TECHNICAL REPORT

Experimental Campaign on the in-vitro platelet-endothelial cells interactions

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In this document we describe planning and execution of the in-vitro experiments. They focused on analyzing the interaction between sCD40L and CD40 on the human umbilical vein endothelial cells (HUVEC) surface. We have used Human Citrated venous whole blood, centrifuged at 120g for 15 minutes, to obtain platelet-rich plasma (PRP). Platelets have been obtained by gel-filtration of PRP onto Sepharose 2B (Sigma Chemical, St Louise, MO, USA) columns equilibrated with a calcium free Tyrode's buffer (HEPES 3.1mM, $NaH_2PO_4 \cdot H_2O$ 4mM, NaCl 137mM, KCl 2.6mM, $MgCl_2$ 1mM, dextrose 5.6mM, BSA 0.1%, pH 7.4).

Then, gel-filtered platelets have been centrifuged at 1000g for 10 minutes, after the addition of 0.02μM prostacyclin (PGI_2, ICN Biomedicals, Aurora Ohio, USA), and the pellet was resuspended at a concentration of 1.5×10^8/ml in Tyrode's buffer.

HUVECs have been cultured in EBM-2 medium (Lonza) supplemented with the EGM-2 Bullet Kit (Lonza) on the bottom wells of a 96 well-plates (75.000 cells per well) (Corning® HTS Transwell® 96 well permeable supports, USA).

Human gel-filtered platelets were stimulated with 0.1 U/ml bovine alpha thrombin for 10 minutes. Thrombin was neutralized with 10 U/ml of hirudin (Knoll AG, Ludwigshafen, Germany) and the stimulated platelet suspension (7.5×10^6 platelets per transwell) was added to

transwells with 0.4 μm pores, placed on the 96-well plate where HUVECs were cultured and incubated for different times, from 10 minutes to 4 hours, without agitation at 37°C and in 0.5% CO_2 atmosphere.

Transwell allows studying the effect of sCD40L released from platelets on HUVECs, without direct platelet interaction with endothelial cells at different times starting from the thrombin neutralization, for 10, 30, 60, 120 and 240 minutes.

After incubation, endothelial cell supernatants have been collected and stored at -80°C for later sCD40L assay by ELISA (R&D System, following producer instructions) [1].

Endothelial cells have been detached with mild trypsinization, centrifuged for 5 minutes at 250g, resuspended in PBS and incubated for 30 min in the dark with a saturating concentration of a FITC-conjugated anti-VCAM-1 mAb (CD106, BD Pharmingen) or PE-conjugated anti-CD40L (TRAP-1, BD Pharmingen) mAb. Samples have been diluted by adding 0.5 ml of PBS and finally analyzed in a EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, Florida, USA). At specific times (10, 30, 60, 120 and 240 minutes), a sample of 1500 HUVEC cells has been analyzed by flow cytometry [2]. This technique collects all events where the considered cell fluorescence is higher than basal fluorescence and this proves that some sCD40L bind to the considered HUVEC cell.

Flow cytometry showed a relevant presence of CD40L on the HUVEC surface after 10 minutes of co-incubation, indicating that sCD40L released from platelets binds HUVECs CD40 receptor within 10 minutes (Figure 1). A significant VCAM-1 expression on HUVECs was visible only starting from 2 hours after co-incubation with platelets (Figure 2), thus showing that CD40/sCD40L interaction is early but it takes lower time to activate endothelial cells. In addition, at the same time instants, the concentration of free sCD40L has been measured (Figure 3), allowing to estimate also the number of sCD40L carriers by using the molecular weight of sCD40L (Figure 4).

