PHA eludes macrophage suppression to activate CD8$^+$ T cells

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ABSTRACT

Tumors may include a high proportion of immune modulatory cells and molecules that restrain the anti-cancer response. Activation of T cells to eliminate cancer cells within the immune-suppressive tumor microenvironment remains a challenge. We have shown that C57BL/6 J peritoneal cell culture models features of macrophage-dense tumors as TCR ligation fails to activate T cells unless IFNγ is neutralized or iNOS is inhibited. We tested other forms of T cell activation and found phytohemagglutinin (PHA) distinctive in the ability to markedly expand CD8 T cells in this model. IFNγ or iNOS inhibition was not necessary for this response. PHA triggered less IFNγ production and inhibitory PD-L1 expression than TCR ligation. Macrophages and CD44^{hi} T cells bound PHA. Spleen T cell responses to PHA were markedly enhanced by the addition of peritoneal cells revealing that macrophages enhance T cell expansion. That PHA increases CD8 T cell responses within macrophage-dense culture suggests this mitogen might enhance anti-tumor immunity.

1. Introduction

There is growing understanding that the immune system not only controls tumor growth, but also facilitates cancer development within tumor microenvironments (TMEs) (Renner et al., 2017; Gajewski et al., 2013). Both malignant cells and atypical ratios of white blood cell (WBC) subpopulations comprise the TME (Wang et al., 2017). In certain tumor types, a considerable fraction of WBCs are macrophages (Mφs) that can block productive, anti-tumor immunity (Gurusamy et al., 2017). In organized lymphoid tissue, such as the lymph nodes (LN) or the spleen (SP), Mφs are a minor population of cells apportioned within the evolved architecture of each particular organ. This cellular distribution ensures normal lymphocyte biology and subset collaboration that maintains homeostasis (Turk, 1977). Within TMEs, however, this cooperative response is lost to immune suppression fostered by aberrant cellular composition (*e.g.*, increased Mφs, T$_{regs}$), regulatory receptor-ligand interactions (*e.g.*, PD-1/PD-L1), and anti-inflammatory cytokine production (*e.g.*, TGF$_{β1}$, IL10) (Kelly et al., 1988; Gooden et al., 2011). There is considerable interest in therapeutic approaches to subvert this suppression, particularly strategies that can increase the number and effectiveness of cytotoxic T cells in the TME (He et al., 2015).

We model several features of the TME by the culture of peritoneal cavity (PerC) cells. Distinct from organized lymphoid tissue, the peritoneum harbors an immune cell composition marked by a large fraction of CD11b^{hi} F4/80$^+$ Mφs, as well as activated (CD44^{hi}) T and B cell subsets (Composto et al., 2011). The increased proportional representation of Mφs is essential for the immune suppression observed in PerC cell culture (Composto et al., 2011; Matlack et al., 2006; Silberman et al., 2012; Goldman et al., 2017a). Following TCR ligation, PerC T cells produce IFNγ, which triggers Mφ iNOS expression (Composto et al., 2011; Matlack et al., 2006; Silberman et al., 2012). Inhibition of iNOS by N^G-monomethyl-L-arginine (1-MA) revealed that amino acid catabolism is responsible for reduced T cell expansion (Matlack et al., 2006; Rath et al., 2014). Conventional sources of lymphocytes, *i.e.*, murine SP cells or human peripheral blood, lack these key features of TMEs (Bronte and Pittet, 2013; Sen et al., 2018).

In our search for forms of T cell activation that might circumvent Mφ suppression, we found the mitogen phytohemagglutinin (PHA) particularly effective in this capacity (Matlack et al., 2006). A lectin extract from the red kidney bean (*Phaseolus vulgaris*) with potent mitogenic and cell agglutinating properties, PHA consistently stimulated PerC T cell proliferation (Sharon and Lis, 2004). Particularly exciting was the marked expansion of CD8$^+$ T cells. Neither IFNγ neutralization or iNOS inhibition were required for this response. Compared to TCR
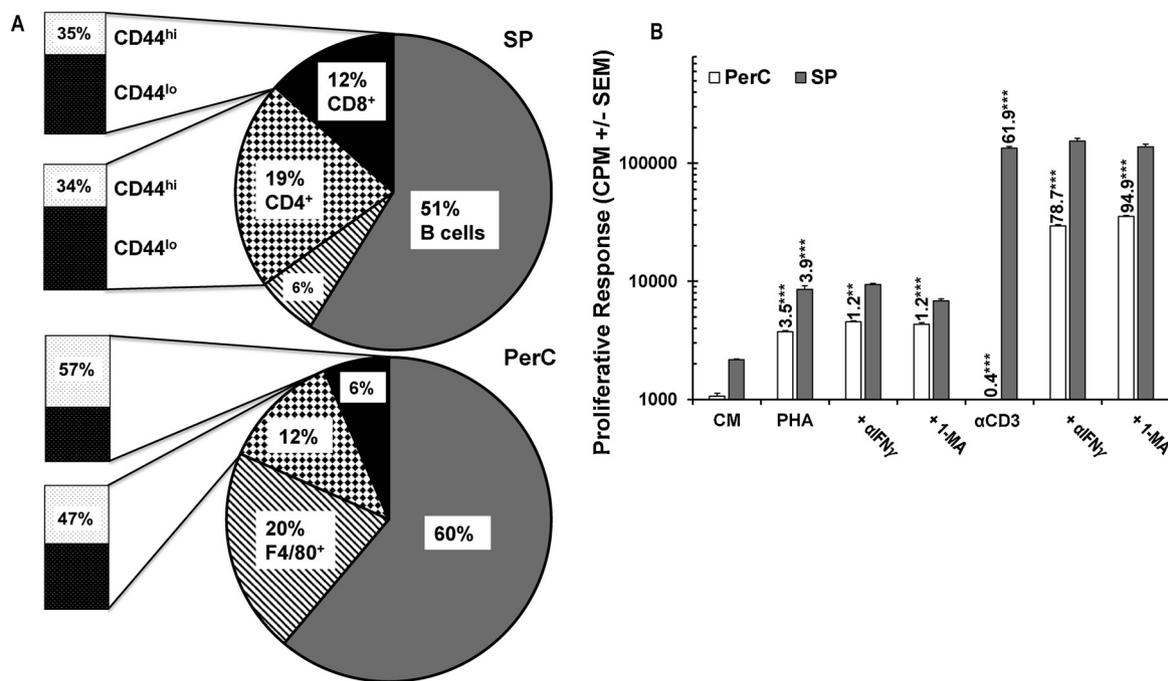
Fig. 1. Panel A: Lymphoid composition of spleen (SP) and peritoneal cavity (PerC) cells. Mφs defined by F4/80 and B cells by CD45R expression. Data are averages from 8-10 analyses of 8-16 wk old C57BL/6 J mice. Panel B: PerC T cells respond to PHA stimulation without IFN neutralization or iNOS inhibition (1-MA). CM = complete media (unstimulated control). Numbers above histograms represent stimulation indices (SI) as described in *Methods*. Asterisks indicate significant differences in the experimental vs control conditions.

