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VLA-4 Affinity Assay for Murine Bone Marrow-derived Hematopoietic Stem Cells

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[Abstract] Hematopoietic stem cells (HSCs) are defined by their functional ability to self-renew and to differentiate into all blood cell lineages. The majority of HSC reside in specific anatomical locations in the bone marrow (BM) microenvironment, in a quiescent non motile mode. Adhesion interactions between HSCs and their supporting BM microenvironment cells are critical for maintaining stem cell quiescence and protection from DNA damaging agents to prevent hematology failure and death. Multiple signaling proteins play a role in controlling retention and migration of bone marrow HSCs. Adhesion molecules are involved in both processes regulating hematopoiesis and stem- and progenitor-cell BM retention, migration and development. The mechanisms underlying the movement of stem cells from and to the marrow have not been completely elucidated and are still an object of intense study. One important aspect is the modification of expression and affinity of adhesion molecules by stem and progenitor cells which are required both for stem cell retention, migration and development. Adhesion is regulated by expression of the adhesion molecules, their affinity and avidity. Affinity regulation is related to the molecular binding recognition and bond strength. Here, we describe the *in vitro* FACS assay used in our research to explore the expression, affinity and function of the integrin $\alpha_4\beta_1$ (also termed VLA-4) for murine bone marrow retained EPCR$^+$ long term repopulation HSC (LT-HSC) (Gur-Cohen *et al.,* 2015).
Keywords: Hematopoietic stem cells, HSC mobilization, Bone marrow retention, VLA-4, aPC/EPCR/PAR1 signaling

[Background] Integrins are type I transmambrane glycoprotein receptors that mediate cell-cell and cell-matrix adhesion interactions, signaling and communication. All integrins are heterodimers of non-covalently associated α and β subunits. In humans integrin heterodimers are formed from 9 types of β subunits and 24 types of α subunits. This diversity is further increased by alternative splicing of some integrin RNAs. Each heterodimer consists of a large extracellular domain which binds proteins in the extracellular environment, a single transmembrane domain, and an intracellular cytoplasmic tail domain. The largest integrin subfamily is composed by integrin β_1 (CD29) that is able to associates with 12 different α subunits ($\alpha_{1\text{-}11}$ and α_v). Integrin β_1 together with integrin chains α_4, α_5, α_6 and α_9 are expressed by murine hematopoietic stem and progenitor cells (HSPCs) and play important roles in regulating their BM retention, migration and development.

Integrins, like other transmembrane receptors, display an ‘outside-in signaling’, *i.e.*, to transduce the signal intracellularly after the binding with their ligand. Moreover, integrins have a peculiar feature: they are able to shift between high- and low-affinity conformation states for ligand binding (‘inside-out’ signaling) (Takagi and Springer, 2002). According to the cell type, integrins can be either basically

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activated or basally inactive. In the inactive state, the integrin extracellular domains are not bounded to the ligands, and are in a bent conformation. Following intracellular activation signals, the extracellular domain is straightened, stabilizing the extended active conformation. Thus, the external ligand binding site, is now exposed to the ligand binding, allowing the transmission of the signals from the outside to the inside (Luo *et al.*, 2007).

Very Late Antigen-4 (VLA-4, also known as CD49d/CD29 or $\alpha_4\beta_1$) is a member of the integrin α_4 family together with $\alpha_4\beta_7$. Within the integrin family, VLA-4 has some unique features. In contrast to related members of β_1 subfamily, VLA-4 is predominantly expressed on hematopoietic lineage cells (Hemler, 1990) and is functionally involved in both cell-cell and cell-ECM adhesive interactions. Moreover, despite sequence homology with other integrin α subunits, the α_4 strand because of the lack of the inserted I-domain doesn't undergo post-translational cleavage near the transmembrane region. Finally, the α_4 chain contains a trypsin-like cleavage site, constitutively expressed on most leukocytes and on hematopoietic stem and progenitor cells (Hynes, 1992).

