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Impairment of PD-L2 positive B1a cells enhances susceptibility to sepsis in RasGRP1-deficient mice

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ABSTRACT

RasGRP1 is a key molecule that mediates antigen-initiated signaling for activation of the RAS-MAPK pathway in lymphocytes. Patients with aberrant RasGRP1 expression experience lymphocyte dysfunction and are afflicted with recurrent microbial infections. Yet, the underlying mechanism that accounts for microbial infection remains unknown. We previously reported that B1a cells are heterogeneous with respect to PD-L2 expression and that RasGRP1 deficiency preferentially impairs PD-L2+ B1a cell development. In the present study, we show that PD-L2+ B1a cells exhibit increased capacity for differentiation to CD138+ plasma cells that secrete natural IgM antibody, as well as IL-10 and GM-CSF, in response to TLR stimulation. In keeping with this, we show here that RasGRP1-deficent mice are much more susceptible to septic infection triggered by cecal ligation and puncture than wild type mice, and that reconstitution of RasGRP1-deficient mice with wild type PD-L2+ B1a cells greatly rescues RasGRP1-deficient mice from sepsis. Thus, this study indicates a mechanism for the association of RasGRP1 deficiency with predispostion to infection in the loss of a particular B1a subpopulation.

1. Introduction

Ras guanine-releasing protein 1 (RasGRP1) is a member of the guanine nucleotide exchange factor family, members of which are involved in Ras activation. Among RasGRP1 family members, only RasGRP1 and RasGRP3 are expressed in lymphocytes to any great extent. RasGRP1 is highly expressed and plays a dominant role in T lymphocytes and it is exclusively required for T cell activation and development [1]. However, the expression profile and role of RasGRP1 are quite different in B lymphocytes. Conventional B2 cells express abundant amounts of RasGRP3, but very little RasGRP1, and RasGRP1 expression is dispensable for B2 cell activation and development [2]. In contrast, among B1 cells, RasGRP1 is abundantly expressed with very little RasGRP3, and RasGRP1 links PLCy2 and Ras for Map kinase ERK activation. As a consequence, loss of RasGRP1 specifically impairs B1 cell activation and development [3]. In line with this, the antigen specific BCR repertoire is also significantly altered in RasGRP1-deficient B1 cells.

In animal models, loss of RasGRP1 has been demonstrated to be closely associated with immune dysfunction. RasGRP1-deficient mice produce substantial anti-nuclear antigen autoantibody [2,4], a typical feature of lupus autoimmune disease. In humans, aberrant RasGRP1 protein expression is frequently observed in patients with systemic lupus erythematous (SLE), where T cell activation is severely impaired [5–7]. RasGRP1 mutation can lead to a failure of activation-induced T cell apoptosis that results in autoimmune lymphoproliferative syndrome (ALPS) [8]. Recently, patients with RasGRP1 mutation were shown to manifest impaired immune function and to experience recurrent infections [9,10]. Although these patients have deficient natural killer (NK) cell function that can play a role in protection against microbial infection, the underlying mechanism accounting for the failure of bacterial protection remains unclear.

B1 cells are a distinct B cell subpopulation that protects against bacterial infection by secreting natural IgM antibody [11,12], IL-10 [13], and GM-CSF [14,15]. Mouse B1a cells are divided by PD-L2 expression into 2 roughly equally sized subpopulations of positive and negative B1a cells [16]. PD-L2 is a stable marker—PD-L2− B1a cells do not acquire PD-L2 expression after stimulation, and its constitutive expression in B1a cells is regulated differently than its inducible expression in macrophages [16,17]. Although many features of PD-L2+ B1a cells (also known as L2pB1 cells) match those of PD-L2− B1a cells (also known as L2nB1 cells), there are some differences with respect to antibody repertoire and T cell interaction [18,19]. We have shown that RasGRP1 is a key signaling molecule for B1 cell activation and development, and that loss of RasGRP1 results in a significant loss of PD-L2+ B1a cells [3]. In the current study, we find that in response to TLR

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stimulation PD-L2+ B1a cells secrete more IgM, IL-10 and GM-CSF than PD-L2− B1a cells. Further, we show that RasGRP1 deficient mice are highly susceptible to sepsis triggered by cecal ligation puncture (CLP), a very severe bacterial infection model. Adoptive transfer of PD-L2+ B1a cells from WT animals largely reverses the susceptibility to sepsis of RasGRP1-deficient mice. These results establish the role of the PD-L2+ subset of B1a cells in providing for microbial defense.

