Blockade of the PD-1/PD-L1 immune checkpoint pathway enhances host defense against Mucorales and improves outcomes in a murine model of invasive pulmonary mucormycosis

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Background

Emerging experimental evidence suggests that immune checkpoint inhibitors (ICIs) can enhance antifungal immunity. In addition, there is anecdotal evidence of a potential benefit of adjunct PD-1 pathway blockade in patients with intractable mucormycosis. However, proof-of-concept data in animal models are lacking. Therefore, we compared the efficacy of PD-1 and PD-L1 inhibition in an immunosuppressed murine model of invasive pulmonary mucormycosis (IPM).

Methods

Mouse model and morbidity/mortality end points: 8-week-old female BALB/c mice were immunosuppressed with cyclophosphamide (Cyc, 150 mg/kg on days -4 and -1, 100 mg/kg on day +3) and cortisone acetate (CA, 300 mg/kg on day -1). Mice were infected intranasally with 50,000 *Rhizopus arrhizus* spores (clinical isolate Ra-749, day 0). On days 0, +2, +4, and +6, mice received intraperitoneal injections of 250 µg/kg PD-1 or PD-L1 blocking antibodies versus 250 µg/kg of the corresponding isotype antibodies (all antibodies from Leinco Technologies). Survival was monitored for 7 days post-infection. Infection severity was scored using the murine sepsis score (MSS, 0 = healthy to 3 = moribund, Mai et al., 2018, Intensive Care Med Exp). Mice dying prior to the day of assessment were scored as 4.

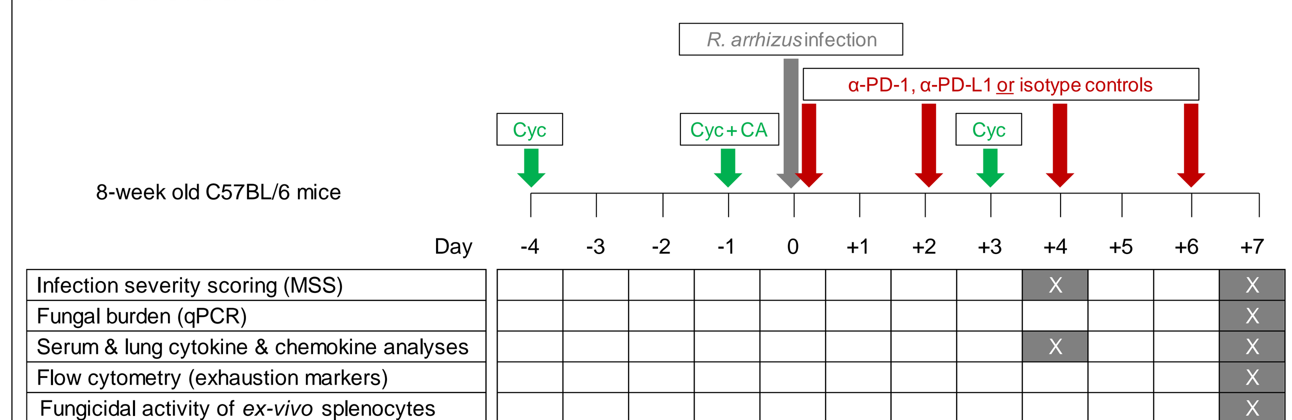


Figure 1: Overview of experimental procedures and end points

Fungal burden in lung tissue: Fungal burden was determined in lung tissue homogenates on day +7 using an 18S qPCR assay.

IncuCyte assay: Splenocytes were isolated on day +7 from mice with IPM that received ICI antibodies or isotype treatments. Two-hundred FTR1-GFP *R. arrhizus* spores were co-cultured in a 96-well flat bottom plate with 100,000 splenocytes (effector/target ratio [E:T] = 500), 20,000 splenocytes (E:T ratio = 100), or 4,000 splenocytes (E:T ratio = 20). Plates were imaged hourly in the IncuCyte ZOOM HD/2CLR time lapse microscopy system (Sartorius). Mycelial expansion was quantified using the "Green Channel Neurite Length" NeuroTrack feature, as described in Wurster et al., 2019, mBio.

