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Inhibition of T-cell responses in tumor microenvironments by myeloid-derived suppressor cells (MDSCs) is widely accepted. We demonstrated augmentation of monocytic MDSCs whose suppression of not only T-cell, but also B-cell, responsiveness paralleled the immunodeficiency during LP-BM5 retrovirus infection. MDSCs inhibited T cells by inducible nitric oxide synthase (iNOS)/nitric oxide (NO), but uniquely, inhibition of B cells was ~50% dependent each on iNOS/NO and the MDSC-expressed negative-checkpoint regulator VISTA. Blockade with a combination of iNOS/NO and VISTA caused additive or synergistic abrogation of MDSC-mediated suppression of B-cell responsiveness.

Myeloid-derived suppressor cells (MDSCs) inhibit the generation and/or effector activities of antitumor T-cell responses (1, 2). Limited reports indicate MDSC regulation of autoimmunity (3) and selected viral infections (4–7), including only recently, retroviral infections and murine and human AIDS (8–11). Murine MDSCs are relatively immature and heterogeneous, but all express Gr-1 and CD11b. Two subsets, monocytic Ly6G$^{+/-/lo}$ Ly6C$^{+/hi}$ and granulocytic/polymorphonuclear-leukocyte-like Ly6G$^{+/hi}$ Ly6C$^{+/-/lo}$, use differential suppressive mechanisms to inhibit T cells (12, 13). MDSC inhibition of B-cell responses is studied rarely, if at all.

Retroviruses are adept at co-opting immunoregulatory mechanisms. Human immunodeficiency virus type 1/simian immunodeficiency virus induction of PD-1 downregulates T effector cells (14, 15), and murine Friend retrovirus infection-induced PD-1 and Tim-3 affect pathogenesis and retroviral loads (16, 17), sometimes with "functionless" T cells occurring (14, 15). Viral infections can also induce CD4$^+$ FoxP3$^+$ regulatory T (Treg) cells (18), including in LP-BM5 murine retroviral pathogenesis (19–21). By 5 weeks postinfection (wpi), LP-BM5 causes profound immunodeficiency, with increased susceptibility to "opportunistic" infections and B-cell lymphomas (22, 23). Immunodeficiency requires "pathogenic" CD4$^+$ T-effector cell expression of CD154 and ligation of CD40 (22, 24, 25), and PD-1/PD-L1 and IL-10 downregulate effector T-cell activity (21, 26).

A CD11b$^+$ FcRγIII/II$^+$ myeloid cell subset expands during LP-BM5 pathogenesis (26, 27). We recently defined these monocytic MDSCs as Gr-1$^+$ Ly6C$^{+/hi}$ Ly6G$^{+/-/low}$ CD11b$^+$ with strong *ex vivo* inhibition of T- and B-cell responses used to measure LP-BM5-induced immunodeficiency (10). This robust direct MDSC-induced inhibition of B-cell responsiveness is novel for murine retrovirus-induced immunosuppression, if not generally. Also, a new negative-checkpoint regulatory ligand, VISTA (V-domain Ig suppressor of T-cell activation) (28–30), also designated PD-1H (31), with homology to PD-L1 has been defined. VISTA can be highly upregulated on myeloid-derived cells and can inhibit T-cell responses in autoimmunity and antitumor immunity in a nonredundant manner with PD-L1 (28).

At 5 wpi with LP-BM5, regarding cell surface VISTA expression, the percentage of VISTA$^+$ spleen cells had not expanded but VISTA mean fluorescence intensity (MFI) increased and the shape of the positive peak changed, consistent with the dominance of CD4 T-cell-expressed VISTA in uninfected B6 mice (28) and with CD11b$^+$ VISTA$^+$ cell expansion. Comparison of cells from wild-type (WT), iNOS$^{-/-}$, and VISTA$^{-/-}$ B6 mice (32) at 5 wpi confirmed VISTA and CD11b coexpression by the highly enriched monocytic Ly6C$^+$ MDSC population we have previously described (10), as depicted in the representative experiment in Fig. 1 (consistent with the average MFI and percent positivity over three experiments [legend to Fig. 1]). Of note, there was minimal contamination with other cells, particularly CD4$^+$ Treg cells (legend to Fig. 1). Interestingly, similar monocytic MDSCs could be isolated from the spleens of uninfected mice. These MDSCs expressed levels of VISTA approaching (and, over three repeat experiments, not significantly statistically significantly different from) that of their