VLA-4 plays a major role in the regulation of immune cell recruitment to inflamed endothelia and sites of inflammation through its interactions with two alternative ligands, vascular cell adhesion molecule-1 (VCAM-1) and the alternatively spliced connecting segment 1 (CS-1) of fibronectin (Hemler, 1990; Papayannopoulou *et al.,* 1998). It also participates in many cellular events and is crucial for BM retention and mobilization of immature stem and progenitors cells from the bone marrow (Lapidot and Petit, 2002; Peled *et al.,* 2000).

Migration of hematopoietic stem cells to the bone marrow is a regulated multistep process that requires precise regulation and activation of various molecules including chemoattactants, selectins and integrins. While the initial steps of hematopoietic stem and progenitor cells tethering and rolling along BM blood vessel endothelium are primarily regulated by selectins, various integrins have been shown to be involved in the next stages of this process. VLA-4 plays an important role in homing, lodgment and retention of HSCs within the marrow microenvironment (Rettig *et al.,* 2012). Previous studies demonstrate that treatment of donor BM cells with a neutralizing anti α_4 integrin antibody before injection into lethally irradiated recipients, inhibits their homing to the femurs of recipient mice, increasing the number of HSPCs in the peripheral blood and spleen. Moreover, recipient mice pretreated with neutralizing antibodies against VCAM-1 gave similar results, adding evidences to the important role of the VLA-4/VCAM-1 axis in HSPC homing to the bone marrow (Papayannopoulou *et al.,* 1995).

Recently, factors traditionally related to coagulation and inflammation have been shown to independently control long term (LT) HSCs retention in the bone marrow and their recruitment to the blood (Aronovich *et al.,* 2013; Gur-Cohen *et al.,* 2015). Adult murine BM LT-HSCs, endowed with the highest repopulation and self-renewal potential, express endothelial protein C receptor (EPCR) which is used as a marker to isolate them (Balazs *et al.,* 2006).

Protease-activated receptor-1 (PAR1) is functionally expressed by bone marrow stromal and endothelial cells as well as HSC and immature and maturing leukocytes (Gur-Cohen *et al.,* 2016). Activated protein C ([aPC], the major ligand for EPCR)-EPCR/PAR1 signaling facilitate LT-HSC BM repopulation, retention, survival, and chemotherapy resistance by restricting nitric oxide (NO) production.

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Inhibition of NO generation by aPC/EPCR/PAR1 signaling on LT-HSC, inhibits downstream CDC42 activity and induces CDC42 polarity, as well as increasing VLA4 expression, affinity and adhesion. Conversely, acute stress and clinical mobilization up-regulate thrombin generation and activate different PAR1 signaling leading to NO generation that overcomes BM EPCR^{+}LT-HSC retention, inducing TACE mediated EPCR and VLA-4 shedding, up-regulation of CXCR4 and PAR1 on LT-HSC, stromal PAR1 mediated CXCL12 secretion, resulting in stem and progenitor cell recruitment to the blood stream (Gur-Cohen *et al.,* 2015). VLA-4 is expressed at higher level by bone marrow EPCR^{+}LT-HSC together with higher affinity to its ligands, inducing their BM retention and protection from DNA damaging agents. The restriction of NO by EPCR/PAR1 signaling increase the affinity of VLA-4 regulating anchorage and bone marrow retention of LT-HSC and chemotherapy resistant (Gur-Cohen *et al.,* 2015).

Multiple small molecules have been developed in an attempt to regulate integrin dependent adhesion. The affinity states of human VLA-4 can be recognized by monoclonal antibodies sensitive to its molecular conformation (Masumoto and Hemler*,* 1993). Importantly, changes in VLA-4 affinity can be detected in real-time and on a physiologically relevant time frame using a ligand mimicking LDV-containing fluorescent small molecule (LDV-FITC) by FACS (Chigaev *et al.,* 2001). VLA-4 recognize with high affinity a peptide sequence within fibronectin, which comprises 25 amino acid, termed CS-1 (Hynes, 1992). LDV (leu-asp-val) is the tripeptide identified as the minimal sequence for specific VLA-4 recognition of CS-1 segment of fibronectin. Here we describe a method to detect VLA-4 affinity monitoring mean fluorescent intensity through flow cytometry using LDV-FITC.

