae* were kept, grown, and used under strict biosafety containment conditions throughout the experiments in the BSL3 facility. *B. abortus* strains were grown on

tryptic soy agar plates, and 50 µg/mL kanamycin for the DsRed strain. For infection, strains were grown for 16 h at 37 °C under shaking in tryptic soy broth until OD at 600 nm reached 1.

Mouse infection and pharmacological treatments

Mice were inoculated intraperitoneally with 1×10^6 CFU of the *B. abortus* chosen strain or with 1×10^9 CFU for gavage infection⁴⁷. When specified, mice were injected i.p. with 200 µg/mL of neutralizing anti-PD-L1 (BioXcell, clone 10 F.9G2) or isotype control (BioXcel, LTF2) antibodies at the indicated time points right after infection (or i.v. at the indicated time points upon Ba-wt LPS challenge). At the indicated times after infection, the peritoneal cavity was opened under aseptic conditions. The peritoneal fluid exudate was harvested by injection of 4 mL sterile PBS, followed by a gentle massage of the peritoneum to dislodge any attached cells and fluid aspiration, and centrifuged 10 min at 400 G at 4 °C. Omentum and spleen were then harvested and weighted. Peritoneal fluid exudate pelleted cells, omentum, and spleen were incubated for 1 h in 50 µg/mL gentamicin (Sigma Aldrich, G1397) and washed 3 times with sterile PBS to remove extracellular bacteria. For colony forming unit (CFU) enumeration, organs were dissociated into sterile PBS by crushing with a syringe plunger in a 70 µm nylon cell strainer. Omental cells were recovered by centrifugation, and half of the spleen was used. Serial dilutions in sterile PBS were plated in triplicates onto TSB agar and incubated for 3 days at 37 °C. The second half of the spleen was processed for cell population determination by flow cytometry. When CFU was found equal to zero, value 1 was used arbitrarily to detect the individual point on a graph with a Log₁₀ scale.

Lipopolysaccharides

LPS from Ba-wt, *Ba-wadC*, and *E. coli* ATCC 35218 (Ec) were purified as described²¹. All preparations were above 98% purity and devoid of contaminant proteins, free lipids, nucleic acids, and cyclic glucans. A stock of 2 mg/mL of each LPS was prepared in pyrogen-free sterile water, sonicated briefly, and sterilized by autoclaving. The molar mass ratio of the different LPS (Ec LPS: Ba-wt LPS: *Ba-wadC* = 1:3:2) was calculated based on the KDO content⁷¹. Equal molarity of LPS was intraperitoneally injected in mice, with a dosage of 10 µg, 30 µg or 20 µg per mouse for Ec, Ba-wt, and *Ba-wadC* LPS, respectively.

Cytometry

Omentum was digested for 8 min at 37 °C with agitation in RPMI, 2 µg/mL collagenase type II (Worthington Biochemical Corporation, LS004174), 0.2 µg/mL DNase I (Sigma, DN25100MG). Digestion was stopped in RPMI, 10 mM EDTA, and 2% Bovine Serum Albumin (BSA). Pieces of omentum were then minced with scissors, crushed with a syringe plunger on a 70 µm nylon cell strainer, and filtered in the same buffer. Total single-cell suspensions finally proceeded for FACS analysis. For peritoneal fluid exudates, suspensions were prepared as mentioned above by injecting 4 mL of cold PBS into the peritoneal cavity, and red blood cells were removed with the red blood cell (RBC) lysis buffer (eBioscience, 00-4333-57). Cell suspensions were spined at 400 G at 4 °C and pelleted cells proceeded to FACS analysis. Spleen tissue was cut into small pieces and incubated in gentleMACS with a mixture of 500 µL RPMI, 2 µg/mL type II collagenase, 2 µg/mL DNase I for 20 min at 20 °C and 10 mM EDTA was added to stop digestion. Splenic cell suspensions were filtered on a 70 µm nylon cell strainer, washed once with RPMI, 5% fetal bovine serum (FBS, PAN Biotech, P30-3033), and red blood cells were removed with the RBC lysis buffer in FACS buffer (PBS, EDTA 2 mM, FBS 2%) to a cell concentration of 2×10^6 for 100 µL before staining. Absolute numbers for each population were obtained using beads (eBioscience, BD556296). Generally, cells were preincubated on ice for 10 min with the 2.4G2 hybridoma supernatant to block Fc receptors and stained for surface markers for 30 min at 4 °C. Cell viability was evaluated using LiveDead Fixable Blue Dead Cell Stain Kit (Life Technologies, L34961) for 15 min at 4 °C. When needed,

