Peer Review File

Manuscript Title: Autoimmune-associated T cell receptors recognize HLA-B*27-bound peptides

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have studied the structural basis of HLA-B27.5 recognition by TCRs isolated from patients with ankylosing spondylitis, a disease with a strong association with the HLA-B27.5 allele. A particular TCR beta chain motif was previously reported to be enriched in such patients, and the authors isolated TCR alpha-beta pairs with this motif and then identified the peptide motifs recognized by these TCRs using yeast display. Five TCRs were studied: four from PBMC of one AS patient (clones AS4.1 to 4.4) and one from synovial fluid of another AS patient (clone AS3.1). Based on the selection criteria, these TCRs shared the TRBV9 V-region and a Y/FSTDTQ-BJ2.3 CDR3 beta motif. Yeast display identified human and microbial peptides recognized by these TCRs that shared a similar peptide motif. Structural analysis showed that these TCRs also shared a similar overall binding pattern. The biochemical and structural studies have been elegantly performed, but significant questions remain on the disease relevance of the identified peptides and the generality of the observed 'structural kernel' for TCR recognition in AS.

1. T cells with this TCR beta motif were analyzed from only two patients, thus the general relevance of these findings for AS remains unclear.
2. The authors were highly selective in choosing a particular TCR beta chain motif. It remains highly uncertain that only CD8 T cells with this particular motif are involved in the disease. Thus, the 'structural kernel' may be relevant for T cells with this motif, but not other CD8 T cell populations that could very well also be involved in this disease.
3. The investigators attempted to isolate TCRs with the pre-specified TCR beta motif in two sets of experiments. In the first series, CD8+ TRBV9+ T cells were sorted, but only one sequence with the pre-specified motif was isolated from one of the three patients, which was not further pursued (AS2 TCR). In a second series, CD8+ TRBV9+ T cells were sorted from four patients, and in one patient four TCRs were isolated (AS4.1 – 4.4) that were analyzed by yeast display. A more interesting TCR (AS3.1) was sequenced from synovial fluid where this TCR came from an expanded clone. Given that TCRs from only two patients were studied here, the identified AS2 TCR should also be characterized. Specifically, does it recognize some of the human/microbial peptides identified for the other TCRs?
4. The stated frequency of blood TCRs with the TCR beta motif is very low, approximately 1:100,000, which is within the range of naïve T cell populations. Furthermore, the TCRs in the second series were not isolated from PBMC, but rather T cells that were first expanded with anti-CD3 and IL-2 for 28 days. In vitro expansion could have biased the representation of TCRs in this sample. Thus, the disease relevance of the four studied AS4.1 to 4.4 TCRs remains unclear. Do the investigators have frozen PBMCs available on which they could perform deep TCR beta chain repertoire sequencing (on

hundreds of thousands of TCR beta chains)? It would be useful to define the actual frequency of the AS4.1 to AS4.4 clones among CD8 T cells from this patient.
5. The investigators have not validated the identified human and microbial peptides on blood samples from other AS patients. This will be essential to confirm the disease relevance of these antigens. It will be critical to also analyze PBMCs from healthy donors with the HLA-B27.5 allele to demonstrate whether there is a disease-related difference in the recognition of these peptides.
6. It remains to be determined whether the identified peptides are properly processed from full length antigens. The authors should transfect their APCs with cDNA construct to evaluate T cell stimulation.
7. The expression patterns of the corresponding human genes were not described. Are some of the human genes expressed in the synovia?

Minor points:
Figure 1K: The pie chart should include patient AS1455 from whom the AS4.1 - 4.4 TCRs were isolated for structural analysis.
The TCR sequences are listed for the AS3.1 TCR in Extended Table 2, but it is unclear which of the listed sequences in Extended Table 1 correspond to AS4.1 – 4.4 TCRs.

Referee #2 (Remarks to the Author):

This manuscript provides evidence that CD8+ T cells bearing certain TCRs that have been reported previously to be over-represented in AS and ReA might be recognizing self as well as microbial peptides presented by HLA-B*27. The work clearly advances the field in part by providing the first identification of actual peptides that are recognized by disease-associated TCRs using a high-throughput yeast display screen. If correct, this would provide the strongest evidence to date to support the arthritogenic peptide hypothesis, one of several mechanisms proposed to explain the role of HLA-B*27 in disease, although many questions would remain.

I have several concerns. Firstly, the SCT construct used to screen for peptides recognized by the TCRs in question does not encode HLA-B*27:05. It was necessary to introduce mutations into the heavy chain to enhance expression. Of concern would be mutations that affect TCR recognition or peptide binding specificity. One that was likely to affect TCR recognition was changed back to wild type (R169S to R), while at least one other mutation (H114Y) that is highly likely to alter peptide binding specificity was left unchanged. Residue 114 differs in key HLA-B*27 subtypes, most notably HLA-B*27:06, which is not associated with disease. This is a significant flaw that raises concerns about the disease-specificity of the entire screen. Retaining the ability to bind some known B*27:05 ligands (e.g. SRYWAIRTR) does not diminish these concerns as overlapping peptide binding specificity would be expected.

Secondly, the authors provide no evidence that HLA-B*27+ patients with AS/ReA (vs. HLA-B*27+ or HLA-B*27- healthy controls) have CD8+ T cells that recognize any of the identified self or microbial peptides, either by T cell activation or tetramer-based assays. This is critical for closing the loop in

terms of establishing CD8+ T cell cross-reactivity and arthritogenic peptide plausibility. Additional lines of evidence for alternative plausible mechanisms underlying the role of HLA-B*27 in disease developed over the last 2 decades have set a high bar for establishing arthritogenic peptides. While this is the type of approach that is needed, evidence for CD8+ T cell cross-reactivity in patients is crucial.

The lack of citations and reference to other work on the role of HLA-B*27 in disease serves to diminish what has been done relative to what is being shown here (e.g. no citations for 'unusual HLA-B*27 heavy chain biochemical properties' and 'disparate mechanisms'). HLA-B*27 has recently been shown to impact bone formation in AS (JCI 2019;129:5357) and potentially alter TGFb signaling (ARD 2019; 78:1653), both of which are mechanisms unrelated to antigen presentation that may not impact inflammation, yet remain highly disease relevant. In addition, there is strong GWAS-identified predisposition for IL-23/IL-17 axis involvement in AS, including CD4+ rather than CD8+ T cells, and of course IL-17A is now recognized as a major treatment target. How this relates to putative CD8+ autoreactive T cells is not at all clear. Animal models of HLA-B*27-mediated disease have no requirement for CD8+ T cells, and HLA-B*27-independent models that best recapitulate the spnodyloarthritis phenotype are mediated by CD4+ T cells or entheseal resident CD4/CD8-negative T cells responsive to IL-23. There is no evidence from animal models that I am aware of that supports an auto reactive CD8+ T cell mechanism.

This is a complex field and no single mechanism fits all of the existing data and cumulative observations, and arthritogenic peptides remain an attractive component of this mix. However, this complexity is something that consumers of novel research results need to understand.