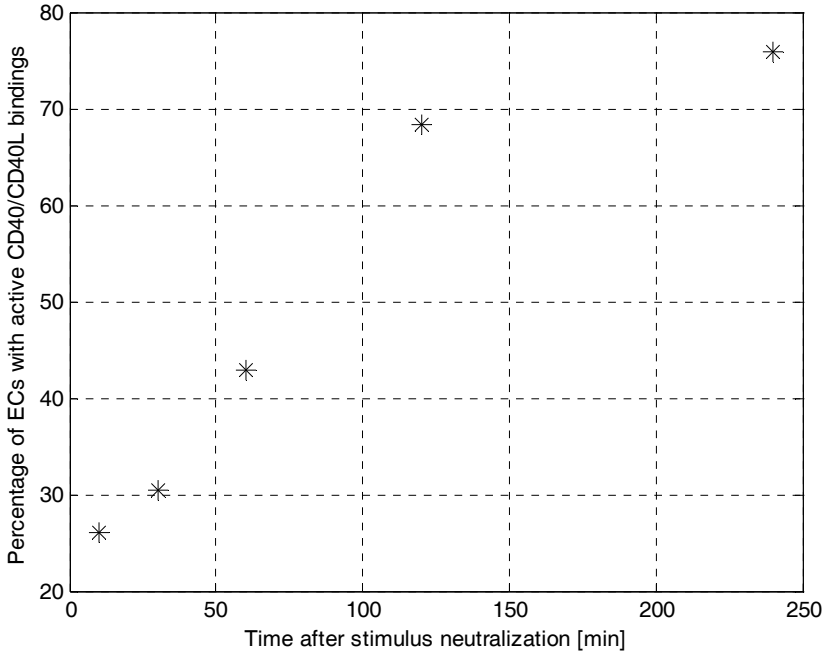

Figure 1: Estimated percentage of endothelial cells (ECs) with active CD40/CD40L bindings at different times after platelet stimulus neutralization. Method: flow cytometry.

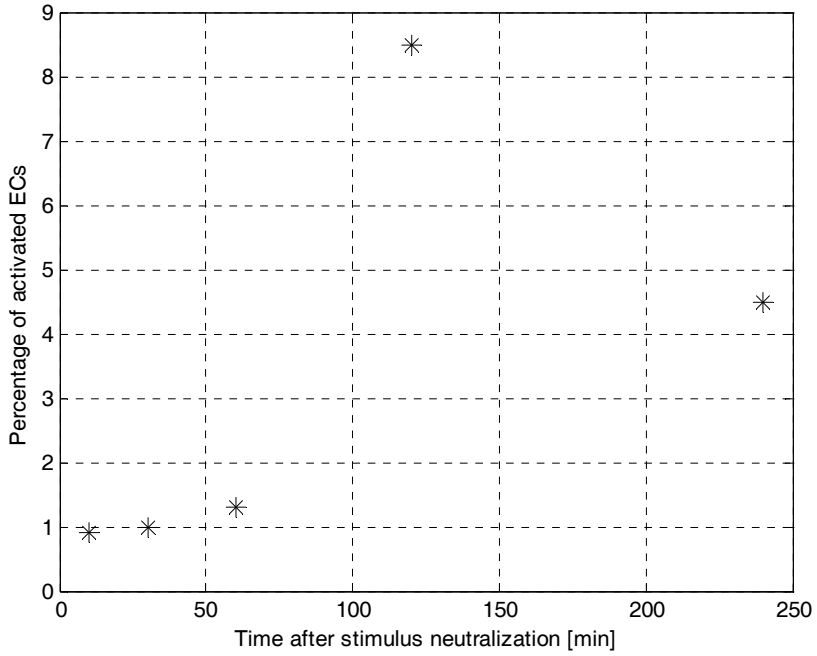

Figure 2: Estimated percentage of endothelial cells (ECs) expressing VCAM-1 after platelet stimulus neutralization. Method: ELISA assay.