ligation, PHA stimulation led to less IFN production and lower inhibitory PD-L1 expression by Mφs. These data encourage evaluation of PHA as a CD8 T cell agonist to promote anti-tumor immunity.

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/6 J and IFNγR$^{-/-}$ (*B6.129S7^{Ifngr}/J*) mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

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% fetal bovine serum (FBS) (Hyclone, Logan, UT). Viable cell counts were determined by Trypan blue exclusion. For proliferation assays, dilutions of cells ($3.0 - 4.0 \times 10^6$/ml) in RPMI 1640 culture media (Life Technologies) supplemented with 10% FBS (< 0.3 EU or < 0.06 ng /ml of endotoxin), 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 incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 48 hrs. CFSE experiments plated cells at 4×10^6/ml in 48 well, flat-bottom plates. Endotoxin testing was done per manufacturer's (Pierce LAL Chromogenic Endotoxin Quantitation Kit) instructions. For anti-CD3 stimulation soluble anti-CD3ε mAb (clone 145-2C11; < 0.001 ng/ug endotoxin) (eBioscience, San Diego,

CA) was added at 1.0 μg/ml. To inhibit arginine catabolism, the inducible nitric oxide synthase (iNOS) inhibitor N^G-monomethyl-L-arginine (1-MA; CalBiochem) was added[10]. Neutralizing anti-mouse mAb for IFNγ (clone XMG1.2), IL-10 (clone JES5-16E3), IL-4 (clone 11B11), IL-2 (clone JES6-1 A12), IFNAR1 (clone MAR1-5 A3), and PD-L1 (clone MIH5), were added at 7.5 μg/ml (eBioscience, all MAbs < 0.001 ng/ug endotoxin). All neutralizing mAbs were added at culture initiation. Phytohemagglutinin (Sigma-Aldrich) was added at 2-16 μg/ml. Optimal concentrations of all reagents were determined in titration experiments. After 44 hours, 1 μCi of [3 H] thymidine (Moravek Inc., Brea, CA) was added to each well. Four hours later the plates were frozen 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).

