Altered Repertoire Diversity and Disease-Associated Clonal Expansions Revealed by T Cell Receptor Immunosequencing in Ankylosing Spondylitis Patients

Aimee L. Hanson, Hendrik J. Nel, Linda Bradbury, Julie Phipps, Ranjeny Thomas, Kim-Anh Lê Cao, Tony J. Kenna, and Matthew A. Brown

Objective. Ankylosing spondylitis (AS) is a common spondyloarthropathy primarily affecting the axial skeleton and strongly associated with HLA–B*27 carriage. Genetic evidence implicates both autoinflammatory processes and autoimmunity against an HLA–B*27–restricted autoantigen in immunopathology. In addition to articular symptoms, up to 70% of AS patients present with concurrent bowel inflammation, suggesting that adverse interactions between a genetically primed host immune system and the gut microbiome contribute to the disease. Accordingly, this study aimed to characterize adaptive immune responses to antigenic stimuli in AS.

Methods. The peripheral CD4 and CD8 T cell receptor (TCR) repertoire was profiled in AS patients (n = 47) and HLA–B*27–matched healthy controls (n = 38). Repertoire diversity was estimated using the Normalized Shannon Diversity Entropy (NSDE) index, and univariate and multivariate statistical analyses were performed to characterize AS-associated clonal signatures. Furthermore, T cell proliferation and cytokine production in response to immunogenic antigen exposure were investigated in vitro in peripheral blood mononuclear cells from AS patients (n = 19) and HLA–B*27–matched healthy controls (n = 14).

Results. Based on the NSDE measure of sample diversity across CD4 and CD8 T cell repertoires, AS patients showed increased TCR diversity compared to healthy controls (for CD4 T cells, P = 7.8 × 10−6; for CD8 T cells, P = 9.3 × 10−4), which was attributed to a significant reduction in the magnitude of peripheral T cell expansions globally. Upon in vitro stimulation, fewer T cells from AS patients than from healthy controls expressed interferon-γ (for CD8 T cells, P = 0.03) and tumor necrosis factor (for CD4 T cells, P = 0.01; for CD8 T cells, P = 0.002). In addition, the CD8 TCR signature was altered in HLA–B*27+ AS patients compared to healthy controls, with significantly expanded Epstein-Barr virus–specific clonotypes (P = 0.03) and cytomegalovirus–specific clonotypes (P = 0.02). HLA–B*27+ AS patients also showed an increased incidence of “public” CD8 TCRs, representing identical clonotypes emerging in response to common antigen encounters, including homologous clonotypes matching those previously isolated from individuals with bacterial-induced reactive arthritis.

Conclusion. The dynamics of peripheral T cell responses in AS patients are altered, suggesting that differential antigen exposure and disrupted adaptive immunity are underlying features of the disease.

INTRODUCTION

Ankylosing spondylitis (AS) is a seronegative immune-mediated arthritis particularly affecting the axial skeleton, in which enthesitis leads to joint erosion and reactive bone formation. Despite its high prevalence (1) and an extensively studied genetic architecture (2–6), the cause of AS remains incompletely understood. Carriage of the class I HLA allele HLA–B*27 is observed in >80% of cases (7,8), contributing ~20% to disease heritability. Up to 70% of AS patients manifest subclinical bowel inflammation, 2

Matthew A. Brown, MBBS, MD, FRACP, FAHMS, FAA: Queensland University of Technology and Translational Research Institute, Brisbane, Queensland, Australia, and Guy’s and St Thomas’ NHS Foundation Trust and King’s College London NIHR Biomedical Research Centre, King’s College London, UK.

Drs. Kenna and Brown contributed equally to this work.

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Address correspondence to Matthew Brown, MBBS, MD, FRACP, FAHMS, FAA, Guy’s and St Thomas’ NHS Foundation Trust and King’s College London NIHR Biomedical Research Centre, 16th Floor Tower Wing, Guy’s Hospital, Great Maze Pond, London SE1 9RT, UK. Email: matt.brown@kcl.ac.uk.

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and 5–10% have concomitant inflammatory bowel disease (IBD) (9,10). In addition, evidence of bacterial invasion of the intestinal epithelium (11) and the demonstration that the AS gut microbiome is disturbed, leading to increased carriage of peptide pools presentable by HLA–B*27 (12), suggests that exposure to gut-derived bacterial components is important in driving the pathogenesis of AS.

The immunologic processes contributing to inflammation in AS are unclear. Studies have indicated that the disease has genetic associations with the TLR4 (13) and MEFV genes (14), among others, thereby suggesting that autoinflammation is involved, and other evidence has suggested that AS is an autoimmune disease driven by the recognition of an HLA–B*27–restricted arthritogenic peptide by CD8 T cells (15–17). Disease-associated polymorphisms in the endoplasmic reticulum aminopeptidase genes ERAP1 and ERAP2, which are genes of the antigen-processing pathway, support an autoimmune mechanism (2,4). AS-associated variants in these genes alter trimming of the pHLA affinities (22,23). Despite fractional overlap in the TCR encoding the heterodimeric TCRαβ (21). Nontemplated insertion/deletion of nucleotides at the V(D)J junction (termed the third CDR3 rearrangements (out-of-frame) T cell rearrangements were excluded. All results with the exception of 1, were residing in Australia at the time of the study. Blood samples were obtained from subjects in Brisbane, and the samples were processed in our laboratory within 6 hours of collection. PBMCs were extracted from the blood in a manner as previously described (4) and stored until analyzed.