2. Materials and methods

2.1. Mice

C57BL/6J mice and B6(Cg)-Il10tm1.1Karp/J mice at 8–12 weeks of age were obtained from the Jackson Laboratory. 8–10-week-old RasGRP1-deficient mice and littermate control mice were used in this study. All mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines, and studies with these mice were approved by the Institutional Animal Care and Use Committee.

2.2. Antibodies and reagents

Anti-mouse monoclonal antibodies against CD19 (clone 1D3), B220 (clone RA3-6B2), CD138 (clone 281–2), CD5 (clone 53–7.3), CD43 (clone S7), GM-CSF (clone MP1-22E9), and PD-L2 (clone TY25) were obtained from BD pharmingen. Anti-mouse polyclonal antibody against IgM was obtained from SouthernBiotech. LPS and Lipid A were obtained from Sigma Aldrich.

2.3. Cecal ligation and puncture

8–10 week-old RasGRP1-deficient or littermate wild type control mice were anesthetized with isoflurane and underwent CLP, as previously described [13]. Briefly, a 1.5-cm incision was made to the abdominal wall, and the cecum was exposed and ligated 1-cm from the tip with 4-0 silk suture. The ligated cecum was punctured with a 22-gauge needle, and a small amount of fecal material was extruded. The cecum was replaced into the abdominal cavity, and the abdomen was closed in 2 layers with running 4-0 nylon suture. The sham mice underwent the same procedure with the exception that the cecum was neither ligated nor punctured. Animals were subcutaneously injected with 1 mL of normal sterile saline (0.9% NaCl) and imipenem (25 mg/kg). Furthermore, mice were subcutaneously injected with meloxicam (10 mg/kg) at day 0 and day 1. Mouse survival was monitored for 10 days.

2.4. B1 cell subset isolation and in vitro stimulation

Peritoneal cells were obtained by injecting 5 mL HBSS into the peritoneal cavity. Red cells in the collected peritoneal cells were depleted using ACK buffer. The resultant cells were incubated with antibodies. PD-L2+ and PD-L2− B1a cells (CD19+ CD5+ CD43+) were sorted (FACSMelody; Becton Dickinson). Cells were incubated in RPMI-1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME with 1 μg/ml LPS or 10 μg/ml Lipid A for 24 h. Culture supernatant was harvested for ELISA assay, after which cells were washed, and subjected to intracellular flow cytometric analysis and Elispot assay.

2.5. Elispot analysis

Lps- or Lipid A-stimulated PD-L2+ and PD-L2− B1a cells were washed twice with warm PBS. 1000 cells were then suspended in 200 ul RPMI medium (supplemented with 10% FCS and 20 μM 2-ME) and incubated in 96-well Elispot plates at 37 °C with 5% CO2 for 8 h. After culture, the plates were washed three times with washing buffer followed by incubation with HRP-conjugated goat-anti-mouse IgM antibody at room temperature for 2 h. Elispots were visualized by ImmunoSpot (Cellular Technology Limited).

2.6. IL-10 analysis

Supernatant from cultured B1 cells was assayed for IL-10 by Elisa (R &D Systems).

2.7. GM-CSF analysis

Cultured B1 cells were washed twice with cold PBS. The resultant B1 cells were then stained with antibodies followed by flow cytometric analyses using a LSR-Fortessa x-20 (BD Biosciences). For intracellular GM-CSF staining, single cell suspensions of cultured B1 cells (supplemented with brefeldin A at 5 μM for the last 5 h) were fixed and permeabilized.

