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A Case-Control Study Reveals Immunoregulatory Gene Haplotypes That Influence Inhibitor Risk in Severe Hemophilia A

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Abstract

Introduction—Several genes that modify risk of factor VIII inhibitors in hemophilia A patients have been identified. Aside from the underlying mutations that cause hemophilia A, inhibitor risk appears to be modified by polymorphisms in various cytokines and immunomodulators, including *IL10*, *TNFA*, and *CTLA4*. HLA haplotypes have not been strong determinants of inhibitor risk.

Aim—We sought to confirm previous observations on factor VIII inhibitor risk-modifying genes and to test new candidate genes encoding various other TH1/TH2 cytokines. We also sought to determine whether normal factor VIII gene polymorphisms affect inhibitor risk in Caucasians.

Methods—We studied 915 Caucasian, severe hemophilia A patients (282 inhibitor cases and 633 non-inhibitor controls). Genes were analyzed using 368 tagging SNPs starting 20kb 5' and ending 10kb 3' of each gene's coding sequence; four other polymorphisms (factor V Leiden & prothrombin 20210 polymorphisms and two in *HFE*) were also evaluated.

Results—Haplotypes that increased inhibitor risk were found in *IL10* (OR 1.33, $P = 0.04$), *IL12* (OR 1.31, $P = 0.04$), and *IL1 α* (OR 2.16, $P = 0.034$). Protective haplotypes were seen in *IL2* (OR 0.69, $P = 0.008$) and *IL1 β* (OR 0.75, $P = 0.02$). One rare haplotype in the factor VIII gene increased the risk of inhibitor development by nearly four-fold (OR 3.8, $P = 0.004$).

Conclusion—We replicate previous findings for *IL10*; identify new associations with *IL1*, *IL2* and *IL12*; and identify a rare factor VIII haplotype in Caucasians that is associated with increased inhibitor risk.

Keywords

hemophilia A; inhibitor; factor VIII; IL10; IL2; IL12; IL1

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Authorship Contributions

JL secured funding for this project, designed experiments, assisted with data analysis and interpretation, and wrote the manuscript. PSR helped to secure funding for this project, designed experiments, oversaw data analysis and interpretation, and helped write the manuscript.

JJG helped to secure funding for the Multicenter Hemophilia Cohort Studies, helped secure additional funding for this project, designed experiments, secured patient samples, helped with data analysis, and helped to edit the manuscript.

IM designed experiments, did the data analysis and most of its interpretation, and helped write the manuscript.

Disclosure of Conflicts of Interest

There are no conflicts of interest relevant to this report to disclose.

Introduction

Treatment of hemophilia A with factor VIII has improved the life of patients with severe disease [1]. Epidemics of hepatitis and human immunodeficiency virus (HIV) from concentrates in the 1970's and 1980's were a setback, but virus-free plasma-derived concentrates and recombinant DNA products re-established the upward trend in life expectancy [2]. Patients with hemophilia A (without HIV) now experience a nearly normal life expectancy (64 years) [3].

Neutralizing antibodies to factor VIII ("inhibitors") still complicate factor VIII therapy for approximately 20% of severe hemophilia A patients. Mutations in factor VIII are the chief risk determinants of inhibitor development predominantly in patients with severe disease [4]. However, discordance for inhibitors in related patients provides evidence that other genetic factors play a role [5]. Polymorphisms in immunoregulatory genes such as *IL10*, *TNF α* , and *CTLA4* have been associated with altered risk for inhibitor development [6-8]. In contrast, *HLA*, *IL1 β* and *IL4* genes have shown weak or nonexistent association with inhibitor development [9,10].

African-Americans and Latinos with hemophilia A have higher inhibitor risk than Caucasians [11]. Viel *et al* recently showed that inhibitor status among African-Americans was associated with factor VIII haplotypes that are rarely seen in Caucasians [12]. For the 24% of African-Americans with an uncommon factor VIII isoform (H3 or H4), the inhibitor risk was 3.6 times (95% confidence interval, 1.1 to 12.3; $P=0.04$) that seen in those who had one of the common isoforms H1 or H2.

