# The role of endocytic pathways in cellular uptake of plasma non-transferrin iron

Yang-Sung Sohn,[1] Hussam Ghoti,[2,3] William Breuer,[1] Eliezer Rachmilewitz,[2] Samah Attar,[3] Guenter Weiss,[4] and Z. Ioav Cabantchik[1]

[1]Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Israel; [2]Department of Hematology, Wolfson Medical Center, Holon, Israel; [3]The European Hospital, Gaza, Palestinian Authority; and [4]Department for Internal Medicine I, Clinical Immunology and Infectious Diseases, Medical University of Innsbruck, Austria

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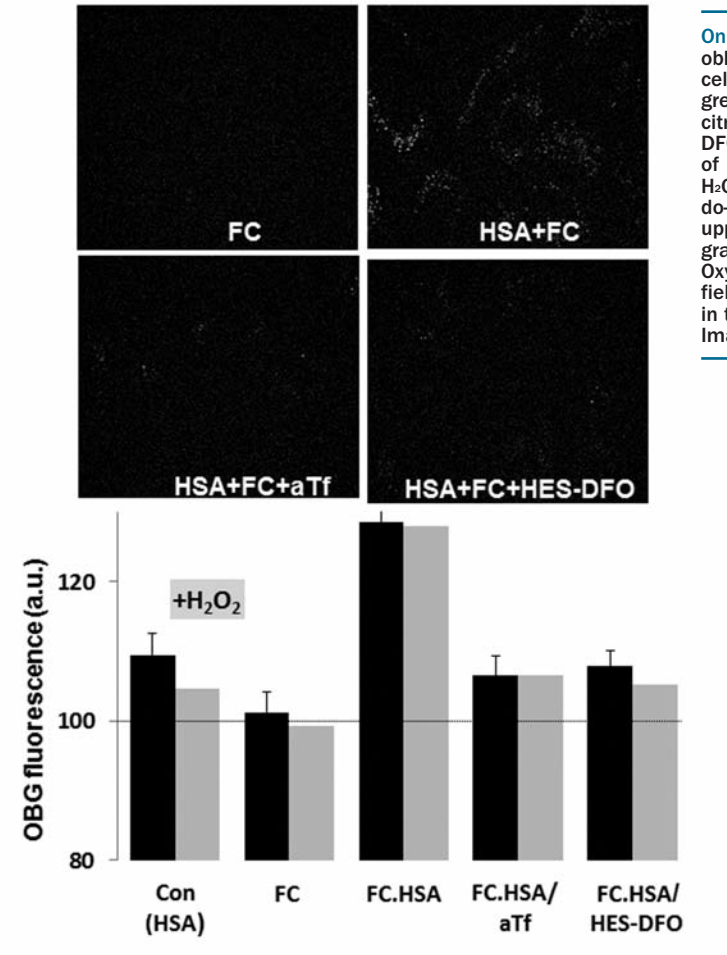
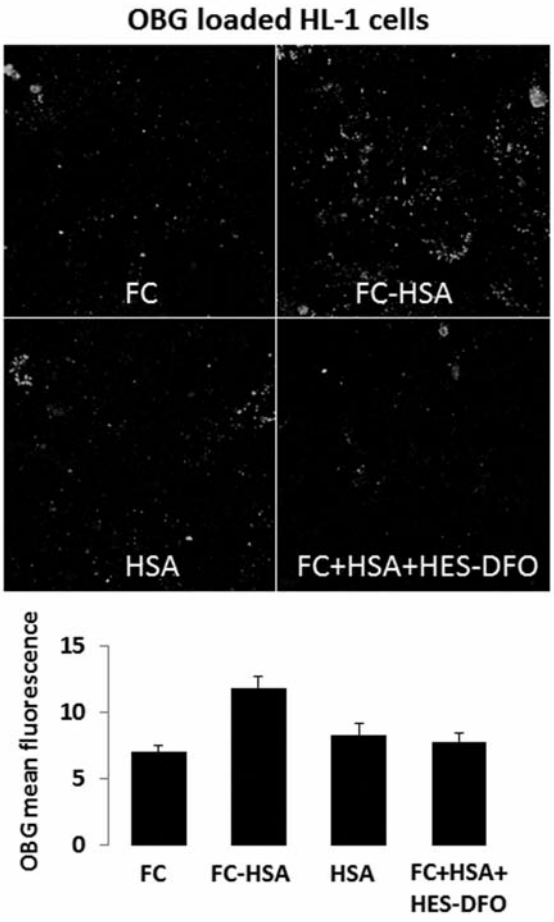
## Online Supplementary Appendix