2.8. Cell adoptive transfers

PD-L2+ B1a cells (CD19+ CD5+ CD43+) were sorted (FACSMelody; Becton-Dickinson) from peritoneal cavity washouts and 0.5 million cells of each sub-population were injected (i.p.) into RasGRP1-deficient mice immediately after CLP surgery.

2.9. Statistical analysis

Graphpad Prism was used for survival curve calculation; results with p values below 0.05 were considered significant.

3. Results

3.1. PD-L2+ B1a cells preferentially differentiate in response to TLR signals

B1 cells are primarily located in the peritoneal and pleural cavities of mice where they play a critical role in maintaining homeostasis. Upon encountering invading pathogens, B1 cells rapidly respond to protect against infection [20]. After toll-like receptor (TLR) activation, a high percentage of, but not all, B1 cells become CD138+ cells [21], suggesting that B1 cells are heterogeneous. B1 cells can be divided into two sub-populations by PD-L2 expression [16]. To test whether these two sub-populations equally respond to TLR signals, PD-L2+ B1a cells and PD-L2− B1a cells were sorted and stimulated with LPS or Lipid A. Consistent with previous reports, we found that B1a cells of both subsets responded to LPS [22] and Lipid A [23] and differentiated into CD138+ B cells. However, many more PD-L2+ B1a cells became CD138+ cells after stimulation than PD-L2− B cells (Fig. 1). These results indicate that B1a cells are heterogeneous in responsiveness to TLR signaling, and CD138+ responders are enriched in the PD-L2+ subpopulation.

3.2. PD-L2+ B1a cells preferentially secrete IgM antibody in response to TLR signals

B1 cells generate natural IgM polyreactive antibody, which recognizes microbial antigens and provides first-line protection against invading pathogens. At an early stage of infection, B1 cells are rapidly activated by TLR stimulation and produce large amounts of IgM antibody [24]. This stimulated B1 cell IgM is thought to play a critical role in eliminating pathogens. CD138 is a marker for antibody-secreting plasma cells [25]. The finding that more PD-L2+ than PD-L2− B1a cells become CD138+ after TLR stimulation suggests that the former has stronger IgM secretory capacity than the latter. To examine this, PD-L2+ B1a cells and PD-L2− B1a cells were sorted and cultured with LPS and Lipid A. After incubation, IgM secretion was tested by Elispot assay. We found that B1a cells differentiated into IgM-secreting cells