We sought to confirm association of immune response modifier gene polymorphisms on inhibitor development and to extend this work to other TH1 and TH2 immune response genes. We also looked at polymorphisms in the factor VIII gene itself to see if the findings in African-Americans with hemophilia could be found in Caucasians. We replicated previous family study findings for *IL10*, found association of inhibitors with other immune response modifier genes, and showed an association with an infrequent factor VIII gene haplotype in Caucasians.

Materials and Methods

Human Subjects

Subjects were non-Hispanic Caucasians with severe hemophilia A (factor VIII <1%), recruited to the Multicenter Hemophilia Cohort Studies I/II between 1982 and 2005, under IRB-approved protocols (Clinical Trial registration number NCT00341705). Subjects were from 48 hemophilia centers located primarily in North America and Europe. All subjects were at least 13 years of age and extensively treated with factor VIII.

Case-Control Study Design

Cases (n=302) were patients with factor VIII inhibitors, defined as having at least one Bethesda inhibitor assay titer of > 1.0 Bethesda Inhibitor Assay Unit. Controls (n= 633) were a simple random sample of all severe hemophilia A subjects without factor VIII inhibitors. The prevalence of HIV was 41% in Cases and 46% in Controls ($P=0.95$). The prevalence of chronic HCV was 80% in Cases and 77% in Controls ($P=0.98$).

DNA Purification & SNP Genotyping

Genomic DNA was purified from leukocytes using Qiagen chromatography columns (Qiagen, Valencia, CA). Tag SNP genotypes were determined by Sequenom (Boston, MA),

using PCR and template-dependent single base pair extension. Extended nucleotides were identified using MALDI-TOF mass spectroscopy.

Candidate Genes

Twenty candidate genes (Table 1) were selected for analysis based on previous associations with factor VIII inhibitor development in humans [6-8], animal studies of factor VIII [13] or factor IX immunogenicity [14], effects on hemophilia A severity [15], or their role in the TH1/TH2 immune response.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms that are within blocks of DNA that do not undergo homologous recombination during meiosis are termed “tag SNPs”. Tag SNPs that define haplotypes in Caucasians were selected from the HapMap database (Public Release #26), using the Haploview program. SNPs were located between 20 kb 5' upstream to and 10 kb 3' downstream to the gene's coding sequence. In all, 372 SNPs were selected, consisting of tag SNPs chosen to cover all possible common (greater than 1% prevalence) haplotypes in each genomic region ($r^2 = 1.0$) [16].

SNP-based Association Analysis

The risk of inhibitor development associated with individual SNPs was determined using unconditional logistic regression models adjusted for HIV and HCV status. SNP genotypes were evaluated using a co-dominant model with a three-level ordinal variable coded 0, 1, and 2 for the number of rare alleles. Likelihood-ratio test was used to assess the relative improvement in model fit after the inclusion of individual SNPs. False discovery rate (FDR) adjustment was used to control for the number of tests (SNPs) in each region.

Haplotype-based Association Analysis

Haplotype phasing was inferred using Haploview (version 4.1), and analyzed for significant association with inhibitor status, using the HaploStats package in R [16]. We calculated odds ratios (OR) and 95% confidence intervals (CI) estimating the relative risk of inhibitor in relation to specific haplotypes within a haplotype block region using a logistic regression model adjusted for HIV and HCV status. The global additive effect of common haplotypes (>1% prevalence) within a block was also calculated using a global score test [17].

Results

Replication of Association between *IL10* Polymorphisms and Inhibitor Development

Inhibitor status was associated with six SNPs located ~3,700 to 11,900 nucleotides 5' to the *IL10* Met translation start site (raw P values <0.05, Figure 1). This region also contains the *IL10G* microsatellite dinucleotide CA repeat DNA sequence [18], shown previously to be associated with inhibitor risk. [6] The global effect of haplotypes in this region was also significant ($P = 0.016$, Figure 1). The signal mostly reflected one common (32% prevalence) haplotype consisting of three tag SNPs (at rs6667202, rs4072226, and rs4072227) that was associated with increased inhibitor (OR = 1.23, CI 1.01-1.5, individual P value = 0.04).