HLA typing. Subjects were genotyped using an Illumina Immunochip or CoreExome version 24 SNP microarray. Class I and class II HLA types were imputed using HLA*IMP:03 (27), with HLA–B*27 typing confirmed by HLA–B*27–specific polymerase chain reaction (28).

Antibody staining and cell sorting. In samples from 47 AS patients (of whom 37 were HLA–B*27+) and from 38 healthy controls (of whom 20 were HLA–B*27+), ~1 x 10^7 PBMCs were stained with 1 μl phycoerythrin–CF594–conjugated anti-CD3 (OKT3), 1 μl allophycocyanin (APC)–Cy7–conjugated anti-CD8 (HI/8a), and 5 μl APC-conjugated anti-CD4 (RPA-T4) antibodies (all from BioLegend). CD3+CD4+ and CD3+CD8+ T cells were isolated by fluorescence-activated cell sorting on a MoFlo Astrios (Beckman Coulter).

Library preparation and immunosequencing. TCR DNA libraries were prepared for sequencing in accordance with the hsTCRB kit protocol (Adaptive Biotechnologies), at the "Survey" level of resolution (~30,000 T cell genomes/reaction, run in duplicate) and sequenced on an Illumina MiSeq (using the version 3 reagent kit, 150 cycles) or NextSeq (using the mid-output reagent kit, 300 cycles). Further details are available in the Supplementary Methods (on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

Data handling. Raw sequence reads were transferred to Adaptive Biotechnologies for custom demultiplexing, V–D–J annotation, and quantification of TCR β-chain CDR3 rearrangements at nucleotide and amino acid resolutions. Nonproductive (out-of-frame) T cell rearrangements were excluded. All results were generated using productive TCRβ CDR3 rearrangements only. Immunosequencing data are available from the corresponding author upon request.

T cell proliferation assay and antigen-presenting cell phenotyping. Two million PBMCs from a secondary cohort of 19 HLA–A*02+ AS patients and 14 HLA–A*02+ healthy controls were labeled using a CellTrace Violet cell proliferation kit and cultured in complete medium, in which the cells were either left unstimulated or cultured in the presence of 10 μg/ml cytomegalovirus (CMV)–p65 HLA–A*02–specific peptide (NLVPMATV), 10 Lf/ml tetanus toxoid (AVaccines), or 0.1 μg/ml soluble anti-CD3 (UCHT1; BioLegend). After 7 days, PBMCs were restimulated, and cell proliferation and intracellular cytokine production from CD4 and CD8 T cells were measured (as described in Supple-
mentary Methods, Supplementary Table 1, and Supplementary Figure 1, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

To assess dendritic cell (DC) and monocyte subset frequencies and constitutive or inducible levels of costimulatory receptor expression, ~2 × 10^6 PBMCs from the same individuals were stained immediately or at 20 hours after stimulation with a Toll-like receptor (TLR) ligand cocktail, for flow cytometry analysis (see Supplementary Methods, Supplementary Table 2, and Supplementary Figure 2 at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

Statistical analysis. CD4 and CD8 immunosequencing data were analyzed independently using custom scripts in R, version 3.4.4 (29). TCR repertoire diversity was quantified using the Normalized Shannon Diversity Entropy (NSDE) index to correct for differences in profiling depth across samples (see Supplementary Figure 3 at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract), and disease-associated changes in the NSDE score were assessed using a generalized linear model (GLM) with adjustment for study covariates (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract]). For each individual, the percentage of all profiled T cells contained in the top 100 (most expanded) clonotypes was calculated by summing total TCR rearrangements within the top 100 unique nucleotide clones and dividing by the total number of productive rearrangements. Group comparisons were conducted using a GLM with beta distribution, adjusting for study covariates (as described in Supplementary Methods).

To identify putative CMV epitope–restricted clones as well as Epstein-Barr virus (EBV) epitope–restricted clones, the command line tool “VDJmatch” was used to query a local download of the VDJdb database (30). CD8 clones sharing 100% TCRβ CDR3 amino acid sequence identity with a published clonotype that was specific for a CMV or EBV epitope, and for which the HLA restriction of the published clonotype matched an HLA class I allele carried by the individual (detected at 2-digit resolution), were retained. The percentage of the total CD8 T cell repertoire contained in putative CMV and EBV epitope–responsive clones was compared between groups by Wilcoxon’s rank sum test.