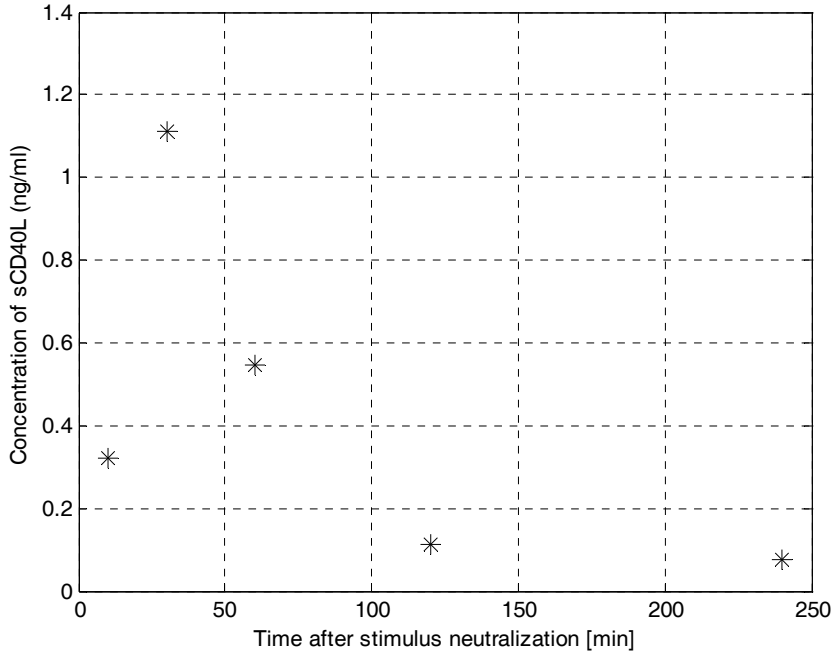

Figure 3: Measured concentration of sCD40L at different times after platelet stimulus neutralization

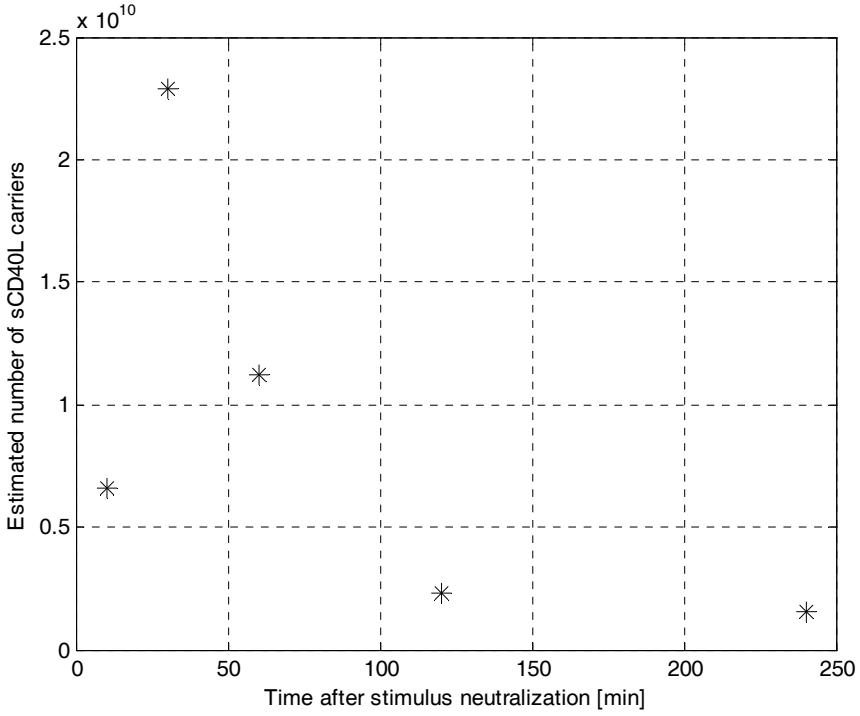

Figure 4: Estimated number of free sCD40L carriers at different times after platelet stimulus neutralization, using data from Figure 1 and molecular weight.

References

[1] R. M. Lequin, “Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA),” Clinical Chemistry, 51(12), pp. 2415–2418, December 2005.

[2] M.G. Ormerod, "Flow Cytometry — A basic introduction", 2008, ISBN 978-0955981203.