The global effect of *IL10* haplotypes on inhibitors was stronger in HIV-positive subjects ($P = 0.0007$). Most of this effect came from two rare haplotypes defined by tag SNPs rs4072227, rs1701586, rs880790, rs885334, and rs12565617 (Table 2), that conferred protection against inhibitors (prevalence of 4.7-6.7% and odds ratio $\leq 2 \times 10^{-9}$ for each, not shown in Figure 1).

Novel Associations: IL2, IL1, and IL12A

Three SNPs in the *IL2* gene had significant association with inhibitor development, two of which (rs2069762 and rs4833248) remained significant after correction for multiple hypothesis-testing (Figure 2). One haplotype with 28% prevalence among controls (Table 2) conferred protection against inhibitor development (OR 0.69, CI 0.53-0.91).

The associations with *IL2* tended to be stronger in HIV-infected patients (Figure 2). The haplotype with significant protective effect in the overall analysis increased further (OR 0.57, CI 0.38-0.86, $P = 0.009$), and a second haplotype (13% prevalence among controls) was also significant in the HIV-positive subgroup (OR 0.56, CI 0.32-0.98, $P = 0.04$). The global effect of all *IL2* haplotypes was significant only in the HIV-positive subgroup ($P = 0.03$).

A series of SNPs in the *IL12A* gene were associated with inhibitor (Figure 3). One *IL12A* haplotype with a prevalence among controls of 24% was associated with increased risk of inhibitor development (OR = 1.31, CI 1.02-1.68, $P = 0.04$). The global effect of all *IL12A* haplotypes on inhibitor risk did not achieve significance ($P = 0.10$). No difference in the association with inhibitor risk was seen in HIV-infected patients.

Multiple individual tag SNPs in the genes for the $IL\alpha$ and $IL\beta$ chains had marginal associations with inhibitor risk (Figure 4), but none of these was significant after correction for multiple testing. Analysis of haplotypes in these genes showed a rare haplotype (1.3% prevalence among controls) in *IL1\alpha* that conferred significantly increased risk for inhibitor (OR = 2.2, CI 1.1-4.3, $P = 0.03$). Other relatively common haplotypes in the *IL1\alpha* gene were weakly associated with increased risk but were not significant, as shown in Table 2. One common *IL1\beta* haplotype with 28% prevalence among controls, was associated with decreased inhibitor risk (OR 0.75, CI 0.58-0.96, $P = 0.02$).

Polymorphisms in the Factor VIII Gene

Initial analysis of 10 tag SNPs in the 216,928 bp region containing the factor VIII gene suggested no significant associations with inhibitor development (Figure 5). In light of the findings of Viel *et al* [12], we also analyzed factor VIII haplotypes in this Caucasian population. One rare haplotype was associated with significantly increased risk for inhibitor (OR 3.8, CI 1.5-9.5, $P = 0.004$), as it was seen predominantly among cases (3.1%) and was extremely rare among controls (0.25%). This haplotype was the strongest risk factor detected in our study.

Other Genes

Non-significant associations were seen with individual SNPs in genes previously associated with inhibitors in human (e.g., *TNFA*) or in vitro laboratory studies (i.e., *IL17A*) [7,29]. No association was found with factor V Leiden or prothrombin 20210 gene polymorphisms that are associated with prothrombotic states thereby moderating the severity of the hemophilia A bleeding phenotype [15] and potentially decreasing intensity of factor VIII exposure and inhibitor development. The relative rarity of the factor V (4.3% prevalence) and prothrombin 20210 (3.0% prevalence) polymorphisms in our subjects limits our power to detect their effects.