Flow cytometry: Splenocytes were labelled with fluorescent antibodies according to the manufacturer's protocol. Panel 1: CD3-FITC (Miltenyi Biotec, "MB"), CD279 (PD-1)-PE (MB), CD49b-PE-Vio 770 (MB), CD152 (CTLA-4)-APC (MB), and CD366 (Tim-3)-Brilliant Violet (BioLegend, "BL"). Panel 2: CD11b-PE (MB), CD274 (PD-L1)-PE/Cyanine7 (BL), CD273 (PD-L2)-APC (MB), and CD366 (Tim-3)-Brilliant Violet (BL). 50,000 viable cells per panel were acquired with a Gallios flow cytometer (Beckman Coulter).

Cytokine assays: Cytokine and chemokine concentrations per gram of lung tissue and per mL of serum were determined using a custom-designed 19-plex magnetic Luminex assay (R&D Systems). The assay was performed according to the manufacturer's instructions and analyzed using a Luminex 200 device (Luminex Corporation).

Results

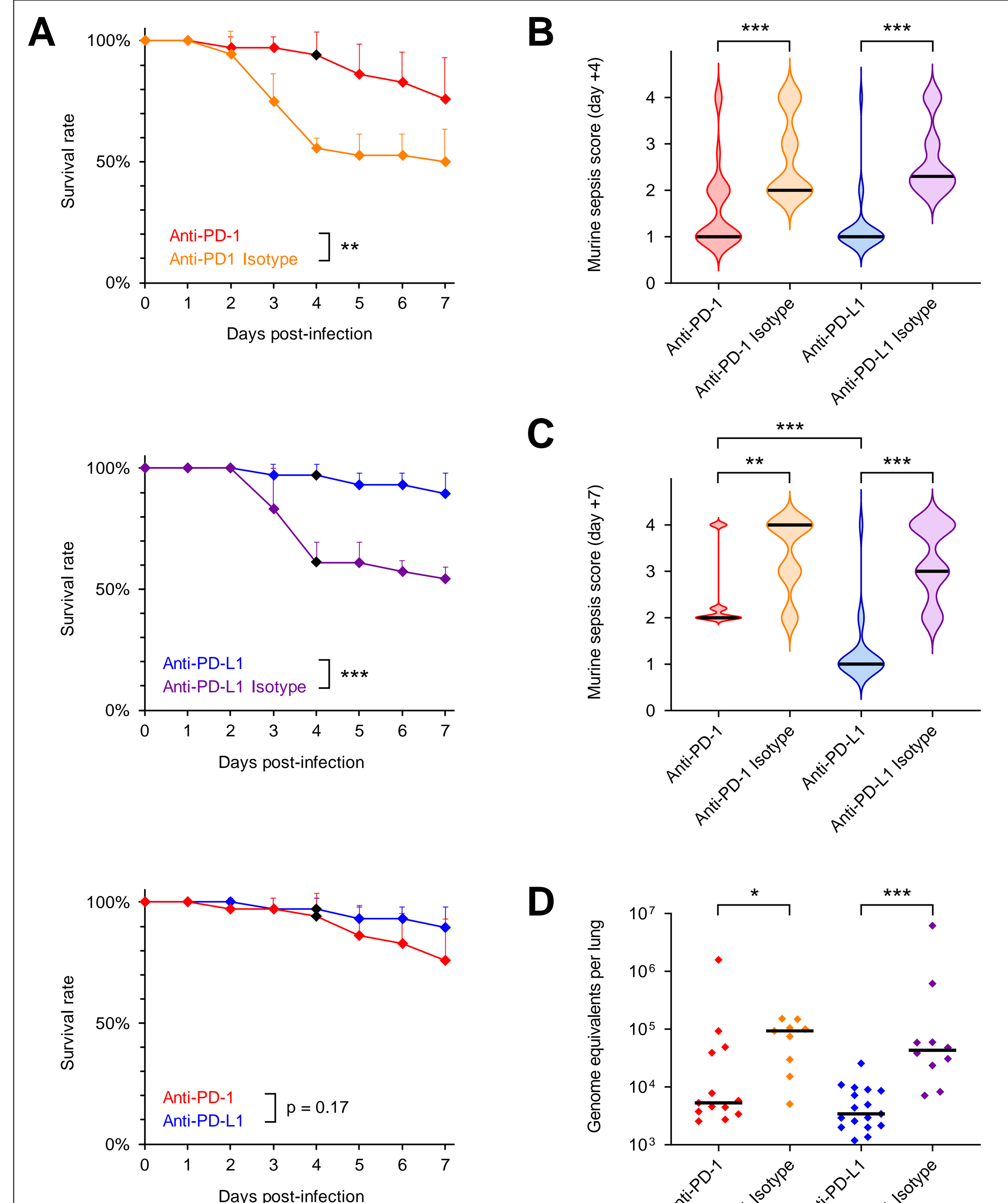


Figure 2: Inhibition of the PD-1/PD-L1 immune checkpoint pathway improves infection outcomes in immunosuppressed mice with IPM. (**A**) Survival rates of mice with IPM depending on the treatment received (n = 34-36 animals per group). Error bars indicate inter-replicate standard deviations based on 3 independent experiments. Mice euthanized in non-moribund condition for biomaterial sampling on day +4 were censored (black diamonds). Mantel-Cox log rank-test. (**B-C**) Violin plots summarizing the distribution of murine sepsis scores on day +4 (**B**, n = 24-26 per group) and day +7 (**C**, n = 19-26 per group). (**D**) Fungal burden per lung depending on the treatment, as determined by quantitative PCR on day +7 (n = 9-16 survivors per group). (**B-D**) Black bars indicate medians. Mann-Whitney-U test. * p<0.05, ** p<0.01, *** p<0.001.