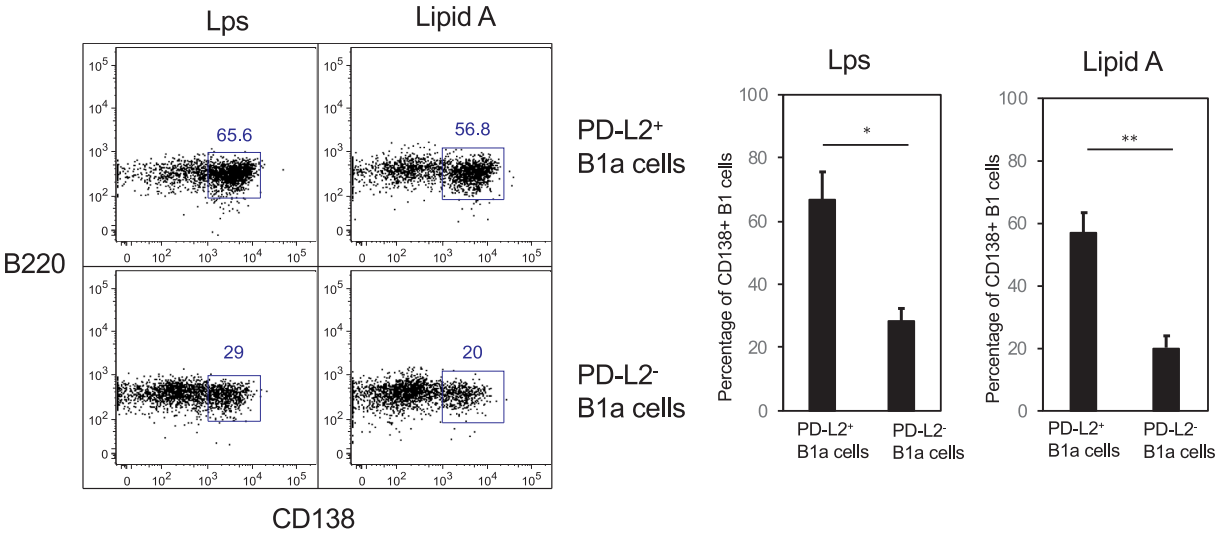

Fig. 1. PD-L2 + B1a cells preferentially differentiate in response to TLR signals. Peritoneal PD-L2 + B1a (CD19 + CD5 + CD43 +) cells (top panel) and PD-L2 − B1a cells (bottom panel) were sorted from C57BL/6 mice and stimulated with LPS (left panel) or Lipid A (right panel) for 24 h. CD138 expression on activated B1a cells was evaluated by flow cytometry. Results represent one of three comparable experiments.

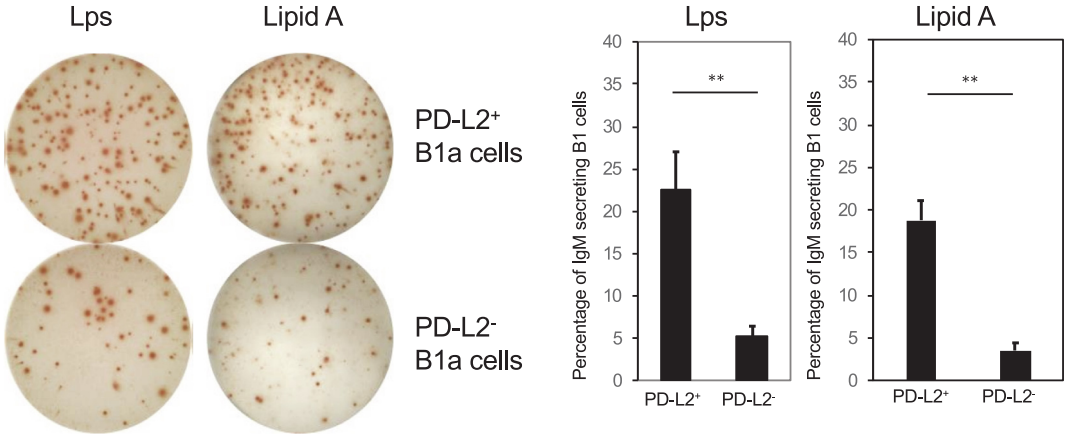

Fig. 2. PD-L2 + B1a cells preferentially secrete IgM antibody in response to TLR signals. Peritoneal PD-L2 + B1a (CD19 + CD5 + CD43 +) cells (top panel) and PD-L2 − B1a cells (bottom panel) were sorted from C57BL/6 mice and stimulated with LPS (left panel) or Lipid A (right panel) for 24 h. IgM-secreting capacity was evaluated by Elispot assay. Results represent one of three comparable experiments.

after culture with LPS and Lipid A. In line with the profile of CD138 + B1a cells, we found a much higher fraction of PD-L2 + B1a cells actively secreted IgM after LPS/Lipid A culture as compared to PD-L2 − B1a cells (Fig. 2). These results indicate that PD-L2 + B1a cells exhibit much stronger TLR-stimulated IgM-secretory capacity than PD-L2 − B1a cells.