Author Rebuttals to Initial Comments:

Referees' comments:

Dear Referees: We wish to highlight the major change to this resubmitted manuscript. In a completely independent set of experiments, the Yokoyama lab found certain TCRs were expanded in the anterior chamber of the eye in HLA-B27+ patients with anterior uveitis (AU). Uveitis is also associated with HLA-B27 and is relatively frequent in AS patients, though these disorders can appear separately. Strikingly we found that these TCRs bear the same CDR3β motif we found in AS patients, so we tested uveitis TCR transfectants for activation by the AS peptides. We found that the AS candidate peptides also activated the AU T cells, leading us to conclude that both diseases are likely seeing the same autoantigens. We have included these data in our revised manuscript and added Yokoyama and Paley as co-authors. We think this new finding strongly supports our contention that we have: 1- shown that HLA-B27+ spondyloarthropathies and uveitis are likely triggered by T cells cross reacting between microbial and autoantigens. We also note that a recent abstract where T cells with the same BV9-AV21 TCRs have been recently found in gut biopsies from AS patients with Crohn's disease and Ulcerative Colitis (https://ard.bmj.com/content/80/Suppl_1/14.3). These TCRs almost certainly will be reactive to the same antigens we describe here, further strengthening the disease relevance. 2- We have identified candidate self and microbial peptides that could very well be autoantigens. Indeed, some of these microbes represent known triggers for Reactive Arthritis, a limited inflammatory condition also linked to carriage of HLA-B27. 3- These peptides contain a strikingly identical sequence and structural motif that results in identical recognition of all of these peptides by the TCRs. We wish to emphasize that the near-atomically superimposable structures showing the TRBV9 and TRAV21 recognition of the shared motif from different triggering peptides is highly implausible to rationalize as having occurred by chance. These findings do not exclude a secondary role for HLA B27 misfolding or other mechanisms, as the collective disease etiology is very likely to be complex.

Referee #1 (Remarks to the Author):

The authors have studied the structural basis of HLA-B27.5 recognition by TCRs isolated from patients with ankylosing spondylitis, a disease with a strong association with the HLA-B27.5 allele. A particular TCR beta chain motif was previously reported to be enriched in such patients, and the authors isolated TCR alpha-beta pairs with this motif and then identified the peptide motifs recognized by these TCRs using yeast display. Five TCRs were studied: four from PBMC of one AS patient (clones AS4.1 to 4.4) and one from synovial fluid of another AS patient (clone AS3.1). Based on the selection criteria, these TCRs shared the TRBV9 V-region and a Y/FSTDTQ-BJ2.3 CDR3 beta motif. Yeast display identified human and microbial peptides recognized by these TCRs that shared a similar peptide motif. Structural analysis showed that these TCRs also shared a similar overall binding pattern. The biochemical and structural studies have been elegantly performed, but significant questions remain on the disease relevance of the identified peptides and the generality of the observed 'structural kernel' for TCR recognition in AS.

1. T cells with this TCR beta motif were analyzed from only two patients, thus the general relevance of these findings for AS remains unclear.

As mentioned above we have extended our study to include TCRs from HLA-B*27+ patients with Acute Anterior Uveitis (AAU), an inflammatory condition of the iris and ciliary body of the eye. Up to half of AS patients experience at least one episode of AAU during their lifetime. While AAU can occur in isolation from AS, it is still associated with HLA-B*27. Indeed, two patients in our study with HLA-B*27+ AAU had no clinical evidence of AS.

We performed an unbiased interrogation of the TCR repertoires of the eye and blood of HLA-B*27+ AAU patients (Fig. 1a). Remarkably, we identified similar BV9-AV21 pairings to the populations we initially identified in the blood and synovial fluid of AS patients. These TCRs were identified in 2 of the 5 patient samples and were selectively expanded in ocular fluid over the blood in one patient, suggesting antigen-specific recruitment to the anterior chamber of the eye (Fig. 1b). Two AAU-derived ocular BV9-AV21 TCRs were included in TCR transduced T cell stimulation studies, and both recognised specific self- and bacterial-peptide candidate predicted from the original AS3 and AS4-TCR led yeast display screens (Figure 4l, m, n). We also extended the number of AS patients screened and we now include a further 3 BV9-AV21 pairs identified from the synovial fluid (SF) of 3 additional AS donors. These SF-derived TCRs also demonstrated reactivity against the peptides mined on A3/AS4 TCR-led screen, again demonstrating the shared reactivity, despite subtle differences in the CDR3β and more so for the CDR3α motifs, amongst the various BV9-AV21+ TCRs. So, in summary, despite differences in terms of the breadth and magnitude of responses, specific patterns of reactivity against self, most notably GPER1, PRPF3 and RNASEH2B as well as bacterial (e.g. YEIH) candidate peptides were apparent for both AS- and AAU-derived TCRs. These data suggest a shared autoimmune T-cell-based pathology of HLA-B*27 for these anatomically distinct diseases. Furthermore, with the abstract reporting isolation of BV9-AV21 TCRs from gut biopsies of patients with Crohns' and UC, where GPER1 is highly expressed, we feel the case for disease relevance of our findings has become much stronger.

2. The authors were highly selective in choosing a particular TCR beta chain motif. It remains highly uncertain that only CD8 T cells with this particular motif are involved in the disease. Thus, the 'structural kernel' may be relevant for T cells with this motif, but not other CD8 T cell populations that could very well also be involved in this disease.

We completely agree that other T cells, with different sequences, are involved in AS an AAU, but the striking expansion of the TRBV9 subset served as a "smoking gun" to further investigate their specificities. The rationale for choosing the candidate BV9+ TCR was based on the independent reporting of near identical TCR BV9 populations, in relatively large ankylosing spondylitis (AS) patient cohorts[1-3], and absent or very infrequent in controls. In one study, the BV9+ population was enriched in synovial fluid samples compared to blood during acute synovitis, suggesting recruitment to the joint[1]. Similar clonotypes were reported previously in ReA patients[4]. We do not exclude the possibility that CD8+ T cells bearing different V segments-CDR3 motifs are also involved in disease pathology, but we considered it would be highly informative to screen this robust candidate in initial yeast display screening runs. The previously published information provided strong justification to screen these candidate CD8+ BV9+ TCRs.

We would also like to add that the near-atomically superimposable structures showing the TRBV9 and TRAV21 recognition of the shared motif from different triggering peptides is highly implausible to rationalize occurring by chance

3. The investigators attempted to isolate TCRs with the pre-specified TCR beta motif in two sets of experiments. In the first series, CD8+ TRBV9+ T cells were sorted, but only one sequence with the pre-specified motif was isolated from one of the three patients, which was not further pursued (AS2 TCR). In a second series, CD8+ TRBV9+ T cells were sorted from four patients, and in one patient four TCRs were isolated (AS4.1 – 4.4) that were analyzed by yeast display. A more interesting TCR (AS3.1) was sequenced from synovial fluid where this TCR came from an expanded clone. Given that TCRs from only two patients were studied here, the identified AS2 TCR should also be characterized. Specifically, does it recognize some of the human/microbial peptides identified for the other TCRs?

As discussed in Point 1, we now include T cell stimulation data for a total of 6 patients in the updated manuscript. 13 BV9-AV21 TCRs pairs, including receptor derived from the peripheral blood (n=4 TCRs) and synovial fluid samples (n =7 TCRs) of AS patients, and from the ocular fluid of AAU patients (n = 2 TCRs) (Fig. 4). Despite differences regarding the magnitude and breadth of TCR reactively, clear patterns of shared reactivity to specific self and bacterial candidate peptides were apparent, particularly against the GPER1, PRPF3, RNASEH2B and the YEIH peptides.