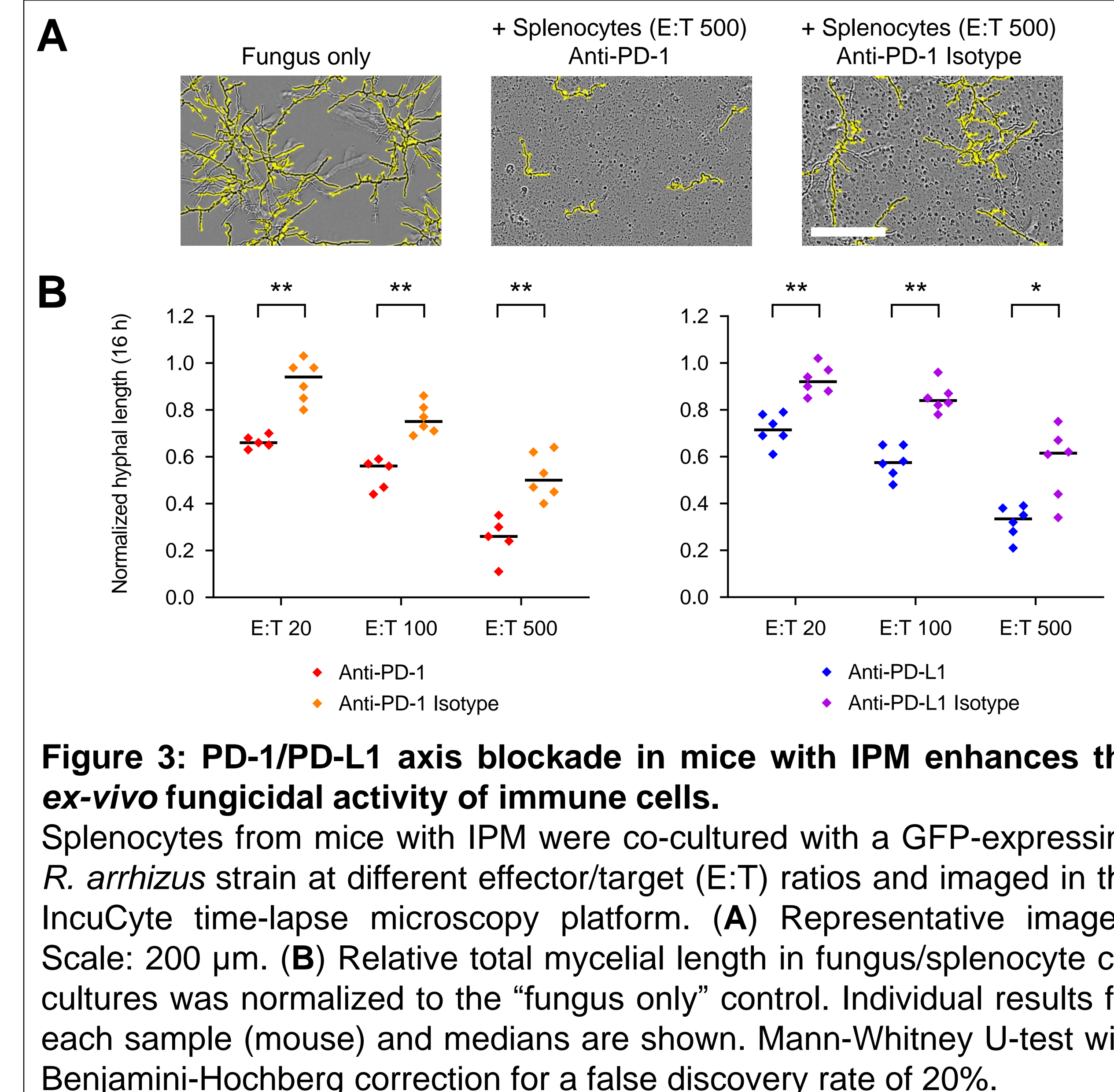
