Novel 6p21.3 Risk Haplotype Predisposes to Acute Coronary Syndrome

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Background—The HLA-DRBI*01 allele of the human leukocyte antigen has been associated with acute coronary syndrome. Genome-wide association studies have revealed associations with human leukocyte antigen and non–human leukocyte antigen genes of 3 major histocompatibility complex gene classes but not at allelic level.

Methods and Results—We conducted a large-scale genetic analysis on a case–control cohort comprising 5376 acute coronary syndrome cases and 4852 unrelated controls from 4 populations of 2 European countries. We analyzed the risk candidate allele of HLA-DRBI*01 by genomic real-time polymerase chain reaction together with high-density single nucleotide polymorphisms of the major histocompatibility complex to precisely identify risk loci for acute coronary syndrome with effective clinical implications. We found a risk haplotype for the disease containing single nucleotide polymorphisms from BTNL2 and HLA-DRA genes and the HLA-DRBI*01 allele. The association of the haplotype appeared in 3 of the 4 populations, and the direction of the effect was consistent in the fourth. Coronary samples from subjects homozygous for the disease-associated haplotype showed higher BTNL2 mRNA levels (r=0.760; P<0.00001). We localized, with immunofluorescence staining, BTNL2 in CD68-positive macrophages of the coronary artery plaques. In homozygous cases, BTNL2 blocking, in T-cell stimulation assays, enhanced CD4+FOXP3+ regulatory T cell proliferation significantly (blocking versus nonblocking; P<0.05).

Conclusions—In cases with the risk haplotype for acute coronary syndrome, these results suggest involvement of enhanced immune reactions. BTNL2 may have an inhibitory effect on FOXP3+ T cell proliferation, especially in patients homozygous for the risk alleles.

Clinical Trial Registration—https://www.clinicaltrials.gov; Unique Identifier: NCT00417534.

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Key Words: acute coronary syndrome ■ association studies ■ BTNL2 ■ expression experiments ■ genetics ■ haplotype ■ human leukocyte antigen ■ MHC ■ regulatory T cell ■ single nucleotide polymorphism

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frequencies among populations. The number of alleles at the HLA-DRB1 [MIM: 142857] locus is markedly high. No single universal single nucleotide polymorphism (SNP) tags the HLA-DRB1*01 allele. We recently showed that in the Finnish population, SNP imputation of the HLA-DRB1*01 allele showed only an ≈50% success rate because of the narrow gene pool showing HLA haplotype frequencies typical of the Finnish population when compared with those of Europeans. This may explain why the HLA-DRB1*01 association has emerged only in studies with direct HLA allele typing, but in no GWAS studies. HLA-DRB1*01 may play a role in promoting coronary artery disease by affecting antigen presentation. Presentation of peptide fragments of oxidized low-density lipoprotein cholesterol may connect the cholesterol pathway to inflammation and immunology in atherosclerosis progression.

Clinical Perspective on p 63

Because of the promising results from previous GWA and candidate gene studies and the fact that the MHC contains many genes with putative immune functions, we hypothesized that analysis of other MHC genes using high-density SNPs stratified according to the HLA-DRB1*01 allele will show novel associations with acute coronary syndrome (ACS) with effective clinical implications.

Materials and Methods

Patient Populations

Our entire study comprised 1 discovery population (from Finland) and 3 replication populations (I and II from Finland and III from Spain) for a total of 5376 cases and 4852 control subjects. The discovery population included 2090 ACS cases. Replication population I included 742 cases either with ST-elevation myocardial infarction or non-ST-elevation myocardial infarction. Replication population II included 762 either ST-elevation myocardial infarction or non-ST-elevation myocardial infarction cases. Replication population III included 1768 first myocardial infarction cases. Control subjects for discovery and replication populations I and II were selected from The National FINRISK Study (FINRISK) 1992, 1997, 2002, and 2007 participants from the same geographic region as the cases. Initially, age, sex, and area were matched before any selection of cases and control subjects for replication population III. For case and control characteristics, see Table 1. Geographical areas of recruiting were shown in Figure I in the Data Supplement. The final numbers of cases and controls in each step of the analyses are in Figure IIA–IID in the Data Supplement. Samples passing the typing were grouped according to their HLA-DRB1*01 positivity and negativity. In all study populations, a certain number of those samples failed the evaluation of HLA-DRB1*01 allele numbers or the SNPs and were thus excluded (data in Figure IIA–IID in the Data Supplement).