2.3. Immunofluorescence staining and flow cytometric analyses

For carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assays cells were labeled with the CellTrace CFSE Cell Proliferation Kit as described by the manufacturer (Thermo Fisher) prior to culture. For cell surface staining, *ex vivo* or cultured PerC and SP cell suspensions were first treated 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-, PerCP-Cy5.5-, or PE-labeled rat anti-mouse CD8, CD4, CD44, PD-L1, CD11b, CD45R/B220, and/or F4/80 mAbs (eBioscience). Isotype- and fluorochrome-matched, nonspecific mAb controls were employed to establish analysis gates. To identify PHA-binding cells, biotinylated PHA (b-PHA) was added at 0.2 – 10.0 μg/ml (Vector Labs, Burlingame, CA) concurrent with FITC- and PerCP-Cy5.5-labeled leucocyte subset-specific mAbs. After incubation and washing, Streptavidin-PE (StrAv-PE; R&D Systems, Minneapolis, MN) was added. Intracellular IFNγ staining was conducted as described by
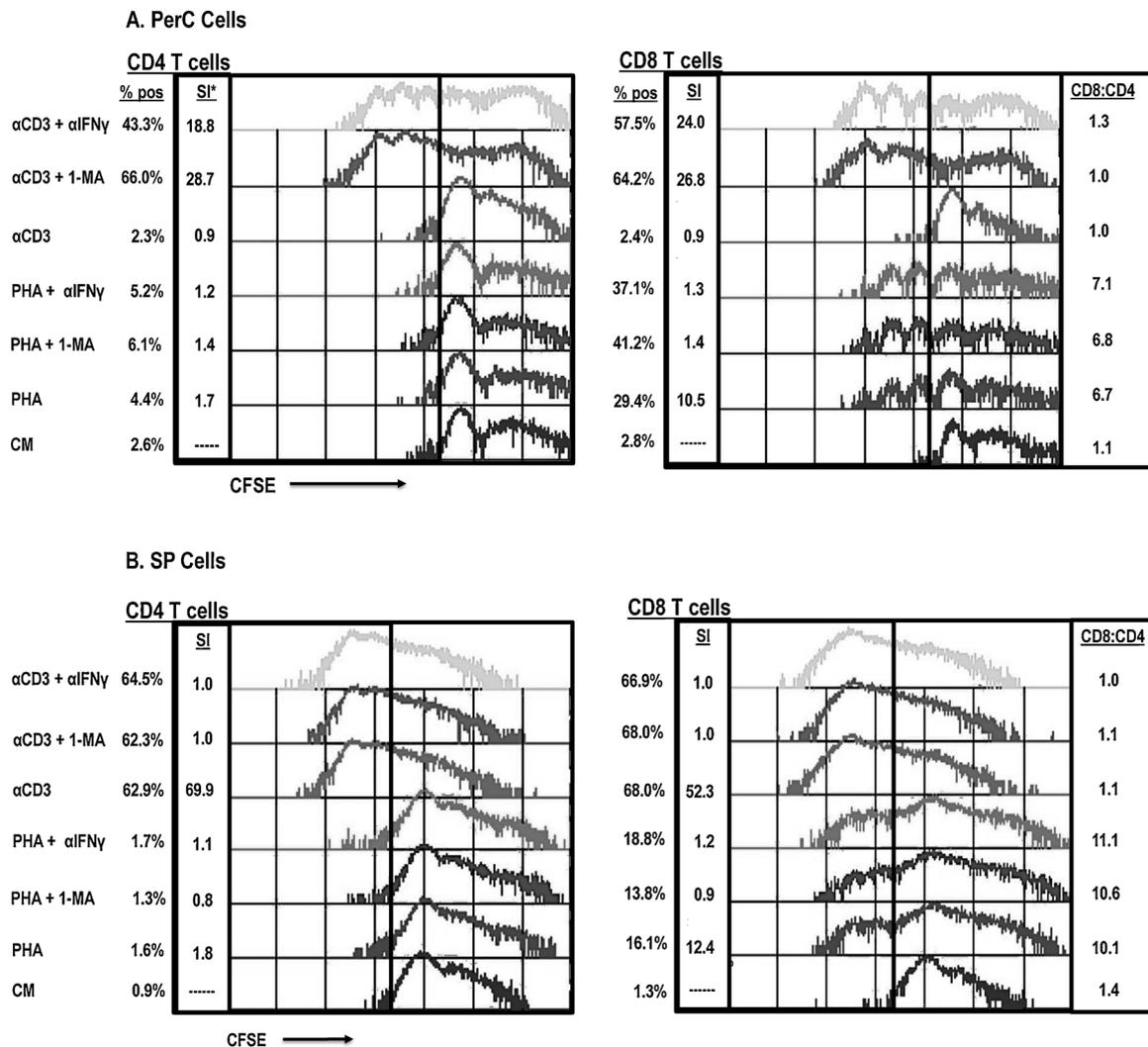
Fig. 2. PHA stimulates SP and PerC CD8 T cell proliferation. Panel A: PHA, but not TCR ligation stimulates PerC T cell proliferation. PerC T cells stimulated by TCR ligation require IFNγ neutralization or iNOS inhibition to respond. Panel B: SP T cells respond better to TCR ligation than PHA stimulation. CFSE-labeled PerC or SP cells, cultured with PHA or αCD3 +/- 1-MA or αIFNγ, were resolved into CD4$^+$ and CD8$^+$ T cell subsets by flow cytometry. *SI = stimulation index as described in methods. CD8:CD4 represents % CD8$^+$ cells / % CD4$^+$ cells. Data shown are representative of 8-10 experiments.

the manufacturer (eBioscience, San Diego, CA). Isotype-matched control mAbs were used to monitor nonspecific binding. The percentage of lymphocytes or myeloid cells expressing 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. All experiments were done a minimum of 3 times, the majority more than 5 times.

2.4. Statistical analyses, stimulation index (SI), 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 media {CM} alone). Indices that fall within the 0.8 – 1.2 range were not considered statistically significant. The Mean Fluorescence Intensity (MFI) index is defined as the average MFI for the treatment group (cultured, stimulated) divided by the average MFI for the appropriate

control (*ex vivo*, unstimulated).