cells were fixed for 15 min in 2% PFA at 20 °C. Antibodies for the staining mixes are listed in Supplementary data 1. For intracellular staining, isolated cells or omentum were incubated for 3 h in the presence of 10 µg/mL Brefeldin A (Sigma, B7651-5MG) and then BD Cytofix/Cytoperm solution (BD Biosciences, 554722) was used according to the manufacturer's instructions. Multiparameter flow cytometry up to 14 channels was performed using a FACS Fortessa (BD Biosciences), and data were analyzed with the FlowJo v10 software. Multiparameter spectral cytometry with 45 channels was performed using an Aurora machine (Cytek) and data were analyzed with the SpectroFlo software (Cytek).

Cytokine/chemokine dosage

For mouse studies, peritoneal fluid exudates were spined at 400 G at 4 °C, and supernatants were put at −80 °C. Murine-isolated omenta were digested for 30 min at 4 °C with agitation in PBS, 0.5% Triton X-100, 0.5% NP-40, and protease inhibitors; after centrifugation at 9300 G for 20 min, omental supernatants were put at −80 °C. The dosage of murine CXCL1 (Cusabio, CSBE17286m) or IL-1RA (ThermoFisher, EMIL1RN) was done by ELISA following the manufacturer's instructions. Murine CXCL1 levels were also measured in serum, and those of IL-1RA in T cell-neutrophil coculture supernatants after 2 days of culture. For comparison purposes, all values were normalized to a 2 mL final volume.

For human studies, cytokine/chemokine concentrations were determined at the initial visit in serum from acute, acute with relapse, chronically infected patients, and healthy donor control groups by Luminex using the Human Cytokine/Chemokine/Growth Factor 45-Plex ProcartaPlex Panel 1 (ThermoFisher, EPX450-12171-901) according to supplier's recommendations. The resulting data were normalized by Log₁₀ transformation and Pareto (mean-centered and divided by the square root of the standard deviation for that variable), and then analyzed by sPLS-DA (sparse Partial Least Squares Discriminant Analysis) algorithm to produce an easy-to-interpret model that can discriminate groups and rank the chemokine/cytokine variables by the absolute values of their loadings that were used to define the latent components. sPLS-DA and supporting heat map analysis was carried out using the MetaboAnalyst platform version 5.0. For this cytokine/chemokine analysis, number and features of patients enrolled in each clinical group are the following: Acute treated, $n = 20$ (15.78% women; age range 4–64 years with a median age of 42); Acute with relapse, $n = 6$ (16.66% women; age range 17–59 years with a median age of 39); Chronic, $n = 5$ (40% women; age range 28–56 years with a median age of 42); Healthy donor, $n = 20$ (with similar median age and sex ratio).

Immunostaining

Omenta were harvested and fixed immediately with Antigenfix (Diatech, P0016) for 25 min at 4 °C, and washed in PBS. For cytokine/chemokine in situ visualization, whole omenta were first incubated in RPMI, 5% FBS, 10 µg/mL Brefeldin A for 3 h at 37 °C, then fixed with Antigenfix. After permeabilization and blocking of non-specific sites with 0.5% saponin, 2% BSA, 1% FBS, and 1% donkey or goat serum for 2 h, the whole organs were labeled for 24 h–48 h at 4 °C with primary antibodies (Supplementary Data 1) followed by incubation for 2 h at 20 °C with secondary antibodies. Whole organs were mounted in ProlongGold (Invitrogen, P10144). Images were acquired on a Confocal laser scanning microscopy Zeiss Fastairyscan LSM880. Images of 2048 × 2048 pixels were then assembled using Adobe Photoshop 2024. 3D reconstruction of the confocal image was done on Imaris.