SNP Genotyping, Data Cleaning, and Imputation

All samples of the discovery population were genotyped with the Illumina 610K genotyping chip (Illumina HumanHap 610-Qud SNP Array, San Diego, CA) at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). All the SNPs in replication populations I, II, and III and those for expression studies were genotyped with the Sequenom platform (iPLEX MassARRAY, San Diego, CA) at the Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland). Because of the promising results from previous GW A and candidate gene studies and the fact that the MHC contains many genes with putative immune functions, we hypothesized that analysis of other MHC genes using high-density SNPs stratified according to the HLA-DRB1*01 allele will show novel associations with acute coronary syndrome (ACS) with effective clinical implications.

Genotyping

HLA-DRB1*01 Genotyping

HLA-DRB1*01 genotypes (negative, heterozygous, and homozygous) were analyzed from genomic DNA (concentration 10 ng/μL, purity minimum of A260/A280=1.6) by real-time quantitative polymerase chain reaction (Rotor-Gene 6000; Corbett Research, Mortlake, Australia) with SYBR Green labeling (ABgene, Epsom, UK, and Stratagen, AH Diagnostics, Skärholmen, Sweden) at the HLA laboratory having international European Federation for Immunogenetics accreditation. This method determines the amount of the DNA product during the polymerase chain reaction (double-stranded DNA detected by SYBR Green). The resulting number of alleles is compared with control samples with a known number of the HLA-DRB1*01 allele. Specificity relies on rigorous primer selection, and before the HLA-DRB1*01 analyses, DNA concentration comparability between samples and controls was assured by amplification of a housekeeping gene (β-actin) in parallel with standard dilutions of 8, 10, and 14 ng/μL. The method has been adapted for HLA-DRB1*01 based on our earlier method for the complement C4 copy number analyses and annually validated with international proficiency testing samples. In all study populations, a certain number of the initial samples failed the HLA-DRB1*01 typing and were excluded. These excluded samples are listed in Figure IIA–IID in the Data Supplement. Samples passing the typing were grouped according to their HLA-DRB1*01 positivity and negativity. In all study populations, a certain number of those samples failed the evaluation of HLA-DRB1*01 allele numbers or the SNPs and were thus excluded (data in Figure IIA–IID in the Data Supplement).

mRNA Analyses of Human Coronary Artery Specimens

Samples from human left anterior descending coronary artery came from recipient hearts at the time of cardiac transplantation (n=47) or from the hearts of organ donors (n=85) that were excluded from transplantation because of size or tissue-type mismatch or coronary atherosclerosis. Samples came from one center: Helsinki University Central Hospital, Helsinki, Finland, between 1997 and 2008. Coronary artery samples snap-frozen and stored at −80°C were dissected free of excess periarterial tissues. (More detailed information is given in the Data Supplement.) Detailed information for immunohistochemistry of human coronary artery specimens and for T cell proliferation against vaccinia antigens is also given in the Data Supplement.
### Table 1. Characteristics of Cases and Controls in Discovery and Replication Populations

