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Research paper

High macrophage PD-L1 expression not responsible for T cell suppression

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ABSTRACT

Tumors are often comprised of microenvironments (TMEs) with a high proportion of cells and molecules that regulate immunity. Peritoneal cavity (PerC) cell culture reproduces key features of TMEs as lymphocyte proliferation is suppressed by PerC macrophages (Mϕs). We monitored the expression of T cell stimulatory (Class II MHC, B7) and inhibitory (PD-L1) molecules by PerC APCs before and after culture and report here that IFNγ-driven PD-L1 expression increased markedly on PerC Mϕs after TCR ligation, even more so than seen with direct APC activation by LPS. Considering the high APC composition of and pronounced PD-L1 expression by PerC cells, it was surprising that blocking PD-1/PD-L1 interaction by mAb neutralization or genetic ablation did not relieve suppression. This result parallels TME challenges observed in the clinic and validates the need for further study of this culture model to inform strategies to promote anti-tumor immunity.

1. Introduction

Homeostasis in the immune system is reflected in the strict control of the initial activation and duration of a response ensuring that inflammation is acute. Body-wide, innate housekeeping functions, e.g., apoptotic corpse clearance by phagocytes, operate in a non-inflammatory manner [1]. Inflammatory responses arise in organized lymphoid tissues that have evolved a particular architecture and cellular composition that permits activation and concomitant regulation [2]. Essential cellular and molecular braking systems, e.g., regulatory surface receptor (CTLA-4 or PD-L1) expression and antagonistic cytokine (IL-4, IL-10, IFNγ) production, arise concurrent with the commitment to activate and temper immunity to ensure organism homeostasis. This orderliness evolved to address external ("nonself") transient infectious challenges, not to address cancer; an internal, enduring challenge that reflects lifespan extension with coincident mutation accrual ("altered self"). Cancer involves persistent, homeostatic immune-regulatory mechanisms that become counter-productive, cloaking the tumor from immune detection. By eliminating overtly dangerous cells in the early phases of disease, the immune system also contributes to tumor evolution by creating conditions that foster the emergence of immune-resistant variants [3]. Epithelial tissue-derived tumors often harbor microenvironments (TMEs) with an aberrant cellular composition (high Mϕ:T cell ratios) and constitutive expression of anti-inflammatory molecules (PD-1/PD-L1, IL-10) [4–6]. High Mϕ composition and PD-L1 expression have become prognostic criteria for staging cancer [7–9]. Such tumors may also have a "simmering" inflammatory signature with effector CD8 T cell infiltration and IFNγ production [9]. These tumor types have been treated successfully with novel immune-modulatory drugs; initially checkpoint inhibitors negating CTLA-4 ligation, then PD-1 or PD-L1 blockade, and most recently combinations of these drugs [10–13]. However, we are at an early stage with these T cell-focused medicines as they impact a small fraction of a growing patient population [14]. Further understanding of the TME and identification of additional drug targets will be essential to build upon this early success.

We model the TME by studying Mϕ-rich peritoneal cavity (PerC) cells *in vitro* and have reported on how Mϕs suppress PerC B and T cell proliferation following B/TCR ligation [15–18]. IFNγ is essential for suppression of PerC T cells and is a key driver of inhibitory PD-L1 expression [17–19]. We investigated PD-L1 expression in the PerC/TME model system and found rapid and marked upregulation of this molecule on Mϕs. TCR ligation triggered the greatest increase in PD-L1, even more so than by direct APC activation via TLR-4 ligation. However, we were surprised to find that PD-1/PD-L1 blockade fails to reverse suppression. Thus, as observed in the clinic, high PD-L1 expression is not always the reason for failed immunity [14]. These results validate further study of this culture model to advance understanding of how to liberate immunity restrained by persistent immune regulating, homeostatic mechanisms.