Isolation of T cells and Neutrophils

Peritoneal or omental neutrophils were recovered from respectively the peritoneal fluid exudates or omentum of C57BL/6J mice at 48 h post-Ba-wt or post-Ba-wadC LPS in vivo challenge, harvested and prepared as described above; cell suspensions were washed twice in

PBS at 4 °C. Bone marrow neutrophils (Bm neutrophils) were obtained from the femurs and tibias of C57BL/6J naïve mice. Briefly, bone ends were cut off, and bone marrow was flushed with RPMI, 5% FBS, and 50 μ M 2-mercaptoethanol. From both cell suspensions, red blood cells were removed as described above. Afterward, neutrophils were purified by negative magnetic isolation using the mouse neutrophil Isolation kit (Miltenyi Biotec, 30-097658) plus the anti-F4/80 MicroBeads UltraPure (15 μ L per mix, Miltenyi Biotec, 130-110443), according to the manufacturer's instructions.

T cells were extracted from the spleens of naïve C57BL/6J mice and isolated using the Dynabead Untouched Mouse T Cells Kit (Invitrogen Life Technologies, 11413D) according to the manufacturer's protocol. Cells were resuspended in RPMI, 10% heat-inactivated FBS, and 50 μ M 2-mercaptoethanol, further referred to as a complete medium. Proper isolation was validated by staining the resulting cell suspensions with anti-CD11b, anti-CD3, anti-CD5, anti-Ly6G, anti-F4/80, anti-PD-L1, and anti-Sca-1 antibodies from Biolegend (Supplementary Data 1).

Neutrophil and T cell co-cultures

Splenic murine total T cells were labeled with 5 μ M CellTrace Violet (ThermoFisher Scientific, 100,000 cells per well). Proliferation was induced by coated anti-CD3 (5 μ g/mL, clone 145-2C11, ThermoFisher) and anti-CD28 (1 μ g/mL, clone 37.51, ThermoFisher), or Phorbol 12-Myristate 13-Acetate (PMA, 10 ng/mL, Sigma, P1585) and ionomycin (250 ng/mL, Sigma, I9657). Nonproliferative controls were incubated in the presence of 50 ng/mL murine rIL-7 (PeProtech, 217-17). Peritoneal neutrophils (pN) or omental (oN) from Ba-wt or Ba-wadC-LPS injected mice or bone marrow neutrophils (BmN) from non-treated mice were added at the indicated ratios (1:1, 100,000, or 1:0.5, 50,000 neutrophils per well). When specified, pNeutrophils were pre-incubated with anti-PD-L1 (200 nM, clone 10 F.9G2, BioXcell, PB0101) or isotype-matched (anti-rat IgG2b isotype control, BioXcell, BP0090) antibodies for 15 min before co-culture with total T cells. When indicated, recombinant murine IL-1RA (10 ng/mL, PeProtech, 200-01RA) was added to purified total T cells stimulated or not with anti-CD3/anti-CD28.

For transwell assays, pNeutrophils were cultured at a 1:1 T cell ratio on 6.5 μ m polycarbonate filter inserts with 0.4 μ m pores (Corning, 3413) in 24-well flat-bottom plates (Falcon, 353047). T cells were cultured on the bottom (300,000 T cells per well) and stimulated with anti-CD3/anti-CD28 antibodies or PMA/ionomycin as described above.

In all conditions, cells were harvested after 3 days of co-culture, stained with monoclonal antibodies against CD5, CD4, CD8, Ly6G, CD25, CD69, PD-1, Sca-1, and CD44 (Supplementary Data 1) and analyzed on a FACS Fortessa flow cytometer (BD Biosciences). T cells with CellTrace violet intensity below stained IL-7-cultured controls were counted as proliferating T cells.

Human RNA-seq Analysis

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll from whole blood of healthy volunteers or brucellosis patients at the first visit, and RNA sequencing was performed following a procedure established by the Benaroya Research Institute, Seattle, USA. Number and features of patients enrolled in each clinical group: Acute treated, $n = 56$ (14.54% women; age range 4–73 years with a median age of 43); Acute with relapse, $n = 6$ (16.66% women; age range 17–59 years with a median age 39); Chronic, $n = 11$ (18.18% women; age range 28–70 years with a median age of 38); Healthy donor, $n = 32$ (with similar median age and sex ratio). Serum from some of these patients and healthy donor control blood were kept at -80°C for cytokine/chemokine dosage. Original RNA-seq data were deposited on gene expression omnibus (GEO-NCBI-NIH; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69597>). Patients were handled at the University Clinic of Infectious Diseases and Febrile Conditions, Skopje, from 2007 to 2015. 217 men