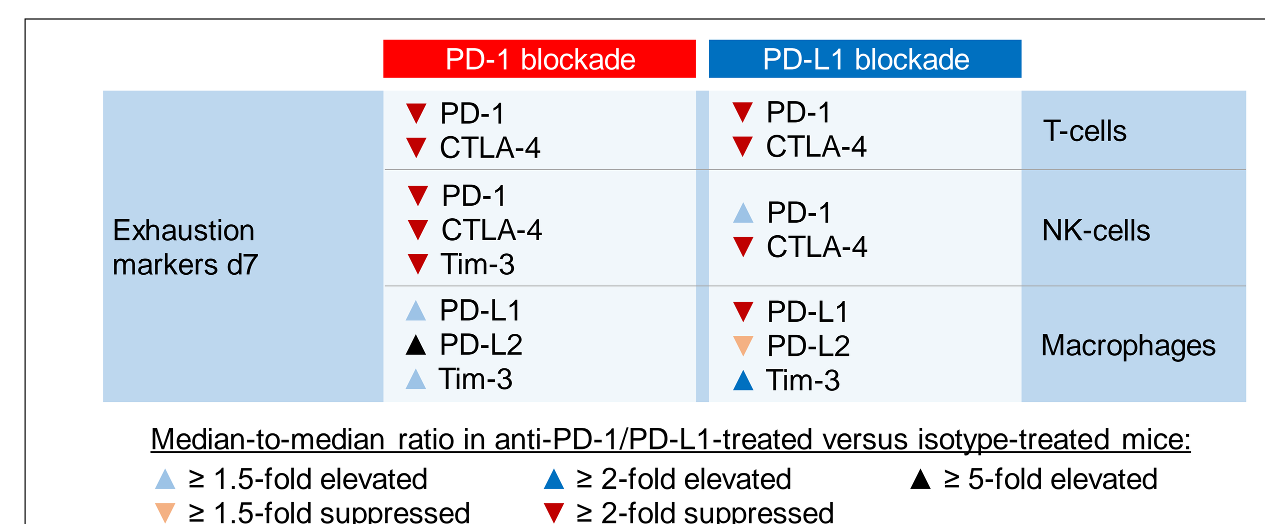