|                      | Population | Subjects | Male Subjects, % | Age (y) ±SD | Body Mass Index, (kg/m²) ±SD | Subjects With Diabetes Mellitus, % | Subjects With Hypertension, % | Subjects With Hypercholesterolemia, % | Current Smokers, % |
|----------------------|------------|----------|------------------|-------------|----------------------------|----------------------------------|-----------------------------|-------------------------------------|------------------|
| **Discovery**        |            |          |                  |             |                            |                                  |                             |                                     |                  |
| Cases                | Corogene   | 2090     | 1455 (69.6)      | 66.1±11.8   | 27.4±4.8                  | 397 (19.0)                      | 1376 (65.8)                  | 1170 (56.0)                       | 651 (31.1)       |
| Controls             | FINRISK    | 1580     | 847 (53.6)       | 55.8±12.2   | 26.5±4.5                  | 119 (7.5)                       | 882 (55.8)                   | 787 (49.8)                        | 384 (24.3)       |
| **Replication I**    |            |          |                  |             |                            |                                  |                             |                                     |                  |
| Cases                | Tacos      | 742      | 457 (61.6)       | 69.3±11.8   | NA                        | 176 (23.7)                      | 411 (55.4)                   | NA                                  | 131 (17.7)       |
| Controls             | FINRISK    | 613      | 302 (49.3)       | 50.1±12.9   | 26.9±4.5                  | 43 (7.0)                        | 301 (49.1)                   | 276 (45)                          | 116 (19.0)       |
| **Replication II**   |            |          |                  |             |                            |                                  |                             |                                     |                  |
| Cases                | Artemis    | 670      | 510 (76.1)       | 64.1±9.7    | NA                        | 237 (35.4)                      | 404 (60.3)                   | NA                                  | 275 (41.0)       |
| Cases                | Fincell I  | 65       | 59 (90.7)        | 59.6±9.8    | NA                        | 8 (12.3)                        | 25 (38.5)                    | NA                                  | 25 (38.5)        |
| Cases                | Fincell II | 27       | 26 (96.3)        | 59.3±8.9    | NA                        | 1 (3.7)                         | 8 (29.6)                     | NA                                  | 11 (40.7)        |
| Controls             | FINRISK    | 910      | 682 (74.9)       | 65.3±11.9   | NA                        | 83 (9.2)                        | 568 (62.5)                   | 578 (63.5)                        | 191 (21.0)       |
| **Replication III**  |            |          |                  |             |                            |                                  |                             |                                     |                  |
| Cases                | Regicor    | 1786     | 1451 (81.2)      | 58.2±11.9   | 27.9±4.7                  | 449 (25.1)                      | 972 (54.4)                   | 895 (50.1)                        |                  |
| Controls             | Regicor    | 1749     | 1174 (67.1)      | 60.4±9.9    | 27.8±4.6                  | 258 (14.8)                      | 615 (35.2)                   | 358 (20.5)                        |                  |

### Statistical Analysis

#### Heterogeneity

Testing of between-study heterogeneity for the HLA-DRB1*01 allele was done by calculation of $F$. The Cochran’s $Q$ test is $18.56$, corresponding to a $P$ value of $0.0003$ with 3 degrees of freedom. The $F$ is $83.84\%$, which is the percentage of the variation because of heterogeneity rather than chance. Thus, we can conclude that heterogeneity exists between the studies, which is caused by the lack of any HLA-DRB1*01 association in replication populations I and III (Figure III in the Data Supplement).

#### Association Analyses

**HLA-DRB1*01** information was treated as a dichotomous variable. Those individuals with at least one HLA-DRB1*01 allele were considered as positive and were selected for the association study. The association analyses used PLINK software.21 SNPs were excluded from association analysis if they had minor allele frequency of $<1\%$, they had an individual genotyping rate $<95\%$ (missingness), or they showed deviation from Hardy–Weinberg equilibrium with a $P$ value $<0.01$. We used $5×10^{-8}$ as threshold for significance in the first stage and then 0.004 for the replication as a result of the Bonferroni correction for the 12 SNPs. Odds ratios and $P$ values were estimated with logistic regression in an additive model adjusting for age and sex. The 10 first dimensions of the multidimensional scaling on the matrix of identity-by-state also served as covariates.