Discussion

Our case-control study replicates the association of factor VIII inhibitors with polymorphisms in the *IL10* promoter region seen in family studies [6]. This region is located upstream to both *IL10* and *IL19* promoter regions. *IL19*, is a member of the *IL10* gene

super-family [19], and may have resulted from duplication of the *IL10* gene. IL 10 increases B-cell proliferation and maturation into immunoglobulin-producing plasma cells. *IL19* increases expression of IL10, IL4, and IL13, cytokines critical to the TH2 immune response and antibody development [20]. The possibility that inhibitor development is partially mediated by the effects of IL19 should be investigated through in vivo and in vitro mechanistic studies.

The increased significance of the *IL10* gene association in HIV seropositive subjects suggests an interplay between viral infection and the inhibitor response. Although chronic HIV infection may cause loss of factor VIII inhibitors and anti-factor VIII CD4 T-lymphocytes [21], during acute HIV infection there is increased IL10 expression from various immune cells [22]. The latter may promote the Th2/antibody response and increase factor VIII inhibitor risk. Certain *IL10* promoter haplotypes are known to accelerate the progression to AIDS in HIV-infected persons of European/Caucasian ethnicity [23,24]. Further, polymorphic microsatellite dinucleotide repeats in the same region are associated with lower concentrations of IL10 [25]. We speculate that *IL10* haplotypes associated with lower expression may be critical both to AIDS progression as well as the factor VIII inhibitor response. We do not know the dates for HIV seroconversion and inhibitor development in our subjects, so we cannot say with certainty what the effect of HIV infection on inhibitor formation was in our study. As reported previously for our cohort and others, inhibitor prevalence was slightly lower among HIV+ subjects (30%, vs. 34% in HIV- subjects). The HIV+ and HIV- subjects born during the epidemic have essentially the same inhibitor rates (26-27%). We suspect that subjects born before 1977 developed inhibitors prior to HIV infection. For these subjects, the inhibitor rate is marginally higher (43%) in HIV + subjects than for HIV – subjects (39%), but we doubt the significance of that finding.

Individual SNPs and haplotypes in other immune genes modified inhibitor risk (Table 2). There was decreased risk of inhibitor associated with one haplotype encompassing the *IL2* gene, and this negative association was stronger in HIV positive subjects (Figure 2), as seen for *IL10*. No differential effect in HIV- or chronically HCV-infected subjects was seen for any other genes that modified inhibitor risk. Inhibitors also were associated with haplotypes in the *IL1β* and *IL1α* genes. A previous study of one SNP in the *IL1β* gene showed no association with inhibitor risk [6], underscoring the importance of looking at haplotypes. Interestingly, there was increased inhibitor risk (OR 2.16) in one rare (1.7% prevalence) haplotype in the *IL1α* gene, whereas a fairly common haplotype in the *IL1β* gene (27% prevalence) had a protective effect (OR 0.75).

Interleukin 12, a heterodimeric cytokine expressed from the *IL12A* and *IL12B* genes, is secreted by dendritic cells. It stimulates synthesis of interferon- γ critical to the TH1 immune response [26]. *IL12A* polymorphisms were significantly associated with increased inhibitor risk (OR = 1.3) but *IL12B* polymorphisms were not (data not shown). The association of certain *IL12A* haplotypes with increased inhibitor risk may relate to its role in stimulating TH1 lymphocyte development [27]. Conversely, IL-12 is suppressed by the activity of IL-10, and modulation of inhibitor risk may depend on the balance between the effects of these cytokines [28].