Sparse partial least squares discriminant analysis (sPLS-DA) of the V-gene family, V-J gene, and CDR3 clone counts was conducted on the total sum scale (TSS)–normalized, central log ratio (CLR)–transformed feature count matrix, using the splsda function from the R package mixOmics (31) (see Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract]). Univariate comparison of the frequencies of the V-gene family and V-J gene pairs between AS patients and healthy controls was conducted using a Wilcoxon’s rank sum test applied to TSS-normalized, CLR-transformed count data. The Benjamini and Hochberg method was used to adjust the P values from comparisons of the V-gene family frequencies, with an adjusted P value of less than 0.05 considered significant. A significance threshold of P < 0.01 was set for comparisons of V-J gene frequencies (false discovery rate [FDR] ~0.36 in CD4 T cells, and FDR ~0.42 in CD8 T cells) (FDRs were calculated as described in Supplementary Methods). In testing the differential incidence of CDR3 amino acid clones between HLA-B*27+ AS patients and healthy controls, TCRs were assigned a value of 0 if they were absent or a value of 1 if they were present at ≥1 copy within each repertoire. Clone incidence between groups was compared using Fisher’s exact test, which was applied to the contingency table of present/absent counts for those TCRs observed in ≥4 individuals, with significance set at P < 0.01.

Statistical comparison of the frequencies of monocytes, DCs, and divided T cell subpopulations between AS patients and healthy controls was conducted using a linear model, with correction for age and sex. The cell division index (CDI) was calculated as previously described (32).

RESULTS

Study cohort and sequencing metrics. TCRβ immunosequencing was conducted in PBMCs from 47 AS patients (of whom 37 were HLA-B*27+) and 38 healthy controls (of whom 20 were HLA-B*27+) (Table 1). All subjects had paired CD4 and CD8 TCR repertoire data available, with the exception of 3 samples (from 1 HLA-B*27– control and 2 HLA-B*27+ controls), with the exception of 3 samples (from 1 HLA-B*27– control and 2 HLA-B*27+ controls), which had insufficient genetic material available for library preparation of 1 of the 2 cell populations. Patients and healthy controls were matched by age, but not by sex, reflecting the predominance of male patients with the disease. Twenty patients were receiving treatment with a tumor necrosis factor (TNF) antagonist at the time of sampling, which resulted in significantly lower disease activity according to the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI; scale 0–10) (33), as compared to patients who had not received biologic treatment (Table 1). Sample metadata and sequencing metrics are included in Supplementary Table 3 (on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

Increased TCR repertoire diversity and reduced size of T cell expansions in AS patients. TCR repertoire diversity was quantified using the NSDE index (34), a score bounded between 0 (monoclonality) and 1 (maximum diversity). When assessed using a GLM fitted with study covariates, the NSDE score of sample diversity across the CD4 and CD8 repertoires was negatively associated with increasing age (Pearson’s correlation = −0.4, P = 4.2 × 10−4), male sex (P = 0.04), and HLA-B*27 carriage (P = 0.004) in the CD8 TCR repertoire only (see Supplementary Figure 4, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract). After correction for HLA-B*27 status, age, and sex, AS
patients had a higher mean NSDE score than healthy controls, in both the CD4 T cell population (NSDE score 0.99 in AS patients versus 0.97 in controls; $P = 7.8 \times 10^{-4}$) and CD8 T cell population (NSDE score 0.86 in AS patients versus 0.81 in controls; $P = 9.3 \times 10^{-3}$) (Figures 1A and B).

In analyses stratified by HLA–B*27 status, the association of disease status with the NSDE score remained significant in the HLA–B*27+ cohort (for CD4 T cells, $P = 5.6 \times 10^{-5}$; for CD8 T cells, $P = 0.02$) but was lost in the HLA–B*27− cohort (for CD4 T cells, $P = 0.33$; for CD8 T cells, $P = 0.11$). This is likely attributable to reduced statistical power in the latter cohort, as the trend toward an increased NSDE in AS patients was evident in both HLA status groups (see Supplementary Figure 5, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract). There was no effect of TNF antagonist treatment on the NSDE score in AS patients, for either the CD4 TCR repertoire ($P = 0.63$) or the CD8 TCR repertoire ($P = 0.31$) (see Supplementary Figure 6, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

The increased diversity of the TCR repertoires in AS patients was attributed to a marked absence of highly expanded clones (>1% frequency) in the patients’ blood, particularly in the CD4 compartment (Figure 1C). To quantify the degree of T cell expansion, the percentage of all profiled CD4 or CD8 T cells contained in the top 100 (most expanded) nucleotide clones was calculated per sample (Figure 1D). After correction for HLA–B*27 status, sex, and age, AS patients had a lower mean percentage of T cells contained in the top 100 clonal expansions in both the CD4 TCR repertoire ($P = 0.31$) (Figures 1E and F). There was no effect of TNF antagonist treatment on the percentage of T cells in the top 100 rearrangements for either the CD4 TCR repertoire ($P = 0.42$) or CD8 TCR repertoire ($P = 0.11$) (Figures 1E and F). Carriage of HLA–B*27 (in patients and healthy controls combined) was associated with a significantly greater percentage of T cells in the top 100 rearrangements in the CD8 TCR repertoire only ($P = 0.007$) (Figure 1F). There was no effect of TNF antagonist treatment on the percentage of T cells in the top 100 rearrangements in either the CD4 TCR repertoire ($P = 0.42$) or CD8 TCR repertoire ($P = 0.20$) in AS patients (see Supplementary Figure 7, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