Abbreviations: APC, antigen presenting cell; CM, complete media control; iNOS, inducible nitric oxide synthase; M, macrophage; MZ, marginal zone; PerC, peritoneal cavity; SP, spleen; 1-MA, N^G-monomethyl-L-arginine; TCR, T cell receptor; TME, tumor microenvironment

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2. Materials and methods

2.1. Mice

Two-to-four month old male and female mice, bred and maintained at Rider University, were handled in accord with NIH, Animal Welfare Act, and Rider University IACUC guidelines. Breeding pairs of C57BL/6J, IFNγR$^{-/-}$ (*B6.129S7^{Ifngr}/J*), and iNOS$^{-/-}$ (*B6.129P2-Nos2^{tm1Lau}/J*) mice were obtained from the Jackson Laboratory, Bar Harbor, ME. PD-L1$^{-/-}$ mice initially came from the laboratory of Dr. Arlene Sharpe, Harvard Medical School, Cambridge, MA.

2.2. Preparation of cell suspensions, cell culture, and cytokine ELISA

Spleen (SP) cell suspensions were obtained by gentle disruption of the organ between the frosted ends of sterile glass slides. Red blood cells were removed from SP cell preparations by hypertonic lysis followed by washing with Hanks Balanced Salt Solution (HBSS) (Life Technologies, Grand Island, NY). Peritoneal cavity (PerC) cells were obtained by flushing the peritoneum with 10 mls of warm (37 °C) HBSS supplemented with 2–3% fetal bovine serum (FBS) (Hyclone, Logan, UT). Viable cell counts were determined by Trypan blue exclusion. Various dilutions ($1.0–4.0 \times 10^6$/ml) of cells, in RPMI 1640 culture media (Life Technologies) supplemented with 10% FBS containing < 0.3 EU (< 0.06 ng)/ml of endotoxin (Invitrogen), 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM l-glutamine, 2×10^{-5} M 2-ME, and 10 mM HEPES, were plated in 96-well "V"- or flat-bottom microtiter plates (Corning Costar, Fisher Scientific, Pittsburgh, PA) and then incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. Most experiments plated cells at 3 x 10^6/ml in a V-bottom plate unless otherwise specified. Endotoxin testing (Pierce LAL Chromogenic Endotoxin Quantitation Kit) was done per manufacturer's instructions. For anti-CD3 stimulation soluble anti-CD3 mAb (clone 145-2C11; < 0.001 ng/μg endotoxin) (eBioscience, San Diego, CA) was added at 1.0 μg/ml. LPS from *E. coli*, serotype R515 (Enzo Life Sciences, Farmingdale, NY or Sigma) was added at 5 μg/ml. To inhibit arginine catabolism, the inducible nitric oxide synthase (iNOS) inhibitor N^G-monomethyl-l-arginine (1-MA; CalBiochem) was added. Neutralizing anti-mouse mAb for IFNγ (clone XMG1.2; < 0.001 ng/μg endotoxin), IL-10 (clone JES5-16E3; < 0.001 ng/μg endotoxin), GM-CSF (clone MP1-22E9; < 0.001 ng/μg endotoxin), IFNAR1 (clone MAR1-5A3; < 0.001 ng/μg endotoxin), PD-1 (clone J43; < 0.001 ng/μg endotoxin), and PD-L1 (clone MIH5; < 0.001 ng/μg endotoxin), were added at 7.5 μg/ml (all from eBioscience). All neutralizing mAbs were added at culture initiation. Optimal concentrations of all reagents were determined in titration experiments. After 44 h, 1 μCi of [^{3}H] thymidine (Moravek Inc., Brea, CA) was added to each well. The plates were frozen 4 h after labeling, and then thawed for harvesting onto filter paper mats using a semi-automated cell harvester (Skatron Instruments, Richmond, VA). Radioactivity was measured by liquid scintillation spectrometry. For each experiment 5 wells were established for each test group. IFNγ production in tissue culture supernatants was measured by sandwich cytokine ELISA as specified by the manufacturer (Thermo Fisher). Each experiment was repeated 3–5 times.

