MHC class II super-enhancer increases surface expression of HLA-DR and HLA-DQ and affects cytokine production in autoimmune vitiligo

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Contributed by Charles A. Dinarello, December 6, 2015 (sent for review November 2, 2015; reviewed by Betty Diamond and Robert D. Nicholls)

Genetic risk for autoimmunity in HLA genes is most often attributed to structural specificity resulting in presentation of self-antigens. Autoimmune vitiligo is strongly associated with the MHC class II region. Here, we fine-map vitiligo MHC class II genetic risk to three SNPs only 47 bp apart, located within a predicted super-enhancer in an intergenic region between *HLA-DRB1* and *HLA-DQA1*, localized by a genome-wide association study of 2,853 Caucasian vitiligo patients. The super-enhancer corresponds to an expression quantitative trait locus for expression of HLA-DR and HLA-DQ RNA; we observed elevated surface expression of HLA-DR ($P = 0.008$) and HLA-DQ ($P = 0.02$) on monocytes from healthy subjects homozygous for the high-risk SNP haplotype. Unexpectedly, pathogen-stimulated peripheral blood mononuclear cells from subjects homozygous for the high-risk super-enhancer haplotype exhibited greater increase in production of IFN-γ and IL-1β than cells from subjects homozygous for the low-risk haplotype. Specifically, production of IFN-γ on stimulation of dectin-1, mannose, and Toll-like receptors with *Candida albicans* and *Staphylococcus epidermidis* was 2.5- and 2.9-fold higher in high-risk subjects than in low-risk subjects, respectively ($P = 0.007$ and $P = 0.01$). Similarly, production of IL-1β was fivefold higher in high-risk subjects than in low-risk subjects ($P = 0.02$). Increased production of immunostimulatory cytokines in subjects carrying the high-risk haplotype may act as an "adjuvant" during the presentation of autoantigens, tying together genetic variation in the MHC with the development of autoimmunity. This study demonstrates that for risk of autoimmune vitiligo, expression level of HLA class II molecules is as or more important than antigen specificity.

inflammation | antigen presentation | autoimmunity | vitiligo | MHC transcription

Autoimmune diseases are a group of over 80 disorders that together affect 3–5% of the United States population (1, 2). Many autoimmune diseases are associated with genetic variation in the *HLA* class I and class II gene regions of the major histocompatibility complex (MHC) on chromosome 6p21.3. HLA class I molecules present peptide antigens on the surface of almost all cells, whereas HLA class II molecules present antigens on the surface of antigen-presenting cells, such as dendritic cells, mononuclear phagocytes, and B cells. Contributions of HLA molecules to autoimmunity have almost exclusively focused on antigenic diversity and specificity. Although polymorphisms in intergenic regions of the MHC might additionally affect the complex transcriptional regulation of *HLA* genes, the potential role of these noncoding regions in the pathogenesis of autoimmune diseases has received much less attention.

Vitiligo is an autoimmune disease in which white spots of skin and overlying hair result from progressive destruction of melanocytes by autoreactive T cells (3). In previous genome-wide association studies of autoimmune vitiligo in European-derived Caucasian (EUR) populations, we have identified association with 27 different loci (4–6), most strongly with MHC class II region SNPs in the vicinity of the *HLA-DRB1* and *HLA-DQA1* genes. Here, we refine genetic mapping of vitiligo risk in the MHC class II region to a haplotype of three SNPs that span just 47 nucleotides between *HLA-DRB1* and *HLA-DQA1*, carried on an *HLA-DR53* haplotype. This high-risk SNP haplotype is within a predicted transcriptional super-enhancer active primarily in immune cells, and is functionally active in increasing the cell surface expression of HLA-DQ and HLA-DR molecules. Carriage of the high-risk SNP haplotype is also associated with increased production of immune-stimulatory cytokines on exposure to pathogens.

These findings indicate that susceptibility to autoimmune vitiligo in the MHC class II region involves a primary quantitative effect of increased levels of surface expression of HLA molecules. In addition, a secondary qualitative effect of antigenic specificity is conferred by coding variation in linkage disequilibrium with the variants that affect transcriptional regulation.

Results

Refined Genetic Mapping of Vitiligo Susceptibility in the MHC Class II Region to an Intergenic Super-Enhancer. We previously reported

Significance

Vitiligo is a classic autoimmune disease genetically associated with SNPs in the MHC class II region. To date, the impact of HLA molecules on autoimmunity has focused on structural diversity of antigen presentation. Here, we describe the properties of a 47-nucleotide high-risk haplotype of three SNPs within an intergenic "super-enhancer" located between the *HLA-DRB1* and *HLA-DQA1* genes, localized by a genome-wide association study of 2,853 subjects with vitiligo. Monocytes from healthy subjects homozygous for the high-risk haplotype have increased surface expression of HLA-DR and -DQ, and peripheral blood mononuclear cells from high-risk subjects produce more IL-1β and IFN-γ upon engagement of dectin-1, mannose, and Toll-like receptors. This study underscores the importance of transcriptional regulation of *HLA* genes to the risk of developing an autoimmune disease.

