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Research paper

Macrophage regulation of B cell proliferation

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ABSTRACT

Unlike organized lymphoid tissue, the tumor microenvironment (TME) often includes a high proportion of immunosuppressive macrophages. We model the TME by culturing peritoneal cavity (PerC) cells that naturally have a high macrophage to lymphocyte ratio. Prior studies revealed that, following TCR ligation, PerC T cell proliferation is suppressed due to IFNγ-triggered inducible nitric oxide synthase expression. In this study we assessed the ability of PerC B cells to respond to surrogate activating signals in the presence of high numbers of macrophages. Surface IgM (BCR) ligation led to cyclooxygenase-mediated, and TLR-4 ligation to IL10-mediated, suppression of PerC B cell proliferation. In contrast, PerC B cells had a robust response to CD40 ligation, which could overcome the suppression generated by the BCR or TLR-4 response. However, the CD40 response was suppressed by concurrent TCR ligation. These results reveal the challenges of promoting B and T cell responses in macrophage-rich conditions that model the TME.

1. Introduction

Lymphoid organs harbor white blood cell subsets apportioned in a manner, *e.g.*, *follicles*, *marginal zone*, *etc.*, that facilitates surveillance of lymphatic fluid, blood, and mucosal surfaces. A general feature of all such tissues is a high lymphoid (B and T lymphocytes) to myeloid, (macrophages, dendritic cells) ratio that is essential for initiating immunity. In contrast, the leucocyte distribution found in many forms of neoplasia, *e.g.*, carcinomas of the breast, colon, or ovaries, is distinctive for having an increased representation of immunosuppressive myeloid cells [1–3]. Immunity within such tumor microenvironments (TMEs) is often constrained and altered myeloid/lymphoid ratios have been found prognostic of further cancer development [2,4].

Considerable effort has been invested in defining how the cellular and molecular composition of the TME tempers T cell function. This work has revealed a role for regulatory receptor-ligand pairs (PD1/PDL1, CTLA-4/B7, *etc.*), cytokines (TGF-β, IL-1, IL-6, IL-10, IL-17, *etc.*), enzymes (iNOS, ARG, IDO, COX, *etc.*), and subsets of T (T_{regs}, TH17) and myeloid cells (TAMs, MDSCs, *etc*) in the suppression of anti-tumor T cell function [5–9]. These efforts have informed novel clinical approaches and fostered an exciting era of T cell-focused immunotherapies [5,8]. Myeloid cells, essential for many of the immunosuppressive elements found within the TME, are key targets in current immunotherapeutic drug discovery [8]. Focus upon T cells and regulatory APCs is logical as their interaction is central to the initiation of adaptive anti-tumor immunity. In contrast, B lymphocytes within TMEs, functioning as humoral or APC effectors, or as regulators of cell-mediated immunity, have been less studied [10].

Pliable, *in vitro* models that mimic the TME are essential to dissect the complex cellular and molecular interactions that lead to compromised anti-tumor immunity. We have shown that peritoneal cavity (PerC) T cell proliferation is suppressed by the increased representation of macrophages present in this cell source [11]. T cell proliferation is regulated via catabolism of limiting amino acids, production of anti-inflammatory cytokines, and prostaglandin production [11–14]. PerC cell preparations include a significant fraction of B cells, notably the B1 subset distinctive for antibody responses to commensal microflora [15]. B1 cells, like the more recently described B10 or B_{reg} subset, are also distinctive for autocrine IL-10 production, a cytokine that antagonizes cytotoxic T lymphocyte (CTL) generation [15,16]. TMEs have been reported to include B cells with this and other immunosuppressive (TGF-β production, PD1/PDL1 expression) characteristics [10,17–20].

Abbreviations: APC, antigen presenting cell; ARG, arginase; COX, cyclooxygenase; IDO, indoleamine 2,3-dioxygenase; INDO, indomethacin; iNOS, inducible nitric oxide synthase; PerC, peritoneal cavity; ROS, reactive oxygen species; SP, spleen; 1-MA, N^G-monomethyl-L-arginine; 1-MT, 1 methyl tryptophan; 1-NA, *N-w*-hydroxy-nor-L-arginine.

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In this report we characterize PerC B cell susceptibility to macrophage suppression and show that the agent used to activate B cells dictates their response. The PerC B cell response to BCR ligation was suppressed by cyclooxygenase (COX) activation and to LPS stimulation by IL-10 production. In contrast, CD40 ligation triggered PerC B cell proliferation regardless of macrophage density. However, this response could be suppressed by concurrent activation of PerC T cells. This hierarchy of lymphoid suppression is discussed in the context of strategies to revitalize adaptive immunity within macrophage-rich TMEs.