The strongest risk factor in our study was one rare haplotype of the factor VIII gene that nearly quadrupled the risk of inhibitor development. This finding would be consistent with the hypothesis offered by Viel et al that rare haplotypes in patients of African ancestry represent “mismatches” with factor VIII product derived from predominantly Caucasian blood donors or recombinant products with “Caucasian” protein coding sequences [12]. This “factor VIII mismatch” mechanism, although more common in African Americans, presumably may occur at a lower frequency in the Caucasian population. If confirmed, this

would support development of factor VIII products with amino acid sequences matching that of each patient's factor VIII sequence. Our study design differs from that of Viel *et al* [15], in that we chose to analyze tag SNPs, rather than polymorphisms leading to changes in the factor VIII amino acid coding sequence. However, their polymorphisms are clustered near one of our tag SNPs (rs6643622) that has a marginal signal for inhibitor risk (Figure 5). The genetic component of inhibitor risk is certainly complex, and different polymorphisms in different immune response genes may have additive or opposing effects on risk in the same patient. The factor VIII gene defect and severity of disease will likely remain the most important inherited determinant of inhibitor risk for patients with hemophilia

Our study has inherent limitations. Our subjects were treated in a unique era when HIV- and HCV- infected plasma-derived concentrates were commonly used. Recombinant products and modern, virus-free concentrates from pooled plasma make this unlikely to occur again. Another inherent limitation of our study is that genes were chosen on the basis of *a priori* mechanistic assumptions regarding the immune response. A genome-wide association analysis should be employed to discover genes whose association with factor VIII inhibitors is not expected on mechanistic grounds. Such an approach imposes the obligation to consider and control for false-positive findings from an enormous number of genes, SNPs, and their derivative haplotypes. Recently, Astermark and colleagues presented data from a collaborative trial involving 680 predominantly Caucasian hemophilia patients that surveyed more than 16,000 SNPs in 385 immunomodulatory and coagulation related genes [30]. They found associations of inhibitor risk with polymorphisms in the putative immunomodulatory genes *DOCK2*, *MAPK9*, *PTPRR*, and *CD36*, as well as the coagulation factor XIII gene [30]. These genes would not likely have been tested except in a systematic survey of all immunomodulatory genes. Our studies serve as stepping stones to comprehensive, genome-wide surveys of genetic and other risk factors for inhibitor development. Nonetheless, our data, together with other targeted genetic association studies to date, firmly suggest that polymorphisms that influence the TH1/TH2 balance may be key determinants of risk. The ultimate goals of these studies are to predict inhibitor risk for individuals, to identify novel targets for risk modulation, and to design lower risk recombinant factor VIII products.

Multicenter Hemophilia Cohort Study Center Locations

United States

University of Arizona, Tucson, Arizona; University of California, San Francisco, San Francisco, California; University of California, Davis, Davis, California; Children's Hospital of Orange County, Orange, California; University of Colorado, Denver, Colorado; Christiana Hospital, Wilmington, Delaware; Children's National Medical Center, Washington, District of Columbia; Georgetown University, Washington, District of Columbia; Emory University, Atlanta, Georgia; University of Illinois, Chicago, Illinois; Children's Memorial Hospital, Chicago, Chicago, Illinois; St. Vincent's Hospital, Indianapolis, Indiana; Tulane University, New Orleans, Louisiana; Wayne State University Hutzell Hospital, Detroit, Michigan, 48201; University of Minnesota, Minneapolis, Minnesota; University of Mississippi, Jackson, Mississippi; St. Louis University, St. Louis, Missouri; University of New Mexico, Albuquerque, New Mexico; North Shore Long Island Jewish Health System, Lake Success, New York; University of Buffalo, Buffalo, New York; Cornell University, New York, New York; Mt. Sinai Medical Center, New York, New York; University of North Carolina, Chapel Hill, North Carolina; Wake Forest University, Winston-Salem, North Carolina; Wright State University, Dayton, Ohio; University of Cincinnati, Cincinnati, Ohio; Children's Hospital, Cincinnati, Cincinnati, Ohio; Ohio State University, Columbus, Ohio;

University of Oklahoma, Oklahoma City, Oklahoma; Milton Hershey Medical Center, Hershey, Pennsylvania; University of Pennsylvania, Philadelphia, Pennsylvania; Thomas Jefferson University, Philadelphia, Pennsylvania; Palmetto Health Alliance, Columbia, South Carolina; University of Tennessee, Memphis, Tennessee; Vanderbilt University, Nashville, Tennessee; University of Texas, Houston, Houston, Texas; University of Texas, San Antonio, San Antonio, Texas; University of Utah, Salt Lake City, Utah; Children's Hospital of Wisconsin, Milwaukee, Wisconsin.

