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Author manuscript

*Genes Immun.* Author manuscript; available in PMC 2017 September 16.

Published in final edited form as:

*Genes Immun.* 2017 March ; 18(2): 82–87. doi:10.1038/gene.2017.2.

## Genetic basis for variation in plasma IL-18 levels in persons with chronic hepatitis C virus and human immunodeficiency virus-1 infections

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### Abstract

Inflammasomes are multi-protein complexes integrating pathogen-triggered signaling leading to the generation of pro-inflammatory cytokines, including interleukin-18 (IL-18). Hepatitis C virus (HCV) and human immunodeficiency virus-1 (HIV) infections are associated with elevated IL-18, suggesting inflammasome activation. However, there is marked person-to-person variation in the

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**Conflict of Interest:** The authors declare no conflict of interest.

inflammasome response to HCV and HIV. We hypothesized that host genetics may explain this variation. To test this, we analyzed the associations of plasma IL-18 levels and polymorphisms in 10 genes in the inflammasome cascade. 1538 participants with active HIV and/or HCV infection in 3 ancestry groups are included. Samples were genotyped using the Illumina Omni 1-quad and Omni 2.5 arrays. Linear regression analyses were performed to test the association of variants with logIL-18 including HCV and HIV infection status and HIV-RNA, in each ancestry group and then meta-analyzed. Eleven highly correlated SNPs ( $r^2=0.98-1$ ) in the *IL18-BCO2* region were significantly associated with logIL-18; Each T allele of rs80011693 confers a decrease of 0.06 log pg/mL of IL-18 after adjusting for covariates (rs80011693; rs111311302  $\beta=-0.06$ , P-value= $2.7\times 10^{-4}$ ). In conclusion, genetic variation in *IL18* is associated with IL-18 production in response to HIV and HCV infection and may explain variability in the inflammatory outcomes of chronic viral infections.

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## Introduction

Inflammasomes are multi-protein cytosolic complexes that integrate several pathogen-triggered signaling cascades, ultimately leading to caspase-1 activation and the generation of pro-inflammatory cytokines including interleukin (IL)-18 and IL-1 $\beta$  (1,2). While there are inflammasome-dependent and -independent means to induce mature IL-1 $\beta$  production (3-5), to date, inflammasome activation has been the only mechanism described to convert pro-IL-18 to mature IL-18 (6). Thus, higher levels of IL-18 are indicative of inflammasome activation. Both hepatitis C virus (HCV) and human immunodeficiency virus-1 (HIV) are known to activate a specific inflammasome, the NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome, as measured by increases in IL-18. For example, plasma IL-18 levels increase sharply during the acute phase of HCV infection, remain elevated in those with persistent infection, and return to the pre-infection level with spontaneous resolution of HCV (7). Similarly, levels are elevated in patients with chronic hepatitis and cirrhosis, and reduced after the infection is resolved either spontaneously (8) or after antiviral treatment (9). IL-18 levels are also elevated in HIV infection, an effect that is partially reversed by antiretroviral therapy (10,11).

Genetic determinants of IL-18 levels have been demonstrated in cohorts of individuals from different age groups without chronic viral infections (12-14). Several independent loci have been correlated with serum IL-18 levels in multiple genome-wide association studies including the *interleukin 18 and beta-carotene oxygenase 2 (IL18-BCO2)* gene region of chromosome 11 and, the *NLR family, and caspase activation and recruitment domains (CARD) domain containing 4 (NLRC4)* gene on chromosome 2 (12-14). Associations of serum IL-18 levels and SNPs in the *IL18* gene were replicated in several populations of European ancestry with different demographic characteristics including women with no history of chronic illness (14), women with and without Type 2 diabetes (14), individuals with and without premature coronary heart disease (15) and older adults (12). Strong genetic effects in *cis* location have been suggested, however the biological mechanisms underlying the genetic associations are unknown.

