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Cutting Edge: Chronic NF-κB Activation in CD4+ T Cells in Rheumatoid Arthritis Is Genetically Determined by HLA Risk Alleles

Charles F. Spurlock, III,* John T. Tossberg,* Nancy J. Olsen,† and Thomas M. Aune*‡

Of identified genetic variants, HLA polymorphisms confer the greatest risk for developing autoimmune diseases, including rheumatoid arthritis (HLA-DRB1*04). There are strong influences of HLA polymorphisms on cell type-specific gene expression in B cells and monocytes. Their influence on gene expression in CD4+ T cells is not known. We determined transcript and proteins levels of target genes in lymphocyte/monocyte subsets in healthy controls and rheumatoid arthritis subjects as a function of HLA-DRB1*04 haplotype. We identified gene expression dependent on HLA-DRB1*04 genotype in CD4+ T cells. NF-κB activity in CD4+ T cells was also dependent on HLA-DRB1*04 genotype, and blocking HLA-DR inhibited NF-κB activity in CD4+ T cells and normalized gene expression, as did pharmacologic inhibition of NF-κB. We conclude that interactions between TCR and HLA haplotype. If so, underlying signaling mechanisms conferring these traits are also unknown and are the subject of the present study.

Materials and Methods

Subject recruitment

PBMCs were analyzed using a three-laser BD LSR II flow cytometer and FlowJo software (Tree Star) as described (9). Primary Abs were anti-Apc1 (Novus Biologicals, NBP1-77375), anti-phospho–NF-κB p65 (Ser536, P-p65) (Cell Signaling Technology, 9718), and anti-phospho–NF-κB p65 (Ser536, P-p65) (Cell Signaling Technology, 3033). Secondary Abs were FITC goat anti-mouse Ig and FITC goat anti-rabbit Ig (BD Biosciences). Cell surface markers (BD Biosciences) were Pacific Blue mouse anti-human CD4, Alexa Fluor 700 mouse anti-human CD8, allophycocyanin mouse anti-human CD19, and PE mouse anti-human CD14. Mouse anti-human HLA-DR was from BD Pharmingen (G46-6). Cyclosporin A (30024), Bay 11-7085 (B5681), and BI-78D3 (3063) were from Sigma-Aldrich.

Genotyping

Genomic DNA was isolated from whole blood as described (10). TaqMan single nucleotide polymorphism (SNP) assays were used for genotyping (see Ref (*1)).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CTRL, healthy control; eQTL, expression quantitative trait loci; PGK, phosphoglycerate kinase; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism.

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Genome-wide genetic association studies identify polymorphisms within the HLA locus as major risk factors for developing autoimmune diseases. Given the known function of HLA proteins to present peptide Ags to T cells, it is assumed that these associations are explained by differences in presentation of self-antigen to T cells by polymorphic HLA proteins (1). The HLA-DRB1*04 allele is a major risk factor for developing rheumatoid arthritis (RA) (2). Variations in three amino acids contained within the peptide-binding groove almost completely explain RA disease risk conferred by the HLA-DRB1 haplotype (3). One interpretation is that these amino acids directly impact the HLA-DRB peptide-binding groove and define repertoires of foreign- and self-antigens presented to CD4+ T lymphocytes. The molecular basis for this association may be preferred binding of MHC class II molecules encoded by HLA-DRB1*04 to citrullinated rather than arginine-containing peptides (4). As such, citrullinated neoantigens may represent self-antigens.

In the absence of RA, HLA-DRB1*04 risk alleles are associated with accelerated telomere erosion in CD4+ T cells and other signs of immunosenescence such as increased numbers of CD4+, CD28− T cells (5). Immunosenescence also promotes inflammation (6). HLA haplotypes, especially disease-associated haplotypes, are associated with variations in gene expression patterns in B cells and monocytes, which are termed expression quantitative trait loci (eQTL) (7, 8). It is not known whether CD4+ T cells that recognize Ag in the context of HLA-DRB1*04 also exhibit eQTL conferred by HLA haplotype. If so, underlying signaling mechanisms conferring these traits are also unknown and are the subject of the present study.

Materials and Methods

Subject recruitment

Blood samples were obtained from control (CTRL) subjects without medical conditions or family history of autoimmune disease and subjects meeting the American College of Rheumatology/European League against Rheumatism criteria for RA from three U.S. sites (Tennessee, Texas, Pennsylvania) (Supplemental Table I). Institutional Review Board approval was obtained from all sites.

Flow cytometry, Abs, and reagents

PBMCs were analyzed using a three-laser BD LSR II flow cytometer and FlowJo software (Tree Star) as described (9). Primary Abs were anti-Apc1 (Novus Biologicals, NBP1-77375), anti-phosphoglycerate kinase (PGK)-1 (Abcam, ab67355), anti–γH2AX (Cell Signaling Technology, 9718), and anti-phospho–NF-κB p65 (Ser536, P-p65) (Cell Signaling Technology, 3033). Secondary Abs were FITC goat anti-mouse Ig and FITC goat anti-rabbit Ig (BD Biosciences). Cell surface markers (BD Biosciences) were Pacific Blue mouse anti-human CD4, Alexa Fluor 700 mouse anti-human CD8, allophycocyanin mouse anti-human CD19, and PE mouse anti-human CD14. Mouse anti-human HLA-DR was from BD Pharmingen (G46-6). Cyclosporin A (30024), Bay 11-7085 (B5681), and BI-78D3 (3063) were from Sigma-Aldrich.