**Differential abundance of viral epitope-restricted clones in AS patients.** Given the decreased percentage of CD4 and CD8 T cells contained in large expansions in the peripheral blood of AS patients, we examined whether the TCR repertoires in AS patients also demonstrated evidence of reduced peripheral T cell
Figure 1. Increased diversity of the CD4 and CD8 T cell receptor (TCR) repertoires in ankylosing spondylitis (AS) patients attributable to the reduced size of clonal expansions. A and B, Comparison of the Normalized Shannon Diversity Entropy (NSDE) index of the CD4 (A) and CD8 (B) TCR repertoire in the blood of AS patients (n = 47) and healthy controls (CONT.) (n = 38). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. C, Frequency of CD4 and CD8 nucleotide clones in the profiled repertoire assessed in the blood of AS patients and healthy controls. Lowly expanded clones (LECs) (<1% frequency) and highly expanded clones (HECs) (>1% frequency) are shown. D, Percentage of the total CD4 and CD8 profiled repertoires contained in the top 100 most common nucleotide clones in the blood of AS patients and healthy controls. E and F, Group average measures for the CD4 (E) and CD8 (F) TCR repertoires in samples stratified by disease status (left), HLA-B*27 status (middle), or both (right). Results are the mean ± SEM. Values above the bars are the number of individuals in each group. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract.
expansion in response to common viral infection. A total of 926 of the HLA-specific, CMV epitope–restricted CDR3 clones and 273 of the HLA-specific, EBV epitope–restricted CDR3 clones annotated in the VDJdb database were identified within the sequenced CD8 TCR repertoire from the peripheral blood of 64 individuals (see Supplementary Tables 4 and 5, available on the Arthritis & Rheumatology website).

Figure 2. Greater percentage of peripheral CD8 T cells contained within viral epitope–restricted clones and evidence of reduced inflammatory cytokine production upon stimulation in the blood of AS patients compared to healthy controls. A, Percentage of the total CD8 TCR repertoire contained in putative cytomegalovirus (CMV) or Epstein-Barr virus (EBV) epitope–restricted clonotypes (amino acid rearrangements). B and C, Group average measures for CMV (B) and EBV (C) epitope–restricted clonotypes in samples from AS patients and healthy controls. Results are the mean ± SEM. Values above the bars are the number of individuals. D and E, Percentage of AS patient– and healthy control–derived peripheral blood CD4 (D) and CD8 (E) T cells expressing CD25, interferon-γ (IFNγ), and tumor necrosis factor (TNF) without stimulation (Unstim) or upon stimulation with 10 μg/ml HLA-A*02–restricted CMV-p65 peptide, 10 Lf/ml tetanus toxoid (Tet. Tox), or 1 μg/ml anti-CD3. Percentages are based on the population of divided cells. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. NS = not significant (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract.
Altered cytokine production upon TCR stimulation of T cells from AS patients. To further investigate apparent differences in the dynamics of antigen-restricted T cell responses between AS patients and healthy controls, T cell proliferation and cytokine production upon stimulation of PBMCs with CMV-p65, tetanus toxoid, or anti-CD3 were assessed in a secondary cohort of 19 AS patients and 14 healthy controls (see Supplementary Table 6, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract). There was no difference in T cell proliferation between AS patient-derived and control-derived CD4 or CD8 T cells stimulated with antigen or anti-CD3, as calculated using the CDI (see Supplementary Figure 8, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract). Following stimulation of PBMCs with tetanus toxoid, the percentage of divided CD8 T cells expressing CD25 was higher in AS patients compared to healthy controls (linear model \( P = 0.02 \); however, the percentage of tetanus toxoid–stimulated divided CD8 T cells expressing IFN\( \gamma \) was lower in AS patients compared to healthy controls (\( P = 0.03 \) (Figure 2E)).

Following stimulation of PBMCs with anti-CD3, fewer TNF+ CD4 T cells and TNF+ CD8 T cells were detected in PBMCs from AS patients compared to healthy controls (\( P = 0.01 \) and \( P = 0.002 \), respectively) (Figures 2D and E). No differences in either T cell proliferation or cytokine production were seen in response to CMV-p65 stimulation in either cell subset (Figures 2D and E). There was no differential effect of T cell stimulation on interleukin-17–producing cells (data not shown).