3.3. PD-L2 + B1a cells preferentially secrete IL-10 in response to TLR signals

IL-10 is a key cytokine for regulation of inflammation. Sources of IL-10 include regulatory T cells [26], regulatory B cells [27], and B1 cells [28]. In particular, PD-L2 + B1a cells have been reported to be a key source of IL-10 in the peritoneal cavity [29]. To further elucidate IL-10 secretory capacity in B1a cell subsets, we assayed supernatant from sort-purified PD-L2 + B1a cells and PD-L2 − B1a cells stimulated with LPS and Lipid A for 24 h. In line with previous reports, B1a cells secreted a large amount of IL-10 after stimulation with LPS. Interestingly, we found that IL-10 secretory capacity was heterogeneous, and that PD-L2 + B1a cells had greater activation of IL-10 secretion than PD-L2 − B1a cells in response to LPS (Fig. 3). The magnitude of the difference in IL-10 secretion between PD-L2 + and PD-L2 − B1a cells was similar to the difference in LPS-stimulated IL-10 gene expression reported after depletion of PD-L2 + B1a cells [29]. Although significant, the difference in IL-10 secretion between PD-L2 + and PD-L2 − B1a cells was smaller after stimulation with Lipid A as compared to the difference after stimulation with LPS.

3.4. PD-L2 + B1a cells preferentially generate GM-CSF in response to TLR signals

B1 cells are capable of differentiating into GM-CSF-secreting cells in response to TLR signaling [14]. These activated, GM-CSF-secreting IRA (innate response activator) cells play an essential role in protection against sepsis [14]. To determine the origin of IRA cells, PD-L2 + B1a cells and PD-L2 − B1a cells were sorted from C57BL/6J mice and stimulated with LPS and Lipid A. After stimulation, intracellular GM-CSF expression in activated B1a cells was evaluated by immunofluorescent staining and flow cytometry. In line with the previous reports, B1a cells expressed GM-CSF after stimulation with LPS or Lipid A. We further found that PD-L2 + B1a cells had much greater GM-CSF-expressing capacity than PDL-2- B1a cells in response to LPS or Lipid A (Fig. 4).

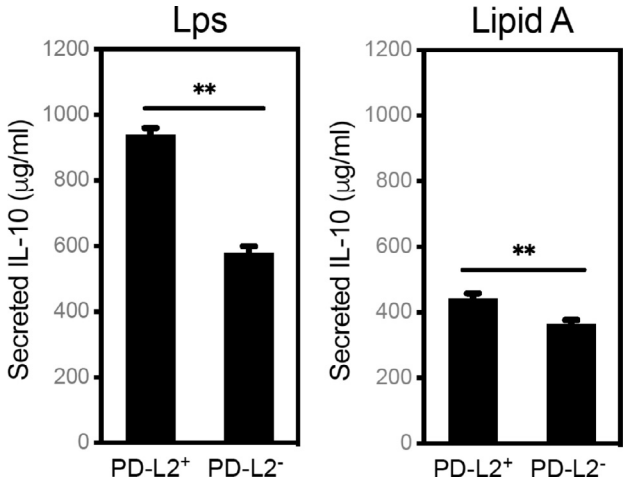

Fig. 3. PD-L2+ B1a cells preferentially secrete IL-10 in response to TLR signals. Peritoneal PD-L2+ B1a (CD19+ CD5+ B220lo) cells (top panel) and PD-L2− B1a cells (bottom panel) were sorted from C57BL/6 mice and stimulated with LPS (left panel) or Lipid A (right panel) for 24 h. Supernatants were collected and assayed for IL-10 by ELISA. Results represent mean +/− SEM values for 4 independent experiments.

3.5. RasGRP1-deficient mice are susceptible to sepsis induced by CLP

Sepsis is a leading cause of death. It is an extreme reaction to intense infection that includes release of cytokines, a process termed "cytokine storm", that triggers severe inflammation and tissue damage. Both B1a cell-derived IgM [11] and IL-10 [13] have been demonstrated to protect against sepsis. The impairment of IgM antibody secretion and IL-10 production associated with the loss of PD-L2+ B1a cells in RasGRP1-deficient mice suggest that these mice may be unusually susceptible to sepsis. To test this, RasGRP1-deficient mice and littermate WT control mice were subjected to CLP to trigger sepsis. Mouse survival was observed for 10 days and the survival rate was calculated. In line with previous reports, around 80% WT mice survived after CLP surgery. In contrast, RasGRP1-deficient mice were much more sensitive to sepsis and only 25% of these mice survived (Fig. 5). These results indicate that RasGRP1-deficient mice are especially susceptible to sepsis.