4. The stated frequency of blood TCRs with the TCR beta motif is very low, approximately 1:100,000, which is within the range of naïve T cell populations. Furthermore, the TCRs in the second series were not isolated from PBMC, but rather T cells that were first expanded with anti-CD3 and IL-2 for 28 days. In vitro expansion could have biased the representation of TCRs in this sample. Thus, the disease relevance of the four studied AS4.1 to 4.4 TCRs remains unclear. Do the investigators have frozen PBMCs available on which they could perform deep TCR beta chain repertoire sequencing (on hundreds of thousands of TCR beta chains)? It would be useful to define the actual frequency of the AS4.1 to AS4.4 clones among CD8 T cells from this patient.

CD8+ T cell expansion prior to 10x VDJ single cell sequencing (SCS) served as a means to non-specifically enrich the candidate BV9-expressing T cells which are present at low frequencies in the blood of AS patients (~$1/10^5$)[1,2], enabling us to identify the AV sequences paired with the BV9 CDR3 motif. A prior SCS run performed using freshly isolated $BV9^+$ T cells lead to the identification of a single barcode from one patient only, thus prompted this pre-expansion-based approach for subsequent SCS runs. This expansion strategy was successful given that for donor AS4, 4 separate BV9 expressing barcode expressing the AV21 chain coupled to different CDR3 α motifs were identified, thus providing good coverage of *in vivo*-derived BV9-AV21 clonotypes.

Furthermore, we believe that the additional identification of BV9-AV21 clonotypes from the eye of HLA-B27 AAU (Fig. 1), particularly the evidence for *in vivo* and tissue-specific clonal expansion as described in Point 1 strengthens the disease relevance of the BV9-AV21 TCRs.

Although it would be interesting to define the frequencies of the AS4.1 to AS4.4 clones among patient derived CD8 T cells, this is not feasible due to a lack of follow-up patient sample material. Additionally, previous work has clearly highlighted the greater frequency of the TCR BV9 CDR3 motif in AS patients compared to HLA-B*27 positive and negative healthy controls[1]. We would argue that this is more informative than measuring actual frequencies given that the initiating immunologic event may have happened many years previously.

5. The investigators have not validated the identified human and microbial peptides on blood samples from other AS patients. This will be essential to confirm the disease relevance of these antigens. It will be critical to also analyze PBMCs from healthy donors with the HLA-B27.5 allele to demonstrate whether there is a disease-related difference in the recognition of these peptides.

The presence of almost identical, expanded T cell receptors in the AAU patients with very similar specificities, as well as from the inflammatory lesions of AS patients with UC and Crohn's, and the rarity of such sequences in healthy controls[1] makes it very likely that these T cells with the peptide specificities defined here are involved in the disease process. As mentioned, we also think that the near atomic superimposition of the structures strongly supports physiological relevance, and that the structural convergence is being underappreciated as validation. Although it would be interesting to explore the functionality of these T cells in more detail, there are significant challenges to this work. The frequencies of BV9-AV21+ T cells in the blood of AS and AAU patients are low, and although expanded in tissue-specific sites, they are not particularly abundant. This probably reflects the considerable time that may have elapsed since the disease initiating immune response, given that the chronic disease is likely a sustained secondary inflammatory response similar to that in rheumatoid arthritis[5,6]. Added to this inflammatory environment of progressive disease, medical therapy such as NSAIDS and/or biologics could affect the functionality of these T cells. We discuss in the manuscript that the functionality of these T cells might be less prominent at the point of diagnosis when sample material is acquired, potentially years following an initial trigger that activates these cells. We propose that a prospective study aiming to evaluate such T cells prior/during acute onset to the establishment of clinical disease, albeit challenging, would carry substantial benefit.

6. It remains to be determined whether the identified peptides are properly processed from full length antigens. The authors should transfect their APCs with cDNA construct to evaluate T cell stimulation.

In the revised manuscript we provide evidence that minigenes expressing 60 amino acid stretches encompassing the epitopes contained within both the GPER1 and YEIH gene products are recognised by a test BV9-AV21 TCR (AS8.4 TCR) (Extended Data Figure 6b). These data provide evidence that the peptide can be processed from longer minigene products, and therefore very likely from their respective full-length gene products *in vivo*.

7. The expression patterns of the corresponding human genes were not described. Are some of the human genes expressed in the synovia?

As discussed in the manuscript, the GPER1 protein is expressed in cartilage, bone and the iris/ciliary body in the eye. However, we cannot be certain that the peptides we describe comprise the specific disease triggers for AS or AAU, or whether additional peptides containing the shared structural motif we found in the crystal structures (Fig. 5). A number of self-peptides might also be involved in the autoimmune process, with some antigens specific to the iris/ciliary body in the eye and others to the enthesis in the joint. Equally, we propose that putative disease-causing antigen(s) should bear similar features to these peptides, and multiple microbial triggers with the shared 'kernel' features. Nonetheless, our findings expand the concept of the 'arthritogenic peptide', and importantly, we define a minimal motif that is recognised by these disease-candidate BV9+ T cells. This information could provide invaluable to future efforts (e.g. Mass Spectrometry-based approaches) aimed to define putative 'arthritogenic peptide' repertoires.

Minor points:
Figure 1K: The pie chart should include patient AS1455 from whom the AS4.1 - 4.4 TCRs were isolated for structural analysis.

The new Extended Data Fig.1 has included the AS1455.

The TCR sequences are listed for the AS3.1 TCR in Extended Table 2, but it is unclear which of the listed sequences in Extended Table 1 correspond to AS4.1 – 4.4 TCRs.

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1. I have several concerns. Firstly, the SCT construct used to screen for peptides recognized by the TCRs in question does not encode HLA-B*27:05. It was necessary to introduce mutations into the heavy chain to enhance expression. Of concern would be mutations that affect TCR recognition or peptide binding specificity. One that was likely to affect TCR recognition was changed back to wild type (R169S to R), while at least one other mutation (H114Y) that is highly likely to alter peptide binding specificity was left unchanged. Residue 114 differs in key HLA-B*27 subtypes, most notably HLA-B*27:06, which is not associated with disease. This is a significant flaw that raises concerns about the disease-specificity of the entire screen. Retaining the ability to bind some known B*27:05 ligands (e.g. SRYWAIRTR) does not diminish these concerns as overlapping peptide binding specificity would be expected.

We understand the referee's concerns and have two responses. First, amino acid mutations are occasionally required to drive the stable expression of human MHC subtypes on the surface of yeast cells[7,8]. In this study, three mutations were required, with one, H114Y, located in the peptide binding groove. As H114Y is a conservative mutation from both a physiochemical/size perspective we predict this would have limited impact on the canonical HLA-B*27:05 peptide repertoire. Our small-scale thermostability screens of both self and bacterial peptides support this prediction, with comparable thermal stability observed for HLA-B*27:05 and the HLA-B*27:05^{H114Y} in complex with GPER1, PRPF3, and the YEIH peptides (Extended Date Figure 7o). The disease protective B*27:06 subtype also expresses a different amino acid at 114; this substitution - to aspartate - represents a more dramatic change and is coupled to a nearby tyrosine substitution at position 116 (from aspartate in B*27:05) in the F pocket. We argue that both mutations could alter the chemistry of the F pocket and the peptide's C terminal anchor residue. Additional changes, located on the alpha 1 (at position 77) and 2 helices (at position 152) are also unique to HLA-B*27:06, and distinguish this subtype from canonical HLA-B*27:05 and the yeast expressing variant.

Second, and key to note here, is that all subsequent studies - T cell stimulation, SPR and structural work - were performed using non-mutated HLA-B*27:05. Our T cell stimulation studies clearly illustrate that both the self and bacterial peptides, predicted based on peptides libraries evolved using this HLA-B*27:05 yeast display variant, activated AS and AAU-derived BV9-AV21 transduced T cell lines when presented by HLA-B*27:05 antigen presenting cells, thus validating the utility of the minimally mutated HLA-B*27 platform as a means to adequately interrogate the canonical HLA-B*27:05 peptide repertoire in this setting.