Materials and Reagents

1. Syringe with needle 1 ml 25 G x 5/8 in. (0.5 x 16 mm) (BD, catalog number: 300014)
2. Dishes 35 x 10 (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 153066)
3. 70 µm nylon strainer (Sinun Tech, catalog number: Polymer Screens)
4. FACS tubes (Corning, Falcon™, catalog number: 352054)
5. Cells of interest (here murine bone marrow cells obtained from 8 weeks old mice)
6. Dulbecco's phosphate-buffered saline (PBS$^{+/+}$) (Biological Industries, catalog number: 02-020-1A)
7. EDTA (5 mM final concentration in water, pH 7.4) (Avantor® Performance Materials, J.T.Baker, catalog number: 8993-01)
8. Wet ice
9. Antibodies to detect LT-HSCs by flow cytometry:
   a. Sca-1 PEcy7 (clone D7) (Biolegend, catalog number: 108114)
   b. c-kit APC (clone 2B8) (Biolegend, catalog number: 105812)
   c. CD150 Brilliant Violet (clone TC15-12F12.2) (Biolegend, catalog number: 115922)
   d. CD48 Pasific Blue (clone HM48-1) (Biolegend, catalog number: 103418)
   e. EPCR PE (clone eBio1560) (Affymetrix, eBioscience, catalog number: 12-2012-82)

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f. Lineage: CD4 (clone GK 1.5), CD8a (clone 53-6.7), GR1 (clone RB6-8C5), B220 (clone RA3-682), Ter119 (clone TER-119), CD11b (clone M1/70)

10. Calciumchloride dihydrate (EMD Millipore, catalog number: 102382)
11. Magnesium chloride hexahydrate (EMD Millipore, catalog number: 105833)
12. HEPES buffer solution (Biological Industries, catalog number: 03-025-1B)
13. Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific, Gibco™, catalog number: 14175095)
14. Bovine serum albumin solution (10%, BSA) (Biological Industries, catalog number: 03-010-1B)
15. 2.4-((*N*'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine (LDV-FITC) (R&D System, catalog number: 4577)
16. Deionized water (DDW)
17. Gentian violet (Sigma-Aldrich, catalog number: 548-62-9)
18. Acetic acid (Sigma-Aldrich, catalog number: 64-19-7)
19. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: 30525-89-4)
20. LDV medium (see Recipes)
21. LDV-FITC stock and working solutions (see Recipes)
22. Turk's solution (see Recipes)
23. 4% PFA (see Recipes)

Note: Flourochrome should be chosen according to the flow cytometry machine.

Equipment

1. Forceps (Kent Scientific, catalog number: INS700100-2)
2. Scissors (Kent Scientific, catalog number: INS750046)
3. Centrifuge (Eppendorf, model: 5810R)
4. Centrifuge swing-bucket rotor A-462 4 x 250 ml rectangular buckets (Eppendorf, catalog number: 5810709008)
5. Adapters (Eppendorf, catalog number: 5810752000)
6. Inverted light microscope (Olympus, model: CHK2-F-GS)
   Note: This product has been discontinued by the manufacturer.
7. Hemocytometer (Sigma-Aldrich, Bright-Line™, catalog number: Z359629)
8. Incubator 37 °C, 5% CO_2 (Thermo Fisher Scientific, Thermo Scientific™, model: 150i)
9. Cold room or refrigerator (4 °C)
10. Flow cytometer (*i.e.,* Macs Quant instrument [Miltenyi, BergischGladbach, Germany] or BD LSR II flow cytometer)
11. 2 L glass flask or bottle (Kimble Chase Life Science and Research Products, catalog number: 26500-2000)
12. Plastic weigh boat (Sigma-Aldrich, catalog number: Z186856)

Note: Color combinations can be adjusted to match the laser combinations available.

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Software

1. FlowJo V10 software (Tree Star, optional)

Procedure

A. Obtain murine bone marrow cells

Note: All animal experiments have to be approved by local animal care and ethics authorities.