To determine whether the loss of highly expanded clonotypes and reduced inflammatory cytokine production in T cells from AS patients could be attributed to differences in the antigen presentation environment, DCs and monocyte subsets were quantified in AS patient– and healthy control–derived PBMCs, with the functionality of DCs characterized using analyses of costimulatory marker expression pre- and post–24-hour TLR activation. There were no differences in the frequencies of plasmacytoid DCs (pDCs) or CD1c+ or CD141+ DC subsets between AS patients and healthy controls; however, CD14++CD16+ intermediate monocytes were more frequent in the blood of AS patients compared to healthy controls (linear model \( P = 0.0005 \)) (see Supplementary Figure 9, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

There were no differences in the percentages of pDCs or CD1c+ DCs expressing activation markers (CD40, CD80, CD83, and CD86) or inhibitory markers (programmed death ligand 1 [PD-L1] and immunoglobulin-like transcript 3 [ILT-3]) pre- or post–TLR stimulation (see Supplementary Figures 10A and B, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract). PBMCs from AS patients contained a higher percentage of CD141+ cross-presenting DCs expressing CD40, CD80, and CD83 (\( P = 0.01, P = 0.04, \) and \( P = 0.05 \), respectively) (see Supplementary Figure 10C at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

Upon activation of PBMCs with a TLR ligand cocktail, expression of these maturation markers in control CD141+ DCs reached levels equivalent to those seen in CD141+ DCs from AS patients. CD141+ DCs coexpressing the inhibitory markers PD-L1 and ILT-3 were detected at higher frequency after TLR activation in the blood of AS patients compared to healthy controls (\( P = 0.007 \)) (see Supplementary Figure 10C). These data indicate that cross-presenting DCs are constitutively activated in AS patients, and more likely to acquire a regulatory phenotype upon TLR exposure.

Differences in the TCR repertoire between HLA-B*27+ AS patients and healthy controls. Differential V-gene family and V–J-gene usage across the CD4 and CD8 TCR repertoires of HLA-B*27+ AS patients and healthy controls was assessed using multivariate statistical analyses. Conducting sPLS-DA (35) on V-gene family counts revealed that the CD4 TCR repertoires in AS patients were best distinguished by the increased frequency of TRBV11, V13, V21, V5, and V3 rearrangements (Figure 3A), and the CD8 TCR repertoires in AS patients were best distinguished by the increased frequency of TRBV5 rearrangements (Figure 3B). However, classification of disease status based on V-gene family usage alone was no more accurate than random chance (minimum classification error rate, mean ± SD 0.44 ± 0.04 in CD4 T cells and 0.48 ± 0.04 in CD8 T cells), indicating that there were no large informative differences in global V-gene family usage between patients and healthy controls. Comparison of the frequencies of V-gene family usage by univariate statistical analysis revealed results that were comparable to those revealed by multivariate analyses (Figure 3E).

The disease classification error rate was marginally improved when receptors were defined by both V-gene and J-gene usage (minimum classification error rate, mean ± SD 0.38 ± 0.04 in CD4 T cells and 0.36 ± 0.05 in CD8 T cells). For both the CD4 and CD8 T cell populations alike, 65 of 490 unique V–J combinations were selected by the model as the most informative in differentiating HLA-B*27+ AS patient–derived TCR repertoires from healthy control–derived TCR
Control HLA–B*27+ CD4 TCR repertoires were enriched with clones using TRBV7–2 or TRBV7–9 paired with multiple J- genes (Figure 3C). Differences in the CD8 TCR repertoires of HLA–B*27+ AS patients and healthy controls were similarly driven by increased representation of TRBV7–2 and V7–9 rearrangements in the latter group, whereas TRBV14–1, V6–1, V7–7, and V4–1 paired with various J- genes were enriched in the blood of AS patients (Figure 3D).