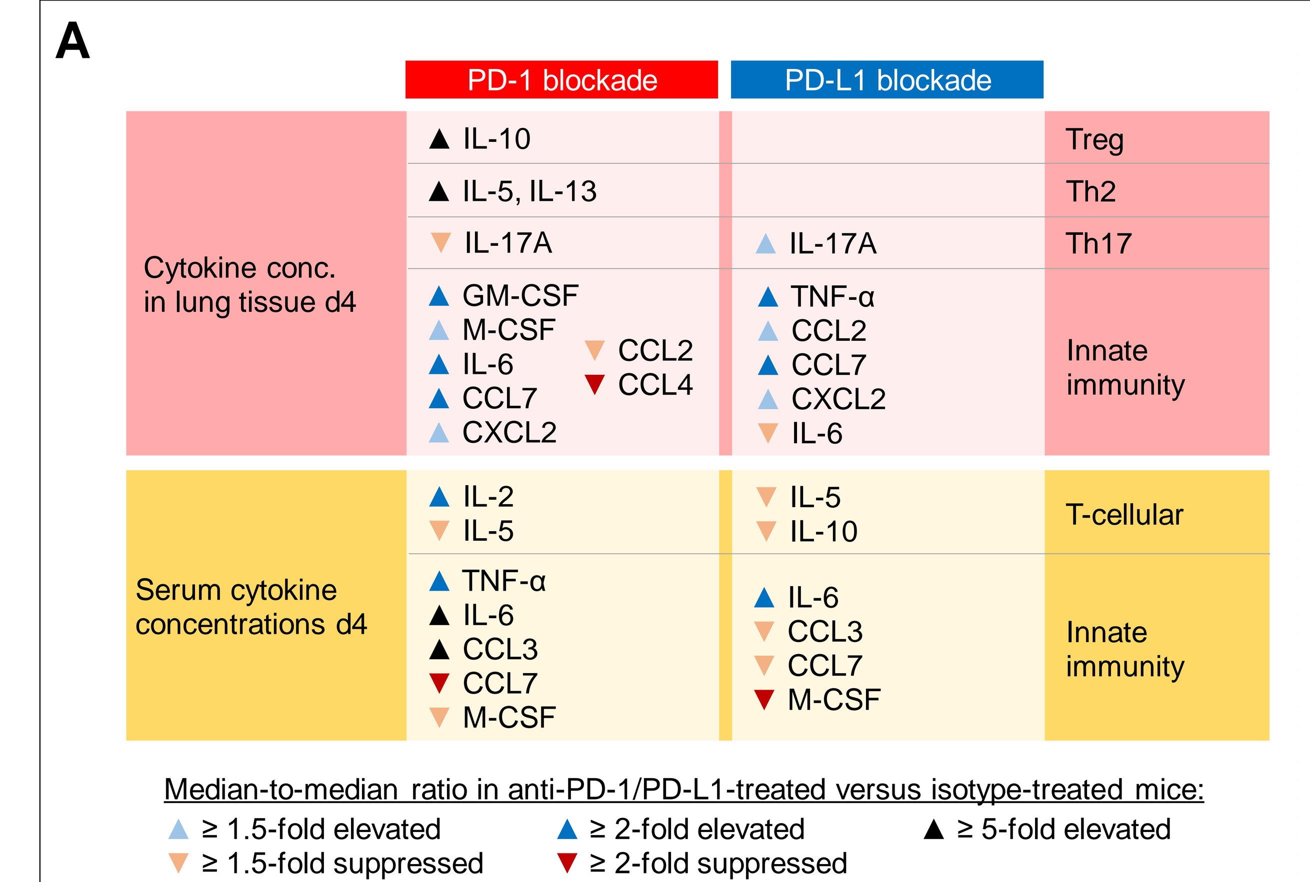
Figure 3: PD-1/PD-L1 axis blockade in mice with IPM enhances the *ex-vivo* fungicidal activity of immune cells.
Splenocytes from mice with IPM were co-cultured with a GFP-expressing *R. arrhizus* strain at different effector/target (E:T) ratios and imaged in the IncuCyte time-lapse microscopy platform. (**A**) Representative images. Scale: 200 µm. (**B**) Relative total mycelial length in fungus/splenocyte co-cultures was normalized to the "fungus only" control. Individual results for each sample (mouse) and medians are shown. Mann-Whitney U-test with Benjamini-Hochberg correction for a false discovery rate of 20%.