3. Results

3.1. Unlike TCR ligation, PHA stimulates T cells in a suppressive, Mφ-dense environment

Due to the increased fraction of Mφs in the PerC, culture of these cells can serve as an *in vitro* model of Mφ-rich TMEs (Fig. 1A). Although PerC cell preparations have fewer T cells than organized lymphoid tissue, they have a significant portion of T cells with the CD44^{hi} effector/memory phenotype (T$_{E/M}$) found in "hot" tumors (Fig. 1 A) (Composto et al., 2011; Lanitis et al., 2017). PerC T cells respond poorly to TCR/CD3 ligation (αCD3) unless IFNγ, a trigger for iNOS expression, is neutralized or iNOS is inhibited by N^G-monomethyl-L-arginine (1-MA) (Fig. 1B) (Matlack et al., 2006). Testing other forms of T cell activation (ConA, Staphylococcal enterotoxin B, not shown (Matlack et al., 2006)), we found that only PHA could stimulate PerC T cell proliferation without requiring IFNγ neutralization or iNOS inhibition to break suppression (Fig. 1B) (Matlack et al., 2006; Goldman et al., 2017b). The greatest PHA response was consistently lower than that of the liberated αCD3 response, indicating that PHA is a less potent stimulator than that found following TCR ligation.
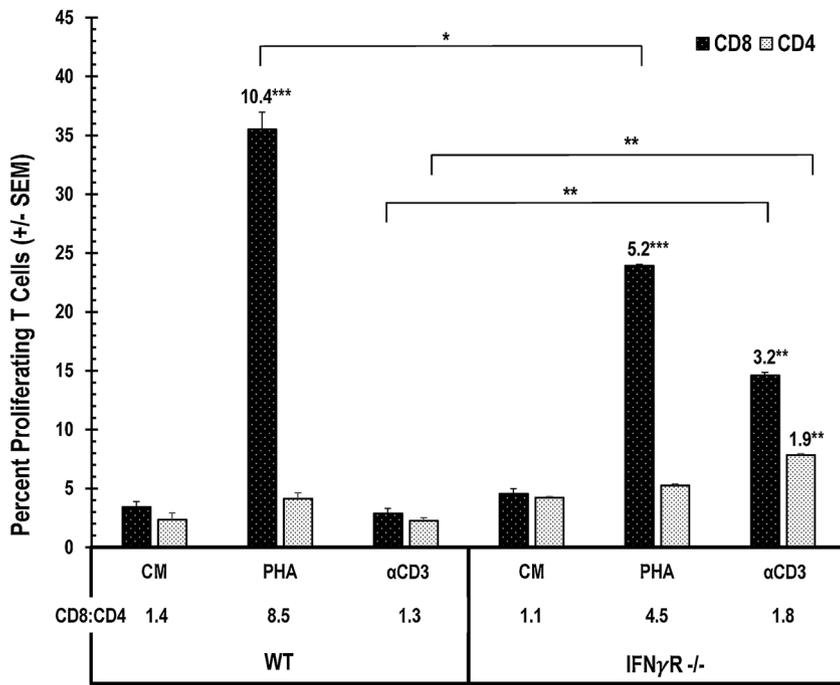
Fig. 3. Optimal PHA-induced CD8 T cell expansion is IFN-dependent. WT or IFNγR$^{-/-}$ PerC cells were CFSE-labeled, cultured with PHA or αCD3 and resolved into CD4$^+$ and CD8$^+$ T cell subsets. SI indicated above histograms, adjacent asterisks indicate significant differences in experimental vs control conditions. Data representative of 3 experiments.
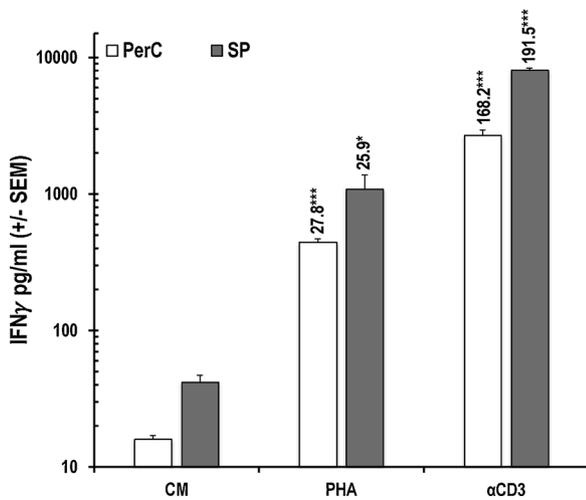

Fig. 4. PHA triggers less IFNγ production. Unstimulated (CM) and stimulated (αCD3, PHA) PerC or SP cell culture supernatants (SNs) were tested for IFNγ production. Numbers above histograms indicate SI. Average values from 6 experiments.

3.2. PHA stimulates CD8$^+$ T cell proliferation in Mφ-dense culture

To determine which cells were responding to PHA, CFSE-based flow cytometry was conducted. Unlike TCR ligation, PHA stimulation resulted in proliferation of both PerC CD4$^+$ and CD8$^+$ T cells (Fig. 2A). Intriguingly, the CD8$^+$ T cell response was much greater than that of the CD4 T cell response (> 10.5-fold expansion for CD8$^+$ T cells vs 1.7-fold for CD4$^+$ T cells; CD8:CD4 = 6.7). IFNγ neutralization or iNOS inhibition restored the response to TCR ligation (> 18-fold response), and both CD4 and CD8 T cells proliferated (CD8:CD4 = 1.0 for 1-MA treatment, 1.3 for αIFNγ). In contrast, these treatments only modestly enhanced the PHA response (< 1.5-fold) and had relatively little impact on the CD8:CD4 ratio. However, the PHA response was IFNγ dependent as both the stimulation index (WT = 10.4, IFNγR$^{-/-}$ = 5.2) and the CD8:CD4 ratio were reduced with IFNγR$^{-/-}$ PerC cells (WT = 8.5, IFNγR$^{-/-}$ = 4.5) (Fig. 3). As seen for PerC cells, the spleen (SP) cell response to PHA also favored CD8 T proliferation (CD8:CD4 = 10.1);