Author contributions: G.C., R.A.S., and C.A.D. designed research; G.C., M.H., Y.J., D.Y., S.A.S., C.H., M.R., H.E., I.W.T., and C.P.N. performed research; G.C., S.A.S., C.P.N., B.E.P., R.A.S., and C.A.D. analyzed data; and G.C., L.D., B.E.P., R.A.S., and C.A.D. wrote the paper.

Reviewers: B.D., The Feinstein Institute for Medical Research; and R.D.N., Children's Hospital of Pittsburgh.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523482113/-/DCSupplemental.

that vitiligo in EUR patients is associated with SNPs in the MHC class II region, with strongest association to rs532098 located between *HLA-DRB1* and *HLA-DQA1* (4), which are in opposite transcriptional orientations (Fig. 1). To better localize causal variation in the region, we compared genotypes of 2,853 EUR vitiligo cases and 37,412 unaffected subjects, imputed through the extended MHC (7, 8) using data from the 1,000 Genomes Project. In the MHC class II region, the greatest association was with rs9271597 [chr6:32591291; $P = 3.15 \times 10^{-89}$, odds ratio (OR) 1.77]. Logistic regression analysis conditional on rs9271597 identified two additional SNPs whose effects could not be distinguished, rs9271600 and rs9271601 (both $P = 3.21 \times 10^{-89}$, OR 1.77). These three SNPs are located only 47 nucleotides apart (chr6:32591291–32591337) and are in perfect linkage disequilibrium. Thus, a three-SNP haplotype in the MHC class II region confers primary risk of developing vitiligo. This haplotype is located within a striking ENCODE (9) transcriptional element (chr6:32588500–32597000) approximately midway between *HLA-DRB1* and *HLA-DQA1*. As shown in Fig. 1, among the seven principal cell types tested by ENCODE, activity of this element was only observed in GM12878 lymphoblastoid cells. In this B-cell–derived cell line, this region has an open hypomethylated chromatin configuration, multiple DNase I hypersensitivity sites, multiple clusters of RNA polymerase II, several transcription factor binding sites, and a prominent H3K27ac mark. Together, these features are characteristic of an active transcriptional enhancer (10–12).

Analysis of this region using HaploReg v4.1 (13), which integrates Roadmap Epigenomics data from 111 reference human epigenomes (14), ENCODE data (9), and predicts the effect of SNPs on transcription factor binding, showed that the predicted enhancer is active in all T-cell subtypes, monocytes, B cells, and neutrophils, as well as in keratinocytes and mammary epithelial cells, and corresponds to an expression quantitative trait locus that regulates expression of *HLA-DR* and *HLA-DQ* RNA in many different tissues (15). SNP rs9271597 alters predicted binding motifs for transcription factors E2F and Sox3; rs9271600 alters motifs for Cdx2, Dbx1, Foxa, HDAC2, Hoxa5, Lhx3, Mef2, Ncx, Pou1f1, Pou2f2, Pou5f1, Sox19, Sox2, Sox5, and Zfp105; and rs9271601 (which is only 5 bp from rs9271600) alters motifs for Cdx2, Dbx1, Foxa, GR, Hoxa5, Lhx3, Ncx, Sox19, Sox2, Sox5, and Zfp105. Because these three SNPs occur as a haplotype in perfect linkage disequilibrium, these predicted changes of transcription factor binding would occur in concert.

The Vitiligo High-Risk MHC Class II Super-Enhancer Haplotype Is Carried on an HLA-DR53 Subhaplotype. To assess possible antigenic specificity associated with the high-risk MHC class II region haplotype, we carried out next-generation DNA sequencing of the classic *HLA-DRB3/4/5*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1* genes in the MHC class II region in 20 unrelated EUR vitiligo patients who were homozygous for the high-risk T allele of the original associated SNP rs532098, of whom all but one were also homozygous for the high-risk SNP haplotype rs9271597-rs9271600-rs9271601. As shown in Table S1, all 40 chromosomes carried *HLA-DRB3* ($n = 13$) or *HLA-DRB4* ($n = 27$). Furthermore, all chromosomes carrying *HLA-DRB4* specifically carried the *HLA-DRB4*01:01* allele and additionally carried *HLA-DRB1*04* (principally **04:01*; $n = 11$) or *DRB1*07* (all **07:01*; $n = 12$) alleles. This combination represents a subset of the *HLA-DR53* haplotype group; we observed no *HLA-DR53* chromosomes carrying *HLA-DRB1*09* alleles, which are rare in EUR populations. The frequency of *HLA-DRB1*04:01* among the 40 vitiligo case chromosomes carrying the high-risk allele of rs532098 was 27.5%, three-times higher than in the USA/EUR general population ($P = 0$). There was no significant association with *HLA-DQA1* or *HLA-DQB2* alleles.