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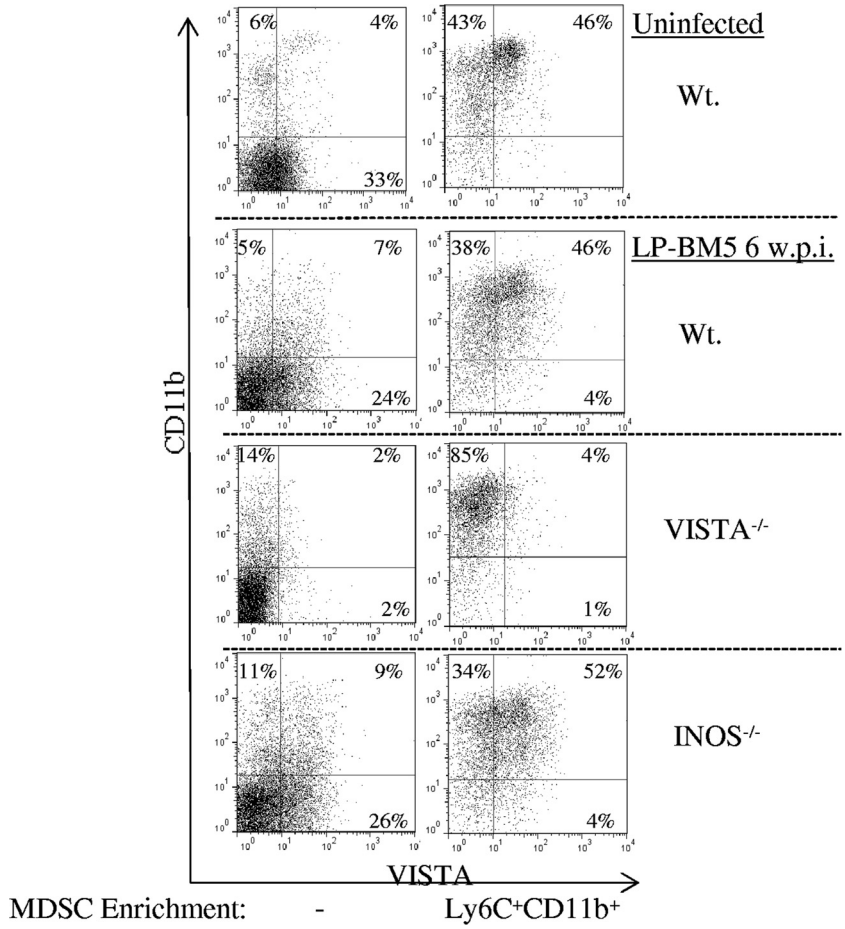

FIG 1 Surface expression of VISTA on unfractionated and Ly6C$^+$ CD11b$^+$ enriched spleen cells from B6 background strains of mice uninfected or infected for 5 weeks with LP-BM5 virus (5×10^4 ecotropic PFU) (33). Contamination with residual CD4$^+$ FoxP3$^+$ Treg cells is ~1.1% of the enriched infected WT and INOS$^{-/-}$ MDSC preparations, with only ~0.2% of the Ly6C$^{+/hi}$ CD11b$^+$ MDSCs which provide the majority of the suppressive activity (not shown). The percentages shown are the proportions of monocytic MDSCs singly or doubly positive for VISTA (13F3) and CD11b (M1/70) expression. The MFI values for VISTA expression on the cells shown here in the upper right quadrants were as follows: WT uninfected/unfractionated, 33; WT uninfected/enriched, 36; WT infected/unfractionated, 30; WT infected/enriched, 56; INOS$^{-/-}$ infected/unfractionated, 32; INOS$^{-/-}$ infected/enriched, 56. The mean percentages for enriched MDSCs (MFIs) for VISTA expression, based on three independent experiments for each mouse strain or condition, were as follows: WT uninfected, 39% ± 8% (41 ± 9); WT infected, 42% ± 4% (60 ± 3); INOS$^{-/-}$ infected, 45% ± 8% (63 ± 6). In four independent experiments, an average of 2.75-fold fewer enriched, monocytic MDSCs were obtained from the spleens of uninfected (versus infected) mice, and on a per-cell basis, the MDSCs from uninfected mice were sufficiently less suppressive (4.3-fold) than those from LP-BM5-infected mice, so that there was approximately 12-fold less total suppressive activity.