and 85 women with a median age of 39 years (range 3–79) were enrolled. Sex was not considered as a variable in this study because the sex of patients reflected the incidence of the disease. Informed consent was obtained from the patients and healthy donors enrolled in the study, based on routine diagnostic blood collection. Brucellosis diagnosis was grounded on clinical signs and symptoms compatible with brucellosis (fever, arthralgia, sweating, malaise, splenomegaly, hepatomegaly, signs of focal disease), and confirmed by a qualitative positive Rose Bengal test and a Brucellacapt assay of $>1/320$.

Statistics

Results were analyzed with the GraphPad Prism v9 and v10 software (San Diego, CA, USA). The error bars show the standard deviation. For all tests, we performed a Shapiro-Wilk test to assess the normality of data distribution. When data homogenization was needed for a figure panel, the test giving the most stringent values was always chosen. The Number of experiments, group size, and statistical tests used are indicated in the figure legends. Values of $p < 0.05$ were considered significant.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Original RNA-seq data were deposited on gene expression omnibus (GEO-NCBI-NIH; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69597>). All raw data generated in this study are provided in the Supplementary Information/Source Data file. Datasets associated with this study are deposited on figshare (<https://doi.org/10.6084/m9.figshare.27247905>). Source data are provided in this paper.

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Acknowledgements

We acknowledge the PICSL imaging facility of the CIML (ImagImm), member of the national infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04), CIML (V. Chatain, M. Pontier) and CIPHE cytometry (H. Luche, P. Grenot) and animal house (C. Napez and P. Hoest) facilities as well as the CIML histology facility (L. Chasson) for expert technical assistance. We thank L. Spinelli (CIML) for help with statistical analyses, M. Sieweke (CIML) for helpful discussion, M. Malissen (CIML) for the gift of mice, B. Malissen (CIML) for his support, R. Kelly and A. Saurin (IBDM) for kind gifts of reagents and J. Tomas for comments on the manuscript. This work was supported by institutional funding from the Center National de la Recherche

Scientifique (CNRS) and the Institut national de la santé et de la recherche médicale (Inserm), and by the Excellence Initiative of Aix-Marseille Université (AMU) (A*MIDEX), a French “Investissements d’Avenir” program, by Laboratory of scientific excellence (labex) initiatives, Labex « INFORM » and « Institut Convergence CenTuri », with the Institute for Development Biology (IBDM) and Aix-Marseille University, by « Blood transcriptional biomarker profiles for category B pathogens » U01-AI082110-01 NIH grant, by Fondation pour la Recherche Médicale (grant DEQ2017033674), and Inserm Transfert (CoPoC, 2020 CITHEB). J.M.P. is a recipient of a post-doctoral fellowship from the Fondation de Coopération Scientifique “Méditerranée Infection”. G.G.E. is a recipient of a doctoral fellowship from the Fondation de Coopération Scientifique “Méditerranée Infection” and Campus France.

Author contributions

S.M. and J.P.G. conceived and supervised the study. J.M.P., G.G.E., R.S., L.H., T.R., V.A.G., S.H., E.M., E. B.C., R.C.A., I.M., J.P.G., and S.M. designed research; J.M.P., G.G.E., R.S., L.H., T.R., V.A.G., H.L., Y.Z., S.H., A.C.Z., M.L.B., and S.M. performed investigation; G.G.E., L.H., T.R., V.A.G., A.C.Z., M.L.B., and R.C.A. performed all BSL3 experiments; J.M.P., G.G.E., R.S., L.H., V.A.G., S.H., A.C.Z., M.L.B., E.M., E.B.C., R.C.A., I.M., J.P.G., and S.M. analyzed data; D.P., C.M., and J.C.G. provided reagents and methodology; M.B. and J.S. were in charge of human study; S.M. and J.P.G. raised funding; S.M. wrote the original manuscript; J.M.P., S.H., H.L., E.M., R.C.A., I.M., J.P.G., and S.M. edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41467-024-55799-2>.

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Peer review information *Nature Communications* thanks Zheng-Fei Liu, and the other anonymous, reviewers for their contribution to the peer review of this work. A peer review file is available.

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