#### Meta-Analysis

We selected the 7 SNPs that were highly associated with ACS in at least 3 of the populations. For these 7 SNPs, association summary statistics from the discovery and replication populations were combined in a random-effects model. Analysis was with PLINK software. The combined meta-analysis comprised 1453 cases and 1302 controls. Heterogeneity within the SNPs was assessed by $F$ index.

#### Linkage and Haplotype Analyses

Linkage disequilibrium analysis was conducted with Haplovew software (version 3.32).22 Haplotype reconstruction was with FAMHAP (version 08/2008)23 and PHASE (version 2.1)24 using the numbers of HLA-DRB1*01 alleles.

#### Alternative GWA Analysis With Data From the Discovery Population

We applied an additional statistical model in the complete discovery population without stratifying for HLA-DRB1*01 to test whether we can obtain the same effect by using the whole data set. This was a logistic regression model adjusted for age, sex, HLA-DRB1*01 allele numbers, and multidimensional scaling covariates, further adding the interaction term of the HLA-DRB1*01 allele number with each SNP.

#### Traditional Risk Factor Analysis

The stepwise logistic regression was performed in SPSS with the following factors included: HLA-DRB1*01, age, sex, diabetes mellitus, hypercholesterolemia, and smoking. We further studied the interaction of all these factors with the susceptible haplotype in a multivariate regression analysis in SPSS.

#### Statistical Power

The total study provides sufficient statistical power (>80%) to detect significant variants in 1600 cases and 1300 control subjects with OR of 1.2 at a 2-tailed $P$ value cut-off of 5×10$^{-8}$ for a SNP with allele frequency at least 15% in a 2-stage analysis, given that 40% of the individuals will be genotyped in the first stage of the analysis. Power calculations were performed with the CATS Power Calculator (http://csg.sph.umich.edu/abecasis/CaTS/) for 2-stage association studies.30

#### T Cell Proliferation Analyses

Statistical analysis for T cell proliferation studies and for coronary sample studies used SPSS. Nonlinked data set comparison used the Mann–Whitney $U$-test, and paired data used Student’s paired $t$ test. The 2-sided $P$ value for statistical significance was $P<0.05$.

The rest of the analyses were performed with PASW Statistics (18.0; SPSS Inc., Chicago, IL) and the statistical software R (R: A Language and Environment for Statistical Computing, 2011).

### Results

#### Association Results of HLA-DRB1*01-Positive Individuals in the Discovery Population

To test our hypothesis, we studied the candidate gene HLA-DRB1*01 with GWA analyses in ACS. We performed the HLA-DRB1*01 genotyping (0, 1, or 2 alleles of HLA-DRB1*01; Figure IIA in the Data Supplement). Subjects with HLA-DRB1*01 were more frequent among cases than among controls (38.9% versus 32.4%; unadjusted OR=1.33; $P=7.75×10^{-5}$; Figure III in the Data Supplement). HLA-DRB1*01 was independently associated with ACS in logistic regression analysis adjusted for age, sex, and multidimensional scaling covariates at the phenotypic
(positivity) (OR=1.42, 95% confidence interval [CI]=1.2–1.7; \( P=2\times10^{-5} \)) and the genotypic level (Table I in the Data Supplement). When we compared 626 cases to 469 controls carrying at least one HLA-DRB1*01 allele, genome-wide analysis produced highly significant associations between ACS and MHC SNPs (Figure 1A; Figure IIA in the Data Supplement). On the basis of the suggestive GWA results in the literature, we chose and analyzed 12 SNPs with the most significant \( P \) values flanking the BTNL2, HLA-DRA [MIM: 142860], and HLA-DRB1 genes (Figure IV in the Data Supplement). Association results conferred susceptibility to ACS in HLA-DRB1*01-positive individuals (Table 2) or protection in negative individuals. Genotypic ORs of BTNL2 and HLA-DRA SNPs stratified by HLA-DRB1*01 showed a dominant pattern in positive, but an additive pattern in negative individuals (Figure V in the Data Supplement). The degree of pairwise linkage disequilibrium in HLA-DRB1*01-positive cases and controls were tested in those 12 SNPs with \( r^2 \) statistics (Figure VI in the Data Supplement).