A. PerC Cells
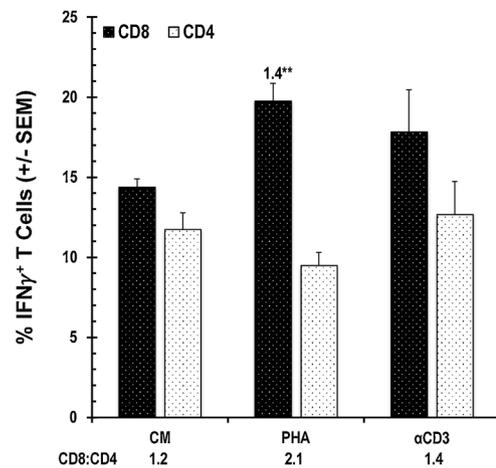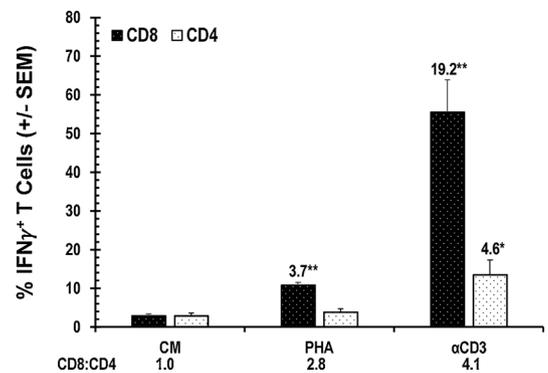

B. SP Cells

Fig. 5. PHA increases IFNγ$^+$ T cells. Panel A, PerC T cells. Panel B, SP T cells. SI above histograms with asterisks to indicate significance. Data representative of 4 experiments.
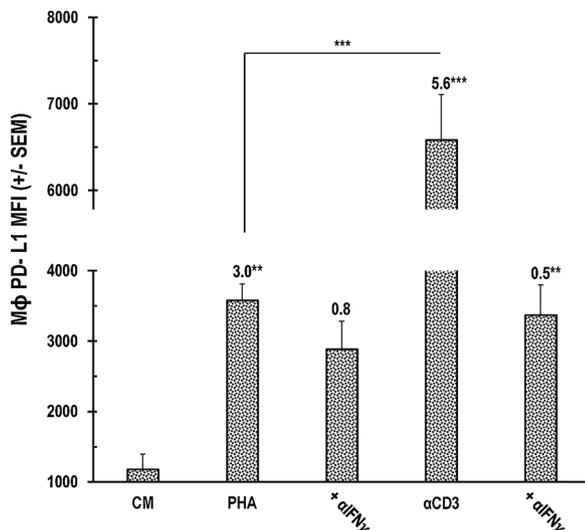
Fig. 6. Less PD-L1 expression by PerC Mφs with PHA treatment. Numbers are SI with asterisks to indicate significance. Data representative of 7 experiments.

however, the response to TCR ligation was not suppressed, and selective CD8 T cell expansion did not occur (Fig. 2B). These data show that PHA promotes CD8 T cell expansion under conditions that suppress the response to TCR ligation.

3.3. PHA increases the number of IFNγ $^{+}$ CD8 $^{+}$ T cells

Produced by both CD4 $^{+}$ and CD8 $^{+}$ T cells, IFNγ is essential for promoting cellular immunity (Mandai et al., 2016). We measured IFNγ production by PerC and SP cells by ELISA and determined the frequency of IFNγ $^{+}$ cells in these cultures by flow cytometry. Consistent with their lower T cell composition (Fig. 1A), PerC cells produced less IFNγ than SP cells when treated with either PHA or αCD3 (30-40% of SP cell values, Fig. 4). PHA increased IFNγ production by both SP and PerC cells (> 25-fold), but the response to TCR ligation was much greater (> 150-fold). Consistent with having more CD44 ^{hi} T$_{E/M}$ cells (Fig. 1A), intracellular cytokine staining revealed that PerC cell culture had more "spontaneous" IFNγ $^{+}$ CD8 $^{+}$ and CD4 $^{+}$ T cells than the SP cell culture (CM values, Fig. 5A vs B). PHA increased the percentage of CD8 $^{+}$ IFNγ $^{+}$ cells in PerC cell culture, generating a higher CD8:CD4 ratio than that following TCR ligation (Fig. 5A). Although both forms of stimulation increased the CD8:CD4 ratio, PHA triggered a smaller fraction of IFNγ $^{+}$ cells within each of the SP CD8 and CD4 T cell pools (Fig. 5B). These data illustrate that, in addition to expanding CD8 T cells under Mφ-dense conditions, PHA increases the number of IFNγ $^{+}$ cells within this important subset that is essential for cellular immunity.

3.4. PHA triggers less PerC Mφ PD-L1 expression than TCR ligation

Although IFNγ is essential for promoting cellular immunity, this cytokine also restrains T cell expansion by promoting APCs and tumor cells to express PD-L1, an immune-suppressive ligand for the PD-1 receptor expressed by activated T cells (Figs. 1, 2) (Mandai et al., 2016). Mφs are particularly sensitive to this cytokine as they rapidly and markedly upregulate PD-L1 expression *in vitro* (Sanmamed and Chen, 2014). However, compared to TCR ligation, PHA stimulation resulted in considerably less PD-L1 expression (~50%, Fig. 6). IFNγ neutralization significantly reduced the PD-L1 increase observed following TCR ligation, but had less impact on the PHA response. Neutralization of other cytokines associated with PD-L1 upregulation (IL4, Type 1 IFN]) did not impact the PHA response (data not shown). These results suggest that PHA could serve as a potent supplement to checkpoint therapy (Marin-Acevedo et al., 2018).

3.5. PHA binds CD44^{hi} effector/memory T cells and macrophages

PHA has been shown to bind glycoprotein motifs found on the TCR and CD2 of T cells, and to ligate TLRs-2/6, -4, and -5 on monocytes, Mφs, and dendritic cells, APCs essential for T cells to respond to this mitogen (Chilson et al., 1984; O'Flynn et al., 1985; Unitt and Hornigold, 2011; Lipsky et al., 1976; Byrne et al., 1988; Ricci-Azevedo et al., 2017). Since these prior studies focused on PHA binding to cells from organized lymphoid tissue and cell lines, we assessed PHA binding to PerC cells by flow cytometry. FSC/SSC analysis revealed that the addition of a standard culture concentration of PHA (2.5 μg/ml) led to the formation of cellular aggregates, particularly reducing the proportion of Mφs (by 42%) and lymphocytes (by 63%) within their gates, to form aggregates that increased by more than 2-fold the percent representation of cells outside of these gates. (Fig. 7). Reducing the PHA concentration 10-fold (0.3 μg/ml) revealed that lymphocytes were still forming aggregates, whereas Mφs were closer to the untreated control. Staining for CD4 and CD8 T cell subsets resolved on the basis of CD44 expression revealed that PHA bound most T cells, particularly those expressing high levels of CD44 (~10-fold higher MFI; Fig. 8). Compared to CD4 $^{+}$ T cells, CD44^{hi}