Surface Expression of HLA-DQ and HLA-DR Is Increased on Peripheral Blood Monocytes from Healthy Subjects Carrying the High-Risk MHC Class II Super-Enhancer Haplotype. We hypothesized that the vitiligo-associated high-risk *HLA-DRB1-DQA1* super-enhancer haplotype results in increased expression of HLA-DR and HLA-DQ in vivo, thereby facilitating an increase in the presentation of vitiligo autoantigens. To test this hypothesis, we first genotyped healthy EUR individuals without known autoimmune disease to identify subjects homozygous for either the vitiligo-associated high-risk or more common low-risk SNP haplotypes. We then measured the surface expression of HLA-DQ and HLA-DR on peripheral blood monocytes from these high-risk and low-risk subjects using flow cytometry (Fig. 2*C*). Because activated monocytes shed the CD14 surface marker, monocytes were identified by gating for $CD3^{-}CD11b^{+}CD14^{+/lo}$ (Fig. 2*A*). We next determined the mean fluorescence intensity (MFI) of HLA-DQ or HLA-DR

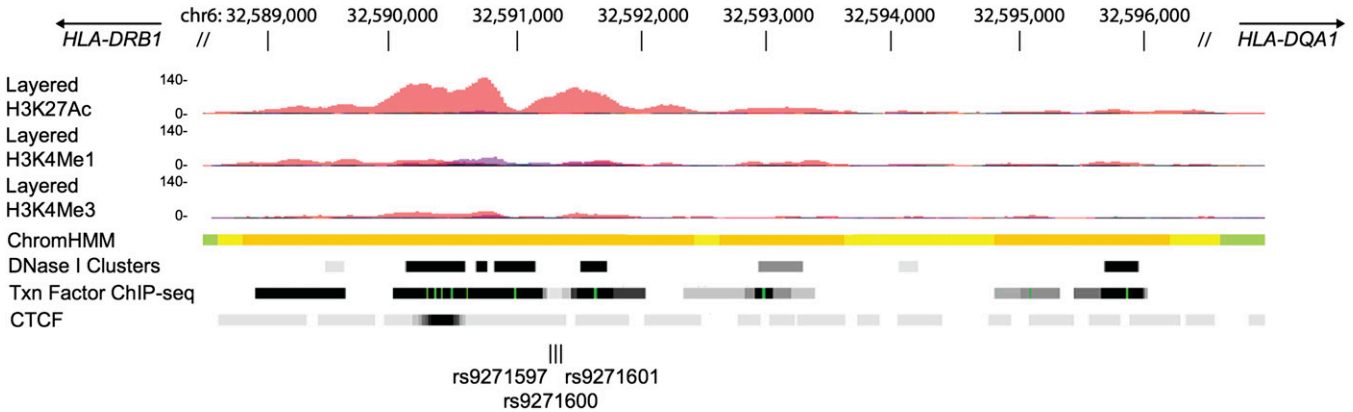

Fig. 1. Vitiligo association in the MHC class II region of human chromosome 6p. Nucleotide positions, *HLA-DRB1* and *HLA-DQA1* genes, transcriptional orientation, and the three SNPs that define the vitiligo high-risk haplotype are shown. Layered H3K27Ac, H3K4Me1, and H3K4Me3 marks, hidden Markov model chromatin state segmentation (ChromHMM), DNase I hypersensitive site cluster (DNase I Clusters), transcription factor chromatin immunoprecipitation sequencing (Txn Factor ChIP-seq), and CTCF ChIP-seq data are from ENCODE (9). For layered H3K27Ac, H3K4Me1, H3K4Me3 marks, data are shown for the seven cell lines studied by ENCODE; red indicates data from GM12878 lymphoblastoid cells. For ChromHMM, data shown are for GM12878; orange indicates strong enhancers, yellow indicates weak/poised enhancers, and green indicates weakly transcribed regions. For DNase clusters, darkness indicates relative signal strength in 125 cell types from ENCODE (V3). For Txn factor ChIP-seq, darkness indicates relative signal strength of aggregate binding of 161 transcription factors and green bars indicate ENCODE Factorbook (42) canonical motifs for specific transcription factors. For CTCF ChIP-seq, data are from GM12878.