Compared to what is observed in studies of persons without chronic viral infection, there is a wider range of IL-18 elevation observed in persons with chronic viral infection that cannot be explained by age, race, or gender (2,7,16,17). This suggests that host genetic differences may partially explain variation in IL-18 response to viral infection. To investigate this hypothesis, we measured IL-18 levels in individuals with well-characterized chronic and active HIV viremia and/or HCV viremia and evaluated the association of single nucleotide polymorphisms (SNPs) in 10 key genes in the viral-NLPR3 inflammasome activation pathway.

## Results

### Participants

Demographic characteristics of the analyzed individuals are presented in Table 1. HIV infected individuals were not under antiretroviral therapy. All individuals had their ancestry defined by principal component analysis using genetic variants. There were 386 individuals of European Ancestry (EA), 796 of African Ancestry (AA) and 356 of mixed or other Ancestry (MOA). Age (P value= 0.94), ancestry (P value = 0.09) and sex (P value= 0.13) were not associated with log IL-18.

### Log plasma IL-18 by infection status

Log IL-18 was significantly higher in 450 persons with both HIV and HCV infections (Mean =2.76 log pg/mL, SD=0.27 log pg/mL) than in the 668 HIV-mono infected (Mean=2.61 log pg/mL, SD=0.30 log pg/mL) and the 420 HCV mono infected participants (Mean=2.50 log pg/mL, SD=0.30 log pg/mL),  $P = 2.2 \times 10^{-16}$  for the three-group comparison, Figure 1. Similar results were observed for each ancestry group independently (Supplementary Figure 1). The multivariable analysis after adjusting for the principal components demonstrates that the level of HIV RNA in plasma is also determinant of IL-18 levels in all individuals (Table 2) as well as in each ancestry group (Supplementary Table 1). Specifically, there was a progressive increase in log IL-18 as HIV RNA increases from low, to moderate and high levels in both the HIV monoinfected and HIV-HCV co-infected groups compared to the HCV monoinfected individuals. However, HIV/HCV infection, HIV RNA and principal components, only accounted for 14% of the variance in IL-18 levels.

### Genetic association analysis

From 10 genes spanning a total of ~519 kb of DNA (Supplementary Table 2), we evaluated 721 genetic variants for EA, 1307 for AA, and 1170 for MOA. We found an association of IL-18 levels and variants in the *IL18-BCO2* region in chromosome 11. The SNPs significantly associated with IL-18 levels after adjusting for the principal components and the HIV and HCV categories are shown in Figure 2. Table 3 displays their allelic frequency, effect size estimates and the annotation of the function and eQTL information described in public databases (<https://genome.ucsc.edu/index.html> and <http://www.gtexportal.org/home/>, respectively). The strongest signals in the *IL18-BCO2* region were in the imputed SNPs rs80011693 ( $\beta=-0.06$ , SE=0.02, P value= $1.9 \times 10^{-4}$ ) and rs111311302 with  $\beta=-0.06$ , SE=0.02, P value= $2.0 \times 10^{-4}$ . Thus, each T allele of rs80011693 confers a decrease of 0.06 log pg/mL of IL-18 after adjusting for HIV-HCV infection, HIV RNA levels and ancestry

(Table 3 and Supplementary Table 3). The direction of the effect of the associated variants is the same across ancestry groups. They were in high linkage disequilibrium (LD) in the three ancestry groups ( $r^2=0.98-1$ ). After conditioning on each of the associated SNPs, the remaining SNPs in the *IL18-BCO2* were not significant (Supplementary Figure 3), highlighting the strong LD across this region. Associations between log IL-18 and polymorphisms in *IL-1 $\beta$* , *ASCC1*, *NLRP3*, *NFKB1*, *NFKB2*, *CASP1*, *TLR7*, *TLR8* and *MYD88* did not reach statistical significance after correcting for multiple tests.