**Association Results Without HLA-DRB1*01 Stratification in the Discovery Population**

The integrated model using all individuals of the discovery population (including as covariates age, sex, the number of HLA-DRB1*01 alleles, multidimensional scaling covariates, and the interaction term of HLA-DRB1*01 allele number with SNPs) confirmed the HLA-DRB1*01 stratification results showing the same most significant associations in BTNL2 and HLA-DRA as the GWA stratified according to HLA-DRB1*01.

In a haplotype analysis, the 12 SNPs of the BTNL2 and HLA-DRA region, together with HLA-DRB1*01, constructed the BTNL2:HLA-DRA:HLA-DRB1*01 haplotype (CGCAGCTCTTA:HLA-DRB1*01) that was more common in ACS patients (14.53%) than in controls (4.23%; OR=4.43, 95% CI=3.57–5.50; \( P=2.98\times10^{-48} \); Figure 2). The BTNL2:HLA-DRA haplotype without HLA-DRB1*01 did not significantly associate with ACS (Table I in the Data Supplement).

We further analyzed the traditional risk factors to evaluate putative interaction with the genetic components that we found. In stepwise logistic regression analyses, all added factors (HLA-DRB1*01, age, sex, diabetes mellitus, hypercholesterolemia, and smoking) were associated with ACS (Table I in the Data Supplement). When we added hypertension, the model was not improved to predict ACS. In multivariate regression analyses, the susceptibility haplotype showed no interactions with traditional risk factors for ACS.

**Figure 1.** Manhattan plots of the discovery population with acute coronary syndrome. The \(-\log P\) values from the association of each single nucleotide polymorphism (SNP) with acute coronary syndrome are based on the logistic regression model adjusted for age, sex, and multidimensional scaling covariates; genotyped and imputed SNPs numbered 2,384,163. Chromosomes are shown from their p-terminal end. The red horizontal line indicates genome-wide significance level \((P<5\times10^{-8})\). **A**, Associations are highly significant \((P=1\times10^{-26})\) on BTNL2 and HLA-DRA SNPs on chromosome 6p21.3 for 626 acute coronary syndrome cases and 469 controls carrying at least one HLA-DRB1*01 allele. **B**, For the total discovery study of 2090 acute coronary syndrome cases and their 1580 controls, no SNPs with genome-wide significance occurred.
Without HLA-DRB1*01 stratification, MHC SNPs revealed an only nominal association (P≥10^-5) between ACS cases and controls based on the logistic regression model adjusted for age, sex, and multidimensional scaling covariates. The combined P value is from the meta-analysis based on random-effects model shows moderate associations (P<2.11×10^-10, respectively), whereas in replication population III, the difference between cases and controls showed a trend toward significance (6.06% versus 5.12%, OR=1.21, 95% CI=0.97–1.50; P=0.0917; Figure 2).

mRNA Expression Analysis and Immunohistochemical Staining of Coronary Arteries

In an independent study, we investigated the expression of BTN2L2 in coronary artery samples by using quantitative mRNA analyses (n=47) and immunohistochemistry (n=24). For mRNA analyses, we selected samples from the coronary area that were without visible signs of lipid accumulation (healthy) or with advanced atherosclerotic lesions (extensively diseased) with the information on BTNL2 mRNA expression analysis and immunohistochemistry.