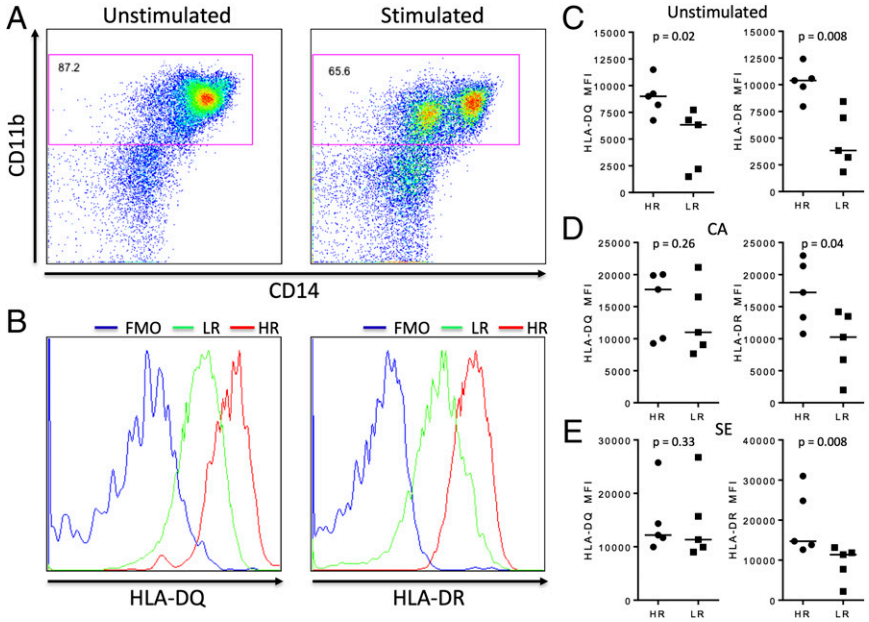

Fig. 2. Expression of HLA-DR and HLA-DQ on blood monocytes of low-risk and high-risk subjects. (*A*) Representative dot plots showing the gating strategy and down-regulation of CD14 of unstimulated compared with *C. albicans*-stimulated monocytes. The frequency of $CD11b^{+}CD14^{+/low}$ monocytes is reported in each dot plot. (*B*) Representative histogram plots of HLA-DQ and HLA-DR expression on unstimulated monocytes from the blood of low risk (LR) and high risk (HR) subjects. Fluorescence minus one (FMO) is shown as a negative staining control. (*C*) Summary of the MFI of unstimulated monocytes from five low-risk and five high-risk subjects. (*D*) Summary of the MFI of *C. albicans*-stimulated monocytes from low-risk and high-risk subjects. (*E*) Summary of the MFI of *S. epidermidis*-stimulated monocytes from low-risk and high-risk subjects. Statistical significance of differences between groups was determined with the unpaired *t* test.

(Fig. 2*B*). As shown in Fig. 2*C*, the MFI of baseline surface expression of HLA-DQ and HLA-DR was significantly higher ($P = 0.02$ and 0.008, respectively) in monocytes from subjects homozygous for the high-risk SNP haplotype compared with subjects homozygous for the low-risk haplotype. These findings demonstrate that the high-risk vitiligo-associated SNP haplotype is associated with increased basal expression of HLA-DQ and HLA-DR.

We next evaluated the surface expression of HLA-DQ and HLA-DR in stimulated peripheral blood monocytes from healthy subjects carrying the high-risk SNP haplotype. Peripheral blood mononuclear cells (PBMCs) from healthy subjects homozygous for the high-risk and low-risk haplotypes were incubated with heat-killed *Candida albicans* or *Staphylococcus epidermidis* for 48 h. These pathogens were chosen based on their ability to induce IFN-γ, which increases expression of MHC class II molecules (16, 17). Surface expression of HLA-DQ and HLA-DR were measured by flow cytometry on the monocyte subpopulation of PBMC, gated for $CD3^{-}CD11b^{+}CD14^{+/lo}$. As shown in Fig. 2 *D* and *E*, compared with unstimulated monocytes the MFI of HLA-DR was markedly increased on pathogen-stimulated monocytes. After stimulation with either *C. albicans* or *S. epidermidis*, cell surface expression of HLA-DR was significantly elevated on monocytes from high-risk compared with low-risk subjects ($P = 0.04$ and $P = 0.008$) (Figs. 2 *D* and *E*). Subjects were 60% males and 40% females, and we observed no significant differences between sexes under either baseline or stimulated conditions.