## Discussion

This investigation extends previous associations of genetic factors with IL-18 plasma levels by demonstrating that the variants in the *IL18-BCO2* region are important determinants of plasma IL-18 levels in the presence of a chronic viral infection. Prior studies demonstrated the importance of this region in persons without an ongoing infection (12-14). Compared to the SNPs, the greater magnitude of the effects ( $\beta$  regression coefficient) associated with HIV mono infection and HIV-HCV co-infection confirm the dominant role of viral infections explaining person to person differences in IL-18 levels (7, 8). However, these dominant viral associations only accounted for a low percentage of the variance in IL-18 levels, suggesting that there are other unmeasured factors responsible for the person-to-person differences in IL-18 levels in each infection group. Our findings suggest that this genetic region has an important role in determining plasma IL-18 levels. It is also notable that in our multivariable model, no other non-genetic factor was as important, including ancestry.

The results of this and previous studies indicate a consistent locus effect in the *IL18* region represented by a SNP to SNP replication across studies. The association of genetic variants in *IL18* on plasma IL-18 levels was studied in older European adults included in the Cardiovascular Health Study (CHS) (12) and European individuals in the In-CHIANTI study (13). They detected an association of IL-18 levels with rs1834481 and rs5744256, two intronic SNPs that were also significantly associated with IL-18 levels in our study. Likewise, rs1834481 was associated with IL-18 levels in a genome wide association study of 1,523 women of European ancestry from the Nurses' Health Study (NHS) and 435 from the Women's Genome Health Study (WGHS) (14). Moreover, rs5744256 and rs2043055 were previously associated with IL-18 levels in candidate gene studies in healthy individuals and in individuals with premature cardiovascular disease, respectively (15,18,19).

We looked at the publicly available RNA expression data (20,21) to investigate the relationship of any of the associated SNPs or other SNPs with expression of IL-18 levels in diverse tissues. Of the 11 variants described in this study, 10 have been described as expression quantitative trait loci (eQTLs) in tissues such as lung, pancreas and skin (21). Other SNPs not included in the present analysis, such as rs7020926, rs7030024, rs13283237, rs7851038 and rs944363, are described as related to IL-18 expression levels in circulating monocytes analyzed in 1,490 unrelated European individuals (22), but no direct biological mechanism has yet been demonstrated.

*BCO2* variants have also been reported as associated with serum IL-18 level. In the CHS and In-CHIANTI cohorts, rs2250417 was significantly associated with IL-18 protein levels (13)

in each individual cohort as well as the meta-analysis (P value= $1.9 \times 10^{-32}$ ). Similarly, He et al describes three different variants (rs2115763, rs12420140, and rs4935984) in this gene as associated with IL-18 levels in the NHS-WGHS (14). The associated variants in the BCO2 gene in the different studies (rs2250417, rs10891343, rs2115763, rs12420140, and rs4935984) are in high LD ( $D' = 0.99-1$ ) with the associated SNPs in our study in populations of African, European, Asian descent as well as admixed populations in The 1000 Genomes project (<http://www.1000genomes.org/>). However, since they were not genotyped or imputed in this study, we cannot rule out their role on IL-18 levels in this analysis.

Even though there is a clear effect of genetic determinants in plasma IL-18 levels, much of the inter-individual differences remain unexplained. Circulating levels and biological activity depends on the level of production of IL-18, its natural inhibitory protein IL-18BP (IL-18 binding protein) as well as the surface expression of IL-18 receptors (IL-18R) on responding cells (23). Thus, variation in the levels of IL-18 might be determined by polymorphisms regulating the expression of these genes or as yet unidentified genes that encode for the proteins that interact with IL-18. Environmental factors such as bacterial and other viral infections stimulating the innate and acquired immune responses concurrently with HCV and HIV may also affect IL-18 levels. However, IL-18 levels have been shown to be stable both before (low levels) and in the chronic phase of (high levels) HCV infection, suggesting that exposure to other infections may play a lesser role than HCV in regulating total levels. Additionally, chronic or acute conditions activating the inflammasome, and epigenetic mechanisms regulating the expression of the IL-18 protein are among other potential modulators of IL-18 variation.