counterparts from infected mice—with respect to both the percent positive and the MFI (legend to Fig. 1). However, such MDSCs from uninfected mice were much less frequent in total cell numbers per spleen and, even compared on a per-cell basis, displayed substantially less suppressive activity—resulting in about 12-fold less MDSC suppressive function than MDSCs from infected mice (legend to Fig. 1).

The possible mechanistic involvement of VISTA was compared to the known differential role of inducible nitric oxide synthase (iNOS)/nitric oxide (NO) in MDSC-mediated suppression (Fig. 2A and B). However, MDSC-mediated suppression of uninfected WT T cells was essentially completely dependent on iNOS/NO, as shown with the iNOS inhibitor N^G-monomethyl L-arginine (L-NMMA). For B-cell responsiveness, L-NMMA blocked MDSC-mediated suppression by ~50% (range, 40 to 65%), as previously shown (10), but an anti-VISTA monoclonal antibody (MAb) blocked WT MDSC-mediated suppression of only B-cell (by ~50%), and not T-cell, responsiveness (Fig. 2A and B). We found the range of anti-VISTA MAb blocking centered around 65%, but a delta of ~55%, by subtracting control hamster immunoglobulin effects (Fig. 2C), a level consistent with the reduced suppression observed with VISTA$^{-/-}$ MDSCs (see Fig. 4). Thus, VISTA appeared to serve differentially, relative to iNOS/NO, for MDSC-mediated suppression of T-cell versus B-cell responses. With VISTA$^{-/-}$ responder cells (Fig. 2D), the anti-VISTA MAb also showed highly significant ($P = 0.003$) but partial specific blocking, confirming that VISTA blocking was directed to the MDSCs; for clarity, follow-up experiments employed VISTA$^{-/-}$ responders. In experiments not shown (three of three), the anti-VISTA MAb also blocked the suppression of B-cell responses by iNOS$^{-/-}$ MDSCs—and, as expected, the