The High-Risk MHC Class II Super-Enhancer SNP Haplotype Is Associated with Increased Production of Immuno-Stimulatory Cytokines. Presentation of the MHC-peptide complex is associated with the production of cytokines that contribute to immune responses targeting different pathogens. We hypothesized that increased HLA-DR and HLA-DQ expression in subjects carrying the high-risk haplotype might result in overexpression of cytokines, which may act as adjuvants and foster autoimmunity following an infection or environmental stress in susceptible subjects. Therefore, we obtained fresh PBMCs from the healthy subjects homozygous for the high-risk and low-risk SNP haplotypes on three separate occasions spanning a minimum interval of 6 wk. PBMCs were cultured under basal and stimulated conditions for 48 h. We used heat-killed *C. albicans* to activate dectin and mannose receptors, and we used heat-killed *S. epidermidis* to activate Toll-like receptor 2 (TLR2). There were no significant differences in basal production of cytokines from cells of homozygous high-risk versus low-risk subjects (Fig. 3; see legend for median and range values). However, on stimulation with *C. albicans* and *S. epidermidis*, cells from subjects homozygous for the high-risk SNP haplotype exhibited a greater increase in the production of IFN-γ and IL-1β compared with cells from subjects homozygous for the low-risk haplotype. Specifically, production of IFN-γ on stimulation with *C. albicans* and *S. epidermidis* was 2.5- and 2.9-fold higher in high-risk subjects than in low-risk subjects, respectively (Fig. 3*A*) ($P = 0.007$ and $P = 0.01$). Similarly, production of IL-1β was fivefold higher in high-risk subjects than in low-risk subjects (Fig. 3*E*) ($P = 0.02$). Conversely, we observed a smaller increase in the production of TNF-α on stimulation with *C. albicans* or *S. epidermidis* in high-risk subjects than in low-risk subjects (Fig. 3*H*) ($P = 0.03$). As shown in Fig. 3, we observed no significant differences in the production of IL-6, IL-2, IL-10, or IL-18. Of particular interest, the increased production of IL-1β in high-risk individuals occurred without a corresponding increase in its natural inhibitor IL-1Ra (Fig. 3*F*), thus leaving the proinflammatory effects of IL-1β unopposed.

Discussion

In this study, we demonstrate that the strong genetic risk for vitiligo associated with the MHC class II region (4) localizes to a haplotype of just three SNPs, which do not encode coding structural variants in the antigen-binding sites of HLA molecules. Instead, the high-risk