On the other hand, pro-IL-18 is constitutively expressed (24,25), its expression increases from baseline levels after the signal 1 and inflammasome assembly results in maturation of IL-18, and its release into extracellular space (26,27). We hypothesize that baseline constitutive expression levels of pro-IL-18 are regulated by genetic determinants represented by the associated variants and, that caspase 1 activation converts pro-IL-18 to mature IL-18 with greater production of mature IL-18 when pro-IL-18 levels are higher. Thus, the higher baseline pro-IL-18 in the individuals with the risk alleles result in higher levels of mature IL-18 production upon inflammasome activation. This would be expected if caspase 1 enzymatic activity is not limiting upon activation and is consistent with the literature on IL-18 regulation (24,28). Our perspective is also influenced by the observation we make in humans infected with HIV or HCV in whom there is a marked increase in plasma total IL-18 that remains elevated for decades or until with HCV, the infection is eliminated, at which time it comes back to baseline (7). Those increases in total plasma IL-18 level following HCV or HIV infection vary markedly from person to person, persist for decades, and undoubtedly are multifactorial but yet poorly understood. Thus, we aimed to study the full range of genes that might contribute, even if the gene doesn't fit the current models of inflammasome activation. We established that there is a genetic basis for differences in plasma IL-18 levels in persons with chronic viral infection that warrants investigation of the molecular mechanisms underlying those observations.

## Materials and Methods

### Study participants

A total of 1538 individuals were selected from 5 different study cohorts: the AIDS Link to the Intravenous Experience (ALIVE) (29), Baltimore Before and After Acute Study of Hepatitis (BBAASH) (30), Boston Area HCV Study Transmission, Immunity, Outcomes Network (BAHSTION) (31), Correlates of Resolved Versus Low-Level Viremic Hepatitis C Infection in Blood Donors (REVELL Study) (32), and Women's Interagency HIV Study (WIHS) (33), as previously described (34). The inclusion criteria were the availability of genome-wide genotyping data and evidence of active HCV and/or HIV infection as determined by presence of both antibodies and detectable RNA levels for more than 6 months prior to the date of IL-18 testing, as described previously (33,34). HCV and/or HIV infected individuals were not included in any anti-HCV or antiretroviral therapy at the time of sample withdraw. Each individual study obtained consent for genetic testing as approved by their governing Institutional Review Board and the Johns Hopkins School of Medicine Institutional Review Board.

### IL-18 plasma levels

Mature IL-18 was measured in plasma from repositied specimens using enzyme-linked immunosorbent assay (MBL International, Woburn, MA, USA) on a sample taken prior to antiretroviral therapy in the HIV-infected participants. The assay was performed per the manufacturer's recommendations using 50  $\mu$ L of plasma. Data were acquired using a SpectraMax M5 (Molecular Devices). The sensitivity of this assay is 12.5 pg/mL and the average intra-assay coefficient of variation is 7.25%.

### Statistical analysis

Plasma IL-18 levels, log-transformed for approximate normalization, were the primary outcome. HIV and HCV infection status, HIV RNA levels, genetic ancestry, gender, and age were each included in the statistical model of linear regression to determine their effect on logIL-18. HCV and HIV infection status and HIV RNA variables were modeled as a unique categorical variable that we refer to as "HIV-HCV infection, HIV RNA". This variable groups the individuals into seven categories: HCV mono infected, HIV mono infected with low (400-10K copies/mL), moderate (10K-50K copies/mL) and high HIV RNA levels (>50K copies/mL) and, co-infected with low, moderate and high HIV RNA levels. ANOVA was used to compare log IL-18 levels between HCV mono infected, HIV mono infected, and HIV-HCV co-infected individuals with all individuals and by genetic ancestry.

### Genetic ancestry analysis

Genetic ancestry was determined by principal components based on 10,340 unlinked SNPs across the genome using the *smartpca* program from the software package *eigenstrat* (35). Principal components analysis is used to summarize the background genetic variation of populations into a few variables that represent ancestral origin. Three distinct ancestry groups emerged including European Ancestry, African Ancestry and mixed or other ancestry, which likely includes Hispanic and Asian ancestry (Supplementary Figure 2).



