A Bacterial Cholesterol Sensor to Assess Cholesterol Accessibility in Red Blood Cells

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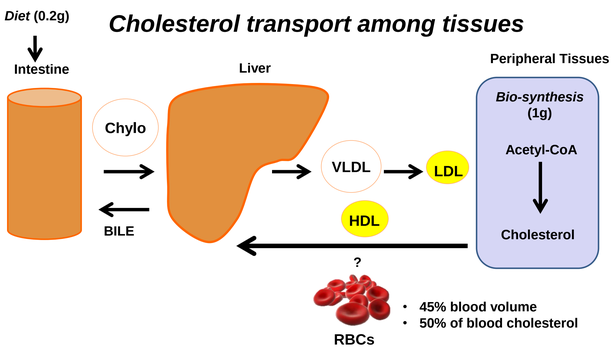
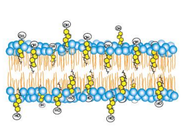
Abstract

The only cells in the body that cannot synthesize cholesterol are red blood cells (RBCs), yet RBCs contain ~50% of circulating blood cholesterol. Whereas HDL is considered the major conduit for reverse cholesterol transport, we hypothesize that RBCs play a role in this pathway. To test this hypothesis, we developed an assay to measure accessible cholesterol in RBCs. We purified and fluorescently labeled domain 4 of a bacterial toxin, Anthrolysin-O (ALOD4), that binds membrane cholesterol. We incubated fALOD4 with RBCs from 164 healthy subjects and measured the fluorescence intensity using flow cytometry. The intra-assay and intra-individual variability were both <10%, whereas the inter-individual values varied over a 10-fold range. No correlation was found between fALOD4 binding and total RBC-cholesterol, hematocrit, or indices of RBC size. fALOD4 binding was inversely related to membrane phosphatidylcholine (PC) (-0.42, $p=6e^{-7}$) and directly related to lyso-phosphatidylcholine levels (LPC) (0.40, $p=6e^{-6}$). Phospholipase A2 treatment, which converts PC to LPC, increased binding 3-fold. fALOD4 binding did not correlate with plasma LDL-C levels, but was directly related to HDL-C (0.30, $p=6e^{-4}$), and inversely related to triglyceride levels (-0.57, $p=2e^{-12}$). Future studies will determine if variability in fALOD4 binding is intrinsic to RBC membranes, is genetically determined, or contributes to atherosclerosis.



Hypothesis

An active form of cholesterol exists in the membrane of RBCs which is able to exchange with lipoproteins, cells, and/or other blood components.



References

1. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol. 2012 Jun;32(6):1460-5.
2. Lange Y, Molinaro AL, Chauncey TR, Steck TL. On the mechanism of transfer of cholesterol between human erythrocytes and plasma. J Biol Chem. 1983 Jun 10;258(11):6920-6..

Assay Development

Anthrolysin-O, a toxin secreted by *Bacillus anthracis*, is a cholesterol dependent cytolysin

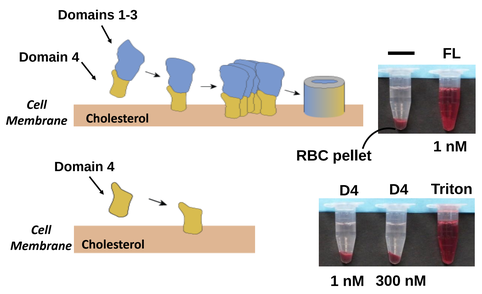


Figure 1: Full length ALO has 4 domains. Domain 4 senses and binds membrane cholesterol and domains 1-3 induce pore formation and RBC lysis.

Isolated domain 4 binds cholesterol in membranes but does not lyse RBCs.

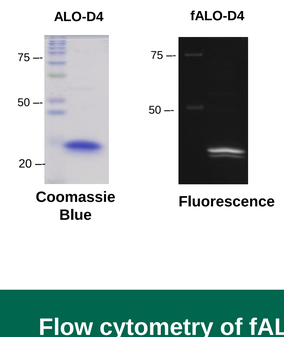


Figure 2: ALO-D4 was over-expressed in bacteria, purified from cell lysate and labeled with a fluorescent tag (Alexa 488).