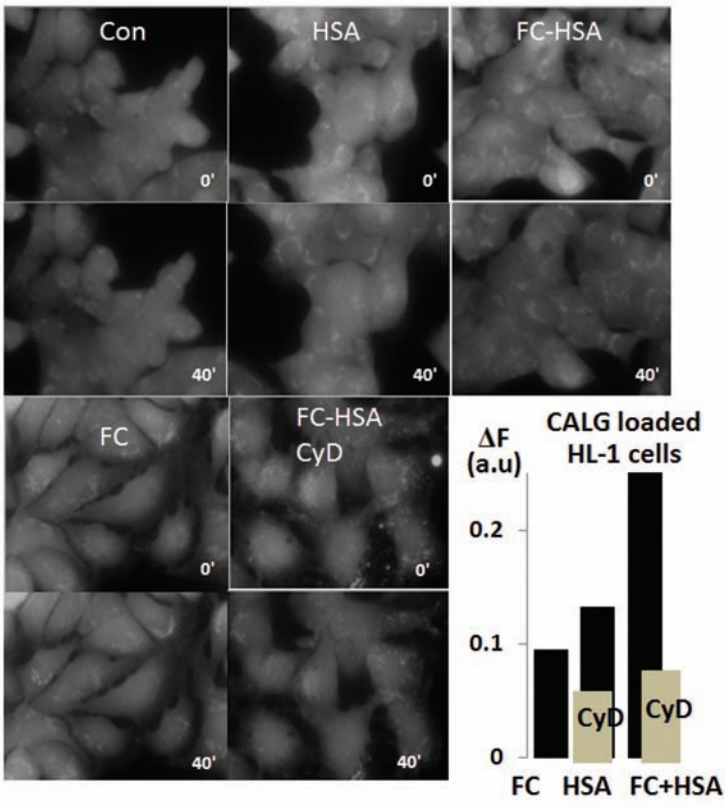
Oxyburst-green (OBG, albumin conjugated dichlorodihydrofluorescein), calcein green (CALG, 3,3'-bis[N,N-bis(Carboxymethyl) aminomethyl] fluorescein), calcein blue (CALB, methylumbelliferone-8-methyleneiminodiacetic acid) and their respective acetomethoxy (AM) precursors CALG-AM and CALB-AM were obtained from Molecular Probes (Invitrogen Corp., Carlsbad, CA, USA); SIH (salicyl isonicotinoyl hydrazine) was a gift from Dr P Ponka (Montreal, Canada), the iron-saccharate polymer Venofer™ was from Vifor International (St Gallen, Switzerland). F-DFO (N-(fluorescein-5-thiocarbamoyl) desferrioxamine was from Molecular Probes (Eugene, OR, USA), deferrioxamine (DFO) was from Novartis-Pharma (Basel, Switzerland). Hydroxyethyl-starch-DFO, M.W. ~ 10 kDa (HES-DFO) was from Biomedical Frontiers Inc., (Minneapolis, MN, USA). Apo-transferrin (aTf) was from Kamada Ltd. (Kibbutz Kama, Israel). Verapamil, Nifedipine, Rhodamine Dextran (R-D) (MW 10 kDa), Wortmannin, W7(N-(6-Aminohexyl)-5-chloro1-naphthalenesulfonamide hydrochloride), chloroquine, nocodazole (N) and ML9 (M), cytocholasin D (cyD) and human serum albumin (Fraction V) (HSA) were from Sigma Chemical Co. (St Louis, MO, USA).



