Materials and Methods:

Framingham Heart Study (FHS)

The FHS offspring study is a large longitudinal community-based observational study of cardiovascular risk factors and CVD[1, 2]. The study participants are the offspring of the Original FHS cohort and their spouses. All of the analyses were based on the participants of examination cycle 8 (n=1625) that had platelet gene expression measured and plasma biomarker analysis[1, 2]. All study participants gave informed consent. The FHS protocol was approved by the Boston University Medical Center Review Board and University of Massachusetts Medical School Review Board.

Risk factor and plasma biomarker assessment

All risk factors analyzed here have been described in detail previously[3]. Plasma levels of CRP (high sensitivity assay by Dade Behring BN100 nephelometer)[4] and soluble P-selectin (R&D Systems, cat # BBE6) were measured as previously described. Serum levels of IL6 (R&D Systems, cat # HS600B), MCP1[4], ICAM1[4], and TNFR1[2, 5] were measured in duplicates as previously described. Fasting glucose, HDL and total cholesterol were assessed as previously published[1].

After removing individuals with extreme elevations of CRP (>10 mg/L, N=106) and/or IL-6 (>10 pg/ml, N=112), the assay ranges in the remaining 1625 subjects was 0.14-9.9 mg/L for CRP and was 0.15 to 9.9 pg/ml for IL-6. The distribution of CRP and IL-6 assay levels in the restricted sample were not normally distributed and were consequently natural log (ln) transformed for statistical analysis.

Platelet and TLR mRNA isolation

Platelet isolation from the participants of the FHS was completed as previously described[1]. Potential platelet contamination with white blood cells was assessed by flow cytometry, confocal microscopy and was determined that there were no more than 1 white blood cell/50,000 platelets.

RNA from platelets was performed as previously described using Qiagen RNeasy kits and cDNA was generated, pre-amplified and analyzed.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

mRNA was evaluated by qPCR using high-throughput RT-PCR instrument (BioMark; Fluidigm, San Francisco, CA) as previously described[1]. The qPCR results cycle threshold (Ct) values were standardized using 3 reference genes: β actin (ACTB), β2-microglobulin (B2M), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were previously found to be highly correlated in the FHS participants. TLRs were considered expressed when Ct values were lower than 25.

Statistical Analysis

Data analysis was performed using STATA 12.0 software. Statistical significance was assessed using two-sided p-values with those <0.05 being considered statistically significant. To evaluate the relative TLR co-expression, Pearson's correlation coefficient was calculated between each pair of TLRs (normalized expression values). Logistic regression models were fit to determine the factors associated with the binary definition of TLR expression as defined earlier (i.e. Ct<25).

The following clinical and demographic factors were included in each model as potential confounding variables (all assessed at the same examination when RNA was collected): age, BMI, smoking status, total cholesterol, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, glucose level, diabetes, coronary heart disease, lipid-lowering therapy, hormone replacement therapy, antihypertensive therapy, and regular aspirin use (at least 3× per week). To evaluate the relationship between inflammatory biomarkers ($\log_e$-transformed values) and co-expression of MYD88 and UNC93B1 (quantitative normalized expression values) further logistic regressions were fit modeling the probability of TLR expression as a function of the potential confounders described earlier with the addition each biomarker in separate models. Finally, we evaluated all models separately in males and females.

References:

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