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Supplemental Information

Surface LSP-1 Is a Phenotypic Marker Distinguishing Human Classical versus Monocyte-Derived Dendritic Cells

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Supplemental Information

Transparent methods

Human Samples. Buffy coats from healthy donors (both male and female donors) were obtained from Etablissement Français du Sang (Paris, France) in accordance with INSERM ethical guidelines. Tumor ascites from ovarian cancer patients were obtained from Hôpital de l'Institut Curie in accordance with hospital guidelines. Tonsils from healthy patients (both male and female) undergoing tonsillectomy were obtained from Hôpital Necker (Paris, France). According to French Public Health Law (article L1121), written consent and IRB approval are not required for human non-interventional studies.

Cell isolation. Tonsil samples were digested as described previously (Durand and Segura, 2016). In brief, samples were cut into small fragments, digested with 0.1 mg mL^{-1} Liberase TL (Roche) in the presence of 0.1 mg mL^{-1} DNAse (Roche) for 40 minutes at room temperature before addition of 10 mM EDTA. Cells were filtered on a 40 μm cell strainer (BD Falcon) and washed. Light density cells were isolated by centrifugation on a Ficoll gradient (Lymphoprep, Greiner Bio-One). DCs and macrophages were enriched by depletion of cells expressing CD3, CD15, CD19, CD56 and CD235a using antibody-coated magnetic beads (Miltenyi). Peripheral Blood Mononuclear Cells (PBMC) were prepared by centrifugation on a Ficoll gradient. Blood $CD14^+$ monocytes were isolated from healthy donors' PBMC by positive selection using anti-CD14-coated magnetic beads according to manufacturer's instructions (Miltenyi). DCs and macrophage populations from ascites were isolated after cell sorting on a FACSAria instrument. Ascites DCs were gated as HLA-$DR^+CD11c^+CD1c^+CD16^-$.

Cell culture. Blood monocytes (1×10^6 cells mL^{-1}) were cultured for 5 days in RPMI-Glutamax medium (Gibco) supplemented with antibiotics (penicillin and streptomicin) and 10% FCS in the presence of 100 ng mL^{-1} M-CSF (Miltenyi), 5 ng mL^{-1} IL-4 (Miltenyi) and 5 ng mL^{-1} TNF-

α (Miltenyi), or 100 ng mL^{-1} GM-CSF (Miltenyi) and 5 ng mL^{-1} IL-4 (Miltenyi). In some experiments, mo-DC and mo-Mac were purified by cell sorting on a FACSAria instrument. Mo-DC were gated as $CD1a^{+}CD16^{-}$ and mo-mac as $CD1a^{-}CD16^{+}$.

Phage display screening. A synthetic phage display library of humanized llama single domain antibodies (NaLi-H1 library) was used as described (Moutel et al., 2016) to select binders. Screening of positive clones was performed using 100 µL of supernatant (80 µL phages + 20 µL PBS/human serum 1%) incubated for 1 h on ice with 1×10^{5} cells from tumor ascites. After washing, phage binding on ascites cells was detected by flow cytometry using an antibody against M13, and ascites DCs were gated as $HLA\text{-}DR^{+}CD11c^{+}CD1c^{+}CD16^{-}$ and ascites macrophages as $HLA\text{-}DR^{+}CD11c^{+}CD1c^{-}CD16^{+}$.

Western blot. Cells were lysed in RIPA buffer (Thermo Scientific) supplemented with cOmplete Mini EDTA-free protease inhibitor cocktail (Roche). Post-nuclear lysates were resolved by SDS-PAGE using 4-12% BisTris NuPAGE gels (Invitrogen) and proteins were transferred to membranes (Immunoblot PVDF membranes, Bio-Rad). Membranes were stained with anti-LSP1 (clone TPD153, Novus Biologicals) or streptavidin-HRP staining (Pierce).

Flow cytometry. For the analysis of tonsil cells, tonsil $CD14^{+}$ macrophages were gated as $HLA\text{-}DR^{+}CD11c^{+}CD14^{+}$, tonsil cDC1 as $HLA\text{-}DR^{+}CD11c^{+}CD14^{-}CD1c^{-}CD141^{+}$ and tonsil cDC2 as $HLA\text{-}DR^{+}CD11c^{+}CD14^{-}CD1c^{+}$. For the analysis of blood cells, B cells were gated as $HLA\text{-}DR^{+}CD19^{+}$, pDC as $HLA\text{-}DR^{+}CD19^{-}CD11c^{-}CD123^{+}$, granulocytes as $HLA\text{-}DR^{-}CD19^{-}SSC^{high}CD16^{+}$, NK cells as $HLA\text{-}DR^{-}CD19^{-}CD14^{-}SSC^{low}CD16^{+}$, T cells as $HLA\text{-}DR^{-}CD19^{-}CD14^{-}SSC^{low}CD16^{-}$, $CD16^{+}$ monocytes as $HLA\text{-}DR^{+}CD19^{-}CD16^{+}CD14^{-}$, $CD16^{+}CD14^{+}$ monocytes as $HLA\text{-}DR^{+}CD19^{-}CD16^{+}CD14^{+}$, $CD14^{+}$ monocytes as $HLA\text{-}DR^{+}CD19^{-}CD16^{-}CD14^{+}CD1c^{-}$, cDC2 as $HLA\text{-}DR^{+}CD19^{-}CD16^{-}CD14^{-}CD1c^{+}$, DC3 as $HLA\text{-}DR^{+}CD19^{-}CD16^{-}CD14^{int}CD1c^{+}$.