Flow cytometry of fALO-D4 bound to RBCs

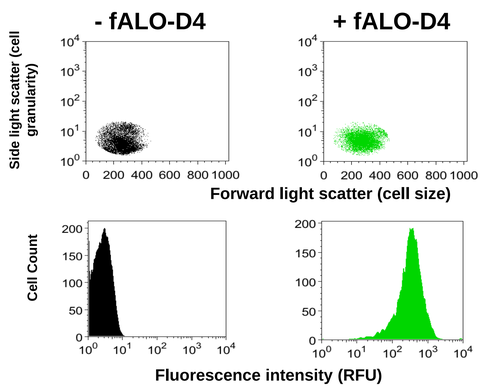


Figure 3: Output from flow cytometer. **Top panel**: Each point represents a single RBC. Side light and forward light scatter are relative measures of cell morphology. The distributions of cell size and shape are similar in the absence and presence of fALO-D4. **Bottom panel**. Fluorescence intensity describes the amount of fALO-D4 bound to cells. Addition of fALO-D4 drives a ~500 fold increase in fluorescence of all cells.

Results

Distribution of fALO-D4 binding in healthy and unrelated individuals

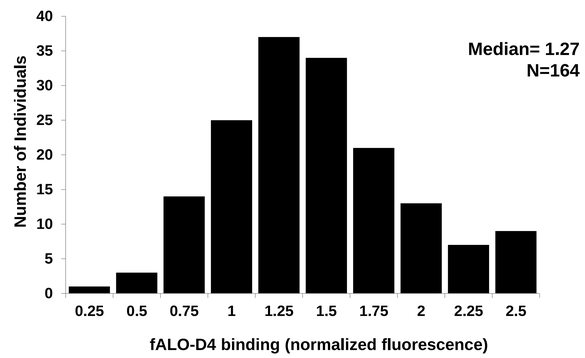


Figure 4: fALO-D4 was used to measure active cholesterol in the RBCs of 164 healthy individuals. The distribution of fALO-D4 binding has a 10-fold range.

Possible Sources of Inter-individual Variation

Intrinsic Property of RBCs
- Lipid Composition
- Protein Composition
- Carbohydrate Modification

Plasma Constituents
- Lipoproteins
- Other proteins

fALO-D4 binding does not correlate with total RBC cholesterol

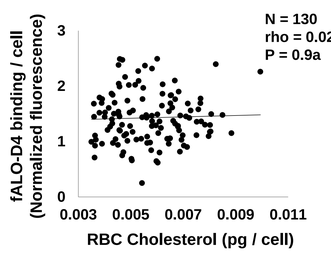


Figure 5: No significant relationship was observed between RBC active cholesterol (fALO-D4 binding) and total RBC cholesterol measured by an enzymatic assay.

fALO-D4 binding correlates with outer leaflet RBC phospholipids

Membrane Lipid (mole %)	Spearman(rho)	P
Phospholipids abundant in outer membrane leaflet		
Phosphatidylcholine (PC)	**- 0.42**	**6.00E-07**
Lyso-PC	**0.39**	**5.89E-06**
Sphingomyelin (SM)	**0.34**	**9.25E-05**
Phospholipids abundant in inner membrane leaflet		
Phosphatidylethanolamine (PE)	0.11	0.22
Lyso-PE	-0.08	0.36
Phosphatidylserine (PS)	-0.06	0.51

PLA2 treatment of RBCs decreases PC:Lyso-PC and increases fALO-D4 binding

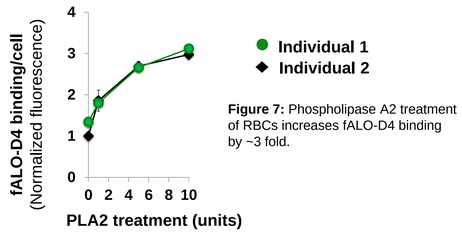


Figure 7: Phospholipase A2 treatment of RBCs increases fALO-D4 binding by ~3 fold.

fALO-D4 binding and plasma lipid & lipoprotein levels

Serum Lipid (mg/dL)	Spearman (rho)	P
Total Cholesterol	- 0.27	0.003
Triglycerides	**- 0.57**	**2.16E-12**
LDL Cholesterol	- 0.17	0.06
HDL Cholesterol	**0.31**	**0.0001**

Conclusions

- **Domain 4 of ALO can be used to assay "accessible" cholesterol in RBC membranes**
- **RBC accessible cholesterol:**
  1. ≠ RBC total cholesterol
  2. Varies over 10-fold range
  3. Related to outer leaflet lipids: ↑SM, lyso-PC ; ↓PC
  4. Related to plasma levels of HDL(↑), TG (↓) and TC (↓)
- **Decreasing the PC:Lyso-PC ratio in RBC membranes increases accessible cholesterol by ~ 3 fold**

Future studies

- **Compare protein & carbohydrate profiles in RBCs from individuals in extremes of activity distribution**
- **Determine if differences in activity persist after treatment with proteases & glycosidases.**
- **Examine mutations that alter HDL levels and PC/LPC ratio affect activity**
  - **LCAT deficiency**
  - **Tangier disease**
- **Analyze segregation of trait in families of individuals in the extremes of distribution**

Acknowledgements

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