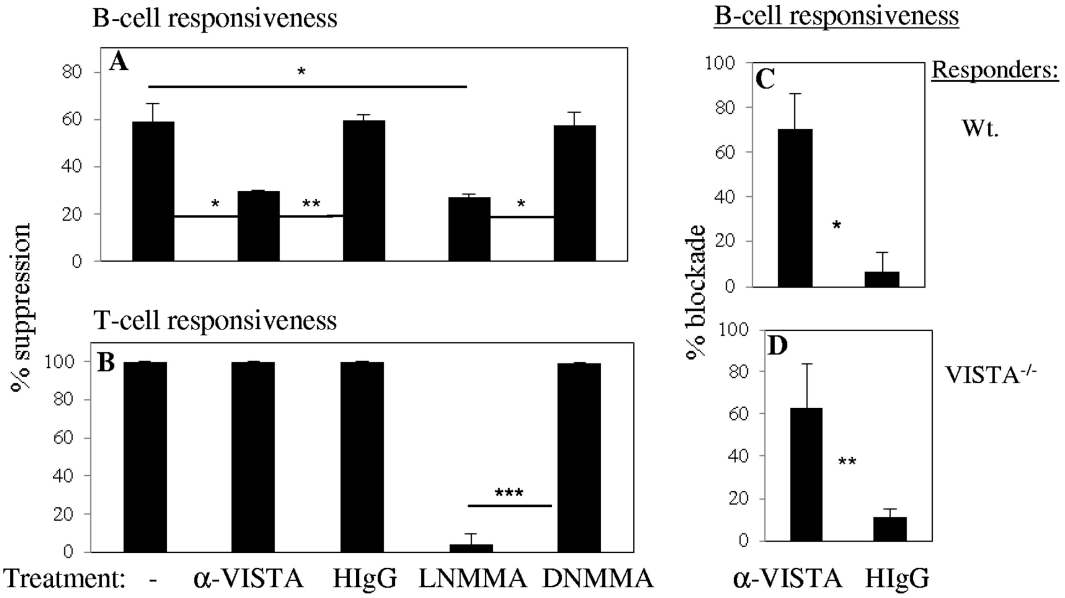

FIG 2 Distinct mechanistic requirements, involving iNOS/NO versus VISTA, differentiate LP-BM5-augmented Ly6C$^+$ CD11b$^+$ MDSC suppression of B-cell versus T-cell proliferation. Three-day cultures terminating with 6-h [^{3}H]thymidine pulses were set up as previously reported (10). WT responder spleen cells stimulated with 50 μg/ml anti-CD40 antibody plus 10 ng/ml IL-4 (A) or 0.75 μg/ml concanavalin A (B) were cocultured with Ly6C$^+$ CD11b$^+$ MDSCs (10) left untreated or treated with anti-VISTA MAb 13F3 (80 μg/ml, the optimal concentration determined by dose-response experiments), 0.8 mM L-NMMA (AG Scientific, San Diego, CA), or analogous controls. The pattern of results presented is representative of two additional experiments for each stimulation. (C, D) Cocultures of WT (C) and VISTA$^{-/-}$ (D) responders with stimulation by anti-CD40 antibody plus IL-4, depicting the average percent blockade of suppression by anti-VISTA MAb-treated MDSCs. Each panel represents the mean values of three independent experiments. Error bars represent the standard deviations. Statistical significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test, with the Holm-Bonferroni *post hoc* test when there are multiple comparisons).

blockade was greater than that obtained with WT MDSCs (see below).

To directly compare the iNOS/NO and VISTA-dependent mechanisms, carboxyfluorescein succinimidyl ester (CFSE)-preloaded responder cells were stimulated in coculture with LP-BM5-augmented monocytic MDSCs (WT versus various knockout origin cells, Fig. 3). By flow cytometry focusing on CD19$^+$ CFSE$^+$ responder B cells, WT MDSC-mediated suppression of stimulated B cells was clear, as revealed by both increased percentages of undivided cells and CFSE geometric means (panels a and b versus panel c). By inclusion of the iNOS inhibitor L-NMMA, the proliferation curve was partially restored toward B cells stimulated in the absence of MDSCs (panel e versus panel b). Alternative use of iNOS$^{-/-}$ MDSCs led to a very similar CFSE profile, as shown by the overlaid proliferation curves of iNOS$^{-/-}$ MDSCs and WT MDSCs treated with L-NMMA (panel f). Full control B-cell responsiveness was not achieved by either inclusion of L-NMMA with WT MDSCs (panel e) or the use of iNOS$^{-/-}$ MDSCs (panel g), consistent with a second, VISTA-dependent suppressive pathway. Indeed, by using the MFI values and defining suppression as the difference between increased MFI for the WT MDSCs (panel c) and the control B-cell responsiveness in the absence of MDSCs (panel b), the proportion of the MDSC suppression due to iNOS/NO could be calculated as ~45% (legend to Fig. 3). When VISTA$^{-/-}$ MDSCs were employed, (i) there was still strong, if not enhanced, MDSC-mediated suppression compared to that obtained with WT MDSCs (panel h versus panel b) and (ii) the blocking profiles of the proliferation curves resulting from genetic removal of MDSC VISTA versus inhibitor or knockout blockade of iNOS/NO were very different (panel h versus panels e to g). These findings confirmed the distinguishable nature of the VISTA versus the iNOS/NO-dependent components of MDSC-mediated suppression, with the results from the VISTA$^{-/-}$ MDSCs also compatible with a possible additional minor mechanism(s).

