Title: **The Genetics of Baseline TH17 Signaling Cytokines: Interleukin-23 and Interleukin-17A**

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**Abstract**

The genetics of chronic inflammation remains poorly understood. Uncovering novel genes underlying for these conditions will greatly facilitate the discovery of the molecular mechanisms responsible for the immuno-pathogenic effects and provide targets for therapeutic intervention. Recent studies have identified dysfunctional TH17 activity as a central process characteristic of autoinflammation, autoimmunity, oncogenesis, and metabolic disease. Here, in a two-staged experiment, we measured baseline circulating levels of the two prototypical cytokines indicative of TH17 activity, IL-23 and IL-17A, in a large, homogeneous population. Genomic DNAs from all individuals were subjected to genome-wide genotyping and a gene-based analysis performed to discover genes significantly correlated with baseline TH17 activity. We identified…

**Introduction**

Substantial evidence has accumulated over the past two decades that numerous common diseases exhibit underlying chronic systemic inflammation.1-5 Indeed, the hand of sustained, elevated, and often misdirected immune response has a pervasive reach: predisposing and driving compromised immunocompetence and a wide variety of diseases. Broadly, sustained immune dysfunction is thought to promote pathogenicity through a number of avenues including tissue damage mediated by innate immune cells, compromised immune tolerance and autoimmunity, fibrosis, vascular leakage, carcinogenesis, gut permeability, neurodegeneration, and possibly accelerated senescence. Circulating markers of inflammation can provide a key, quantifiable intermediate phenotype useful for studying the effects of continually hyperactive inflammatory pathways.6-8 Using this molecular intermediate phenotype approach can dramatically reduce the complexity of clinically-defined phenotypes; and with a more refined, clearer trait, it is reasonable to assume that the underlying susceptibility genetics will likewise be simplified.9 Identifying the genetic variants that underlie variation in circulating inflammatory cytokines can provide insight into the mechanisms and pathways involved in production of these critically important mediators of immune responses.

Clearly, there are numerous immunological pathways involved in response to bacterial, viral and fungal infections and generating chronic systemic inflammation, with CD4+ lymphocytes, including TH17 cells, playing a critical role.10,11 Recent studies have demonstrated that TH17 development, stimulation and signaling plays a fundamental role in atherosclerosis12, response to bacterial infection13, colorectal cancer prognosis14, autoimmune diseases including rheumatoid arthritis15, multiple sclerosis16 and systemic lupus erythematosus17 and carries particularly pronounced effects for autoinflammatory conditions such as psoriasis18, inflammatory bowel disease19 and ankylosing spondylitis.20

Both differentiation of pathogenic effector TH17 cells and memory TH17 cells are dependent on interleukin-23 (IL-23).21,22 IL-23 is a heterodimer composed of p40 and p19, encoded by *IL12B* and *IL23A*, respectively; and binds to the surface receptor interleukin-23R. Stimulated by IL-23 during inflammation, activated TH17 cells produce IL-17A.23 Following IL-17A secretion, this cytokine forms both homodimers and heterodimers with IL-17F, which serve as ligands which bind to IL-17RA/IL-17RC receptor complex on several different types of immune cells. This IL-17 signaling results in downstream ACT1/TRAF6 stimulation and IKK-associated kinase activity, and subsequent activation of NF-kappaB-mediated expression of a variety of inflammatory responses.24-26 This IL-23/IL-17 axis lies at the core of TH17 activity27, with mouse model work, cellular studies, human genetics and transcriptomics work illuminating how (i) differentiation of TH17 cells occurs mediated by IL-23 and increased IL-23R expression28, (ii) Foxp3+ T cells convert to TH17 cells29, and (iii) the interplay between TH1/TH2 balance and Treg populations on TH17 activity generate a pathogenic cascade.30 Importantly, Genome-wide association studies investigating autoinflammatory diseases, such as ankylosing spondylitis, psoriasis, Crohn’s disease and uveitis, have revealed variants at *IL23R*, *IL12A*, *IL12B*, *IL23A*, *STAT3*, and *IL17REL*, thereby highlighting the specific mechanisms by which perturbations in the IL-23/IL-17 axis sets the stage for systemic autoinflammation.31-37 Further, cytokines, receptors and related signaling proteins in the TH17 pathway have been shown to be efficacious targets for therapeutics treating autoinflammatory diseases.38-41 Hence, TH17 activity—as measured by relevant inflammatory cytokines, most notably IL-17A and IL-23—is a key intermediate phenotype suitable for genetic interrogation. TH17 activation is incompletely described and the sustained activity thereof has never been studied genetically. Conversely, absent or hypoactive TH17 response is also a feature in hyper-IgE syndrome42 and autosomal dominant chronic mucocutaneous candidiasis.43

As a first step to dissecting the molecular mechanisms driving susceptibility to TH17-mediated clinical conditions, we conducted the first IL-17A and IL-23 protein-QTL study using samples from a homogenous population from Central Wisconsin. This study is designed to measure baseline cytokine protein levels in a large population and uncover genetic variants and genes correlated with these markers of TH17 activity.