## Genotyping and Imputation

The SNP genotyping data were obtained from a larger genome wide association study (34) using the Illumina Human Omni-Quad array for samples from the ALIVE, BBAASH, BAHSTION and REVELL study groups and using the Illumina Omni2.5 array for the WIHS group. The selected SNPs are part of the NLRP3 inflammasome pathway and include the genes and 10 kilobases upstream and downstream of each gene. The pathway genes included were *Interleukin 18 (IL18)*, *Interleukin 1 beta (IL-1 $\beta$ )*, *Activating Signal Cointegrator 1 Complex Subunit 1 (ASCC1)*, *NLR family, pyrin domain containing 3 (NLRP3)*; *Nuclear Factor Kappa B Subunit 1 (NF- $\kappa$ B1)*, *Nuclear Factor Kappa B Subunit 2 (NF- $\kappa$ B2)*, *Caspase (CASP1)*, *Toll Like Receptor 7 (TLR7)*, *Toll Like Receptor 8 (TLR8)*, *Myeloid Differentiation Primary Response 88 (MYD88)* and *Beta-carotene oxygenase 2 (BCO2)*. Genomic coordinates were based on the Human GRCh37/hg19 assembly (Supplementary Table 2). Genomic imputation was performed for each chromosomal region using IMPUTE2 (36). The reference panel consisted of 2,504 individuals representing four continental populations (Africa, Americas, Asia and Europe) as part of the 1000 Genomes Project (37). Each genetic platform was individually imputed using all individuals and the imputed datasets were merged based on genetic ancestry groups using the software Plink version 1.90 beta (<https://www.cog-genomics.org/plink2>). Only variants with an information score > 0.3 and a genotyping probability greater than 90% were included in the analysis (38). Standard quality control measures were employed pre and post imputation (excluding variants with minor allele frequency (MAF) < 0.01, missing call rate 1%, Hardy Weinberg equilibrium test P value < 1 $\times$ 10<sup>-5</sup>). Genotyped and high quality imputed variants that were common to both platforms were included in the regression analysis.

## Genetic association analysis

Plink was used to analyze linear regression models adjusting for five principal components and HIV-HCV infection, HIV RNA. SNPs were modeled under an additive model in which genotypes are represented as 0, 1, 2 copies of the minor allele. Ancestry groups were tested separately and the results were meta-analyzed by combining ancestry-specific  $\beta$  (regression coefficient) estimates using a fixed effects inverse variance model (39) implemented in META (40). Linkage disequilibrium ( $r^2$ ) was determined in each of the genetic regions by ancestral group. This informed a modified Bonferroni correction using the effective number of independent SNPs (n=103 SNPs for AA, n=38 for EA and n=57 for MOA, n= 197 total variants). Twenty-five of the 197 SNPs were common between at least two ancestry groups, and the remaining were unique. A modified Bonferroni corrected P value < 2.9  $\times$  10<sup>-4</sup> was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We acknowledge support from the National Institutes of Health (NIH) National Institute on Drug Abuse R01DA013324 (Thomas), R01DA12568 (Mehta) and U01DA036297 (Kirk); National Institute of Allergy and Infectious Diseases (NIAID) R01 AI108403 (Cox); NIH K23 AI124913 (Lahiri); NIH U19AI066345,