Flow cytometry stainings were performed at 4°C in PBS containing 2mM EDTA (Gibco) and 0.5% human serum AB male (Biowest). Antibodies used were : FITC anti-CD19 (clone HIB19, BioLegend), FITC anti-CD3 (clone UCHT1, BioLegend), FITC anti-CD16 (clone 3G8, Biolegend), PE anti-M13 (GE healthcare), PE anti-CD19 (clone SJ25C1, eBioscience), PE anti-CD141 (clone AD5-14H12, Miltenyi biotec), Pe/Cy7 anti-CD11c (clone Bu15, Biolegend), Pe/Cy7 anti-CD163 (clone GHI/61, eBioscience), PerCP-eFluor710 anti-CD1c (clone L161, eBioscience), APC anti-CD304 (clone REA774, Miltenyi biotec), APC anti-CD1a (clone HI149, BioLegend), Alexa647 anti-LSP1 (clone TPD153, Novus Biologicals), APC-eFluor780 anti-HLA-DR (clone LN3e, Bioscience), HorizonV500 anti-CD14 (cloneM5E2, BD). In some experiments, cells were stained with Streptavidin-PE (BD), Streptavidin-APC (BD) or Streptavidin-PeCy7 (BD). Fc receptor binding was blocked using TruStain FcX (BioLegend). Dead cells were excluded with 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher) staining. For intracellular staining, cells were first surface stained as above, then incubated with Live/dead fixable Aqua (Thermo Fisher Scientific) for 10 min at 4°C. Then the cells were fixed and permeabilized using Intracellular Fixation & Permeabilization Buffer Set (eBioscience). Unspecific binding was blocked by incubating cells for 30 min at 4°C with blocking buffer (Perm/Wash buffer from Intracellular Fixation & Permeabilization Buffer Set containing 2% of normal mouse serum and 10µg/mL human Fc block (BD)). Cells were stained for intracellular molecules in blocking buffer at room temperature for 30 min. For VHH D4 intracellular staining, cells were further incubated with Streptavidin-APC (BD) for 15 min at 4°C in blocking buffer. Cells were analyzed on a FACSVerse or LSRII (BD Biosciences) instrument. Data was analyzed with FlowJo (Tree Star).

Immuno-precipitation. All the incubation steps were performed rocking the tubes constantly. Cleared cell lysates resuspended in an equal volume of IP buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40) were pre-incubated 1 h at 4°C in the presence of 200 µL of protein

G agarose beads (Thermo Fisher) and successively washed 3 times in IP buffer to eliminate unspecific binding. The supernatant was recovered by centrifugation (3 min × 2500 g), mixed with 200 µg of antibody, and incubated 2 h at 4°C. Finally, 200 µL of washed protein G agarose beads were added and washed after 1 h at 4°C 5 times in 10 mL of IP buffer five times before being resuspended in 50 µL of SDS loading buffer and heated 10 min at 95°C.

Proteomics Mass-spectrometry. Immuno-precipitation was performed with VHH D4 on *in vitro*-generated mo-DC. Two bands were obtained for the immuno-precipitated material after SDS-PAGE migration (molecular weight around 60 kDa and 45-50 kDa respectively). Gel slices were washed and proteins were reduced with 10 mM DTT before alkylation with 55 mM iodoacetamide. After washing and shrinking the gel pieces with 100% (vol/vol) MeCN, we performed in-gel digestion using trypsin (Roche) overnight in 25 mM NH_4HCO_3 at 30 °C. Peptides extracted from each band were analyzed by nanoLC-MS/MS using an Ultimate 3000 system (Dionex, Thermo Scientific, Waltham, MA) coupled to a TripleTOF™ 6600 mass spectrometer (ABSciex). For identification, data was searched against the Swissprot fasta database containing *Homo Sapiens* (2014_10, 20194 sequences) using Mascot™ (version 2.3.02) and further analyzed in myProMS (Poullet et al., 2007). The maximum false discovery rate (FDR) calculation was set to 1% at the peptide level for the whole study (QVALITY algorithm). Only proteins found in two experiments and not in the control IPs were considered candidates. Only one candidate was identified (P33241, Lymphocyte-specific protein 1), with scores of 403.47 and 499.61, and coverage of 23.9% and 26.3%, for the two bands, respectively. Data is available via ProteomeXchange (identifier PXD015647) (Vizcaino et al., 2016).

Supplemental References

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