**Figure 3.** Detection of differential TCRβ V-gene family and V–J- gene usage in the CD4 and CD8 TCR repertoires of HLA–B*27+ AS patients compared to HLA–B*27+ healthy controls. A–D, Sparse partial least squares discriminant analysis identifying V-gene families in the CD4 (A) and CD8 (D) repertoires and V–J gene pairs in the CD4 (C) and CD8 (D) repertoires that best differentiate HLA–B*27+ AS patients from healthy controls. Bar plots indicate the loading coefficient weights of features (ranked from most informative to least informative, from bottom to top) as an indication of feature overrepresentation in AS patients (dark gray) or healthy controls (light gray). Sample cluster plots show clustering of patient and control samples, with values representing the percentage of variability in feature usage explained by each of the first 2 (X and Y) components. Asterisks indicate V–J- gene pairs with differential abundance according to a P value of <0.01 by standard univariate statistical analysis. E, Left, Bar plots showing proportions of V-gene family usage in the CD4 and CD8 TCR repertoires of HLA–B*27+ AS patients and healthy controls. Right, Pie charts and data tables showing the averaged proportions, with differentially abundant V-gene families (prior to multiple testing) annotated. F, Heatmaps showing frequencies of V–J-gene rearrangements in the CD4 and CD8 TCR repertoires of HLA–B*27+ AS patients and healthy controls. Red boxes indicate V–J-gene pairs with differential abundance according to a P value of <0.01 by standard univariate statistical analysis. Red dots indicate the cohort in which frequencies were the highest. Adj. = adjusted (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract.
Figure 4. Association of CD8 TCRβ third complementarity-determining region (CDR3) clones with HLA–B*27+ AS. A, Sparse partial least squares discriminant analysis identifying 10 CD8 CDR3 rearrangements that best differentiate HLA–B*27+ AS patients from healthy controls (CO). Bar plots indicate the loading coefficient weights of features (ranked from most informative to least informative, from bottom to top) as an indication of feature overrepresentation in AS patients (dark gray) or healthy controls (light gray). Sample cluster plots show clustering of patient and control samples, with values representing the percentage of variability in feature usage explained by each of the first 2 (X and Y) components. Asterisks indicate CDR3 clones with differential incidence according to a P value of <0.01 by univariate Fisher’s exact test for comparison of clone counts between groups. B, Dot plots showing incidence of 21,362 CD8 CDR3 rearrangements in HLA–B*27+ AS patients and healthy controls. The opacity and size of each dot is scaled to the natural logarithm of the frequency of clones at each point. CDR3 sequences with increased incidence in AS patients and healthy controls (each at P < 0.01) are indicated in red and blue, respectively. C, Details of the 10 CD8 CDR3 rearrangements seen at increased incidence in HLA–B*27+ AS patients relative to HLA–B*27+ healthy controls (P < 0.01). The V–J-gene subclones comprising each CDR3 identical clonotype are listed, along with the number of individuals in whom that specific V–J combination was observed. The most frequently observed rearrangements are shown in red. D, Cumulative frequencies of the 10 CD8 HLA–B*27+ AS-associated clonotypes in the CD8 repertoire of HLA–B*27+ and HLA–B*27– AS patients and healthy controls. See Figure 1 for other definitions.
When tested independently for a disease association, the top differentially abundant V-J-gene pairs \( (P < 0.01) \) observed in AS patients compared to healthy controls (Figure 3F) were consistent with the repertoire signature observed in multivariate analyses. However, the FDR was high, as a result of testing a large number of independent variables in a relatively small cohort (FDRs \(-0.36–0.42\)).

**CD8 public clonotypes restricted to HLA–B*27+ AS patients.** Selective pressures favoring T cell proliferation in response to a common pHLA can result in public TCR CDR3 usage across individuals. Analyses using sPLS-DA differentiated the CD8 repertoire of HLA–B*27+ AS patients from that of HLA–B*27+ healthy controls, with a mean ± SD minimum classification error rate of 0.29 ± 0.05 based on the abundance of just 10 informative CDR3 rearrangements, with expansions of 3 CDR3 clones (CASSLGRAYEQYF, CASSSSGTGNNOPQHF, and CASSLSGGNTAEFF) indicative of an HLA–B*27+ AS–specific TCR repertoire (Figure 4A). Comparison of the clone incidence between HLA–B*27+ AS patients and healthy controls by Fisher’s exact test supported the sPLS-DA results, revealing 7 additional amino acid acid clones that were significantly associated with AS \((P < 0.01)\) (Figures 4B and C); however, these findings are only suggestive, given the small cohort size and large number of rearrangements tested (FDR >0.5).

Receptors utilizing differing V-genes and lacking nucleotide sequence identity can converge on common CDR3 amino acid motifs during antigen-driven selection. The 10 public CD8 HLA–B*27+ AS–associated CDR3 clones used differing V-genes across individuals. However, all of these clones demonstrated preference for at least 1 V-gene over all others (Figure 4C). Notably, 5 of 10 disease-associated clones demonstrated preferential TRBV7 usage; 25.8% of the 194 nucleotide rearrangements comprising these 10 CDR3 sequences across HLA–B*27+ AS patients utilized TRBV27, compared to only 7.1% of those used by the total CD8 HLA–B*27+ AS patients and healthy controls by Fisher’s exact test supported the sPLS-DA results, revealing 7 additional amino acid acid clones that were significantly associated with AS \((P < 0.01)\) (Figures 4B and C); however, these findings are only suggestive, given the small cohort size and large number of rearrangements tested (FDR >0.5).

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**Detection of CD8 HLA–B*27+ AS-associated clonotypes implicated in spondyloarthritis.** One CD8 CDR3 clone (CASSVGLFSTDQYF) (clone shown in red in Figure 4D) was only detected in the blood of 12 HLA–B*27+ AS patients (absent in all HLA–B*27– patients and all controls), resulting in a strong association with specifically HLA–B*27+ AS \((P = 1.6 \times 10^{-5} \) by Fisher’s exact test). This rearrangement conformed precisely to the CASSVGLFSTDQYF TCRβ CDR3 motif used by CD8 T cell expansions previously detected in the blood and synovial fluid of patients with bacterial-induced reactive arthritis (ReA) and AS (36–39). By scanning all CD8 TCR repertoires for CDR3 rearrangements, that varied only by the substitution or insertion/deletion of 1 amino acid relative to the CASSVGLFSTDQYF sequence, we revealed 23 additional highly homologous rearrangements (Figure 5A); the frequencies of these rearrangements in all HLA–B*27+ samples are listed in Supplementary Table 9, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.41252/abstract).