Combined treatment experiments were conducted. We first employed MDSCs of WT versus VISTA$^{-/-}$ origin in the presence of the iNOS/NO inhibitor L-NMMA. As expected, suppression of B-cell responsiveness by WT MDSCs was partially blocked by either the anti-VISTA MAb or L-NMMA (Fig. 4A, top). But in the same experiment, MDSCs of VISTA$^{-/-}$ origin suppressed B-cell responsiveness in a manner that was blocked only by L-NMMA (Fig. 4A, bottom). And the extent of blocking was then essentially 100%, suggesting that the retained iNOS/NO pathway was the sole major suppressive mechanism. As a follow-up approach, WT MDSCs were treated with the anti-VISTA MAb, L-NMMA, or both reagents (Fig. 4B). Only when the involvement of both VISTA and iNOS/NO was interfered with was there an essentially complete blockade of suppression (Fig. 4B). Considering also repeat experimentation, additive, if not synergistic, blocking by simultaneously interfering with iNOS/NO and VISTA corroborated these two primary independent mechanisms of MDSC-mediated suppression of B-cell responses.

To independently assess the ability of VISTA to inhibit B-cell responsiveness, we employed a VISTA-Ig chimeric fusion protein (28) (legend to Fig. 4). As shown in the representative results in

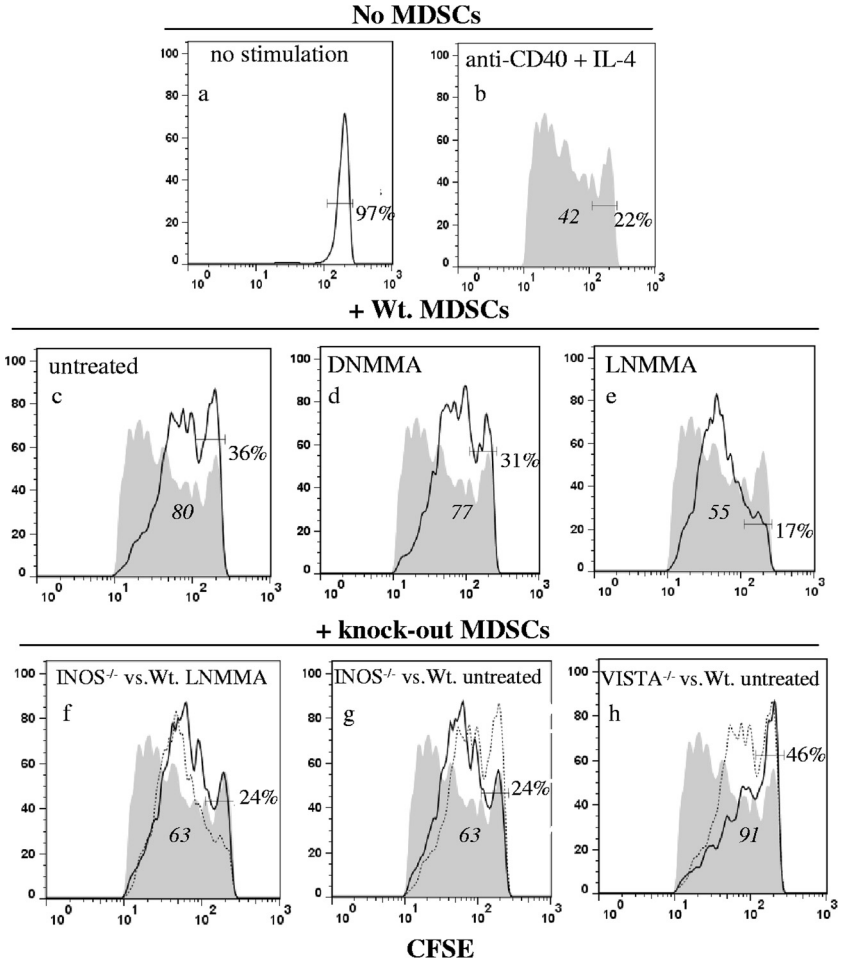

FIG 3 Ly6C$^+$ CD11b$^+$ MDSCs from WT, INOS$^{-/-}$, and VISTA$^{-/-}$ mice infected for 5 weeks with LP-BM5, each uniquely suppress B-cell proliferation of responder spleen cells. CFSE-labeled responder B cells (identified as CD19$^+$) were cultured for 4 days with no stimulation (a) or anti-CD40 antibody plus IL-4 only (b) or with WT MDSCs that were left untreated (c) or treated with D-NMMA (d) or L-NMMA (e). The fully shaded control response in panel b is repeated throughout panels c to h for comparison. For direct comparisons, overlay panels were created for responders with INOS$^{-/-}$ MDSCs (dark line) versus L-NMMA-treated WT MDSCs (dotted line) (f), iNOS$^{-/-}$ MDSCs (dark line) versus WT untreated MDSCs (dotted line) (g), and VISTA$^{-/-}$ MDSCs (dark line) versus WT untreated MDSCs (dotted line) (h). Italicized values are the geometric MFIs of the CFSE$^+$ cells under the dark line in panels b to h, and the nonitalicized values to the right within each panel are the percentages of undivided