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cells represent a greater fraction of the PerC CD8 $^{+}$ T cell pool (47% versus 57%, n = 15; Fig. 1A). Furthermore, all F4/80 $^{+}$ Mφs bound PHA (Fig. 9). These results illustrate that PHA enhances CD8 $^{+}$ T cell activation within Mφ-dense environments and might do so by increasing APC-T cell interaction.
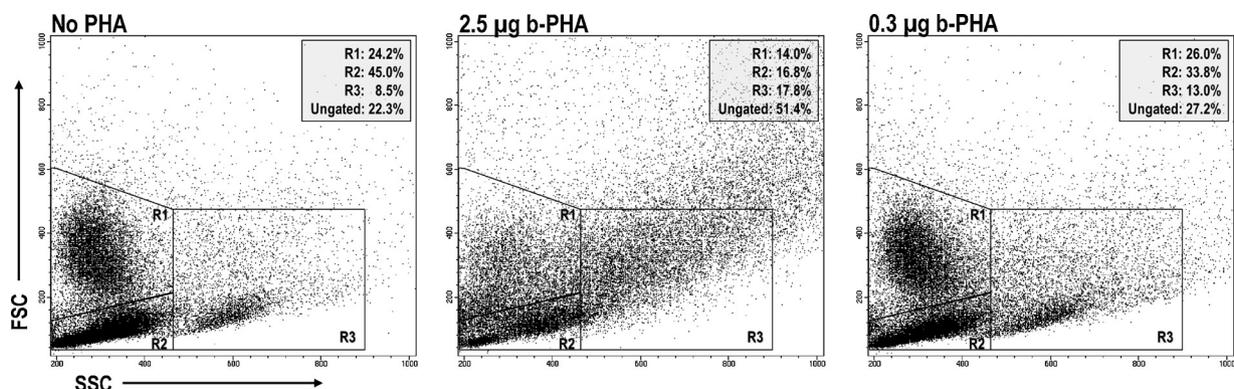

Fig. 7. PHA agglutinates WBCs. FSC/SSC gating for myeloid (R1) and lymphoid cells (R2) and doublets (R3). Ungated data represent that fraction of cells outside of the R1-3 gates. Data shown are representative of 6 experiments.

A. CD4 T Cells

	% Positive	MFI
StrAv-PE + b-PHA	87.1	655
b-PHA Control	2.2	450
StrAv-PE Control	2.7	550
Unstained Control	4.3	631

	% Positive	MFI
StrAv-PE + b-PHA	93.0	45
b-PHA Control	0.1	26
StrAv-PE Control	0.8	17
Unstained Control	0.6	25

B. CD8 T Cells

	% Positive	MFI
StrAv-PE + b-PHA	90.0	636
b-PHA Control	3.1	382
StrAv-PE Control	3.9	400
Unstained Control	3.9	353

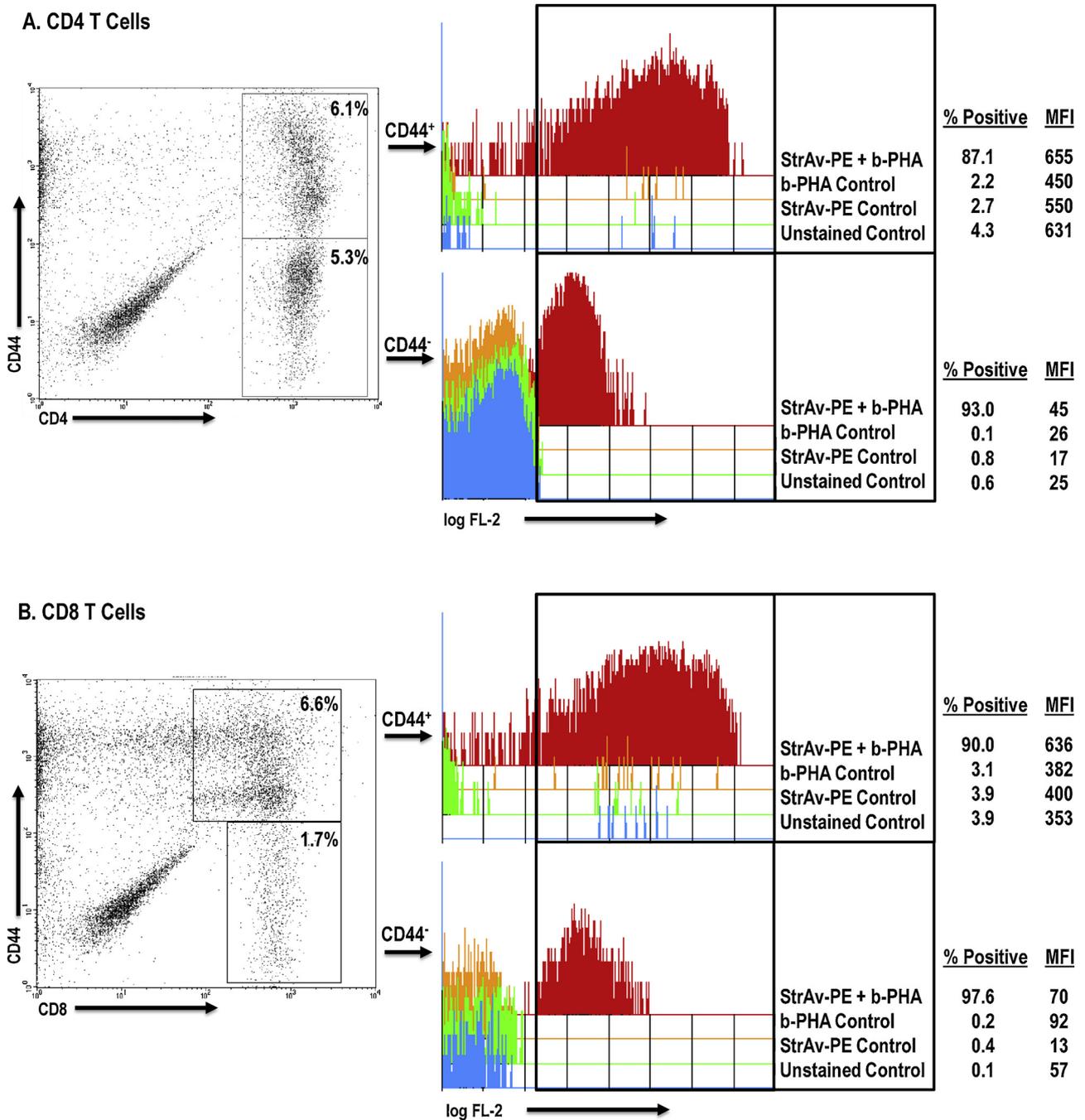|	% Positive	MFI
StrAv-PE + b-PHA	97.6	70
b-PHA Control	0.2	92
StrAv-PE Control	0.4	13
Unstained Control	0.1	57