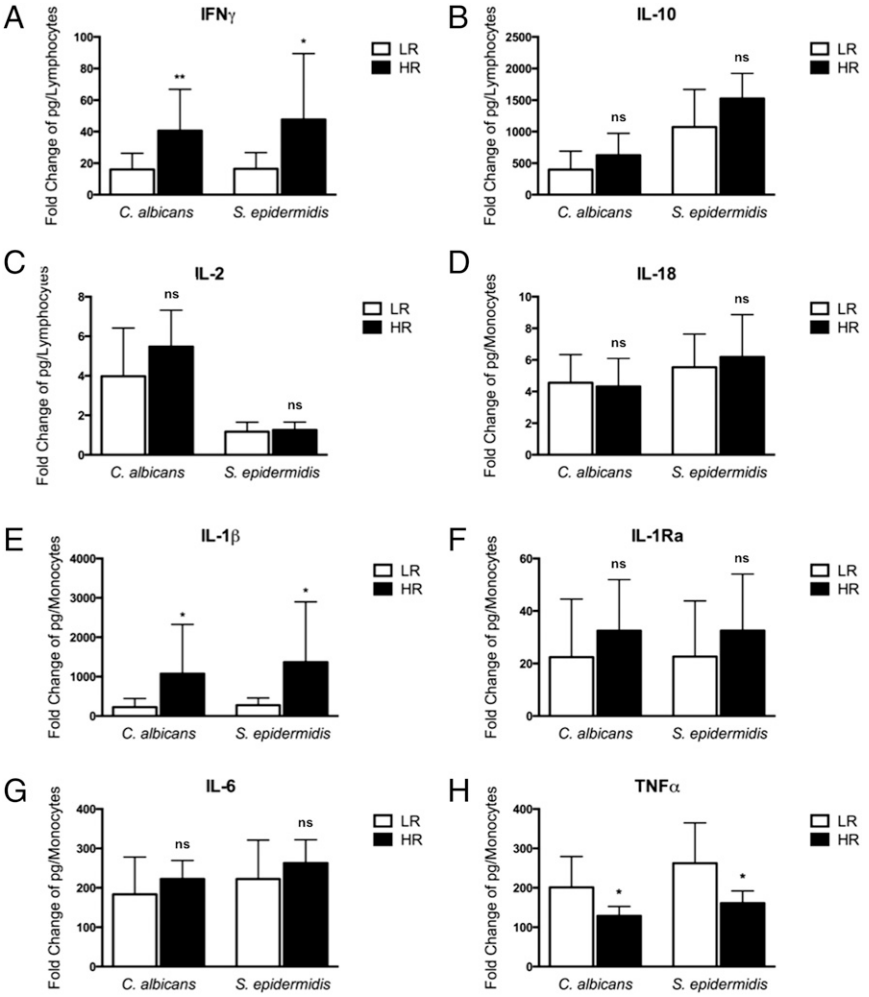

Fig. 3. Production of IFN-γ, IL-10, IL-2, IL-18, IL-1β, IL-1Ra, IL-6, and TNF-α by PBMCs of low-risk and high-risk subjects. Mean ± SEM production of IFN-γ (*A*), IL-10 (*B*), IL-2 (*C*), IL-18 (*D*), IL-1β (*E*), IL-1Ra (*F*), IL-6 (*G*), TNF-α (*H*). Data are expressed as fold-change of baseline cytokine production, which was as follows: IFN-γ: LR median 28 pg/mL, range 15–50; HR median 39; range 15–45; IL-10: LR median 7 pg/mL, range 3–62; HR median 5, range 3–42; IL-2: LR median 10 pg/mL, range 2–17; HR median 8, range 2–20; IL-18: median 21 pg/mL, range 12–97; HR median 31, range 16–184; IL-1β: LR median 23 pg/mL, range 2–56; HR median 16; range 2–77; IL-1Ra: LR median 180 pg/mL, range 14–1016; HR median 232, range 36–829; IL-6: LR median 35 pg/mL, range 9–199; HR median 23, range 14–92; TNF-α: LR median 15 pg/mL, range 7–114; HR median 28, range 13–114. For each determination, data were normalized to the number of lymphocytes or monocytes. Statistical significance of differences between groups was determined with the unpaired *t* test. LR ($n = 12$), HR ($n = 6$); ns, nonsignificant; $^{*}P < 0.05$; $^{**}P < 0.01$.

MHC class II SNP haplotype is entirely intergenic, located between *HLA-DRB1* and *HLA-DQA1*, which are in opposite transcriptional orientations. The haplotype comprises rs9271597, rs9271600, and rs9271601, which are in perfect linkage disequilibrium and span just 47 nucleotides within an ENCODE (9) transcriptional element (chr6:32588500–32597000), which has the characteristics of a super-enhancer (13, 14). This region has been also suggested to harbor a transcriptional insulator (18), and thus the high-risk haplotype might mediate a complex pattern of transcriptional activation or de-repression in permissive cells. Together with correspondence of these SNPs to an expression quantitative trait locus for *HLA-DR* and *HLA-DQ* RNA expression (15), these findings suggested that increased vitiligo risk might result from higher surface levels of HLA-DR or HLA-DQ, possibly resulting in enhanced presentation of self-antigens and associated with increased production of cytokines. The implications of this finding may apply to HLA-associated risk of autoimmunity in general.

These hypotheses were confirmed by analyzing monocytes and PBMCs from healthy subjects homozygous for the high-risk versus low-risk MHC class II SNP haplotypes. We observed that the high-risk haplotype is associated with elevated surface expression of both HLA-DR and HLA-DQ molecules on monocytes, with further increases in the expression of HLA-DR observed on stimulation of dectin, mannose, or TLR2 receptors. HLA class II molecules are essential to generate an immune response to infections, and are highly polymorphic to recognize a great diversity of antigens. Variants promiscuous for antigen presentation have been preserved through evolution and are prevalent in the general population (19); at the same time, some of these alleles are strongly associated with autoimmune diseases. Studies of the mechanistic relationship between HLA polymorphisms and autoimmunity have largely focused on qualitative differences in the polypeptide structure of the peptide-binding groove that mediates presentation of self-antigens to T cells. Thus, genetic localization of the primary determinant of vitiligo risk in the MHC class II region to an apparent intergenic super-enhancer between *HLA-DRB1* and *HLA-DQA1* is truly an unexpected finding, indicating that the number of surface class II molecules is more critical to disease development than structural specificity of a class II molecule. This does not imply no role for

antigenic specificity; the causal SNP haplotype is in strong linkage disequilibrium with the *HLA-DRB4*01:01* allele carried on *HLA-DRB*04:01* and *HLA-DRB1*07:01* haplotypes. Thus, the highest genetic risk of vitiligo in the MHC class II region likely comes from a combination of antigenic specificity and levels of surface expression.

The majority of causal genetic variants underlying complex diseases appear to involve regulatory elements, rather than coding variations (20, 21), and this is particularly the case for autoimmune diseases (22). This paradigm has recently been extended to the MHC: noncoding variation in the *HLA-DPB1* region is associated with enhanced clearance of hepatitis B virus infection (23), and with increased risk of graft-versus-host disease in mismatched hematopoietic cell transplant recipients (24). Similarly, quantitative variation in the expression of *HLA-C* can influence the clinical course of HIV infection and the risk of graft-versus-host disease (25, 26). Our findings expand the importance of quantitative MHC expression to the genetic risk of autoimmune disease, and add a new layer of complexity to traditional considerations of structural specificity of antigen presentation. It is likely that similar phenomena may pertain to a broader range of immunological conditions.