2. Secondly, the authors provide no evidence that HLA-B*27+ patients with AS/ReA (vs. HLA-B*27+ or HLA-B*27- healthy controls) have CD8+ T cells that recognize any of the identified self or microbial peptides, either by T cell activation or tetramer-based assays. This is critical for closing the loop in terms of establishing CD8+ T cell cross-reactivity and arthritogenic peptide plausibility. Additional lines of evidence for alternative plausible mechanisms underlying the role of HLA-B*27 in disease developed over the last 2 decades have set a high bar for establishing arthritogenic peptides. While this is the type of approach that is needed, evidence for CD8+ T cell cross-reactivity in patients is crucial.

We understand these concerns, and this highlights the practical difficulties of obtaining definitive proof of relevance for human autoantigens given the heterogeneity of the diseases even in the most strongly genetically linked diseases such as AS and AAU.

We believe that we do have data that T cells bearing these TCRs, from AS, and now AAU patients recognize and are trigged by the self and microbial peptides, but we demonstrate this with T cell transfectants, not the primary T cells because of the difficulty of obtaining enough patient-derived samples. Nevertheless, the T cell transfectants would have the same specificity as the primary T cells so we feel this represents a valid proxy. We were forced, due to the paucity of the T cells, to essentially 'reconstruct' the patients T cells from the T cell repertoire sequence information in the form of such transfectants which make it very clear that they respond to the peptides we isolated. With the recent abstract that gut biopsies of AS patients with Crohn's and Ulcerative Colitis also express BV9-AV21 TCRs

(https://ard.bmj.com/content/80/Suppl_1/14.3) that will almost certainly share the reactivities we have found, this further strengthens the disease-relevance of our findings.

We have plans for repertoire analyses in naïve and memory T cells in the blood of patients, focusing on the TRBV9-CDR3-TRBJ2.3 – AV21 TCRs defined here. Because these T cells are likely to be low frequency in chronic disease, we cannot carry out direct peptide antigen stimulations to determine functions, but by using single cell sequencing we will be able to determine their likely functions. We would argue that the specificity of the receptors, determined by their TCRs, as described in this manuscript, would remove the need to show peptide specific responses. The scale of this experiment is such that this is beyond the scope of the present study. However, in the meantime, we are confident in our argument made here that the expansion or these T cells in the joint and eye, as well as the published frequencies in blood in patients vs controls, strongly implies antigenic stimulation by these or related peptide immunogens in patients.

A further problem for functional studies is the inflammatory milieu in progressive disease, which when coupled to bouts of treatment regimens with NSAIDS and/or biologics, would likely affect the functionality of these cells. As mentioned in the manuscript (Discussion), the functional prominence of T cells specific for the original epitope may be less apparent at the time of diagnosis and when sample material is acquired, which could be years following an initial trigger event. One solution, also for a future study, would be to prospectively evaluate such T cells prior/during acute onset prior to the establishment of clinical disease, for example following a large salmonella or shigella infection in a closed or semi-enclosed community. Although not without significant challenges given the unpredictable occurrence of such an event and the small percentage of HLA-B*27:05 patients who develop AS, such a study would allow a prospective interrogation of CD8+ reactivity and might adequately address this point.

3. The lack of citations and reference to other work on the role of HLA-B*27 in disease serves to diminish what has been done relative to what is being shown here (e.g. no citations for 'unusual HLA-B*27 heavy chain biochemical properties' and 'disparate mechanisms'). HLA-B*27 has recently been shown to impact bone formation in AS (JCI 2019; 129:5357) and potentially alter TGFb signaling (ARD 2019; 78:1653), both of which are mechanisms unrelated to antigen presentation that may not impact inflammation, yet remain highly disease relevant. In addition, there is strong GWAS-identified predisposition for IL-23/IL-17 axis involvement in AS, including CD4+ rather than CD8+ T cells, and of course IL-17A is now recognized as a major treatment target. How this relates to putative CD8+ autoreactive T cells is not at all clear. Animal models of HLA-B*27-mediated disease have no requirement for CD8+ T cells, and HLA-B*27-independent models that best recapitulate the spnodyloarthritis phenotype are mediated by CD4+ T cells or entheseal resident CD4/CD8-negative T cells responsive to IL-23. There is no evidence from animal models that I am aware of that supports an auto reactive CD8+ T cell mechanism. This is a complex field and no single mechanism fits all of the existing data and cumulative observations, and arthritogenic peptides remain an attractive component of this mix. However, this complexity is something that consumers of novel research results need to understand.

Although in the original manuscript we acknowledged that AS disease pathology is complex and alluded to the other disease models, it was never our intention to diminish the significant

work in the field pertaining to the unusual biochemistry of HLA-B*27. In the revised manuscript, we more specifically acknowledge that both the normal function of HLA-B*27 combined with its unusual biochemistry might uniquely contribute to the autoimmune and inflammatory component of these inflammatory diseases. We also apologise for the omission of key references - these are now cited in this current draft (see line 84).

Additionally, the available animal models may not fully recapitulate human HLA-B*27-driven pathologies in AS or AAU[9]. The rat models require super-physiological HLA-B*27:05 transgenic insertions, e.g., (~ 20-150 copies of the HLA-B*27:05 heavy chain and 45-90 copies of human β_2M[10,11]) to develop disease, while animals remain without disease in the setting of a lower copy-number (6-7 copies), despite preserved surface protein expression. The rat model, therefore, may unintentionally bias mechanistic studies towards the unusual biochemistry of HLA-B*27:05 and away from the arthritogenic peptide model. Additionally, the HLA-B*27 expressing mouse model does not spontaneously develop AS. And although a selection of non-B*27-expressing mouse models permit the study of clinical AS or inflammatory disease, they do not re-capitulate the HLA-B*27 component which is relevant to human disease. The recently described spontaneous Cynomolgus monkey disease model[12] or humanised mouse models (https://www.jax.org/news-and-insights/jax-blog/2020/july/why-humanized-mice) could offer improved platforms to address the arthritogenic peptide model in relation to HLA-B*27:05.

Lastly, we would respectfully note, that in this complex field, the absence of a robust animal model for AS via autoantigen-presentation by HLA-B*27 is not necessarily evidence against the arthritogenic peptide model in human patients. Importantly, there is clinical overlap of multiple disease entities within the spondyloarthritis family, e.g., psoriatic arthritis and IBD-associated arthritis. As a result, while HLA-B*27-independent animal models (such as IL-23 overexpression via minicircles) may appear to recapitulate components of the AS clinical picture, they are also recapitulating components of psoriatic arthritis and IBD-associated arthritis, which occur in the absence of HLA-B*27. Ultimately, it is likely that HLA-B*27-associated disease is a multifactorial process, including genetic factors, the unique biochemistry of HLA-B*27, the composition of the intestinal microbiome, enteric inflammation, and - as our data suggest - antigen presentation by HLA-B*27 to BV9-AV21 CD8 T cells.