Brazil

University of Sao Paulo, Sao Paulo, Brazil; Hospital Brigadeiro, Sao Paulo, Brazil

Canada

South East Health Care Corporation, New Brunswick, Canada

France

Hospices Civils de Lyon, Lyons, France

Germany

University of Bonn, Bonn, Germany

Greece

Laikon General Hospital of Athens, Athens, Greece

Italy

University of Florence, Florence, Italy; University de Milano, Milan, Italy

Sweden

Karolinska Institute St. Gorans Hospital, Stockholm, Sweden

The contribution of subjects from centers in various geographic regions appear in Table 3

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Figure 1. *IL10* Haplotypes are Associated with Increased or Decreased Factor VIII Inhibitor Risk

The upper panel (A) depicts the association of *IL10* tag SNPs and associated haplotypes with inhibitor risk in the overall group and the lower (B) panel depicts the association of *IL10* tag SNPs and haplotypes with inhibitors in the HIV seropositive subjects. 24 tag SNPs are shown along the X axis, in their genomic order. In each panel, the position of the *IL10* and *IL19* genes are indicated by block arrows. The position of the *IL10G* microsatellite CA dinucleotide repeat is indicated by the open block as labeled. Haplotype blocks associated with inhibitor risk are indicated by yellow shading of the constituent tag SNPs. The $-\log_{10}$ of the raw P value for each tag SNP and their corresponding FDR values that accounts for

multiple hypothesis testing are depicted with closed triangles connected with solid line and open circles connected with dashed line, respectively. The global effect of *IL10* haplotypes is depicted with stars connected by a dashed red line in panel B. The dotted horizontal line indicates a significance level of $P = 0.05$. Nucleotides at polymorphic sites in each haplotype are indicated in red. Note that two haplotypes with odds ratios of 2.3×10^{-9} and 1.3×10^{-30} and P values of 0 (seen in 5% and 3% of the HIV positive subjects only) are not depicted on the lower panel. The heat map at the bottom of the figure denotes pairwise linkage disequilibrium (LD) values (D') between tag SNPs. High LD (red color) indicate lack of evidence for recombination between pairs of SNPs, while low LD (green color) indicates recombination between pairs of SNPs. Tracing the squares below a tag SNP in a diagonal direction gives an indication of the LD between that SNP and its neighbors.

Figure 2. *IL2* Tag SNPs are Associated with Decreased Factor VIII Inhibitor Risk

The upper panel depicts the association of *IL2* tag SNPs and associated haplotypes with inhibitor risk in the overall group, and the lower panel depicts the associations in the HIV seropositive subjects. Six tag SNPs are shown along the X axis, in their genomic order. The *IL2* gene is depicted by the block arrow. There is one haplotype block that encompasses the *IL2* gene, based on the linkage disequilibrium “heat map” shown at the bottom. The $-\log_{10}$ of the raw P value for each tag SNP and their corresponding FDR values that accounts for multiple hypothesis testing are depicted with closed triangles connected with solid line and open circles connected with dashed line, respectively. The global effect of *IL2* haplotypes is depicted with stars connected by a dashed red line in each panel. The dotted horizontal line

indicates a significance level of $P = 0.05$. Nucleotides at polymorphic sites in each haplotype are indicated in red, and the global test of haplotype effect for HIV positive subjects is indicated with a dashed red line. The heat map at the bottom of the figure denotes pairwise linkage disequilibrium (LD) values (D') between tag SNPs. High LD (red color) indicate lack of evidence for recombination between pairs of SNPs, while low LD (green color) indicates recombination between pairs of SNPs. Tracing the squares below a tag SNP in a diagonal direction gives an indication of the LD between that SNP and its neighbors.