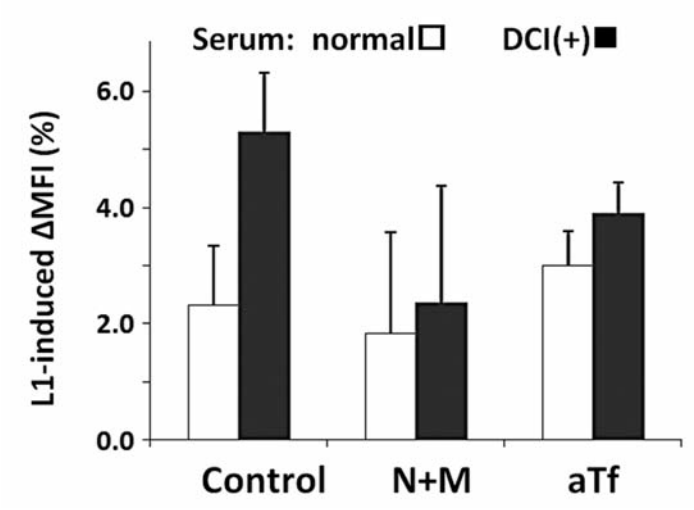

**Online Supplementary Figure S1.** NTBI uptake into H9c2 cardiomyoblasts as revealed with the ROS probe Oxyburst-green (OBG). H9c2 cells were incubated for 18 h in DMEM medium containing Oxyburst-green (400 μg/mL) and HSA (50 μM) supplemented with 20 μM ferric citrate (FC) in the presence and absence of either hydroxyethyl-starch-DFO (HES-DFO, 50 μM) or aTf (25 μM). After washing, the fluorescence of Oxyburst-green was monitored before and 10 min after addition of $H_2O_2$ (50 μM) using live epi-fluorescence microscopy adapted for pseudo-confocal imaging with an Optigrid system. The pictures shown in the upper half are snapshot images taken after addition of $H_2O_2$ and the bar graph in the lower half represents the mean fluorescence intensities of Oxyburst-green (OBG in arbitrary units a.u.) in endosomes of 5 cells per field (±SEM) for one (of 3) representative experiments. Data are given in terms of arbitrary fluorescence units obtained by image analysis with Image J.



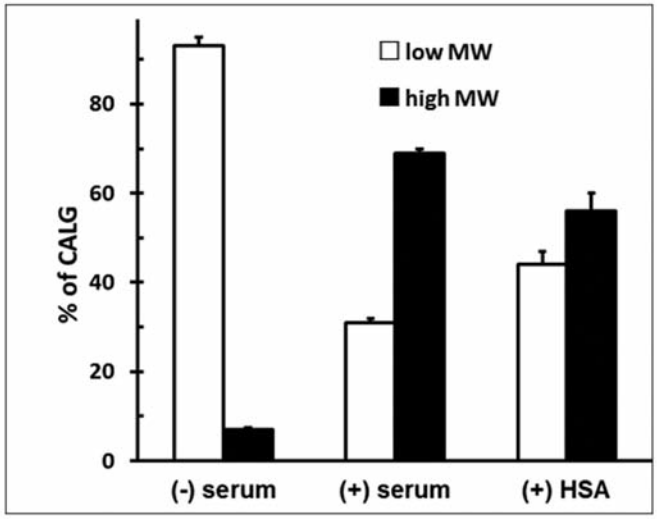

Online Supplementary Figure S2. NTBI uptake into HL-1 cardiomyocytes as revealed with the ROS probe Oxyburst-green (OBG). HL-1 cells were incubated overnight in HL-1 growth medium[16] containing Oxyburst-green (400 μg/mL) and HSA (50 μM) supplemented with 20 μM ferric citrate (FC) in the presence and absence of either hydroxyethyl-starch-DFO (HES-DFO, 50 μM). After washing of cells, the OBG fluorescence was monitored by confocal microscopy. The pictures shown on the upper half are snapshot images taken 10 min after addition of $H_2O_2$ (50 μM) and the bar graph on the lower half represents the mean fluorescence intensities (in arbitrary units a.u.) of 5 cells per field (±SEM) for one (of 3) representative experiments. Data are given in terms of arbitrary fluorescence units (a.u.) obtained by image analysis with Image J.