### Table 2. Significant Associations With Acute Coronary Syndrome

| Gene | SNP | Position, bp | Risk Allele | Discovery Population | Replication I | Replication II | Replication III | Combined |
|------|-----|--------------|-------------|----------------------|---------------|---------------|----------------|----------|
|      |     |              | P Value     | Odds Ratio (95% CI)  | Odds Ratio    | Odds Ratio    | Odds Ratio    | Odds Ratio |
| BTN2*| rs1555115 | 32462498 | C           | 6.67×10^-18 | 5.05 (3.49–7.29) | 1.13×10^-11 | 4.25 (3.14–5.75) | 1.05×10^-8 | 2.73 (2.12–3.53) | 2.3×10^-4 | 1.65 (1.26–2.15) | 7.21×10^-10 | 3.92 (2.65–5.05) |
| BTN2*| rs12525722 | 32466141 | G           | 6.67×10^-18 | 5.05 (3.49–7.29) | NA            | NA            | 3.06×10^-6 | 2.67 (2.07–3.45) | 3.36×10^-4 | 1.62 (1.24–2.10) | 1.0×10^-3 | 2.85 (1.98–4.2)  |
| BTN2*| rs17495612 | 32467409 | C           | 6.67×10^-18 | 5.05 (3.49–7.29) | 1.23×10^-11 | 4.25 (3.14–5.75) | 1.15×10^-8 | 2.7 (2.09–3.48)  | 4.04×10^-4 | 1.61 (1.24–2.10) | 9.31×10^-5 | 3.79 (2.47–4.95) |
| BTN2*| rs16870123 | 32467438 | A           | 6.67×10^-18 | 5.05 (3.49–7.29) | NA            | NA            | NA            | NA            | NA            | NA            | NA            | NA            |
| BTN2*| rs17495626 | 32467621 | G           | 6.67×10^-18 | 5.05 (3.49–7.29) | NA            | NA            | NA            | NA            | NA            | NA            | NA            | NA            |
| BTN2†| rs3763313 | 32484449 | C           | 4.47×10^-15 | 4.12 (2.89–5.88) | 4.98×10^-4 | 3.76 (2.81–5.04) | 1.04×10^-7 | 2.08 (1.59–2.73) | 2.79×10^-4 | 1.76 (1.29–2.39) | 2.16×10^-6 | 2.95 (2.04–4.43) |
| HLA-DRA†| rs3135340 | 32506850 | G           | 7.33×10^-12 | 2.68 (2.13–3.89) | 1.95×10^-9 | 3.24 (2.44–4.31) | 4.65×10^-5 | 2.9 (1.65–2.66)  | 1.14×10^-3 | 1.49 (1.17–1.91) | 7.83×10^-6 | 2.43 (1.32–4.12) |
| HLA-DRA†| rs4988822 | 32506953 | T           | 7.33×10^-12 | 2.88 (2.13–3.89) | 1.95×10^-9 | 3.24 (2.44–4.31) | 4.65×10^-5 | 2.9 (1.65–2.66)  | 1.14×10^-3 | 1.49 (1.17–1.91) | 7.83×10^-6 | 2.43 (1.32–4.12) |
| HLA-DRA†| rs3128659 | 32508917 | C           | 6.60×10^-12 | 2.47 (2.05–3.65) | 6.80×10^-9 | 3.24 (2.44–4.31) | 2.49×10^-9 | 2.9 (1.64–2.65)  | 1.75×10^-3 | 1.46 (1.15–1.86) | 0.29    | 1.61 (0.89–2.48)  |
| HLA-DRA†| rs17496307 | 32509014 | T           | 5.55×10^-15 | 11.5 (7.31–18.15) | 1.99×10^-13 | 5.46 (3.97–7.51) | 1.2×10^-7 | 2.53 (2.05–3.49) | 3.58×10^-5 | 1.82 (1.37–2.41) | 2.32×10^-4 | 5.81 (3.87–6.71)  |
| HLA-DRB1†| rs2395176 | 32513040 | T           | 5.58×10^-12 | 2.68 (2.05–3.5)  | NA            | NA            | NA            | NA            | NA            | NA            | NA            | NA            |
| HLA-DRB1†| rs9269491 | 32650822 | A           | 7.51×10^-15 | 4.04 (2.84–5.70) | NA            | NA            | NA            | NA            | NA            | NA            | NA            | NA            |