	PD-1 blockade	PD-L1 blockade	
Exhaustion markers d7	▼ PD-1 ▼ CTLA-4	▼ PD-1 ▼ CTLA-4	T-cells
	▼ PD-1 ▼ CTLA-4 ▼ Tim-3	△ PD-1 ▼ CTLA-4	NK-cells
	△ PD-L1 ▲ PD-L2 △ Tim-3	▼ PD-L1 ▽ PD-L2 ▲ Tim-3	Macrophages

Median-to-median ratio in anti-PD-1/PD-L1-treated versus isotype-treated mice:
△ ≥ 1.5-fold elevated ▲ ≥ 2-fold elevated ▲ ≥ 5-fold elevated
▽ ≥ 1.5-fold suppressed ▼ ≥ 2-fold suppressed

Figure 4: Inhibition of the PD-1/PD-L1 axis leads to compensatory induction of other checkpoint molecules.
Splenic immune cells were isolated from mice with IPM that received ICI or isotype treatments and survived until day +7. The percentages of PD-1, CTLA-4, and Tim-3 expressing T cells (CD3⁺) and NK cells (CD49b⁺) as well as the percentages of PD-L1, PD-L2, and Tim-3 expressing macrophages (CD11b⁺) were quantified by flow cytometry.



A

	PD-1 blockade	PD-L1 blockade	
Cytokine conc. in lung tissue d4	▲ IL-10		Treg
	▲ IL-5, IL-13		Th2
	▽ IL-17A	△ IL-17A	Th17
	▲ GM-CSF △ M-CSF ▲ IL-6 ▲ CCL7 △ CXCL2 ▽ CCL2 ▼ CCL4	▲ TNF-α △ CCL2 ▲ CCL7 △ CXCL2 ▽ IL-6	Innate immunity
Serum cytokine concentrations d4	▲ IL-2 ▽ IL-5	▽ IL-5 ▽ IL-10	T-cellular
	▲ TNF-α ▲ IL-6 ▲ CCL3 ▼ CCL7 ▽ M-CSF	▲ IL-6 ▽ CCL3 ▽ CCL7 ▼ M-CSF	Innate immunity

Median-to-median ratio in anti-PD-1/PD-L1-treated versus isotype-treated mice:
△ ≥ 1.5-fold elevated ▲ ≥ 2-fold elevated ▲ ≥ 5-fold elevated
▽ ≥ 1.5-fold suppressed ▼ ≥ 2-fold suppressed