Genotyping

Genomic DNA was isolated from whole blood as described (10). TaqMan single nucleotide polymorphism (SNP) assays were used for genotyping (see Ref (*1)).
RNA isolation and quantitative RT-PCR

Total RNA was purified as described (11). cDNA was synthesized from total RNA using oligo(dT) primers (SuperScript III first-strand synthesis kit, Life Technologies) and purified using Qagen PCR purification kits. PCR reactions were in 25 μl with 50 ng cDNA, TaqMan master mix, and TaqMan gene expression assays (Applied Biosystems). Expression levels were calculated using the ΔΔCt method by comparing target gene transcripts to GAPDH transcripts.

Statistical analysis

Statistical analysis was performed using a Student t test with a Welch correction. A p value of <0.05 after correction for multiple testing was considered significant.

Results

CD4+ T cell eQTL defined by RA-associated HLA-DRB1*04 SNP markers

The goal of our studies was to identify eQTL associated with RA HLA-DRB1*04 risk alleles, determine whether eQTL were confined to CD4+ T cells, and determine how a given HLA-DRB1 protein may confer alterations in CD4+ T cell–specific eQTL. Based on results from previous microarray analyses we performed, we designed a TaqMan low-density array to ascertain expression levels of 45 target genes and 3 housekeeping genes with the goal of developing tools to aid in the diagnosis of autoimmune diseases, for example, RA, inflammatory bowel diseases, and multiple sclerosis (12–15). To this end, we analyzed >1500 CTRLs, subjects with different autoimmune diseases, and subjects with chronic noninflammatory diseases (disease controls). It also seemed that we could use these data to identify HLA-DRB1*04-specific eQTL localized to CD4+ T cells.

To initiate our studies we used SNP rs6457620 (chr6:32,771,829, hg18 build) known to tag the RA HLA-DRB1*04 risk allele (2) to genotype CTRL and RA subjects for which we had expression data. This polymorphism is either C or G. In our cohorts, frequencies in white CTRL subjects were ∼25% C/C, 35% C/G, and 40% G/G and ∼40% C/C, 60% C/G, and < 1% G/G in white RA subjects (Supplemental Table I). Presence of the C nucleotide confers RA risk. From these data, we determined whether gene expression levels were associated with HLA-DRB1*04 genotype in CTRL and RA subjects (Supplemental Table II). We identified three categories, which included those genes differentially expressed in CTRL subjects with C/C genotypes compared with C/G and G/G genotypes and in RA with either C/C or C/G genotypes (Fig. 1A, Supplemental Table II), those that differed in CTRL subjects with C/C or C/G genotypes compared with G/G genotypes (Fig. 1B, Supplemental Table II), and those that were independent of genotype but were different between CTRL and RA (Fig. 1C). The first group consisted of ACTRIA, ANAPC1, B2M, EPHX2, GATA3, PGKI, and RANGAP1, the second group consisted of CDKN1A, and the third group consisted of CDKN1B, CSFR, GNBS, JUN, KRAS, and LLGL2. We interpreted these results to demonstrate that expression levels of the first and second groups of genes were determined by presence of HLA-DRB1*04-associated SNP genotype in the absence of RA whereas expression of the third group was determined by the presence of RA rather than genotype.

Because of the high linkage disequilibrium within the HLA genomic region, including the HLA-DRB1 locus, a number of SNPs within this region serve as surrogate tags for the HLA-DRB1*04 RA risk allele (2). Therefore, we used additional SNPs for genotyping that also tag the HLA-DRB1*04 risk allele and performed the same expression analysis. SNPs in-
Inhibition of AP-1 reduced expression of levels equivalent to those in subjects with G/G genotypes. In contrast, inhibition of NFAT or AP-1 did not increase normally repressed in C/C relative to G/G genotypes (Fig. 3).

Expression differences between subjects with G/G and C/C genotypes were reversed or “corrected” in subjects with C/C genotypes by inhibition of these transcription factors. We employed Bay 11-7085, an irreversible inhibitor of IκB, to inhibit NF-κB, cyclosporine A to inhibit NFAT, and the JNK inhibitor BI-78D3 to inhibit AP-1. Inhibition of NF-κB increased levels of ANAPC1, which is normally repressed in C/C relative to G/G genotypes (Fig. 3). In contrast, inhibition of NFAT or AP-1 did not increase levels of ANAPC1. Inhibition of NF-κB in C/C genotypes also reduced transcript levels of PGK1, B2M, and NRAS to levels equivalent to those in subjects with G/G genotypes. Inhibition of NFAT did not alter these gene transcript levels. Inhibition of AP-1 reduced expression of PGK1 to a similar degree as inhibition of NF-κB. One interpretation of these results is that these gene transcript levels are determined, at least in part, by activity of the proinflammatory transcription factor NF-κB and perhaps to a lesser degree by AP-1.