Figure 3. *IL12A* Tag SNPs and Haplotypes Are Associated with Increased Factor VIII Inhibitor Risk

26 tag SNPs are shown along the X axis, in their genomic order. The *IL12A* gene is depicted as a block arrow; one haplotype block is evident in this region, based on the linkage disequilibrium “heat map” shown below the graph. The *IL12A* haplotype block is indicated by yellow shading of reference SNPs on the X axis. The $-\log_{10}$ of the raw P value for each tag SNP and their corresponding FDR values that accounts for multiple hypothesis testing are depicted with closed triangles connected with solid line and open circles connected with dashed line, respectively. The dotted horizontal line indicates a significance level of $P = 0.05$. Nucleotides at polymorphic sites in each haplotype are indicated in red. The heat map at the bottom of the figure denotes pairwise linkage disequilibrium (LD) values (D') between tag SNPs. High LD (red color) indicate lack of evidence for recombination between pairs of SNPs, while low LD (green color) indicates recombination between pairs of SNPs. Tracing the squares below a tag SNP in a diagonal direction gives an indication of the LD between that SNP and its neighbors.

Figure 4. *IL1α* and *IL1β* Haplotypes May Increase or Decrease Factor VIII Inhibitor Risk
 26 tag SNPs are shown along the X axis, in their genomic order. The *IL1α* and *IL1β* chain genes are depicted with block arrows. Two major haplotype blocks in this region are indicated by yellow shading of the tag SNPs on the X-axis. The $-\log_{10}$ of the raw P value for each tag SNP and their corresponding FDR values that accounts for multiple hypothesis testing are depicted with closed triangles connected with solid line and open circles connected with dashed line, respectively. The dotted horizontal line indicates a significance level of $P = 0.05$. Haplotypes are indicated in red. Nucleotides at polymorphic sites in each haplotype are indicated in red. The heat map at the bottom of the figure denotes pairwise linkage disequilibrium (LD) values (D') between tag SNPs. High LD (red color) indicate lack of evidence for recombination between pairs of SNPs, while low LD (green color) indicates recombination between pairs of SNPs. Tracing the squares below a tag SNP in a diagonal direction gives an indication of the LD between that SNP and its neighbors.

Figure 5. A Rare Coagulation Factor VIII Haplotype is Associated with Factor VIII Inhibitor Risk

10 tag SNPs that encompass the factor VIII gene are shown along the X axis, in their genomic order. The factor VIII gene is depicted with a block arrow. One major haplotype block is evident in this region, based on the linkage disequilibrium "heat map" shown below the graph. The $-\log_{10}$ of the raw P value for each tag SNP and their corresponding FDR values that accounts for multiple hypothesis testing are depicted with closed triangles connected with solid line and open circles connected with dashed line, respectively. The dotted horizontal line indicates a significance level of $P = 0.05$. The one haplotype that was found to be associated with inhibitor risk is indicated in red, as are the nucleotides at polymorphic sites in this haplotype. The positions of amino acid coding polymorphisms in the factor VIII gene reported on by Viel et al [12] are indicated in the boxes in the Figure. M2238V = valine substitution for methionine at amino acid 2238, D1241E = glutamic acid substitution for aspartic acid, R776G = glycine substitution for arginine, R484H = histidine substitution for arginine at amino acid 484. The heat map at the bottom of the figure denotes pairwise linkage disequilibrium (LD) values (D') between tag SNPs. High LD (red color) indicate lack of evidence for recombination between pairs of SNPs, while low LD (green color) indicates recombination between pairs of SNPs. Tracing the squares below a tag SNP in a diagonal direction gives an indication of the LD between that SNP and its neighbors.