cells. Calculation for quantitative assessment of responder B-cell suppression: MFI 80 (WT MDSCs) − MFI 42 (control proliferation) = ΔMFI 38 (suppression due to WT MDSCs). As an example, to assess the effect of the INOS knockout on suppression (panel g), MFI 80 (WT MDSCs) − MFI 63 (INOS$^{-/-}$ MDSCs) = ΔMFI 17, or 17 fewer suppression units than for WT MDSCs (Δ38). Thus, Δ17/D38 = 45% (iNOS-associated suppression by WT MDSCs). The pattern of results presented is representative of one additional experiment.

Fig. 4C, VISTA-Ig inhibited polyclonal B-cell responsiveness by 40 to 59%, in a titratable manner, relative to control mIg2a—for both WT and VISTA$^{-/-}$ B-cell responders. Considering repeat experimentation (legend to Fig. 4), such inhibition was observed upon stimulation either with anti-CD40 antibody plus interleukin-4 (IL-4) or with lipopolysaccharide (LPS), complete titration could be achieved, and there was no consistent difference in susceptibility between WT and VISTA$^{-/-}$ responder B cells to blocking by VISTA-Ig.

In conclusion, we describe here for the first time, to our knowledge, the involvement of the novel negative-checkpoint regulator VISTA in MDSC-mediated suppression—in particular, of B-cell responsiveness. Our recent finding (10) that LP-BM5-augmented MDSC-mediated suppression depends on neither PD-L1 nor PD-1 further underscores the uniqueness of VISTA-related function versus its closest relative by sequence homology, PD-L1 (28, 30). The differential involvement of VISTA in the suppression of B-cell versus T-cell responsiveness by the same population of monocytic MDSCs raises the possibility of functional/phenotypic MDSC subpopulations. In addition, the inability to block MDSC inhibition of T-cell polyclonal responses by anti-VISTA MAb treatment here is to be contrasted with previous results demonstrating substantial effects on T-cell function by anti-VISTA MAb blockade in *in vitro* and *in vivo* assays (28, 29). Whether this dichotomy relates to the different myeloid compartment cells studied (e.g., MDSCs here versus myeloid DCs), the differential strength of the stimulatory signal to the responder T cells, and/or differences in the experimental microenvironments from which the myeloid cells were derived is at present unclear but likely insightful.