Infectious triggers may precipitate the development of autoimmunity in predisposed individuals. Microbial stimulation of PBMCs from healthy subjects carrying the high-risk haplotype consistently resulted in increased production of IFN-γ and IL-1β, both of which have been implicated in the pathogenesis of several autoimmune diseases, including vitiligo (27–29). Further studies are needed to evaluate whether IL-1 blocking agents, which are used in the treatment of a broad spectrum of conditions characterized by IL-1–mediated inflammation (29), could represent a useful treatment approach to vitiligo. We additionally observed a relative decrease in production of TNF-α in subjects carrying the high-risk MHC class II haplotype. The role of TNF-α in the pathogenesis of vitiligo is less clear (30–33); in the skin of vitiligo patients, TNF-α is produced by perilesional T cells and is involved in the development of cytotoxic T lymphocytes (34). However, treatment with TNF-α blocking agents, such as adalimumab and infliximab, can be paradoxically associated with de novo vitiligo development in patients with other autoimmune conditions (30–33, 35).

Increased MHC class II expression may predispose to the development of autoimmunity through different mechanisms. For example, irrespective of the nature of the antigen, a threshold of MHC-peptide needs to be presented, and a threshold of T-cell receptors needs to be engaged for T-cell activation and proliferation (36, 37). Also of note, increased expression of MHC class II molecules may alter the T-cell receptor repertoire during thymic development, and affect the survival and expansion of mature T cells (38). The altered cytokine secretion profile of high-risk healthy individuals may indeed be a consequence of increased presentation of MHC-peptide complex to T cells in the PBMC culture. Higher levels of IFN-γ may favor the skewing of differentiating T cells toward a Th1 phenotype (17, 39). Increased production of IL-1β in subjects carrying the high-risk haplotype may act as an "adjuvant" during the presentation of autoantigens, tying together genetic variation in the MHC with the development of autoimmunity.

Materials and Methods

Genotype Imputation. We imputed genotypes through the extended MHC (7, 8) for a total 2,853 generalized vitiligo patients of non-Hispanic and non-Latino European ancestry (EUR) from North America and Europe [NCBI Database of Genotypes and Phenotypes (dbGaP) accession phs000224.v2)] who met strict clinical criteria (3), and 37,412 EUR controls not specifically known to have any autoimmune disease or malignant melanoma (dbGaP; phs000092.v1.p1, phs000125.v1.p1, phs000138.v2.p1, phs000142.v1.p1, phs000168.v1.p1, phs000169.v1.p1, phs000206.v3.p2, phs000237.v1.p1, phs000346.v1.p1, and phs000439.v1.p1; phs000203.v1.p1, and phs000289.v2.p1; phs000196.v2.p1, phs000303.v1.p1, phs000304.v1.p1, phs000368.v1.p1, phs000381.v1.p1, phs000387.v1.p1, phs000389.v1.p1, phs000395.v1.p1, phs000408.v1.p1, phs000421.v1.p1, phs000494.v1.p1, and phs000524.v1.p1). Control datasets were matched to vitiligo case datasets based on platforms used for genotyping.

Quality-control filtering of genome-wide genotype data were carried out using PLINK, v1.9 (pngu.mgh.harvard.edu/~purcell/plink/), excluding subjects with SNP call rate < 98%, sex discordance, duplication, or cryptic relatedness (π-hat > 0.0625). SNPs were excluded based on genotype missing rate ≥ 2%, observed minor allele frequency < 0.01, or significant ($P < 10^{-4}$) deviation from Hardy–Weinberg equilibrium. Genotype imputation was carried out using IMPUTE2 (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html), implemented on the Janus supercomputer (https://www.rc.colorado.edu/resources/compute/janus). The 1,000 Genomes Project phase I integrated variant set v3 (www.1000genomes.org/) was used as reference panel. Only genotypes with imputation INFO > 0.5 were retained, which were combined with prior SNP genotype data.

Statistical Genetic Analyses. We carried out genetic ancestry matching of patients and controls using GemTools (wpicr.wpic.pitt.edu/WPICCompgen/GemTools/GemTools.htm), and performed a Cochran-Mantel-Haenszel (CMH) analysis to test for association. To determine which variants represent the strongest association signal in the MHC class II region, we then applied logistic regression analysis, comparing the fit of a model containing each variant tested and the most significant variant in the region (rs9271597) to a model containing only rs9271597, assuming a multiplicative genotypic effect for the high-risk allele of each variant. We consider rs9271597 and all variants whose effects could not be distinguished from rs9271597 as representing the strongest association signal in the region. Analyses were performed using PLINK v1.9.

HLA Class II Gene DNA Sequencing. We performed amplicon sequencing of exons 2 and 3 of *HLA-DQB1* and exon 2 of *HLA-DRB3/4/5*, *HLA-DRB1*, and *HLA-DQA1* in 20 unrelated EUR vitiligo patients, on the 454 Life Science GS FLX, as previously described (40, 41), except that the GS GType HLA HR primers (Roche Applied Science) were used. The manufacturer's protocol for GS Titanium Sequencing was followed, except that 0.5 copies of DNA per bead were used and 70% of the recommended amount of DNA beads was loaded on the PicoTiter PlateT. *HLA* genotypes were assigned using Conexio Assign ATF 454 software v34 (Conexio Genomics), customized to include data from intronic sequences and International Immunogenetics Information System (www.imgt.org).