Fig. 8. PHA binds CD44^{hi} T cells. PerC cells, stained for CD4/8, CD44, and b-PHA were gated on CD4 (Panel A) or CD8 (Panel B) versus CD44. MFI = Mean Fluorescence Intensity. Data representative of 3 experiments.

3.6. Co-culture of PerC and SP cells reveals synergistic effect of PHA

The APC requirement for PHA to achieve peak T cell activation invited speculation that the modest SP cell response to PHA could be enhanced by the addition of APCs. We tested this premise by co-culture of SP with PerC cells and observed a synergistic proliferative response to PHA stimulation, but suppression with TCR ligation (Fig. 10). While the response to PHA was two-fold greater than the sum of the individual SP and PerC cell responses, the αCD3 response for co-culture was one tenth the response of SP cells cultured alone. IFNγ neutralization recovered the αCD3 response, reinforcing the role of this cytokine in driving suppression in Mφ-dense culture. These results illustrate how PHA enhances APC- T cell interaction under conditions where TCR ligation promotes suppression.

4. Discussion

The results reported here demonstrate that PHA is a superior T cell activator in cultures with a high Mφ composition. PerC Mφs inhibited T-cell activation triggered by TCR (αCD3) ligation unless iNOS was blocked or IFNγ was neutralized. In contrast, PHA stimulated PerC T cell proliferation without IFNγ or iNOS inhibition. PHA bound to Ms and T cells, particularly CD44^{hi} T$_{E/M}$ cells, and generated marked expansion of CD8^{+} T cells. PHA also induced less inhibitory PD-L1 expression by Mφs. These results suggest that PHA might serve in strategies designed to enhance anti-tumor immunity.

PHA has a long history of *in vitro* use as a polyclonal T cell activator and generator of cytokine-rich (IL-2) supernatants (Nowell, 1960; Janossy and Greaves, 1971; Morgan et al., 1976). It has also been tested
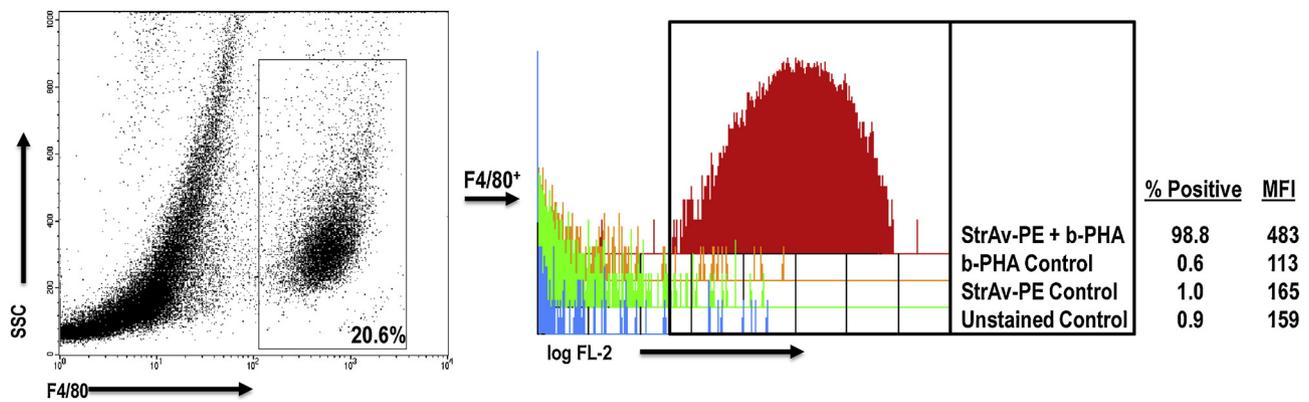
Fig. 9. PHA binds PerC Mφs. PerC cells, stained for F4/80 and b-PHA were gated as shown. Single parameter histogram depicts StrAv-PE/b-PHA binding. Data representative of 3 experiments.
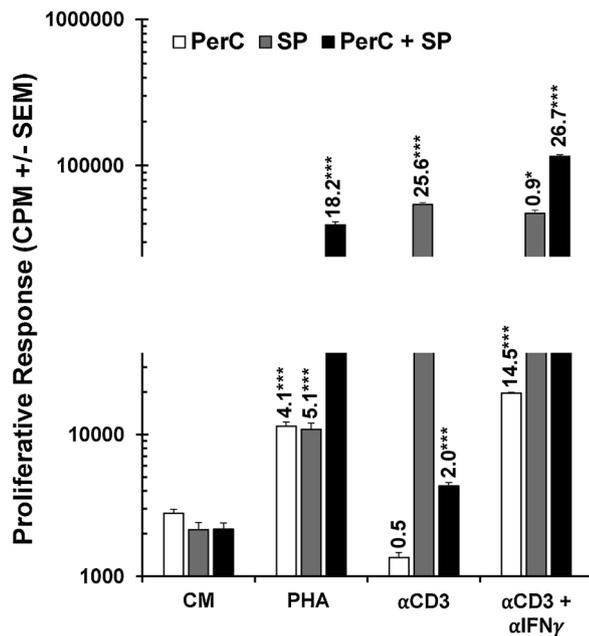