Table 1
Candidate Genes Analyzed for Association with Inhibitor Risk

Gene	# Tag SNPs for $r^2 = 1.0$	# SNPs with 80% Amplification	Gene Coverage	Previous Studies
<i>Previously Studied Genes</i>				
<i>IL10</i>	28	24	86%	Astermark, 20066
<i>TNFα</i>	22	18	82%	Astermark, 20067
<i>IL1A+B</i>	32	26	81%	Astermark, 20066
<i>IL13+4</i>	17	12	71%	Astermark, 20066
<i>CTLA4</i>	13	13	100%	Astermark, 20068
<i>Novel Immunomodulatory Candidate Genes</i>				
<i>IL2</i>	10	6	60%	
<i>IL6</i>	26	17	65%	
<i>IL12A</i>	29	26	90%	
<i>IL12B</i>	28	26	93%	
<i>IL17A</i>	19	16	84%	Ettinger et al, 200829
<i>IL22</i>	31	26	84%	Lozier et al, 200514
<i>IFNγ</i>	13	12	92%	Lozier et al, 200514
<i>TGFβ1</i>	10	7	70%	
<i>CD28</i>	20	17	85%	
<i>IL1RN</i>	42	31	74%	
<i>AZGP1</i>	13	7	54%	Lozier et al, 200513
<i>Factor VIII and Related Coagulation Factors</i>				
<i>Factor VIII</i>	15	10	67%	Viel, et al 200912
<i>FV_{Leiden}</i>	1	1	100%	Kurnik et al, 200815
<i>Prothrombin₂₀₂₁₀</i>	1	1	100%	Kurnik et al, 200815
<i>HFE</i>	2	2	100%	
Sum	372	298	80%	

Table 2
Haplotypes Associated with Increased/Decreased Inhibitor Risk

<i>Gene (tag SNP rs #) Tag SNP Nucleotides</i>	<i>Cases (%)</i>	<i>Controls (%)</i>	<i>OR</i>	<i>95% CI</i>	<i>P Value</i>
<i>IL10</i> (rs6667202,rs407226,rs4072227)					
C-T-T	38	32	1.23	1.01-1.50	0.04
<i>IL10</i> (rs4072227, rs1701586, rs880790, rs885334, rs12565617)					
C-G-C-G-T*	<1	6.7	2.3×10^{-9}	NA	0.0009
T-A-C-A-T*	<1	4.7	1.3×10^{-30}	NA	0.0048
<i>IL2</i> (rs10027390, rs2069772, rs2069779, rs2069778, rs2069762, rs4833248)					
T-G-C-C-T-G	23	28	0.69	0.53-0.91	0.008
<i>IL12A</i> (rs2243115, rs583911, rs2243131, rs568408, rs2243148, rs2243154, rs2133310)					
T-A-A-G-C-G-A	29	24	1.31	1.02-1.68	0.041
<i>IL1A</i> (rs3783557, rs2071373, rs6746923, rs17597976, rs11687624, rs11898680, rs6716046, rs7567619, rs7585707, rs11680809, rs12711742, rs12469600)					
G-T-A-G-T-T-T-T-C-G-T	2.5	1.3	2.2	1.1-4.3	0.034
G-T-A-G-T-C-C-T-T-C-G-T	27	24	1.3	1.0-1.7	0.056 (NS)
A-T-G-G-T-T-T-T-C-A-G-C	24	22	1.3	1.0-1.7	0.077 (NS)
<i>IL1B</i> (rs1143627, rs16944, rs1143623, rs1261220, rs13032029, rs13008855, rs6735739, rs12053091)					
T-A-G-C-C-T-C-C-G	23	28	0.75	0.58-0.96	0.02
<i>Factor VIII</i> (rs5945250, rs17281377, rs4898399, rs6643622, rs7053448, rs1936645, rs5945269, rs5945270, rs17281398, rs6649625)					
T-C-T-G-C-G-C-A-T	3	0.25	3.8	1.50-9.51	0.004

* HIV positive subjects

Table 3
Distribution of Subjects by Geographic Regions

Region of Center	Total Subjects	Case Subjects	Control Subjects
<i>North America</i>	644	228	416
<i>Argentina</i>	61	14	47
<i>Brazil</i>	41	16	25
<i>Greece</i>	56	23	33
<i>Other European</i>	133	21	112
Total	935	302	633