The above results suggest that basal NF-κB activity may be higher in CD4+ T cells from subjects with C/C versus C/G or G/G genotypes. To test this hypothesis we employed flow cytometry to measure NF-κB activity (P-p65) in PBMC subsets from CTRL subjects with C/C, C/G, or G/G genotypes. We found that CTRL subjects with C/C genotypes expressed elevated NF-κB activity in CD4+ T cells compared with CTRL subjects with G/G genotypes (Fig. 4A). NF-κB activity in subjects with RA is shown for reference. P-p65 activity was not increased in CD8+, CD19+, and CD14+ cells in subjects with C/C compared with G/G genotypes (Fig. 4B). We interpret these results to indicate that the type of polymorphic MHC class II molecule expressed by APC contributes to basal NF-κB activity in CD4+ T cells.

To further test this hypothesis, we employed a specific anti–HLA-DR blocking Ab to prevent interactions between HLA-DR and the TCR on CD4+ T cells. PBMC from CTRL subjects with C/C genotypes were treated with either an isotype control Ab or an HLA-DR blocking Ab overnight and intracellular levels of active P-p65 were measured by flow cytometry the next day. Ab treatment reduced detection of HLA-DR protein by >75% as measured by surface staining with a second conjugated Ab (Fig. 5A) and inhibited NF-κB activity in CD4+ T cells from subjects with C/C genotypes by >50% (Fig. 5B). Finally, we asked whether the genetically determined gene expression signature identified above was altered by treatment with anti–HLA-DR. We found that treatment with anti–HLA-DR increased expression of ANAPC1 and decreased expression of B2M, NRAS, and PGK1 (Fig. 5C). We conclude that presence of the C/C genotypes would result in an increase in the expression of ANAPC1 and a decrease in the expression of B2M, NRAS, and PGK1.

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notype increases indices of inflammation, in part, by elevating NF-κB activity.

Discussion

We show that carrying HLA-DRB1*04 risk alleles as measured using surrogate SNP tags regulate gene expression in CD4+ T cells in healthy individuals. Although HLA polymorphisms are known to regulate gene expression and additional phenotypes, such as premature telomere shortening, the underlying mechanisms are not understood (7, 17). Our results demonstrate that the HLA-DRB1*04 risk allele increases activity of a key proinflammatory, prosurvival transcription factor, NF-κB, specifically in CD4+ T cells and this increase is dependent on HLA-DR. We propose that increased NF-κB activity conferred by the HLA-DRB1*04 genotype contributes to RA disease risk.

Major signaling paths activated following productive engagement of the TCR by Ag presented by MHC class II molecules culminate in stimulation of the transcription factors NF-κB, specifically in CD4+ T cells and this increase is dependent on HLA-DR. We propose that increased NF-κB activity conferred by the HLA-DRB1*04 genotype contributes to RA disease risk.

Genetically determined elevated levels of NF-κB activity may also contribute to hyporesponsiveness. We propose a model whereby continuous sampling of peptide–MHC class II complexes by TCR–CD3 complexes on CD4+ T cells creates an intracellular signaling hum that determines basal activities of these three transcription factors. Polymorphisms in HLA alleles have the capacity to alter these baseline activities that in turn alter gene expression programs. These alterations produce cellular liabilities such as autoreactivity or immunosenescence that increase risk for developing specific autoimmune diseases (Fig. 5D). NF-κB, besides being a pro-survival and proinflammatory transcription factor, is also an important driver of immunosenescence and the consequent proinflammatory state (6). Preferential binding of peptide motifs with citrulline rather than arginine in the HLA-DRB1*04 peptide groove may produce altered baseline TCR signaling, resulting in increased NF-κB activity. Alternatively, amino acid variants in HLA-DRB1*04 MHC class II may alter binding to the TCR complex to increase NF-κB activity. Additional studies will be required to distinguish among these or other possibilities.

Disclosures

The authors have no financial conflicts of interest.
References


**Supplementary Table 1.** Demographic and clinical characteristics of CTRL and RA cohorts

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=82)</th>
<th>RA (n=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>34 ± 9</td>
<td>46 ± 11</td>
</tr>
<tr>
<td>Genotype (%)</td>
<td></td>
<td></td>
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<tr>
<td>C/C</td>
<td>24</td>
<td>40</td>
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<tr>
<td>C/G</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>G/G</td>
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<td>0</td>
</tr>
<tr>
<td>Female (%)</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Caucasian</strong></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Characteristics of RA patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Duration, mean ± SD years</td>
<td>10 ± 9</td>
<td></td>
</tr>
<tr>
<td>Active Disease* (%)</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Early RA (disease duration &lt;1 year)(%)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Mean DAS** score</td>
<td>4.9 ± .47</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCQ (%)</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>TNF inhibitors (%)</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

* Defined as the presence of at least 3 of the following: morning stiffness > 45 minutes, > 3 swollen joints, > 6 tender joints, and erythrocyte sedimentation rate > 28 mm / hour.

** DAS = disease activity score