U01AI131314, R01DA033541 and U19AI082630 (Kim). Data in this manuscript were partially collected by the Women's Interagency HIV Study (WIHS). The contents of this publication are solely the responsibility of the authors and do not represent the official views of the NIH. WIHS (Principal Investigators): UAB-MS WIHS (Saag, Kempf, and Konkle-Parker), U01-AI-103401; Atlanta WIHS (Ofotokun and Wingood), U01-AI-103408; Bronx WIHS (Anastos), U01-AI-035004; Brooklyn WIHS (Minkoff and Gustafson), U01-AI-031834; Chicago WIHS (Cohen and French), U01-AI-034993; Metropolitan Washington WIHS (Kassaye), U01-AI-034994; Miami WIHS (Fischl and Metsch), U01-AI-103397; UNC WIHS (Adimora), U01-AI-103390; Connie Wofsy Women's HIV Study, Northern California (Greenblatt, Aouizerat, and Tien), U01-AI-034989; WIHS Data Management and Analysis Center (Gange and Golub), U01-AI-042590; Southern California WIHS (Milam), U01-HD-032632 (WIHS I–WIHS IV). The WIHS is funded primarily by the National Institute of Allergy and Infectious Diseases (NIAID), with additional co-funding from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), the National Cancer Institute (NCI), the National Institute on Drug Abuse (NIDA), and the National Institute on Mental Health (NIMH). Targeted supplemental funding for specific projects is also provided by the National Institute of Dental and Craniofacial Research (NIDCR), the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the National Institute on Deafness and other Communication Disorders (NIDCD), and the NIH Office of Research on Women's Health. WIHS data collection is also supported by UL1-TR000004 (UCSF CTSA) and UL1-TR000454 (Atlanta CTSA).

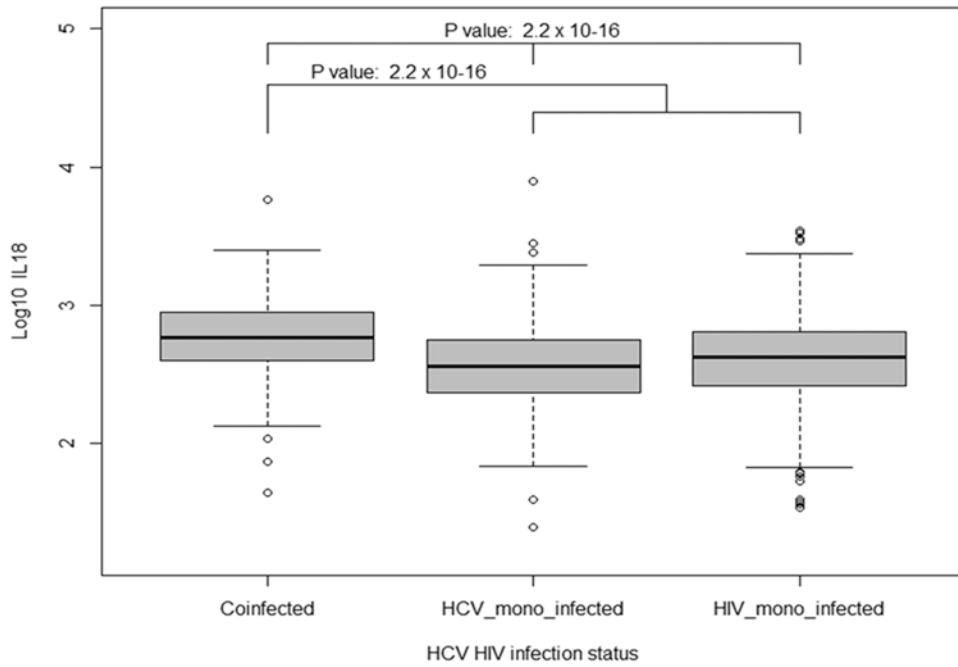
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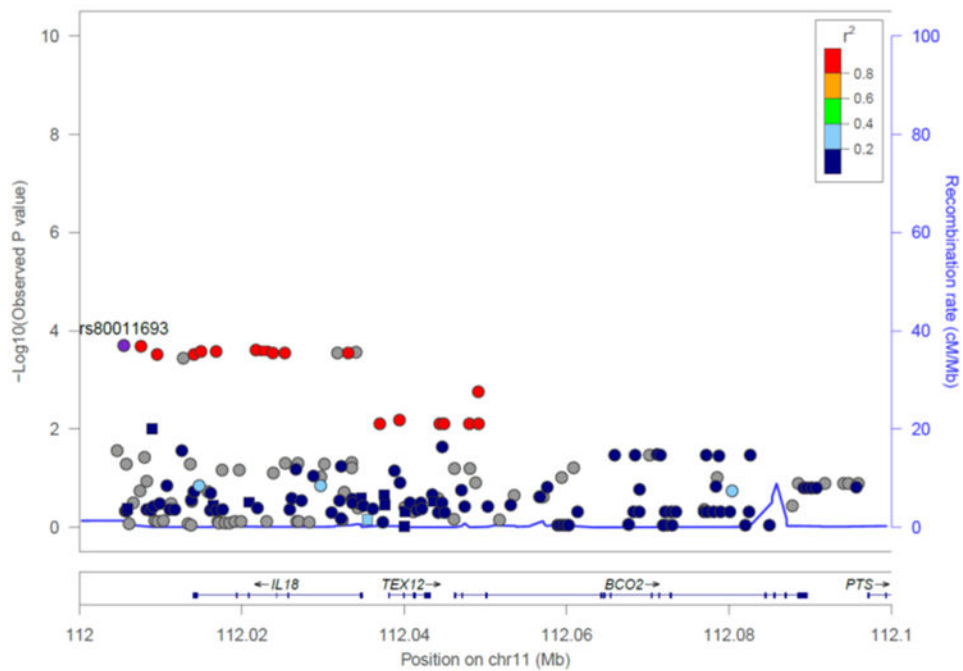