Collectively, the mean percentage of the total productive CD8 TCR repertoire contained within CASSVGLFSTDQYF or highly similar clonotypes was higher in HLA–B*27+ AS patients relative to HLA–B*27+ healthy controls (0.008% in AS patients versus 0.002% in healthy controls; \( P = 0.003 \) by Wilcoxon’s rank sum test) and relative to HLA–B*27– AS patients (mean 0.001%; \( P = 0.023 \)). The CASSVGLFSTDQYF motif was satisfied by a greater percentage of CD8 T cells in TCR repertoires from HLA–B*27+ AS patients compared to those from HLA–B*27+ healthy controls (mean 0.006% versus 0.003%; \( P = 0.002 \)) (Figure 5B); such clones were only detected at 1 count in 2 CD4 T cell samples. Collectively, the 2 homologous CD8 clones of highest incidence, CASSVGLFSTDQYF and CASSVGLYSTDQYF (underline indicates the differing amino acid), were detected in the blood of 18 (49%) of 37 HLA–B*27+ AS patients relative to 2 (11%) of 19 HLA–B*27+ healthy controls (odds ratio for association with HLA–B*27+ AS 8.05, \( P = 0.007 \) by Fisher’s exact test) (Figure 5A). Both rearrangements used the TRBV9–1:TRBJ2–3 gene segments in all samples in which they were detected.

**DISCUSSION**

Despite its strong HLA–B*27 association, an immunologic explanation for the development of AS remains elusive. It is hypothesized that exposure to gastrointestinal-derived bacterial antigens leads to immune activation and the emergence of cross-reactive T cell clonotypes in genetically susceptible individuals. In this study, we investigated the presence of antigen-restricted T cells in AS patients using TCRβ immunosequencing, a method to track and quantify expanded clonotypes by the unique sequence barcode of their rearranged receptors.
Our results reveal fundamental differences in the landscape of antigen-restricted immunity in AS patients, including global reductions in T cell clonal expansion, altered T cell function, expanded viral-specific CD8 clonotypes and public HLA–B*27+ AS-associated CD8 clonotypes, and constitutive activation of cross-presenting DCs. These data suggest that AS is not simply an autoinflammatory disease, but one characterized by profound immunoregulatory imbalance.

The present study is the first to provide evidence that AS patients exhibit significant reductions in the size of CD4 and CD8 T cell expansions globally in the peripheral blood, suggesting that perturbations in T cell survival, senescence, or regulation of clonal proliferation occur in AS patients during adaptive immune responses. It is unclear by what mechanism the disease-associated reduction in T cell clonal expansion arises. Moreover, the consequences of this lowered T cell clonal expansion on immune processes in AS patients remain to be determined. Importantly, such trends were apparent in both HLA–B*27+ and HLA–B*27– disease, although the differences were significant only in HLA–B*27+ AS patients, suggesting that this phenomenon is not purely attributable to properties of HLA–B*27 itself. On a functional level, we observed a reduction in both IFNγ production (in CD8 T cells) and TNF production (in CD4 and CD8 T cells) from patient-derived T cells upon tetanus toxoid or anti-CD3 stimulation through the TCR. Such findings may implicate disruption of the TCR signaling cascade as a means by which cytokine production is dampened, consistent with the reduction in antigen-driven clonal expansion detected by immunosequencing. We cannot, however, rule out the contribution of TNF antagonist treatment of AS patients to these findings, and future investigations of T cell function in AS would be best placed in treatment-naive cohorts.

Perturbed TCR signaling in AS patients has an experimental parallel in the mechanism underlying autoimmune arthritis in the SKG mouse model of spondyloarthritis, in which a mutation in the protein tyrosine kinase ZAP-70 attenuates TCR signaling, thereby enabling self antigen–restricted clonotypes to escape thymic negative selection and populate an autoreactive peripheral repertoire, while simultaneously exhibiting lymphopenia, hyporesponsiveness to bacterial antigen, and delayed bacterial clearance (40–42).

The presence of large putative CMV and EBV epitope-restricted CD8 clonal populations in the blood of AS patients may indicate chronic viral infection, persistent reactivation stemming from T cell exhaustion, and poor pathogen control, or may be indicative of disruptions to the regulatory mechanisms that modulate effector T cell expansion and memory T cell contraction. As previously reported, AS patient–derived mononuclear cells demonstrate deficiencies in the control of EBV infection (43,44), although whether this can be attributed to defective killing by CD8 cytotoxic T lymphocytes is presently speculative. Conversely, studies showing that HLA–B*27:05 can bind the EBV-derived pEBNA3A peptide, in the absence of the canonical pArg2 anchor, have suggested that the flexibility of the HLA–B*27:05 binding groove is an attribute of the molecule that enhances viral protection but predisposes carriers to T cell cross-reactivity and autoimmunity (45). We did not look specifically for expanded...
HLA-B*27–restricted viral epitope–responsive clonotypes in this study; however, the increased abundance of viral-restricted clonotypes in AS patients and healthy controls may suggest a role for endogenous virus–activated cross-reactive T cells in the pathogenesis of AS. Notably, T cell proliferative responses and cytokine production by PBMCs stimulated with CMV-p65 viral peptide in vitro did not differ between patients and healthy controls, although it is to be expected that anti-viral immunity varies substantially between individuals based on prior antigen exposure and HLA background, factors which were not fully controlled for in this study.

