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Expression of an immune checkpoint receptor VSIG4 defines new subsets of mouse peritoneal macrophages

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ABSTRACT

V-set and Immunoglobulin Domain Containing 4 (VSIG4), also known as Complement Receptor of the Immunoglobulin superfamily (CRIg), is a cell surface receptor structurally related to the B7 family of immune regulatory proteins. Expression of VSIG4 on macrophages renders them less responsive to LPS. In addition, VSIG4 promotes immune tolerance by attenuating early T cell activation and supporting the induction and maintenance of Foxp3 in T cells. Expression of VSIG4 on tumor-infiltrating macrophages suggests that it may be implicated in immune evasion. Using our new VSIG4 (CRIg) antibody (clone NLA14), we demonstrate that Large Peritoneal Macrophages (LPM) in mouse consist of two distinct subsets, the VSIG4+ LPM and VSIG4- LPM. These cells cultured in vitro show a spontaneous decrease in the expression of VSIG4, and addition of proinflammatory factors, including IFN-γ and LPS, further reduces VSIG4 expression. On average VSIG4+ LPM express more Arginase 1, LYVE1 and possibly MARCO than the VSIG4- LPM. Further comparative analysis of the two LPM populations using a panel of macrophage specific antibodies revealed that VSIG4+ and VSIG4- LPM show a similar pattern of expression that is distinct from Small Peritoneal Macrophages (SPM). This phenotypic diversity within the LPM most likely reflects differences in function and activation status. Characterization of VSIG4+ LPM might provide an opportunity to better understand the immunoregulatory role of VSIG4 in tumor infiltrating macrophages.

INTRODUCTION

Resident macrophages in mouse peritoneum consist of two distinct subsets: small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM). The LPM cells originate from embryonic progenitors and are normally more abundant than SPM. They express low levels of MHCII, high levels of F4/80, constitutively produce the B-cell attracting chemokine CXCL13, and are known to promote antibody production by B-1 cells. They are also efficient in removing apoptotic cells. Staining with our novel anti-VSIG4 (CRIg) antibody (clone NLA14) revealed that the LPM population consists of two distinct subsets. To shed more light on the phenotypic heterogeneity of LPM we profiled their gene expression and analyzed them using a panel of novel macrophage-specific antibodies. We also addressed the question of phenotypic stability of both LPM populations.

MATERIALS AND METHODS

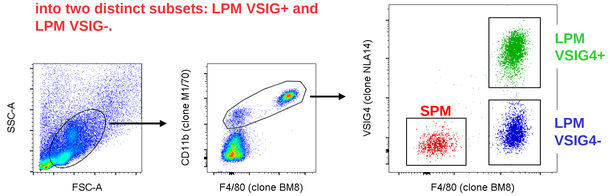
In this study we used 8-12 week old Balb/c, C75BL/6 and SJL female mice, all purchased from Jackson Laboratory and housed for at least 7 days before harvesting tissues. Peritoneal cells were harvested by injecting 5-10 mL of PBS into the peritoneal cavity and aspirating with a 21G needle. The cells were then cultured in RPMI 1640 medium supplemented with Fetal Bovine Serum, penicillin/streptomycin, 2-mercaptoethanol, and sodium pyruvate (all from Gibco) or directly stained and analyzed with a flow cytometer.

Table 1. Non-antibody products used in the study

Product	Catalog #
eBioscience™ Intracellular Fixation & Permeabilization Buffer Set	88-8824-00
eBioscience™ Lipopolysaccharide (LPS) Solution (500X)	00-4976-03
Mouse IL-10 Recombinant Protein, eBioscience™	BMS347
Mouse IL-4 Recombinant Protein, eBioscience™	BMS338
Mouse IFN gamma Recombinant Protein, eBioscience™	BMS326

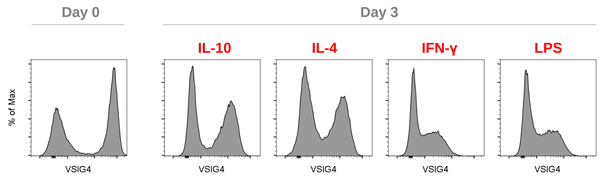
RESULTS

Figure 1. Staining with VSIG4 divides LPM into two distinct subsets: LPM VSIG+ and LPM VSIG-.



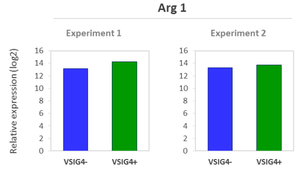
Legend Figure 1. Resident peritoneal exudate cells from Balb/c mice. Cells were stained with F4/80 (clone BM8), CD11b (clone M1/70) and VSIG4 (clone NLA14).

Figure 2. Expression of VSIG4 is down-regulated in cultured LPM. Proinflammatory factors such as IFN-γ and LPS additionally enhance this process.



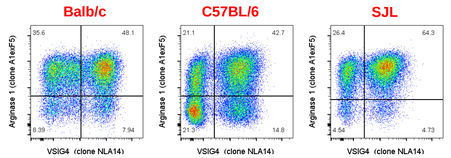
Legend Figure 2. Resident peritoneal exudate cells of Balb/c mice were either analyzed immediately after harvesting (Day 0 panel) or cultured for 72 hours in the presence of IL-10 (20 ng/ml), IL-4 (20 ng/ml), IFN-γ (20 ng/ml) or LPS (200ng/ml) (Day 3 panels). Cells were then stained with F4/80 (clone BM8), CD11b (clone M1/70), Fixable Viability Dye 506 and VSIG4 (clone NLA14). Viable cells in the LPM gate were used for analysis.