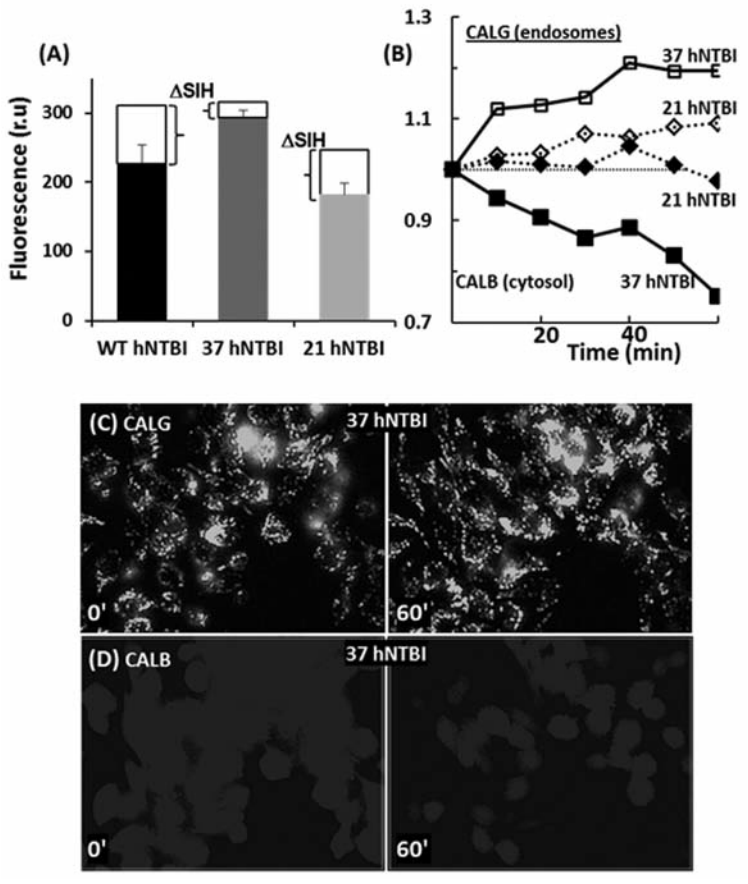

Online Supplementary Figure S3. Uptake of NTBI into the cytosol of HL-1 cardiomyocytes. Effect of human serum albumin and inhibitors of endocytosis. HL-1 cells labeled in the cytosol with CALG via its acetomethoxy precursor CALG-AM as described in the *Design and Methods* section were followed for up to 1 h by epifluorescence microscopy while perfused in DMEM-HEPES supplemented with ferric citrate (Fe:citrate 10: 30 μM) or without (control) and the indicated substances: HSA (50 μM), cytochalasin D (CyD= 100 μM). All incubations and perfusions were carried out at 37 °C. The pictures show snapshots of the same field taken at 0 at 40 min, and the graph shows the mean change in fluorescence (ΔF) between 0 and 40 min of each field (analyzed with NIH Image J, National Institutes of Health, Bethesda, MD, USA).




Online Supplementary Figure S4. Effect of cytoskeleton inhibitors and apo-transferrin on the uptake of NTBI into the cytosol of H9c2 cells. Cultured rat cardiomyocytes (H9c2 line) were cultured for 1 h in medium containing 30% human serum (control serum, empty bars) or the same serum supplemented with 20 μM ferric citrate to generate 8.5 μM NTBI as measured by the DCI assay (DCI(+) serum, filled bars). In systems with cytoskeleton inhibitors, the cells were pre-incubated in serum-free medium for 15 min with nocodazole (20 μM) and ML-9 (100 μM) (N + M) prior to the addition of sera to a final concentration of 30%. In systems with apo-transferrin (aTf), sera were pre-mixed with 2 mg/mL apo-transferrin for 15 min prior to addition to cells. Immediately following incubation with sera, the cells were loaded with 0.125 μM CALG-AM for 10 min, suspended by trypsinization and mean cell fluorescence without and with 0.4 mM deferiprone (L1) was determined by flow cytometry. Data are presented as mean change in fluorescence (ΔMFI) after addition of the chelator L1 (mean±SD) (n=4).




Online Supplementary Figure S5. Binding of CALG to high molecular weight components in thalassemia major sera. CALG (20 μM) was incubated with HBS buffer only, or with 6 different thalassemia major sera with DCI values in the range 0–6.1 μM, or with HBS containing 20 mg/mL HSA for 1.5 h at room temperature, then the samples were diluted 1:5 in HBS and passed through a 30 kD cut-off filter. The fluorescence of the retentate (high MW) and filtrate (low MW) was measured in the presence of 100 μM DFO to obtain the (mean±SD) (n=3) relative CALG concentrations in both fractions.




Online Supplementary Figure S6. NTBI uptake and iron distribution between endosomes and cytosol in RAW cells expressing different levels of NRAMP-1. (A) Endosomal levels of CALG in WT RAW cells and 2 RAW stable mutant cells over-expressing or under-expressing NRAMP1. WT RAW and the sublines 37 (NRAMP-1 over-expressing) and 21 (NRAMP-1 under-expressing) were incubated for 3 h with thalassemia major sera containing high NTBI (hNTBI) in the presence of CALG (50 μM) and washed. Cell CALG fluorescence intensity of 4 cells per field (±SD from 3 independent experiments) was recorded before and 10 min after addition of the permeant SIH (50 μM) in order to reveal the quenched CALG-Fe complexes formed by interaction of CALG with sera-containing NTBI. Data are given in terms of arbitrary fluorescence units (a.u.). (B) Fluorescence changes in endosomal CALG and cytosolic CALB in RAW 37 and 21 sublines following incubation with high NTBI sera. Cells of the RAW 21 and 37 lines (denoted as 21 hNTBI-diamonds and 37 hNTBI-squares) were exposed for 2 h to 30% hNTBI thalassemia major sera supplemented with CALG (50 μM) and subsequently loaded for 10 min with CALB-AM (10 μM). Changes in cytosolic CALB (filled symbols) and endosomal CALG (empty symbols) fluorescence were monitored with time after loading of the probes and are given relative to time 0 (beginning of measurements) for each subline and for each fluorescent probe. The images represent snapshot images taken in the same field at zero and 60 min time points for subline 37: (C) for CALG and (D) for CALB.