3.6. Increased susceptibility to sepsis of RasGRP1-deficient mice is reversed by PD-L2+ B1a cells

We then asked whether the loss of PD-L2+ B1a cells in RasGRP1-deficient mice is responsible for the increased susceptibility of

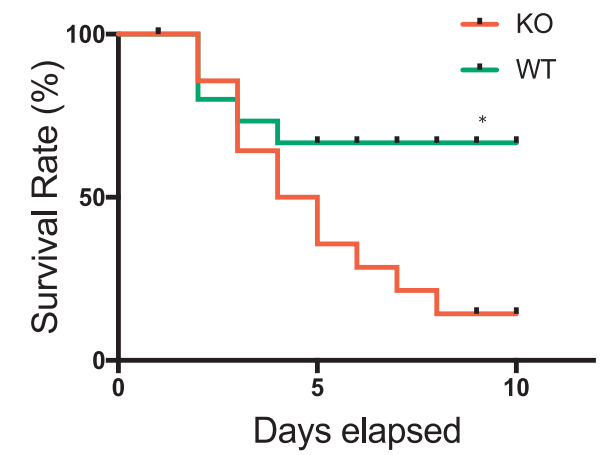

Fig. 5. RasGRP1-deficient mice are susceptible to sepsis induced by CLP. C57BL/6 mice (green, n = 15) and RasGRP1-deficient mice (red, n = 16) were subjected to CLP surgery. The mice were monitored for 10 days after CLP surgery and survival time was noted. ∗, $p < 0.05$.

RasGRP1-deficient mice to CLP-induced sepsis. To examine this, RasGRP1-deficient mice and littermate WT control mice were subjected to CLP surgery. Immediately after surgery, some CLP-RasGRP1-deficient mice were administered WT control PD-L2+ B1 cells. As noted above, RasGRP1-deficient mice were substantially more sensitive to CLP-induced sepsis than were WT control mice. Importantly, we found that reconstitution of RasGRP1-deficient mice with WT PD-L2+ B1a cells immediately after surgery significantly improved the survival rate of CLP-RasGRP1-deficient mice from 20% to 65% (Fig. 6). These results indicate that PD-L2+ B1a cells play a critical role in opposing CLP-induced sepsis, and are capable of improving the viability of RasGRP1-deficient mice that lack this population in the face of infectious challenge.

4. Discussion

RasGRP1 is a key signaling molecule that is essential for lymphocyte function. Expression of RasGRP1 in B cells is subset-specific: B1 cells predominantly express RasGRP1 and not other isoforms, whereas B2 cells predominantly express RasGRP3 [3]. In keeping with this, deletion of RasGRP1 affects B1 cells and does so by preferentially blocking development of PD-L2+ B1a cells [3]. PD-L2+ B1a cells express a repertoire distinct from PD-L2− B1a cells—PD-L2+ B1a cells preferentially express VH11/VH12-containing antibodies that recognize phosphatidyl choline (PtC) [19]. Anti-phosphatidyl choline antibodies