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**Figure 1.** Log plasma IL-18 by HCV and HIV infection status in all the individuals analyzed in the study. The thick horizontal line in each box plot represent the median for each category, the whiskers extend to 1.5 times the interquartile range from the box.



**Figure 2.**

Association of variants in the *IL18-BCO2* region. The variants included 14 genotyped (squares) and 177 imputed SNPs (circles) within the region from 112 Mb to 112.1 Mb on chromosome 11. The recombination in this region is plotted in the background in light blue. Pair wise LDs between rs80011693 and other variants in *IL18-BCO2* were estimated using LD data in EUR population in the 1000 Genomes project (hg19/Nov 2014). The color from blue to red represents the  $r^2$  values.

**Table 1**

Descriptive statistics of the analyzed individuals by genetically estimated ancestry group.

Characteristic	Genetically estimated Ancestry groups		
	European (n=386)	African (n=796)	Mixed or other (n=356)
Female (n, %)	283 (73.3)	703 (88.3)	334 (93.8)
Age (years), mean (SD)	41.37 (11.5)	40.85 (8.5)	37.51 (9.4)
IL-18 (pg/mL), mean (SD)	584.5 (547.4)	546.0 (441.3)	560.3 (410.0)
HIV HCV infection status and HIV RNA			
HCV Mono infected (n, %)	191 (49.5)	156 (19.6)	73 (20.5)
HIV Mono infected, low HIV RNA <sup>I</sup> (n, %)	28 (7.25)	104 (13.1)	58 (16.29)
HIV Mono infected, moderate HIV RNA <sup>I</sup> (n, %)	35 (9.06)	102 (12.8)	62 (17.4)
HIV Mono infected, high HIV RNA <sup>I</sup> (n, %)	56 (14.5)	149 (18.7)	74 (20.78)
HIV HCV Co-infected, low HIV RNA (n, %)	19 (4.92)	75 (9.42)	35 (9.83)
HIV HCV Co-infected, moderate HIV RNA (n, %)	21 (5.44)	76 (9.54)	20 (5.61)
HIV HCV Co-infected, high HIV RNA (n, %)	36 (9.32)	134 (16.8)	34 (9.55)

<sup>I</sup>HIV low, medium and high RNA refers to 400-10K, 10K-50K and >50K HIV copies/mL, respectively.

**Table 2**

Multivariate linear regression analysis of Log IL-18 in all individuals.