2.3. Immunofluorescence staining and flow cytometric analyses

Ex vivo or cultured PerC and SP cell suspensions were first blocked with a "blocktail" of rat anti-mouse CD16/32 MAb (Fc Block, eBioscience) and 2% normal rat serum (Jackson ImmunoResearch, West Grove, PA). Cell suspensions were then stained using titered amounts of FITC-, Cy-Chrome-, or PE-labeled rat anti-mouse IgM, CD11b, CD45R/B220, CD5 and/or F4/80 mAbs (Thermo Fisher, Eugene, OR). Isotype- and fluorochrome-matched, nonspecific mAb controls were employed to establish analysis gates. For carboxyfluorescein succinimidyl ester (CFSE) cell proliferation assays cells were labeled with CellTrace CFSE Cell Proliferation Kit as described by the manufacturer (Thermo Fisher) prior to culture. The percentage of B lymphocytes or myeloid cells co-expressing sets of these markers were determined via multiparameter flow cytometric analyses on a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) by FSC/SSC gating of the lymphoid or myeloid population using CellQuest software.

2.4. Statistical Analyses, stimulation index (SI), and Mean Fluorescent Intensity (MFI) index

Lymphocyte proliferative responses are presented as the average CPM (counts per minute) ± SEM (standard error of the mean). Data sets were compared using the Student's *t*-test with p-values below 0.05 considered statistically significant: * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$ relative to control. The stimulation index (SI) is defined as the average CPM for the treatment (e.g., anti-CD3) divided by the average CPM for the appropriate control response (complete

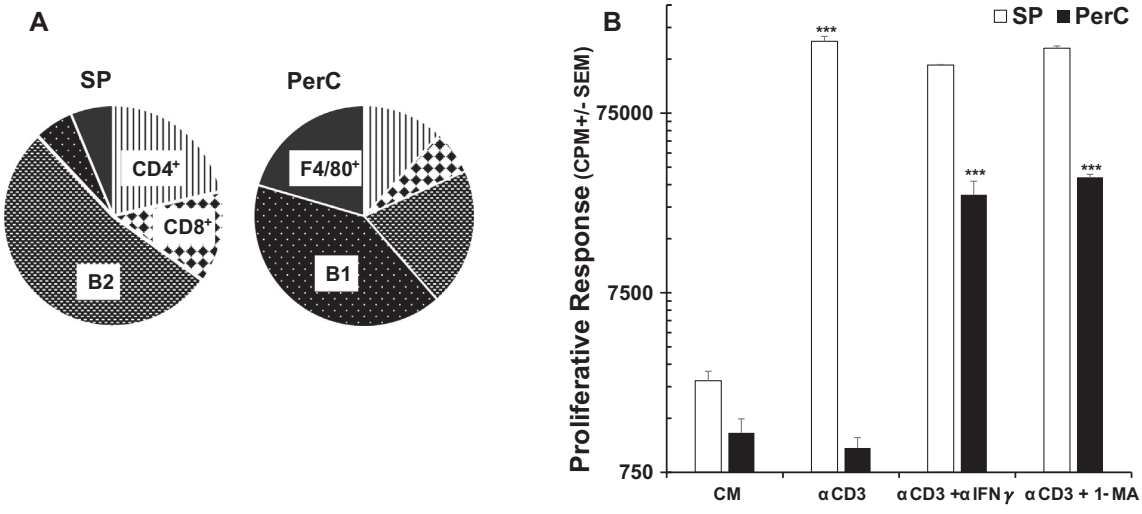

Fig. 1. Panel A: Cellular composition of spleen (SP) and peritoneal cavity cells (PerC). Mϕs were defined as CD11b^{hi}F4/80$^+$ cells; PerC B1 cells = CD45R$^+$CD11b^{med} cells; SP B1 = CD45R$^+$CD5^{lo}; B2 = CD45R$^+$. Data are averages from 8 to 10 analyses of 8–16 wk old C57BL/6J mice. Panel B: PerC T cells fail to proliferate in response to TCR ligation unless IFNγ is neutralized or iNOS is inhibited by 1-MA. Asterisks above histograms indicate significant differences in the experimental and control conditions for each cell source. Panels C & D: PerC CD4 and CD8 T cells respond to TCR ligation if IFNγ is neutralized (Panel C) or iNOS is inhibited by 1-MA (Panel D).