References

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9. Braem, K. & Lories, R.J. Insights into the pathophysiology of ankylosing spondylitis: contributions from animal models. *Joint Bone Spine* **79**, 243-248 (2012).
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11. Tran, T.M. et al. Additional human beta2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats. *Arthritis Rheum* 54, 1317-1327 (2006).
12. Jia, H. et al. A new and spontaneous animal model for ankylosing spondylitis is found in cynomolgus monkeys. *Arthritis Res Ther* **24,** 1. (2022).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have greatly improved the manuscript in this revision. The addition of TCRs from acute anterior uveitis patients (AAU, also associated with HLA-B27:05) significantly strengthens clinical relevance, in particular because these TCRs are strongly enriched/clonally expanded at the site of inflammation compared to the blood. Also, TCRs from AAU patients recognize a similar set of human and bacterial peptides as the AS TCRs.

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B. Originality and significance: novel work is presented which could be significant if corroborated by evidence of expanded auto reactive CD8+ T cells in disease.
C. See comments below
D. There are no statistics applied to comparisons.
E. See below
F. See below
G. Yes
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The initial review of this manuscript identified a number of concerns, most notably (1) the limited number of patients studied, (2) the a priori assumption that the focus should only be on CD8+ T cells, (3) the very low frequency of the TCR beta motif of interest, and (4) the strength of evidence that the 4 TCRs being studied were relevant to disease. In addition, (5) lack of validation of the identified self and microbial peptides was raised – i.e. the need for some evidence that there is autoreactivity against self or microbe in HLA-B27-associated disease, and not in healthy HLA-B27+ donors, was noted. Additional concerns were noted that the HLA-B27 heavy chain used to screen the peptide library was mutated in key residues that made it differ from HLA-B*27:05, including one residue that is altered in a non-disease-associated subtype of HLA-B27. There were also concerns that the findings were not properly contextualized recognizing current research on axial spondyloarthritis and HLA-B27 that directly impact the plausibility of CD8+ T cell autoreactivity as a cause of HLA-B27-associated diseases.

The authors have provided a rebuttal to some of the concerns and a rewritten manuscript with additional results that addresses some, but not all, of the previous shortfalls. The biggest concern is the continued lack of direct evidence that there are expanded autoreactive CD8+ T cells in individuals with HLA-B27-mediated disease – the crux of the arthritogenic peptide hypothesis. Evidence of increased frequency of BV9-BJ2-3 TCRs in synovial fluid and ocular fluid is noted. However, only in one case are the samples are matched – ocular fluid and peripheral blood from the

same individual. In the case of synovial fluid TCR frequencies are compared to peripheral blood from different individuals, making it difficult to draw a conclusion. There is no standard that I am aware of for an expected frequency of potentially autoreactive T cells in active autoimmune disease – yet the data presented here suggest they are extremely rare. Nor is it clear the extent to which TCR frequencies against self MHC increase in inflammatory lesions. In the absence of any assessment of disease-irrelevant HLA controls it remains impossible to know the significance of these findings to disease in terms of a specific HLA-B27-directed immune attack.

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As I have been asked to review this manuscript after the first round of revision from the original submission, my requests for additional data to support their main contention is tempered. Nevertheless, there are elements of the paper that I suggest should be considered by the authors to improve the paper further.

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2) Arguably, the gold standard would be to naturally elute HLA-B27 peptides from disease-associated tissues using mass spectrometry and correlate this with the peptides derived from the library/bio-informatics approach. This is too great an ask, however. Nevertheless, there is a wealth of published immunopeptidomics data available for both self and microbe derived B27-peptidomes,which could be readily used as a resource to fine tune peptide selection and confirm natural presentation of the candidate peptides. Further, one readily achievable experiment would be to show T cell reactivity to naturally presented microbial antigens by examining responses to infected cells – this would

represent a significant advance on the mini-gene expression system used in the revised manuscript.

3) The authors go to some length to discuss the repeated TRBV9 usage. Strangely however, the germline-encoded TRBV9 regions do not appear to play a role at all in mediating contacts with the pMHC, but the authors are silent on this point. What is driving the TRBV9 bias? The molecular basis for TRBV9 bias has been determined previously in author autoimmune-like disorders.

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While this statement holds true for the above cherry-picked example, it does not for some of the other TCRs examined. This needs to be stated.

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Not really. The work actually describes the structural basis of cross-reactivity across self and bacterial peptides

8) can the authors comment on whether the identified peptides are permissive for KIR3DL1 recognition given that B27 is a ligand for this KIR, and perhaps elaborate on this axis.

9) Other structurally-centric studies in the T cell mediated autoimmunity axis have described cross-reactivity across microbial peptides and how this may trigger/drive the disease– this should be discussed in the context of these findings.

10) Consideration for a more generally appealing title.

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We thank the reviewer for their positive reviews.

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The initial review of this manuscript identified a number of concerns, most notably (1) the limited number of patients studied, (2) the a priori assumption that the focus should only be on CD8+ T cells, (3) the very low frequency of the TCR beta motif of interest, and (4) the strength of evidence that the 4 TCRs being studied were relevant to disease. In addition, (5) lack of validation of the identified self and microbial peptides was raised – i.e. the need for some evidence that there is autoreactivity against self or microbe in HLA-B27-associated disease, and not in healthy HLA-B27+ donors, was noted. Additional concerns were noted that the HLA-B27 heavy chain used to screen the peptide library was mutated in key residues that made it differ from HLA-B*27:05, including one residue that is altered in a non-disease-associated subtype of HLA-B27. There were also concerns that the findings were not properly contextualized recognizing current research on axial spondyloarthritis and HLA-B27 that directly impact the plausibility of CD8+ T cell autoreactivity as a cause of HLA-B27-associated diseases.

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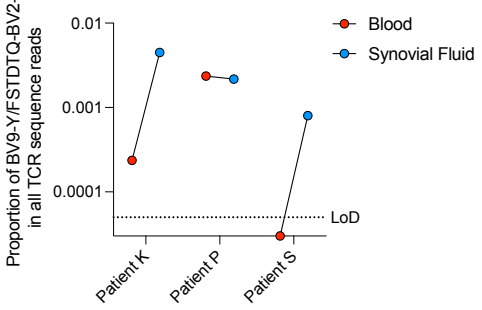
We appreciate the reviewer's concerns and would like to highlight 3 pieces of evidence in response to these concerns.

First, we agree that comparing the frequency of clonotypes across individuals should be done with care. To this end, we have reformatted Figure 1b to better illustrate the frequency of the AS-associated TCRs in the blood and joint of the sampled individuals. However, we would also note prior examples where investigators have used un-paired tissues from different individuals to demonstrate tissue specificity of T cells[1].

Second, we would remind the reviewer that four separate cohorts[2-5] have performed epidemiologic studies and identified the Y/FSTDTQ motifs in AS patients compared to HLA-matched healthy controls. In addition, two of these studies[3,4] also noted enrichment of the Y/FSTDTQ motifs in AS synovial fluid compared to blood. Moreover, we would point to a forthcoming manuscript from our co-authors, posted on *medRxiv* (**https://doi.org/10.1101/2022.05.06.22274633**). In this pre-publication dataset with paired blood and synovial fluid samples, the BV9-Y/FSTDTQ-J2-3 motifs from the synovial fluid had a full log difference in proportion between the blood and synovial fluid for two of three patients (see included figure). We have added this analysis to our manuscript in Extended Data Fig 2b. Coupling our data with prior and contemporaneous studies, the collective evidence strongly supports a tissue-specific enrichment of Y/FSTDTQ motifs for both the joint and eye in patients with HLA-B27-associated disease – and not in healthy HLA-B*27+ control donors.



Proportion of TCRβ sequences containing the BV9-YSTDTQ-J2-3 motif in blood and synovial fluid samples from 3 AS patients. Data from Komech *et al. medRxiv* 2022.