Factor	$\beta$	SE	P value
PC1	0.12	0.16	0.46
PC2	0.24	0.16	0.13
PC3	-0.01	0.16	0.94
PC4	0.19	0.16	0.25
PC5	-0.12	0.16	0.43
HIV Mono infected, low HIV RNA <sup>1</sup>	-0.05	0.02	0.03
HIV Mono infected, moderate HIV RNA <sup>1</sup>	0.02	0.02	0.28
HIV Mono infected, high HIV RNA <sup>1</sup>	0.19	0.02	$2 \times 10^{-16}$
HIV HCV Co-infected, low HIV RNA	0.15	0.02	$7.0 \times 10^{-08}$
HIV HCV Co-infected, moderate HIV RNA	0.18	0.02	$4.2 \times 10^{-10}$
HIV HCV Co-infected, high HIV RNA	0.27	0.02	$2 \times 10^{-16}$

Abbreviations:  $\beta$ : Beta regression coefficient; SE: Standard error; PC: principal component.

<sup>1</sup>HIV low, medium and high RNA refers 400-10K, 10K-50K and >50K HIV copies/mL, respectively.



**Table 3**

Top significant SNPs in IL18 associated with LogIL-18 by genetically estimated ancestry groups and results of the meta-analysis.

Chr	SNP	Position (GRCh37/hg19)	eQTL	Function	Allele Minor/Major	Meta Analysis			European Ancestry			African Ancestry			Mixed or Other Ancestry			Minor Allele Frequency		
						B	SE	P Val	B	SE	P Val	B	SE	P Val	B	SE	P Val	EA	AA	MOA
11	rs80011693	112005483	Yes	Intronic	T/C	-0.06	0.02	0.00020	-0.06	0.02	0.012	-0.08	0.04	0.037	-0.06	0.03	0.066	0.25	0.04	0.11
11	rs111311302	112007619	No	Intronic	G/C	-0.06	0.02	0.00020	-0.06	0.02	0.013	-0.08	0.04	0.035	-0.06	0.03	0.064	0.25	0.04	0.11
11	rs3882892	112014986	Yes	Intronic	T/C	-0.06	0.02	0.00027	-0.06	0.02	0.013	-0.08	0.04	0.052	-0.06	0.03	0.064	0.25	0.04	0.11
11	rs5744276	112016871	Yes	Intronic	G/C	-0.06	0.02	0.00027	-0.06	0.02	0.013	-0.08	0.04	0.052	-0.06	0.03	0.064	0.25	0.04	0.11
11	rs5744258	112021767	Yes	Intronic	G/C	-0.06	0.02	0.00025	-0.06	0.02	0.013	-0.08	0.04	0.055	-0.06	0.03	0.055	0.25	0.04	0.11
11	rs5744256	112022848	Yes	Intronic	G/A	-0.06	0.02	0.00026	-0.06	0.02	0.013	-0.07	0.04	0.064	-0.07	0.03	0.052	0.25	0.04	0.12
11	rs1834481	112023827	Yes	Intronic	G/C	-0.06	0.02	0.00028	-0.06	0.02	0.013	-0.07	0.04	0.066	-0.06	0.03	0.055	0.25	0.04	0.11
11	rs5744249	112025306	Yes	Intronic	C/A	-0.06	0.02	0.00028	-0.06	0.02	0.013	-0.07	0.04	0.066	-0.06	0.03	0.055	0.25	0.04	0.11
11	rs34546088	112031798	No	Intronic	C/CA	-0.06	0.02	0.00028	-0.06	0.02	0.013	-0.07	0.04	0.066	-0.06	0.03	0.055	0.25	0.04	0.11
11	rs11606049	112033129	Yes	Intronic	T/C	-0.06	0.02	0.00028	-0.06	0.02	0.013	-0.07	0.04	0.066	-0.06	0.03	0.054	0.25	0.04	0.11
11	rs5744230	112034062	No	Intronic	T/TA	-0.06	0.02	0.00027	-0.06	0.02	0.013	-0.07	0.04	0.063	-0.06	0.03	0.054	0.25	0.04	0.11