1. Sacrifice desired mice strain by CO_2 euthanization or cervical dislocation.
2. Extract the femurs, tibias and iliac crest bones using forceps and sharp scissors. Place the bones on a small dish supplemented with $PBS^{+/+}$ on ice.
3. Flush total bone marrow cells from the bone cavity with 1-5 ml ice cold PBS (per mouse) using a 1 ml syringe and 25 G needle. Obtain single cell suspension by simply resuspending the cell solution using the same syringe.

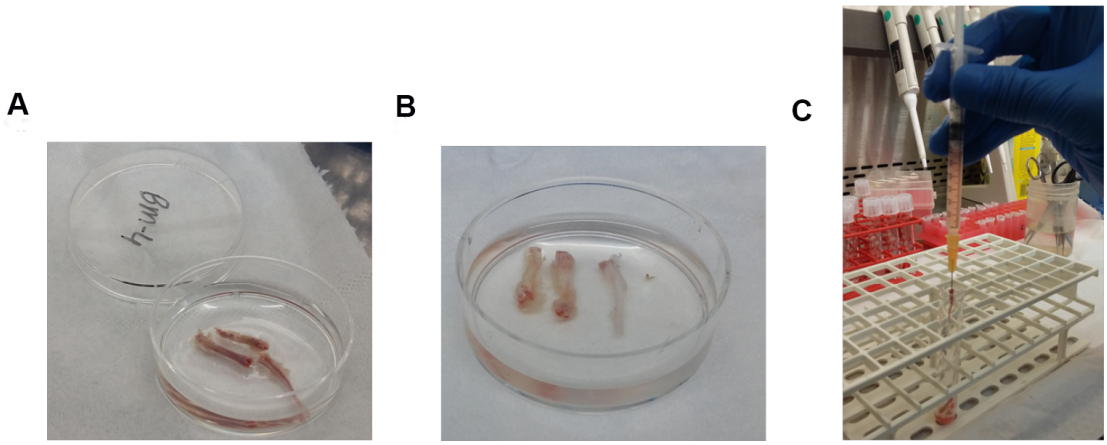

Figure 1. Process of flushing bone marrow out of murine bones. A. Femurs, tibias and iliac crest bones before flushing; B. Femurs, tibias and iliac crest bones after flushing; C. 1 ml syringe with its 25 G needle inserted in the tibia bone cavity.

4. Filter the cells by passing the cell solution through 70 µm nylon strainer to obtain a uniform single-cell suspension.
5. Centrifuge the cells (289.5 *x g*, 5 min) and resuspend in 1 ml LDV medium.
6. Count the cells by diluting the cells 1:10 with Turk's solution. Count the cells under the inverted light microscope using the hemacytometer. From one mouse typically one will get 60-90 million cells from both femurs and tibias and iliac crest bones.

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B. Determination of VLA-4 affinity using flow cytometry (LDV assay)

VLA-4 affinity can be examined on fresh bone marrow cells obtained from treated animals. Alternatively, cells can be obtained from untreated animals and treated *in vitro* with a desired reagent, in the presence of LDV medium, followed by the abovementioned detailed protocol.

Note: It is recommended to continue with the staining after isolation of the bone marrow cells.