Association results are shown for 12 SNPs in discovery population (P<5×10^-8; cases, 626; controls, 469), 6 SNPs in replication population I (cases, 214; controls, 205), 7 SNPs in replication populations II (cases, 296; controls, 291) and III (cases, 317; controls, 337) (P<0.004). HLA-DRB1*01-positive individuals are used for this analysis in a logistic regression model adjusted for age, sex, and multidimensional scaling covariates. The combined P value is from the meta-analysis based on random-effects model of the significant SNPs with acute coronary syndrome in 4 studies (cases, 1453; controls, 1302). CI indicates confidence interval; and SNP, single nucleotide polymorphism.

*The location of the SNP is downstream.
†The location of the SNP is in promoter region, NA not analyzed.

### Replication of the Results in 3 Additional Populations

In replication studies, HLA-DRB1*01 alone was associated with patients with ACS in replication population II, but not in replication populations I and III (Figures III–IID and III in the Data Supplement). Twelve susceptibility SNPs from the discovery population were further analyzed with replication populations I, II, and III. We replicated a total of 6 SNPs for replication population I (P≤4.98×10^-10), 7 for II (P≤5.02×10^-10), and 9 for III (P≤1.75×10^-10), corroborating the disease association found in HLA-DRB1*01-positive cases and control subjects of the discovery population (Table 2). The meta-analysis based on random-effects model shows moderate P values (Table 2) because of heterogeneity of the MHC region (Table II in the Data Supplement). The BTN2L2:HLA-DRA:HLA-DRB1*01 haplotype was significantly more common in patients with ACS than in control subjects in replication populations I and II (16.57% versus 7.32%, OR=2.89, 95% CI=2.16–3.89; P=1.31×10^-13 and 14.98% versus 5.55%, OR=3.42, 95% CI=2.57–4.54; P=2.11×10^-15, respectively), whereas in replication population III, the difference between cases and controls showed a trend toward significance (6.06% versus 5.12%, OR=1.21, 95% CI=0.97–1.50; P=0.0917; Figure 2).
CD68 mRNA expression correlated with atherosclerosis grade of the coronary lesions (r=0.481; P=0.0042), but neither with BTNL2 SNPs nor with BTNL2 mRNA expression (Figure 3E–3G). Immunohistochemical staining of coronary arteries showed that HLA-DRA was present in CD68-positive cells (macrophages; Figure 3I). Similarly, staining for CD68 and BTNL2 showed that BTNL2 was present in macrophages, thus expressing both HLA-DR and BTNL2 molecules (Figure 3H) in coronary artery lesions.

In Vitro T Cell Proliferation Responses in Genetically Selected Patients

We studied the effects of BTNL2 blockage on in vitro T cell proliferation in genetically selected cases by using a pool of vaccine proteins as antigens presented by autologous antigen-presenting cells. Blocking BTNL2 with a monoclonal antibody had little effect on CD4+ T cell proliferation (Figure 4A), but led to a significant increase in proliferation of CD4+FOXP3+ regulatory T cells in cases positive for the haplotype (n=10, difference in T cell receptor beta chain (T cell receptor– normalized expression of FOXP3). The truncating mutation of BTNL2 has been suspected to downregulate its function, leading to inappropriate inflammation. In one Han Chinese population, a chromosomal location near C6orf10-BTNL2 was associated with coronary artery disease at an OR of 1.16.1 In our study, BTNL2 is thought to encode a negative costimulatory-receptor for T cells, possibly inhibiting both T cell proliferation and interleukin-2 production. The difference in HLA-DRB1*01 frequencies is demonstrated by heterogeneity among the Finns and between the Finnish and Spanish study populations. Despite population-specific differences between Finns and Spaniards, we were able to find the same disease-predisposing haplotype in both populations, one enriched among the Finns.