Pertinent to the reduced IFNγ production noted in AS patient–derived CD8 T cells in vitro, a “reverse IFNγ signature” has been observed in macrophages and DCs derived from AS patients (46) as well as DCs from the HLA-B*27 human β2-microglobulin–transgenic rat model of spondyloarthritis (47), whereby expression of IFNγ and classically up-regulated IFNγ response genes were found to be reduced in patients and in transgenic rat cells. In addition, monocytes from AS patients exhibit deficits in IFNγ production upon bacterial lipopolysaccharide stimulation (46). Such data suggest an inherent defect in IFNγ production in AS patients, which may have global effects on pathogen clearance by macrophages, chronic exposure to immunogenic antigens, and immunoregulatory stimulation. The reduced effector cytokine production from patient-derived T cells could not be attributed to a skewing of stimulatory DC subsets, which were present at equivalent frequencies in the blood of AS patients and healthy controls, with no apparent deficit in activation marker expression. Notably, CD141+ cross-presenting DCs were more activated in AS patients than in healthy controls, consistent with the active presentation of CD4 and CD8 epitopes. Furthermore, the proliferating CD8 T cells from AS patients significantly up-regulated CD25 expression in response to bacterial antigen. It is possible that CD8 T cells, when more vigorously stimulated by cross-presenting DCs, maintain a state of heightened activation, potentially through persistent exposure to bacterial adjuvant, and fail to undergo senescence to limit clonal expansion, resulting in less productive responses to foreign antigen in patients (48). Further studies are required to address functional defects in the adaptive immune responses in AS patients that may explain the global repertoire changes observed herein.

Differential receptor usage was evident in both the CD4 and CD8 TCR repertoire of HLA-B*27+ AS patients relative to HLA-B*27+ healthy controls, indicating differential antigen exposure in both populations. At V-J-gene resolution, a CD8 TCR repertoire signature identified by sPLS-DA could be used to predict disease status with ~70% accuracy, indicating that biased TCR usage is a distinguishing feature of the disease, even when assessed at low resolution. At the CDR3 level, public amino acid clones were detected with increased incidence in both the CD4 and CD8 repertoire of HLA-B*27+ AS patients. Five of the 10 AS–associated CD8 clonotypes identified in the present study displayed preferential usage of the same TRBV27 gene, including the 3 CDR3 rearrangements identified by sPLS-DA as most indicative of an HLA-B*27+ AS TCR repertoire. Furthermore, 2 disease-associated rearrangements exhibited near-perfect sequence identity (CASSLGRAYEYQYF and CASSLGSTYEQYF), suggesting an enrichment of structurally similar TCRs facilitating recognition of common pHLA motifs in AS. Whether these shared disease-associated clones are directly involved in AS–associated inflammation or whether they are a hallmark of common immune responses to foreign antigen, tissue destruction, or disease processes cannot be resolved by these data alone.

Finally, the results of this study support the findings of 2 recent independent studies that used comparable TCR profiling approaches to identify a possible role of CD8 CDR3 clones adhering to the CASSVGV[IVIL]{YF}STDTQYF motif in the pathogenesis of AS (38,39). The enrichment of TCRs with high sequence identity to the CASSVGFSTDSTQYF CDR3 clone found exclusively in HLA-B*27+ disease suggests that AS patients have both shared and individual T cell responses to a common immunodominant antigen engaged by receptors of similar structure (49). Results of a previous study demonstrating that CDR3 clones identical to those detected in the present study were increased in abundance in the inflamed synovium relative to the peripheral blood of AS patients (39), coupled with evidence of consistent detection of these CDR3 clones in the inflamed joints of patients with bacterial-induced ReA (36,37), suggest that these HLA-B*27–associated spondyloarthropathies have a common mechanism of CD8 T cell reactivity. Collectively, these findings provide strong supporting evidence to indicate a TCR repertoire profile specific to AS in which HLA-B*27–restricted bacterial antigen–responsive CD8 T cell clonotypic expansions occur on a background of an altered adaptive immune response in AS patients.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Brown had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Hanson, Nel, Thomas, Lê Cao, Kenna, Brown.
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Author/s:
Hanson, AL; Nel, HJ; Bradbury, L; Phipps, J; Thomas, R; Cao, K-AL; Kenna, TJ; Brown, MA

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