Third, we agree that a universal standard for the frequency of putative pathogenic T cells would provide a valuable benchmark for the field of autoimmunity. However, we would like to note prior studies in rheumatoid arthritis and type 1 diabetes have reported similar frequencies of putative pathogenic TCRs in the blood from patients compared to healthy controls[6,7]. In addition, given the variability of potential pathogenic mechanisms across human disease and potentially across

tissues, it is unlikely that absolute frequency of a T cell will be predictive of pathogenic potential without incorporating more relevant attributes of the cell, such as TCR specificity or function. Moreover, additional factors are likely to come into play including very long chronicity and therapeutic interventions. Thus, we would posit that the organ-specific enrichment of putative pathogenic TCRs in our data and that of other groups provide strong evidence for disease relevance.

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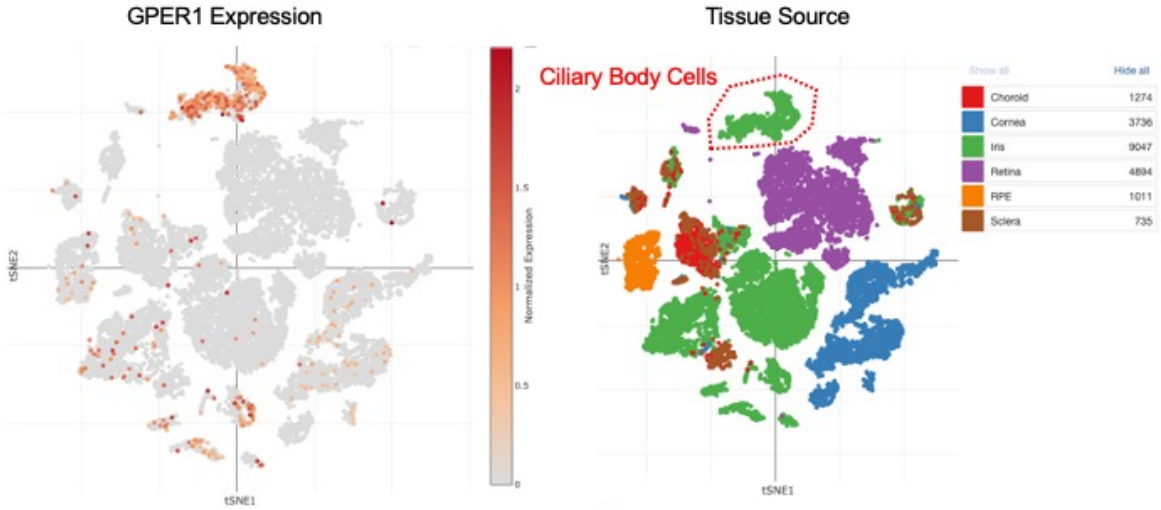
We thank the reviewer for observations and questions. We agree that the candidate antigens, such as GPER1, are expressed more broadly than just the eye and joint. However, the anatomic distribution of GPER1 expression within the eye is consistent with tissue specificity. In a single-cell transcriptomic analysis of porcine and human ocular tissues, expression of *GPER1* is predominantly found within ciliary body cells[8] (Figure below, and mentioned in the discussion: lines 492-493). Importantly, the inflamed structures in HLA-B*27-associated acute anterior uveitis (AAU) are the iris and ciliary body[9]. Thus, the expression of *GPER1* closely aligns with the manifestations of HLA-B*27 AAU within the ocular structure.



Expression of *GPER1* is predominantly found within ciliary body cells. Single cell RNA sequencing from human and porcine ocular tissues. Iris and ciliary body cells are labelled in green with ciliary body cells circled in red. Data from Gautam *et al. Nat. Communications.* 2021.

Website: https://singlecell.broadinstitute.org/single_cell/study/SCP1311/multi-species-single-cell-transcriptomic-analysis-of-ocular-compartment-regulons?genes=GPER1#study-visualize

We would also like to draw the reviewer's attention to an abstract from our co-authors where the same TRBV9-AV21 TCRs were found in gut biopsies from AS patients with Crohn's disease and Ulcerative Colitis. This data adds support to the proposed link between the gut spondyloarthritis[10] and further strengthens the disease relevance of our findings (**https://ard.bmj.com/content/80/Suppl_1/14.3**). And although the antigens we described may not encompass all the tissue-specific antigens relevant to AS, ReA, and AAU, at these distinct anatomical sites, the core structural interactions between the AV21-BV9 TCRswe describe in this manuscript, offers strong evidence that disease causing antigens very likely carry the same structural motif as the putative antigens we report.

With regard to the bacterial peptides, the *yeiH* epitope is found in *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and *Klebsiella* while the *gspD* epitope is found in *Chlamydia*, all of which are associated with Reactive arthritis (ReA). This is now mentioned in the Discussion (line 508). While the epitopes can be found in other species that are not typically associated with

ReA, such as *E. coli*, we would predict that an inflammatory event from an infectious diarrheal illness may be required to activate these cross-reactive TCRs and break peripheral tolerance, whereas commensal gut bacteria would not normally do this. We speculate that the subclinical, microscopic enteric inflammation seen in AS patients could also provide the inflammatory "second hit" needed to initiate or fuel the disease process[11].

2) Arguably, the gold standard would be to naturally elute HLA-B27 peptides from disease-associated tissues using mass spectrometry and correlate this with the peptides derived from the library/bio-informatics approach. This is too great an ask, however. Nevertheless, there is a wealth of published immunopeptidomics data available for both self and microbe derived B27-peptidomes,
which could be readily used as a resource to fine tune peptide selection and confirm natural presentation of the candidate peptides. Further, one readily achievable experiment would be to show T cell reactivity to naturally presented microbial antigens by examining responses to infected cells – this would represent a significant advance on the mini-gene expression system used in the revised manuscript.

We agree that naturally eluting peptides from disease-associated tissues using mass spectrometry would represent a significant milestone for autoimmune-based research. We have searched the bulk of published B*27:05 immunopeptide literature, and unpublished datasets made available to us, but have not identified our candidate self-peptides. However, most of these peptide elution studies were performed using HLA-B*27 transfected B cells, such as C1R or T2-transfected cells, which are likely to differ substantially from the physiological antigen presenting cells *in vivo* and thereby present different self-peptides. Added to this are the ongoing challenges of how well individual peptides fragment in mass spectrometry-based studied, a problem that can lead to the under-representation of target peptides in final datasets (**https://doi.org/10.1002/pro.3919**). Moreover, very few peptide-MHC complexes are needed to trigger antigen-specific T cells such that very low levels of HLA-B*27-associated peptides may be sufficient to trigger autoreactivity and be difficult to detect by mass spectrometry. However, current advances in instrumentation technologies and identification algorithms should hopefully lead to the careful interrogation of synovial fluid cell subsets or even entheseal tissue in the future.

In the current manuscript, we demonstrate that the yeiH peptide is successfully processed from a minigene construct and is presented by HLA-B*27 resulting in the activation of TCR-transduced SKW-3 cells. Although we do agree that an advance on this finding would be to evaluate the processing of bacterial lysate by antigen presenting cells, we suggest that this requires a very comprehensive experimental approach that is best tackled as part of a separate project. We argue that without careful planning and optimisation, a negative result based on a sub-optimal experimental set-up may not be reliable. There are a number of factors to consider for such a study, not least with respect to the antigen-presenting cells for which representation of ERAAP risk and protective genotypes is likely to be key. Added to this is the experimental design set-up relevant to bacterial strains and bacterial lysates, especially in relation to defining

the optimal experimental set-up to compare lysates (e.g. production of lysates from bacteria engineered to either knock-down or knock-out the yeiH gene as well as mutants with peptides that do not bind HLA-B*27). However, we do agree that those experiments are very worthwhile and meaningful undertakings, albeit best tackled outside the scope of this current work.