Prediction of SNP Effects on Transcription Factor Binding Motifs. To predict the effect of the high-risk versus low-risk rs9271597-rs9271600-rs9271601 haplotypes on binding of transcription factor motifs in the region, we analyzed the alternative genotypes at each SNP using HaploReg v4.1 (13). (www.broadinstitute.org/mammals/haploreg/haploreg.php). Settings were $r^2 = 1.0$ to specify the haplotypes, the population was EUR, mammalian conservation used both GERP and SiPhy-omega, base data included both RefSeq and GENCODE genes, and the sources for epigenomes included ChromHMM (core 15-state model and 25-state model using 12 imputed marks), H3K4me1/H3K4me3 peaks, and H3K26ac/H3K9ac peaks.

Selection of Control Subjects for Functional Analyses. To identify subjects for functional studies, we genotyped 85 unrelated, healthy EUR subjects with no known autoimmune diseases for SNPs rs9271597 and rs9271601, which tag the high-risk haplotype. We identified 31 subjects who were homozygous for the low-risk haplotype and 9 homozygous for the high-risk haplotype. This project was approved by the Colorado Multiple Institutional Review Board (COMIRB), and written informed consent was obtained from all subjects.

Immunofluorescence Staining and Flow Cytometry. Healthy individuals homozygous for either the rs9271597-rs9271601 high-risk or low-risk haplotypes were identified, peripheral venous blood was collected into heparinized tubes, and PBMC were prepared by differential centrifugation of blood over Ficoll-Paque (Sigma-Aldrich). PBMC were suspended in RPMI 1640 containing penicillin-streptomycin (Cellgro) and 10% heat-inactivated FBS (HyClone) at a concentration of 5×10^6 cells per milliliter; 500 μL of PBMC suspension was added to 24-well flat-bottom polystyrene plates and incubated at 37 °C in a CO_2 enriched environment. Cells were either left unstimulated or were stimulated with heat-killed *C. albicans* or *S. epidermidis* (1×10^6 micro-organisms in 1 mL total volume). After 48 h, the supernatants were collected and frozen. Adherent cells were treated with ice-cold EDTA for 30 min, detached, and combined with the suspension cells. PBMCs were washed, incubated with FcR-blocking reagent (Miltenyi Biotec), and were surfaced-stained with anti-CD3

(Qdot605, Biolegend), anti-CD14 (EF450, eBiosciences), anti-CD11b (PerCP-Cy5.5, Biolegend), anti-CD11c (APC-Cy7, Biolegend), anti-HLA-DQ (FITC, eBiosciences), and anti-HLA-DR (PE-Cy5, Biolegend) mAbs for 30 min at 4 °C. Cells were washed, fixed, resuspended in 2% formaldehyde, and analyzed using a LSRII flow cytometer (BD Immunocytometry Systems).

Cytokine Studies. Freshly isolated PBMCs were resuspended at a concentration of 5×10^6 cells/mL of RPMI 1640 plus 10% heat-inactivated FBS and differential cell counts were performed. Cells were then transferred to 24-well plates (2.5×10^6 cells per well) and incubated for 48 h at 37 °C in a humidified CO_2-enriched environment. Cells were cultured without the addition of stimulants (baseline) or stimulated with heat-killed *C. albicans* or *S. epidermidis* (1×10^6 micro-organisms per milliliter). Cytokine concentrations were determined in the supernatants by specific ELISA (BioTechne). PBMCs from each subject were cultured on three separate occasions spanning a minimum interval of 6 wk. Because specific cytokines are produced by distinct cell subtypes in the PBMC population (i.e., IL-1β is a monocyte product), and because differential cell counts may vary considerably among donors, picogram levels of each cytokine were normalized by the number of cells responsible for the production. Data were expressed as fold-change of baseline, unstimulated production. Group measures are expressed as the mean ± SEM. Statistical significance was assessed using the unpaired Student *t* test (GraphPad Prism 6.0).

ACKNOWLEDGMENTS. This work was funded in part by Grants R01AR045584, R01AR056292, and R01AI15614 from the National Institutes of Health; National Institutes of Health Grant T32 AI007405 (to C.P.N.); and the Interleukin Foundation and Instituto di Ricovero e Cura a Carattere Scientifico San Raffaele Scientific Institute (G.C.). The Janus supercomputer is supported by the National Science Foundation (CNS-0821794), the University of Colorado Boulder, the University of Colorado Denver, and the National Center for Atmospheric Research, and is operated by the University of Colorado Boulder.

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