Fig. 10. PHA synergizes T cell activation with PerC + SP co-culture. PerC and SP cells were cultured alone or together with PHA or αCD3 +/- αIFNγ. SI and statistical significance indicated over histograms. Data representative of 4 experiments.

as a treatment to expand autologous T cells *in vitro* for subsequent infusion into cancer patients (Cheema and Hersh, 1972; Frenster and Rogoway, 1970; Mazumder et al., 1984). In a study focused upon melanoma treatment, direct tumor injection of *in vitro*, PHA-activated autologous lymphocytes led to a 93% response rate, which was statistically significant relative to treatment with the non-activated control (Frenster and Rogoway, 1970). In a phase I trial monitoring sarcoma patients with considerable tumor burden, large numbers of activated T cells could be safely generated and transfused, and evidence of their migration into tumors was attained, however, no clinical benefit was observed (Mazumder et al., 1984).

Cells of the immune system express distinct glycoprotein signatures that resolve them into functionally distinct subsets (Lemaire et al., 1994; Rodriguez et al., 2018). PHA has been shown to bind specific glycoprotein motifs on the TCR and CD2 of T cells, and to ligate TLRs-2/6, -4, and -5 on monocytes (Chilson et al., 1984; O'Flynn et al., 1985; Unitt and Hornigold, 2011; Lipsky et al., 1976; Byrne et al., 1988; Ricci-Azevedo et al., 2017). In this report, we show that PHA preferentially binds cells expressing high levels of CD44, a receptor for hyaluronic acid, collagens and other cellular matrix molecules (Fig.7). Post-

translational glycosylation of CD44 impacts cellular activation, effector function, and recirculation/homing. CD44 was originally designated phagocytic glycoprotein-1 (Pgp-1) due to high expression by Mφs relative to spleen cells (Colombatti et al., 1982; Hughes et al., 1983). Subsequent work found that $T_{E/M}$ are CD44hi and that the greatest expression of Pgp-1 was by peritoneal exudate, alloreactive, Lyt-2$^+$ (CD8$^+$) T cells (Budd et al., 1987). Cell surface carbohydrate modification is a phenotypic hallmark of CD8$^+$ $T_{E/M}$ cells including tumor-infiltrating lymphocytes (TILs) (Salmi et al., 1995; Galvan et al., 1998; Harrington et al., 2000). High CD8/CD4 T cell ratios in tumors are a positive prognostic factor (Hadrup et al., 2013; Djenidi et al., 2015). Intriguingly, CD44 is also expressed by cancer stem cells and is a key tumor promoter in transformed cells lacking functional p53 (Godar et al., 2008). CD44 neutralization augments T cell activation triggered by TCR ligation, but is not necessary for an optimal PHA response (Denning et al., 1990). *In toto*, these studies suggest that "hot" TMEs, *i.e.*, those comprised of Mφs, an inflammatory T cell infiltrate, and CD44hi cancer cells, likely have a "sugar code" that could provide multiple targets for PHA binding and potent activation of anti-tumor CD8$^+$ T cells (Gajewski et al., 2013; Gurusamy et al., 2017; Kelly et al., 1988; Gooden et al., 2011; Composto et al., 2011; Lanitis et al., 2017; Brooks, 2017).

Complete expression of CTL effector function requires IFNγ. However, this cytokine can also trigger Mφ suppression of T cell expansion via both iNOS and PD-L1 (Composto et al., 2011; Matlack et al., 2006; Silberman et al., 2012; Rath et al., 2014) (Figs. 1–3, 6, 10). This dual role for IFNγ has been noted in cancer and represents a significant clinical challenge (Mandai et al., 2016; Zaidi and Merlino, 2011). Compared with TCR ligation, PHA generates less IFNγ production and less PD-L1 expression - factors that favor T cell activation under Mφ-dense conditions. Notably, this was despite PerC T cells having greater percentages of both CD4$^+$ and CD8$^+$ CD44hi IFNγ-producing T cells (Kambayashi et al., 2003; Moon et al., 2015). Considering the Th1 polarization of the C57BL/6 strain of mice studied herein, it is significant to note that PHA also promoted BALB/c (Th2-prone) PerC T cell activation (data not shown) (Mosmann and Coffman, 1989).

Early studies of PHA demonstrated the absolute requirement for APCs to promote T cell activation and that increasing cell density optimized responses (Lipsky et al., 1976; Ceuppens et al., 1988; Folch et al., 1973). However, this response appears to be costimulation independent as CTLA-4-Ig, which blocked responses to TCR ligation and ConA, did not impact the PHA response (Yeh et al., 2007). Still, studies have shown that large numbers of activated (thioglycollate-elicited) Mφs can suppress the PHA response (Folch et al., 1973). Thus, strategies that debulk tumors in conjunction with checkpoint blockade are likely to optimize therapeutic efficacy (Noy and Pollard, 2014; Topalian et al., 2016; Pardoll, 2012). Perhaps PHA can activate the "exhausted" T cells found in advanced tumors (Thommen and Schumacher, 2018).

Incorporating PHA as a "glyco-conjugate" partnered with an anti-CTLA-4, -PD-1, or -PD-L1 MAb might be a strategy to optimize activation of a polyclonal anti-tumor CTL response.

Conflict of interest

The authors have no competing financial interests.

Acknowledgements

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