3) The authors go to some length to discuss the repeated TRBV9 usage. Strangely however, the germline-encoded TRBV9 regions do not appear to play a role at all in mediating contacts with the pMHC, but the authors are silent on this point. What is driving the TRBV9 bias? The molecular basis for TRBV9 bias has been determined previously in author autoimmune-like disorders.

Based on our structural work, the BV9 germline-encoded CDR1 and CDR2 regions are not involved in pMHC interactions. Thus, it is less clear why BV9 is strongly preferred in AS associated TCRs. This is different from previously published literature describing a celiac disease associated SP3.4 TCR[12] where the BV9 germline-encoded CDR1 L37 and CDR2 Y57 amino acids play important roles for stabilizing the overall complex, and explains the biased BV9 usage. It also likely that non-structural factors including AV-BV chain pairing and BV-BJ joining frequencies (influenced by complex factors relating to convergent and bias V-D-J recombination[13-15]) contribute to their abundance and consequently, the preferential selection of the BV9 segment *in vivo*. These factors are now acknowledged in our revised manuscript (Results: line 434-440).

Other points

1) The crystallography. Somewhat surprisingly, there is a significant disconnect between the statistics reported in the crystallographic table and that reported in the PDB validation reports – some incorrect space groups, wrong Rfac/Rfree values etc. Whilst I am sure that there is a ready explanation for these inconsistencies, I would recommend that the authors tie up these loose ends. However, an Rfree of 38% for a ternary complex is simply far too high and certainly highlights an issue that needs to be corrected. The authors should show omit maps for the peptides within the ternary complexes.

We apologize for the formatting errors in the original table, this has been corrected. The AS4.3-RNASEH2B-B*27 complex crystal suffered radiation damage during data collection. Nevertheless, the statistics for data collection are adequate to process (Rmerge: 0.224; CC1/2: 0.988; Completeness: 91.7). We subsequently solved the complex structure and the electron density in the TCR-pMHC interface is unambiguous and allows us to confidently superimpose this structure with other complex structures in the manuscript to assess structural similarity. In other words, despite the liabilities of this dataset, we found that was useful for the limits of our intended comparative analysis. The density in the TCR constant domain, β2M and the B*27:05 heavy chain α3 domain is relatively poor, and this contributed to the overall high Rfree in the structure. The omit maps for this and the other TCR-pHLA-B*27 complexes are now included as Extended Data Fig. 9 in the re-submitted manuscript.

2) the SPR. Only tables are shown. Sensorgrams need to be presented, and there is no detail on numbers of times the experiments were conducted
The SPR sensorgrams are now shown in Extended Data Figure 8.

3) the steric hinderance argument doesn't fully make sense. Why would a p9-Lys not be disfavoured with His 116?

We felt some attempt at rationalization of the differential effects of HLA-B*27:05 versus HLA-B*27:09 was warranted, albeit with the clear limitations the reviewer expresses. This structural theory was offered as one possible explanation to understand why the P9 K containing PRPF3 peptide drove comparable T cell responses when presented by HLA-B*27:05 and HLA-B*27:09, whereas the P9 R containing peptide, GPER1, only drove T cell stimulation when presented by HLA-B*27:05. Our structural interpretation is based on the examination of existing p-B*27:05 (Asp116) and p-B*27:09 (His116) structures (Fig. A) (PDB: 2BST, 1JGE and 1K5N). We predicted greater steric hinderance and disfavored positive-positive interactions for HLA-B*27:09 when the peptide P9 is Arg (Fig. B). When P9 is Lys, the steric hinderance is measurably less due to the increased atom distance between HLA-B*27:09 H116 and P9 Lys (Fig. C). However, we accept that this is a structural prediction only. In the revised manuscript we have introduced minor revisions to better reflect the theoretical nature of this interpretation and to better acknowledge that despite their differential ability to stimulate T cells on an HLA-B*27:09 background, both the PRPF3 and GPER1 peptides are comparably poor stabilisers (based on their comparable thermal melt data) of *in vitro* refolded HLA-B*27:09 (Results: line 357-382).

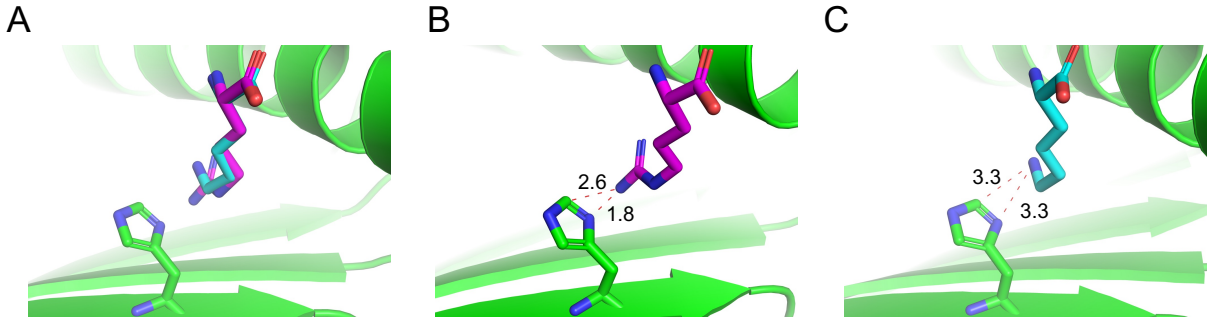


Figure A. P9 Arg and P9 Lys interaction with H116 on B*2709
Figure B. P9 Arg distance to H116
Figure C. P9 Lys distance to H116

4) 'The KD values378 for AS8.4-GPER1-HLA-B*27:05 and AS8.4-YEIH-HLA-B*27:05 are 15 μM and 1.2 μM respectively which are in accord with the stronger activation of the AS8.4 TCR by these two peptides (Fig. 5d).'
While this statement holds true for the above cherry-picked example, it does not for some of the other TCRs examined. This needs to be stated.

In our initial manuscript, we performed SPR for peptides that activated several TCRs derived from (AS3.1, AS4.1, AS4.2, AS4.3) PBMCs. We later tested TCRs derived from patient's synovial fluid and ocular fluid in our revised manuscript. To our surprise, we observed that AS8.4 TCR exhibit superior activity towards GPER1 and YEIH peptide. Thus, we picked AS8.4 to measure SPR affinity. The data shows that the superior activity of AS8.4 could at least in part explained by the relatively higher affinity of the complexes. Other synovial fluid derived and Ocular fluid derived TCRs exhibit comparable level of activity to the PBMC derived TCRs and were not included for further SPR analysis. We fully acknowledge that TCRs with near identical affinities for the same pMHC complexes might not necessarily translate to similar activity in T cells stimulation assays. This has been the subject of previous work done in our lab[16,17]. Our aim was to highlight that our AS TCR-pMHC complexes affinities are within the range of existing studies published by other groups[18], with higher affinities measured towards bacteria derived YEIH peptide compared to self-peptides. However, we accept that it is more appropriate to refer to the range of affinities, and we now also mention this in the revised manuscript (Results: line 391-393).

5) The authors try to use the peptide-mediated interactions mediated by the AV21 chain as an element of novelty however many TCR-pMHC interactions have demonstrated germline-encoded TCR-peptide recognition, and this should be acknowledged.