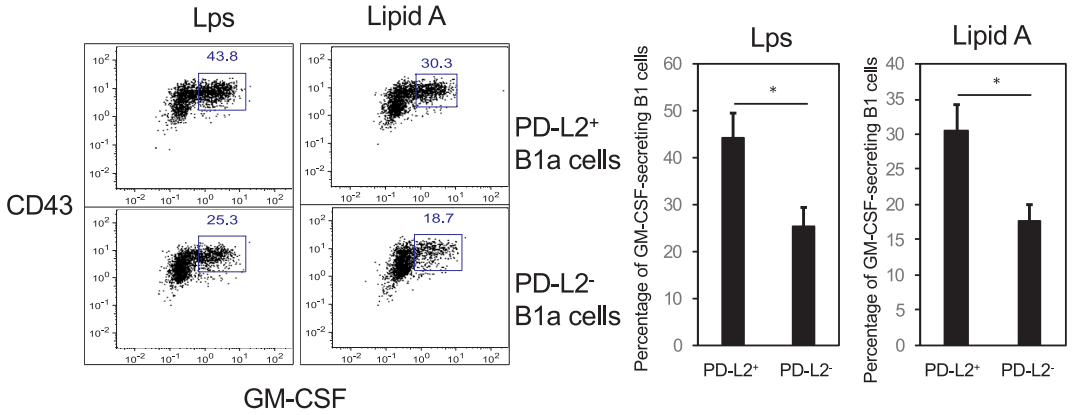

Fig. 4. PD-L2+ B1a cells preferentially generate GM-CSF in response to TLR signals. Peritoneal PD-L2+ B1a (CD19+ CD5+ CD43+) cells (top panel) and PD-L2− B1a cells (bottom panel) were sorted from C57BL/6 mice and stimulated with LPS (left panel) or Lipid A (right panel) for 24 h. Intracellular GM-CSF expression was evaluated by flow cytometry. Results represent one of three comparable experiments.

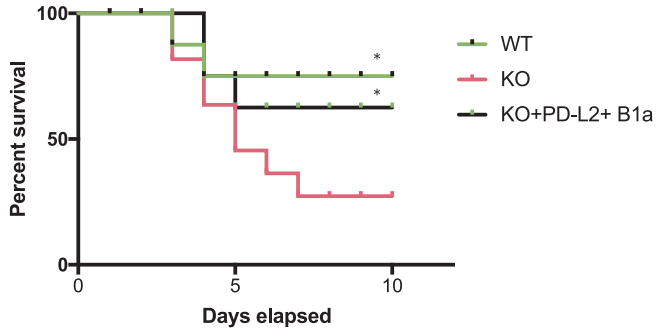

Fig. 6. Increased susceptibility to sepsis of RasGRP1-deficient mice is reversed by PD-L2+ B1a cells. CLP surgery was carried out on C57BL/6 mice (green, n = 18), RasGRP1-deficient mice (red, n = 15), and RasGRP1-deficient mice subsequently reconstituted with PD-L2+ B1a cells (black, n = 15). The mice were monitored for 10 days after CLP surgery and survival time was noted. *, $p < 0.05$.

are important in defense against CLP-induced sepsis [11]. Other features of B1a cells, including B1a cell differentiation and production of IgM, IL-10 and GM-CSF, are also important in sepsis defense. Here, we showed that, after stimulation of TLR by LPS and Lipid A, these activities are more strongly induced in PD-L2+ B1a cells as compared to PD-L2− B1a cells. We hypothesized that, to the extent that antibody repertoire and production of IgM, IL-10 and GM-CSF are important in defending against sepsis, RasGRP1-deficient mice, that lack PD-L2+ B1a cells, would be unusually sensitive to CLP. We confirmed this outcome. We further delineated the lack of PD-L2+ B1a cells as the responsible element accounting for increased sensitivity to CLP of RasGRP1-deficient mice by demonstrating reversal of sepsis-sensitivity through adoptive transfer of PD-L2+ B1a cells from WT mice to RasGRP1-deficient mice. Thus, PD-L2 is a marker for B1a cells that are critical for defense against CLP-induced sepsis. However, it is not clear that PD-L2+ B1a cells are solely responsible for protection against CLP-induced sepsis. Although PD-L2− B1a cells produced less IgM, IL-10 and GM-CSF after stimulation than PD-L2+ B1a cells, they still produced some of each. Thus to the extent that quantity determines activity, adoptive transfer of increased numbers of PD-L2− B1a cells might provide increased protection against sepsis. On the other hand, the quality of IgM in terms of subset-specific repertoire characteristics may be determinative, such that no amount of PD-L2− B1a cells would provide sepsis protection. This issue remains to be resolved. Regardless, it may be that relative lack of a PD-L2+ comparable subpopulation of human B1 cells is pathogenically connected to the increased microbial susceptibility of patients harboring mutations of RasGRP1 [30,31].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103993.

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