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, BALB/c, CB17-*Prkdc*^{scid}/J, IFNγR$^{-/-}$ (B6.129S7^{Ifngr}/J), iNOS$^{-/-}$ (B6.129P2-Nos2^{tm1Lau}/J), and IL4$^{-/-}$ (B6.129P2-*IL4*^{tm1Cgn}/J) mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

2.2. Preparation of cell suspensions and cell culture

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 ml 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 (Hyclone), 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 3×10^6/ml in a V-bottom plate unless otherwise specified. For anti-CD3 stimulation soluble anti-CD3ε monoclonal antibody (mAb) (clone 145-2C11) (eBioscience, San Diego, CA) was added at 1.0 μg/ml. For anti-IgM stimulation F(ab′)$_2$ fragment of goat anti-mouse IgM, μ-chain-specific (Jackson ImmunoResearch, West Grove, PA) polyclonal antibody was added at 10 μg/ml. LPS from *E. coli*, serotype R515 (Enzo Life Sciences, Farmingdale, NY or Sigma) was added at 5 μg/ml. Concanavalin A from *Canavalia ensiformis* (Sigma) was added at 1.0 μg/ml. Anti-CD40 mAb (clone FGK45) (Enzo) was added at 2.0 μg/ml. To inhibit arginine catabolism, the arginase (ARG) inhibitor *N*-ω-hydroxy-nor-L-arginine (1-NA; CalBiochem, San Diego, CA) or the inducible nitric oxide synthase (iNOS) inhibitor N^G-monomethyl-L-arginine (1-MA; CalBiochem) were added. To inhibit tryptophan catabolism via indoleamine-2,3-dioxygenase (IDO), 1-methyl-tryptophan (1-MT; CalBiochem) was added. To inhibit COX, indomethacin (INDO; Sigma) was added. Neutralizing anti-mouse mAb for IFNγ (clone XMG1.2), IL-10 (clone JES5-16E3), or IL-4 (clone 11B11) were added at 7.5 μg/ml (eBioscience). Exogenous, recombinant murine IL-4, IL-10, and IL-13 cytokines were added at 10 ng/ml (Peprotech, Rocky Hill, NJ). All inhibitors, cytokines, or 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. All experiments were done a minimum of 3 times, the majority more than 5 times.

2.3. Immunofluorescence staining and flow cytometric analyses

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 CD4, CD8, IgM, CD11b, CD45R, CD5 and/or F4/80 mAbs (eBioscience). 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, Eugene, OR) prior to culture. The percentage of 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 and stimulation index (SI)

T cell 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 drug response (*e.g.*, anti-IgM + IL-4) divided by the average CPM for the appropriate control response (anti-IgM alone).

3. Results

3.1. Peritoneal macrophages suppress B cells activated by BCR or TLR-4 ligation

Our prior studies of peritoneal cavity (PerC) leucocyte biology focused on how macrophages (Mϕs) regulate the T lymphocyte response [11,12]. Peritoneal resident CD11b^{hi}, F4/80^{+} Mϕs were found to temper the T cell response to either TCR ligation with anti-CD3 MAb or to the mitogen ConA (Fig. 1A, B). In this report we focused upon macrophage regulation of the PerC B cell response and found that both BCR (F(ab′)$_2$ anti-IgM) and TLR-4 (LPS) ligation induced negligible B cell proliferation (Fig. 1A, B). These results contrast the significant proliferative response of spleen (SP) T and B cells to the same stimulation sources (Fig. 1A, B).

SP and PerC cell preparations differ in both their lymphoid and myeloid compartments (Fig. 2A, B). In terms of lymphoid composition, there are considerably more B-1 B cells in the PerC. Although F4/80^{+} macrophages comprise the majority of the myeloid fraction for both tissue sources, the greater representation of myeloid cells in the PerC leads to cultures that parallel tumor microenvironments rather than organized lymphoid tissue. Spleen T and B lymphocyte proliferation is suppressed when SP cells are co-cultured with PerC cells (Fig. 3A). This regulation is due to Mϕs since PerC cells from SCID mice, which lack mature lymphocytes, suppressed both SP T and B cell responses (Fig. 3B). These results illustrate that resident PerC Mϕs have the capacity to suppress both B and T cell proliferation.