Figure 3. VSIG+ LPM appear to express marginally higher levels of messenger RNA for the Arginase 1 gene



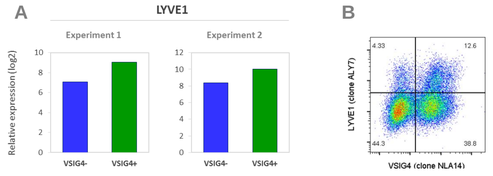
Legend Figure 3. VSIG- and VSIG+ LPM from Balb/c mice were FACS sorted and submitted to gene expression analysis using the Affymetrix Clariom D Mouse gene chip. The experiment was replicated (Experiment 2).

Figure 4. Higher proportion of VSIG4+ LPM appears Arginase 1-positive as compared to VSIG4- LPM. LPM in C57BL/6 express less Arginase 1 than LPM in Balb/c or SJL despite similar ratio of VSIG4+ to VSIG4- LPM.



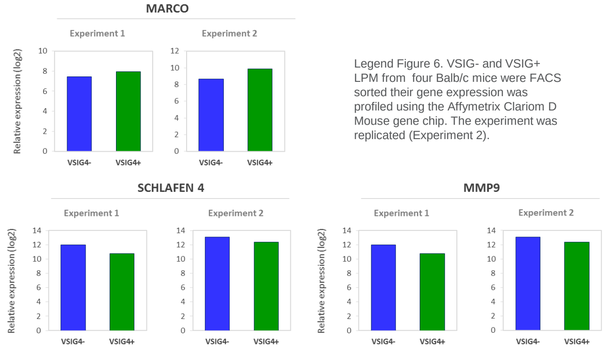
Legend Figure 4. Resident peritoneal exudate cells from indicated mouse strains were co-stained with F4/80, CD11b, VSIG4, and intracellularly stained with Arginase 1 (clone A1exF5). Cells gated on LPM population were used for analysis.

Figure 5. On average VSIG4+ LPM express more hyaluronan receptor LYVE1 as compared to VSIG- LPM. Flow cytometric analysis revealed a heterogenous pattern of LYVE1 expression.



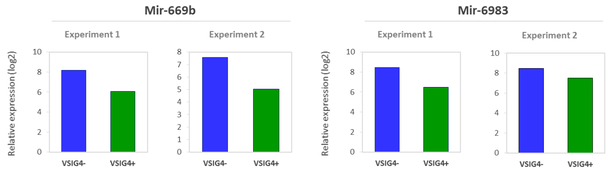
Legend Figure 5. (A) An analysis of LYVE1 gene expression in VSIG- and VSIG+ LPM cells sorted from Balb/c resident peritoneal exudate. The analysis was performed using the Affymetrix Clariom D Mouse gene chip. (B) Flow cytometric analysis Balb/C LPM cells. The cells were co-stained with F4/80, CD11b, VSIG4, and stained with LYVE1 (clone ALY7). Cells gated on LPM population were used for analysis.

Figure 6. The average expression of MMP9 and SCHLAFEN 4 genes is higher in VSIG4- population whereas MARCO is more expressed in VSIG4+ subset



Legend Figure 6. VSIG- and VSIG+ LPM from four Balb/c mice were FACS sorted their gene expression was profiled using the Affymetrix Clariom D Mouse gene chip. The experiment was replicated (Experiment 2).

Figure 7. On average VSIG+ cells expressed less microRNA and small nuclear RNA molecules.



Legend Figure 6. VSIG- and VSIG+ LPM from four Balb/c mice were FACS sorted and their gene expression was profiled using the Affymetrix Clariom D Mouse gene chip. The experiment was replicated (Experiment 2).

Table 2. Antibodies used in the study

Specificity	Clone	Catalog #	Dilution	Staining
F4/80	BM8	48-4801-82	0.25 µg/test	surface
CD11b	M1/70	11-0112-82	0.25 µg/test	surface
VSIG4	NLA14	25-5752-82	0.25 µg/test	surface
Arginase 1	A1exF5	17-3697-82	0.5 µg/test	intracellular
LYVE1	ALY7	12-0443-82	0.06 µg/test	surface
VISTA	MIH64	17-1083-82	0.25 µg/test	surface
Axl	MAXL8DS	17-1084-82	0.06 µg/test	surface
MerTK	DS5MMER	12-5751-82	0.5 µg/test	surface
RELM alpha	DS8RELM	17-5441-82	0.25 µg/test	intracellular

CONCLUSIONS

- Peritoneal macrophages have been traditionally categorized into two populations: SPM and LPM.
- Staining with our novel anti-VSIG4 antibody (clone NLA14) allows to further divide the LPM population into two distinct subsets.
- In cultured LPM the level of VSIG expression decreases, and this process is further accelerated by the presence factors, such as IFN-γ or LPS.
- The VSIG+ LPM tend to express Arginase 1, and LYVE1 more frequently that VSIG- LPM.
- MMP9, Schlafen4 and multiple small RNAs are expressed at lower levels in VSIG+ LPM, suggesting less activated status of these cells.
- Majority of macrophage markers such as: MerTK, Axl, VISTA, CD64, CD204, CD206, CXCL13, and MHCII had similar levels of expression in both LPM populations as analyzed by flow cytometry (data not shown)

REFERENCES

1. Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, Bortoluci KR, Almeida SR, Herzenberg LA, Herzenberg LA. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. Proc Natl Acad Sci USA. 2010 Feb 9;107(6):2568-73.2.
2. Cassado Ados A, D'Império Lima MR, Bortoluci KR. Revisiting mouse peritoneal macrophages: heterogeneity, development, and function. Front Immunol. 2015 May 19;6:225.

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