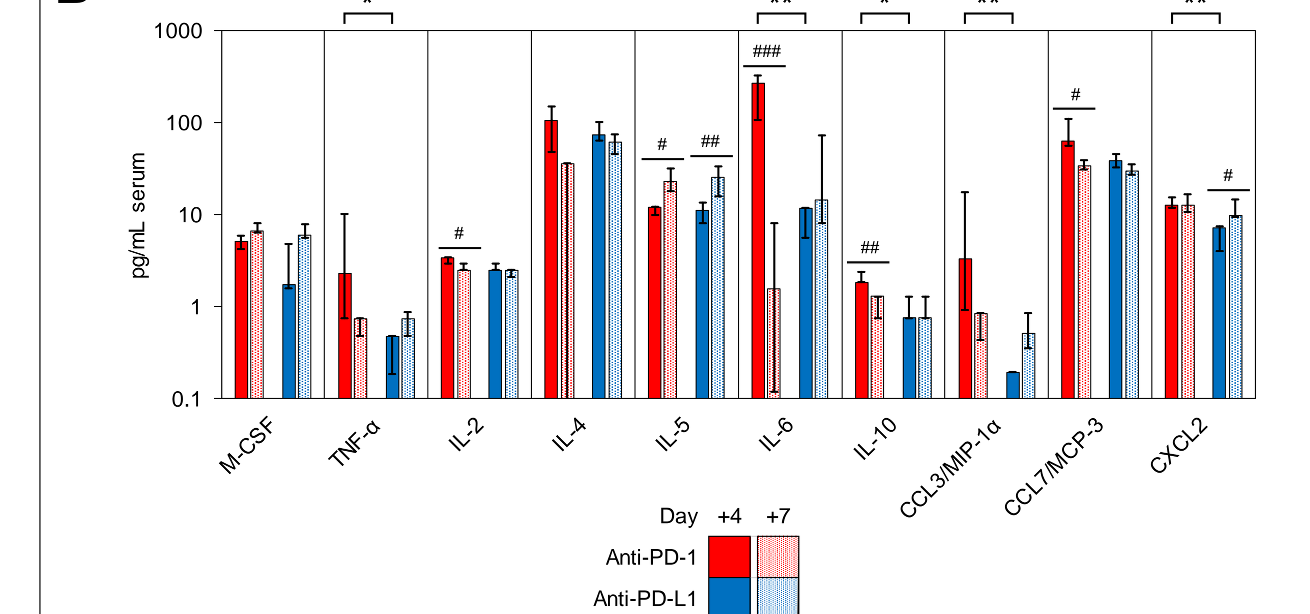


Figure 5: PD-1 blockade induces strongly enhanced production of inflammatory cytokine responses in the lungs and bloodstream, whereas PD-L1 inhibition results in a more balanced cytokine milieu.
Lung tissue homogenates and serum from mice with IPM treated with ICIs or isotype antibodies were obtained on days +4 and +7. Cytokine and chemokine concentrations were quantified using a 19-plex Luminex assay. (**A**) Summary of changes to the cytokine environment in ICI-treated animals versus controls. (**B**) Comparison of median serum cytokine levels of anti-PD-1- and anti-PD-L1-treated mice with IPM on days +4 and +7. Error bars: Interquartile ranges. test with Benjamini-Hochberg correction (false discovery rate 0.2). Cytokines and chemokines with concentrations below the detection limit in a majority of samples are not shown.

Conclusions and Outlook

- **Low-dose therapy with anti-PD-1 and anti-PD-L1 checkpoint inhibitors significantly improved survival outcomes, morbidity, and fungal clearance in immunosuppressed mice with IPM.**
- **A trend toward more consistent and sustained protection was seen in anti-PD-L1-treated mice compared to those receiving anti-PD-1.**
- **PD-1/PD-L1 blockade counteracted immune cell exhaustion and enhanced the fungicidal activity of splenocytes; however, upregulation of Tim-3 suggests compensatory induction of other checkpoint molecules.**
- **Compared to anti-PD-L1, PD-1 blockade resulted in stronger induction of inflammatory cytokine responses in the lungs, including cytokines associated with unfavorable Th2 (IL-5, IL-13) and Treg (IL-10) responses.**
- **Although no apparent signs of hyperinflammatory toxicity were seen, higher serum levels of cytokines linked to ICI toxicity, especially IL-6, were found in anti-PD-1 treated mice compared to PD-L1 inhibition.**
- **Future dose-effect studies in more pathophysiologically representative models of IPM (e.g., leukemia models) are needed to further define the protective therapeutic window of ICIs while avoiding off-target toxicities.**

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