**Materials and Methods**

***Central Wisconsin Population***

Individuals selected for this study were derived from the Personalized Medicine Research Project (PMRP) at the Marshfield Clinic Research Foundation.44 The genetically-homogeneous, founder population in rural Central Wisconsin is the source for PMRP samples. All PMRP participants are adults. This stationary population is largely derived from Bavarian migrants in the late-1800s and carries high utility for genetic mapping efforts through minimization of confounding by population stratification and reduction in allelic heterogeneity. All PMRP individuals have linked longitudinal medical records including ICD9 codes, medical procedures, prescriptions, clinical assessment data, and laboratory test results. Environmental exposures are thought to be relatively uniform across the population and migration rates are low. The PMRP has been effectively used to map disease genes and study clinically relevant traits.45-49

***Selection of Individuals***

Blood samples were collected at the time of PMRP enrollment. Of the PMRP individuals, ~4,500 have been genotyped on the Illumina 660W-Quad array, capturing common genetic variation across the entire genome. All of these genotyped individuals were >50 years old. These data have been subjected to quality control procedures including sample randomization, genotype concordance testing on replicates, PCA analysis for genetic outliers, and Hardy-Weinberg equilibrium testing. Individuals were excluded based on an outlier analysis from the first two principle components derived from the GWAS data. To diminish effects of inflated cytokine expression from transient inflammation stimulated from acute infection, only individuals without evidence of acute infection +/- two weeks of PMRP enrollment were included in the study. Evidence of acute infection consisted of either a temperature reading in excess of 37.7oC, an hs-CRP reading >3.0, or an abnormal white blood cell count of <4.3x109 cells/L or >10.8x109 cells/L. Similarly, a record of immunization within two weeks prior to enrollment was also an exclusion criterion. Lastly, a record of immunosuppressive drug prescription within three months prior to enrollment was also an exclusion criterion. In total, 249 individuals satisfied one or more of these exclusion criteria and therefore removed from the study.

***Experimental Design***

The study was conducted in two phases: a discovery phase with 1,015 subjects and a replication phase with 1,000 subjects. All subjects were genotyped on the Illumina 660W-Quad array. All subjects had plasma concentrations of IL-23 and IL-17A levels determined on the MSD platform. Following the results from Skol at al., a joint analysis was conducted to identify genes associated with IL-23 and IL-17A concentrations.50

***Plasma Collection***

From each PMRP participant, 30mL (3x10mL K3EDTA purple-top tubes) of blood was collected for plasma. Tubes were immediately placed on ice or refrigerated before processing. Within 24 hours, plasma cryovials were stored at -80oC.

***GWAS Assay***

Genomic DNAs from all selected subjects were genotyped on the Illumina Human660W-Quad microarray by the Johns Hopkins University’s Center for Inherited Diseases (CIDR). In excess of 500,000 SNPs were interrogated on this platform.

***Protein Quantification***

Endogenous protein quantification was performed on the Meso-Scale Discovery (MSD) platform. The MSD platform uses preformatted, spatially-arrayed ELISAs in 96-well format. The technology employs electrochemiluminescence detection of proteins, enabling very high sensitivity (lower limits of detection are typically between 0.5 and 10pg/mL), low coefficient of variations (CV), and large dynamic ranges (roughly 3-4 logs). An 8-point standard curve dilution series was plated on each run for back-calculation of protein concentration from optical signal intensities. For another recent study, we observed mean CVs of 1.72% for adiponectin and 2.51% for C-peptide using >600 split samples from PMRP biobank plasmas.46 The MSD platform was used to quantify cytokine proteins levels in plasma from all subjects. For the second phase of the experiment with 1000 individuals, we performed a BSA assay as a method of estimating total protein levels in each sample. These measurements were used to adjust the IL-23 and IL-17A quantifications for total protein levels.

***Statistical Methods***

The optical quantification of IL-23 and IL-17A levels was converted to protein concentration values using the MSD software package. The results were confirmed by fitting power curves to the 8-point standard curve data and adjusting for plate effects. Cytokine concentrations were then transformed to an approximate Gaussian distribution using a quantile method. The resulting values were the endpoint for the statistical genetics tests for the samples analyzed in the first stage of the study. For the second stage analysis, the

**Results**

**Discussion**

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