Second, we combined 2 methods for detecting candidate genes: a direct approach for allele-specific typing of HLA-DRB1*01 and the high density of SNPs in the MHC area. BTNL2 is located in close proximity to the HLA-DRB1 gene (≈200 kb) and has been associated with inflammatory autoimmune diseases and with lipid levels. BTNL2 is thought to encode a negative costimulatory-receptor for T cells, possibly inhibiting both T cell proliferation and interleukin-2 production. The difference in HLA-DRB1*01 frequencies is demonstrated by heterogeneity among the Finns and between the Finnish and Spanish study populations. Despite population-specific differences between Finns and Spaniards, we were able to find the same disease-predisposing haplotype in both populations, one enriched among the Finns.
is merely a tag of another acting gene. However, the HLA-DR and BTNL2 molecules have a close functional relationship, regulating peptide-specific, MHC-restricted T cell recognition: HLA-DRB1 acting as the antigen-presenting receptor and BTNL2 modifying its immune response. At all stages of atherogenesis, macrophage foam cells are present in coronary atherosclerotic plaques and play a central role in inflammatory signaling in the coronary plaque. The histological and mRNA expression analyses of coronary artery samples in relation to number of the BTNL2 single nucleotide polymorphisms (SNPs) and HLA-DRB1*01 allele (n=24). A, mRNA expression of BTNL2 in coronary artery biopsies in patients homozygous (+/+), heterozygous (+/−), or negative (−/−) for BTNL2 and HLA-DRB1*01. B-G, Adjacent sections of the same plaques demonstrating CD68 and BTNL2. Immunohistochemical staining for BTNL2 (B–D) and CD68 (E–G) are from the same patient groups as above. H, Immunofluorescence staining for colocalization of BTNL2 and CD68 in coronary artery macrophages. BTNL2 is in green and CD68 in red, and they merge in yellow. Nuclei are stained blue with DAPI. I, Immunohistochemical staining for HLA-DR alpha chain (brown) of coronary macrophages. RU indicates relative units.

The results of this large study show a strong association between ACS and a common haplotype affecting inflammation or immune reactions, or both. This haplotype occurs in Finland in one patient of 7 patients with ACS and in about one of every 20 healthy controls. In Spaniards, the same haplotype finding was only suggestive, indicating genetic diversity of the predisposing haplotype between different populations. Results should, therefore, be re-evaluated in other populations. Our findings provide a novel basis for studies exploring the role of inflammation and the immune system in ACS.
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Disclosures
None.

Appendix
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**Clinical Perspective**

Coronary artery disease is the health problem worldwide. To date, there is no gene which we could use for to predict early individuals at risk for myocardial infarction. We have shown for the first time that a certain gene combination of major histocompatibility complex is a strong risk factor for CAD. BTNL2, HLA-DRA, and HLA-DRB1*01 haplotype will cause exceptionally high risk for their carrier. Our hypothesis is that in the immunologic process creating risk for coronary artery disease, certain BTNL2 and HLA-MR molecules have a close functional relationship regulating peptide-specific, DRB1*01-restricted T cell recognition. HLA-DR acts as the antigen-presenting molecule in macrophages/foam cells and BTNL2 modifying the immune response. The pathogenetic mechanism caused by the predisposing genes point to human coronary plaque macrophages and foam cells and the limited regulation causing enhancement in inflammatory and immunologic processes. Our results may open a possibility to develop improved preventative measures, such as a test to recognize patients at risk at its early stage, diagnostics and therapeutic strategies for coronary artery disease. Genetic screening for individuals at cardiovascular risk could help to prevent future events through better targeting of statins and other medication.