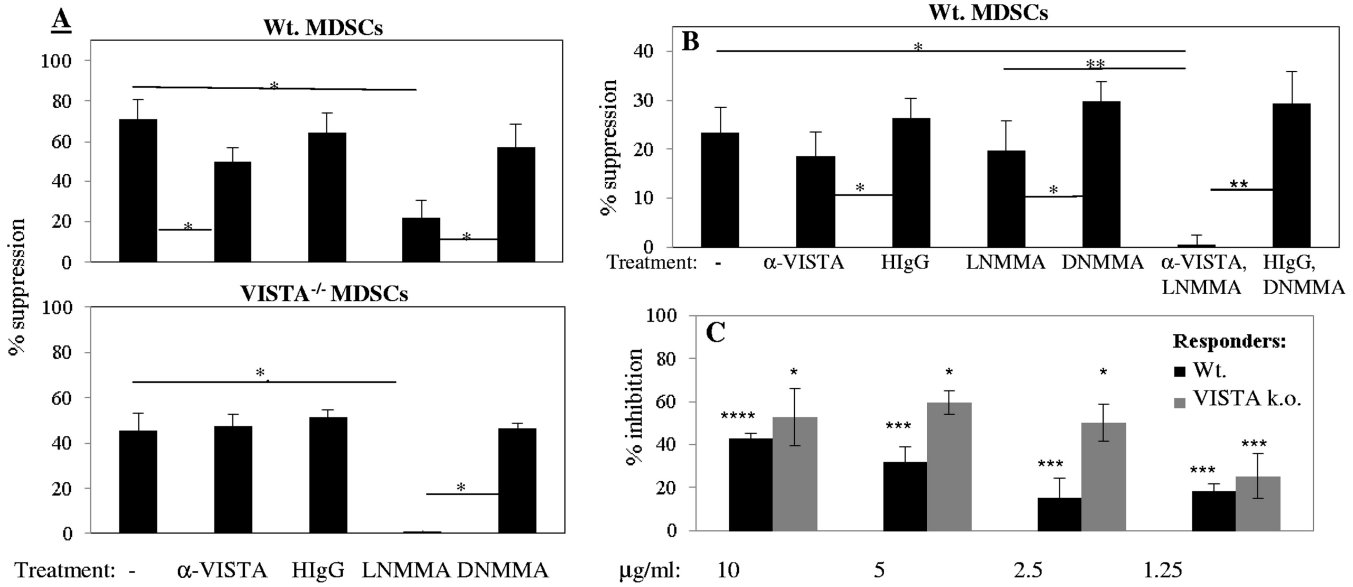

FIG 4 Blockade of LP-BM5-augmented Ly6C$^+$ CD11b$^+$ MDSCs with iNOS/NO and anti-VISTA MAb combined decreases their suppression of *ex vivo* proliferation of B cells stimulated with anti-CD40 antibody and IL-4. Naive VISTA knockout responder cells were cultured with anti-VISTA MAb- or L-NMMA-treated Ly6C$^+$ CD11b$^+$ MDSCs (responder/suppressor [R/S] ratio, 4:1) of either WT (A, top) or VISTA$^{-/-}$ origin (A, bottom) or WT Ly6C$^+$ CD11b$^+$ MDSCs (R/S ratio, 6:1) treated with the anti-VISTA MAb, L-NMMA, or both (B). The pattern of results of additive/synergistic effects is representative of one additional experiment for both panels A and B. (C) Plate-bound VISTA-Ig-effected percent inhibition of splenic B-cell proliferation in response to LPS stimulation (compared to control mIgG2a). The pattern of results presented is representative of repeat experimentation with responder B cells stimulated with LPS, two additional experiments for WT mice, and one additional experiment for VISTA$^{-/-}$ mice or anti-CD40 antibody plus IL-4, two additional experiments for WT mice, and two additional experiments for VISTA$^{-/-}$ mice. All data were derived from 3-day cultures with terminal 6-h [^{3}H]thymidine incorporation assessments. Error bars represent the standard deviations. Statistical significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (Student *t* test with the Holm-Bonferroni *post hoc* test).

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