1. Place 5 million bone marrow cells in a total volume 100 µl of LDV medium (see Recipes) in a FACS tube.
2. Add LDV-FITC to achieve a final concentration of 10 nM (see Recipes) and mix gently by pipetting, do not vortex.
3. To determine LDV-FITC non-specific binding, add EDTA to each LDV-FITC sample at a final concentration of 5 mM.
4. Incubate all the samples for 30 min at 37 °C.
5. After incubation, put the tubes immediately on wet ice and fix the cells by adding ice cold 4% PFA for 10 min (*i.e.*, the reaction is in 100 µl of cells, thus add 4% PFA [1-2 ml] directly to the tube). Since that moment, cells need to be kept under cold conditions on wet ice. Avoid vortex along the procedure.
6. Wash the cells with 2 ml ice cold $PBS^{+/+}$.
7. Centrifuge the cells (289.5 *x g*, 5 min) and gently discard the supernatant.
8. Add 100 µl of ice cold $PBS^{+/+}$ and perform staining of the cell surface markers Lineage (1-2 µl per sample of each antigen), Sca-1 (2 µl/sample), c-Kit (2 µl/sample), CD150 (2 µl/sample), CD48 (1 µl/sample) and EPCR (2 µl/sample). These cell surface markers are only suggestive antigens to detect LT-HSCs, however this can be adjusted according to the research question needs. Color combination should fit with FITC as LDV peptide is conjugated with the FITC fluorochrome.
9. Add the required antibodies into the cell samples and mix gently by pipetting (do not vortex), and incubate for 30 min on ice and protected from light.
10. Wash the cells with 1 ml ice cold $PBS^{+/+}$, centrifuge (289.5 *x g*, 5 min) and gently discard the supernatant.
11. Resuspend the cells in 300 µl $PBS^{+/+}$, do not vortex.
12. Read the samples immediately by flow cytometry and determine LDV binding to LT-HSC by analyzing the intensity of the FITC fluorochrome on gated $Lineage^{-}/c\text{-}Kit^{+}/Sca\text{-}1^{+}/CD150^{+}/CD48^{-}/EPCR^{+}$ cells.

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Data analysis

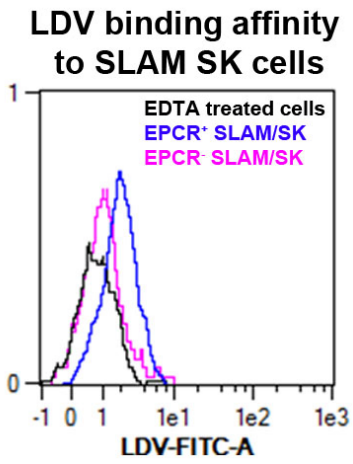

Figure 2. VLA4 affinity measured by LDV probe binding to bone marrow EPCR$^+$ SK (Sca1$^+$ ckit$^+$) SLAM (CD150$^+$ CD48$^-$) cells. In black is the control cells treated with EDTA; in blue the EPCR$^+$ cells gated on SLAM/SK; in pink the EPCR-cells gated on SLAM/SK.

For further analysis information concerning gating strategies and statistical analysis you can consult the article Gur-Cohen *et al.*, 2015 at the following link: http://www.nature.com/nm/journal/v21/n11/full/nm.3960.html.

Recipes

1. LDV medium
   1 mM $CaCl_2$
   1 mM $MgCl_2$
   20 mM HEPES, containing 1% BSA
   Note: It is recommended to prepare stock solutions of 1 M $CaCl_2$ (dilute 1:1,000 directly in the medium) as well as 100 mM $MgCl_2$ (dilute 1:100 directly in the medium).
2. LDV-FITC stock and working solutions
   Dissolve 1 mg LDV-FITC powder in 1 ml DDW (ddH_2O) according to the manufacture instructions (giving a stock concentration of 0.73 mM)
   Note: It is recommended to divide the solution into small aliquots, avoiding repeated freeze/thaw cycles (the solution can be stored at -20 °C for up to 1 year).
   To prepare the working LDV-FITC solution, dilute stock solution with DDW, reaching to a final concentration of 10 nM (for 10 nM working solution dilute 1:730 with DDW and take 1 µl into 100 µl cells supplemented with LDV medium)
3. Turk solution
   50 mg gentian violet
   5 ml acetic acid

495 ml DDW
Dissolve gentian violet in acetic acid and DDW

4. 4% PFA
   Weight out 40 g of powdered paraformaldehyde into a plastic weigh boat
   Pour into a 2 L glass flask or bottle
   Add 1 L DDW, a stir bar and allow to gently agitate while warming to 65 °C, for 5 min
   Add one drop of 10 N KOH or 10 N NaOH base, the solution should then become clear
   Allow the solution to cool at room temperature and adjust the pH to 7.3
   Note: Smaller volumes can be stored at -20 °C for up to one year.

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