We agree that germline-encoded TCR-peptide recognition has been shown previously. Our intention here was to highlight that our structural work clearly explained several phenomena we observed 1. AV21 specifically utilised germline CDR1a sequence to interact with the peptides. 2. The TCR CDR3a are not conserved nor are they involved in peptide-specific interactions. To our best knowledge, this is unusual for TCR-pMHC interactions as the majority of TCRs utilize CDR3a and CDR3b to govern peptide specificity. However, we acknowledge that others have discovered similar germline-somatic TCR-peptide interaction modes[19,20] and we now cite these papers to acknowledge the importance of that work (Results: lines 417-418).

6) the structural kernel phraseology is essentially a focused hot-spot that has been described by Garcia (and others previously), so it is unclear why such elaborate language needs to be used here.

The term hot-spot was originally coined to define peptide amino acids that provide the greatest energetic contribution to a protein-protein interface[21] and is technically a thermodynamic descriptor. In this manuscript, the term 'kernel' was introduced to more generally describe a structural recognition motif shared between sequence-related peptides. Although the 'kernel' motif almost certainly encapsulates the recognition hotspot, we chose not to refer to a specific hotspot because thermodynamic studies were not performed to pinpoint those amino acids. However, we accept that the wording could be simplified, and we have therefore exchanged the world 'kernel' for 'structural motif' in the revised manuscript (throughout the Results and Discussion sections, blue highlighted).

7) 'That the structural superimpositions exhibit near-atomic identity across multiple TCR complexes is highly unusual and suggests that the peptides are being recognized by BV9-CDR3 Y/FSTDTQ in a physiologically relevant manner.'
Not really. The work actually describes the structural basis of cross-reactivity across self and bacterial peptides

We accept this view and have therefore modified our interpretation to reflect the reviewer's comment (Results: line 441-447).

8) can the authors comment on whether the identified peptides are permissive for KIR3DL1 recognition given that B27 is a ligand for this KIR, and perhaps elaborate on this axis.

To address the interesting question posed by the reviewer, we performed an initial experiment to test if HLA-B*27:05 YEIH and HLA-27:05 PRPF3 tetramers bind to HEK293-FT cells transfected with KIR3DL1*002 fused to Green Fluorescent Protein (GFP). Previously characterised HLA-B*57 tetramers that bind KIR3DL1 receptors were included as positive controls[22-24]. Our negative control included a tetramer of HLA-E in complex with a MHC Ia leader sequence peptide (VMAPRTVLL). Our very preliminary data (Figure 2, below) suggests that the PFPF3 and YEIH HLA-B*27:05 peptide complexes also bind KIR3DL1*002, possibly with subtle binding strength differences.

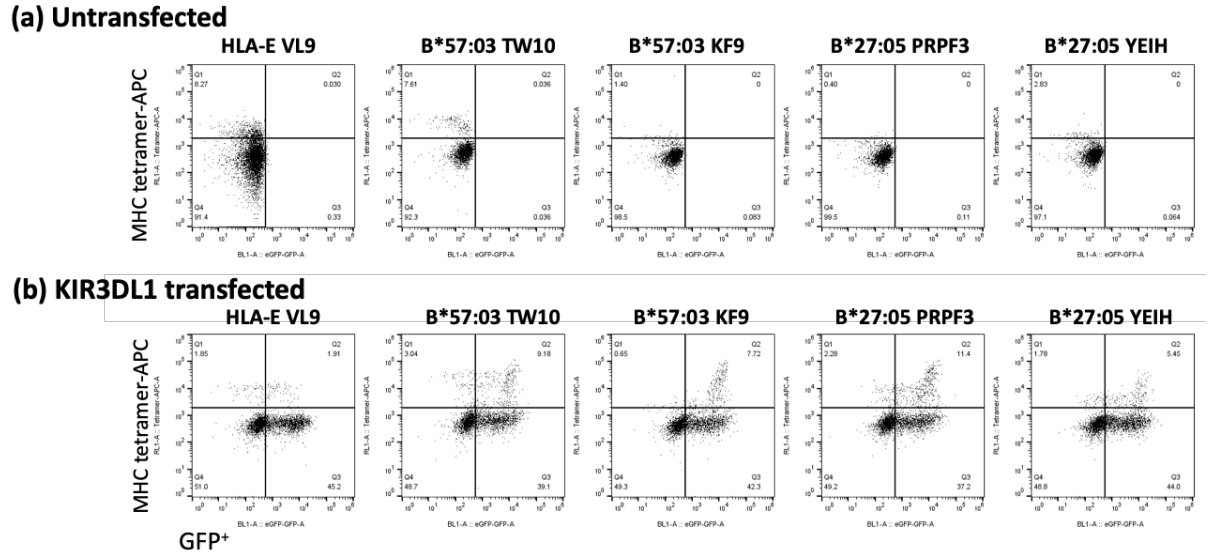


Figure 2: At 24 hours following transfection, both (a) un-transfected and (b) KIR3DL1*002-GFP transfected HEK293-FT cells were incubated with 1μg of APC-conjugated HLA-B*27:05 tetramers refolded with PRPF3 and YEIH, in addition to positive (HLA-B*57:03 TW10 (TSTLQEQIGW), HLA-B*57:03 KF9(KAAFDLSFF)) and negative control (HLA-E VL9 (VMAPRTVLL)) tetramers for 30 minutes on ice. Following subsequent washing and sample resuspension in PBS, staining data was acquired on an Attune NxT flow cytometer. Data processing was performed in FlowJo v9. The Y-axis denotes MHC I tetramer staining, the X-axis denotes GFP positively (relevant to (b)). The Q2 quadrants in (b) denote the percentage of MHC I positive, KIR3DL1-GFP positive cells, plus background staining (HLA-E-VL9 tetramer).

Although KIR3DL1/S1 associations with AS are likely to be complex[25], there is potential for follow-up based on the peptide ligands we describe here. There may be scope to investigate how well other KIR3DL1 allotypes bind the various HLA-B*27:05-peptide complexes, and to what extent these KIR3DL1 allotypes are expressed on disease-relevant T cells and/or NK cells in AS and AAU patients. There is also the question of whether AS-associated ERAAP genotypes facilitate the processing of longer versions of these peptides that bind HLA-B*27:05 but fail to interact with KIR3DL1 receptors - a seminal, and somewhat related observation was made previously for an extended and mutated HIV peptide epitope that retained binding to HLA-B*57:01, but reduced the binding affinity to KIR3DL1*001 relative to the shorter, non-mutated index peptide[24]. These questions could provide new avenues of exploration beyond the scope of our current T cell-based work.

9) Other structurally-centric studies in the T cell mediated autoimmunity axis have described cross-reactivity across microbial peptides and how this may trigger/drive the disease – this should be discussed in the context of these findings.

We acknowledge this point and have now included references to previous findings describing cross-reactivity across microbial peptides triggers/drivers (Results: line 447-448).

10) Consideration for a more generally appealing title.

Although we like the descriptive nature of the current title given the controversy about this topic, we offer the following alternatives:

1. **TCRS SHARED BETWEEN ANKYLOSING SPONDYLITIS AND ACUTE ANTERIOR UVEITIS PATIENTS RECOGNISE HLA-B*27-RESTRICTED, SEQUENCE-RELATED BACTERIAL AND SELF PEPTIDES**

2. **HLA-B27 LINKED INFLAMMATORY CONDITIONS SHARE TCRS THAT RECOGNISE SEQUENCE-RELATED, HLA-B*27-RESTRICTED PEPTIDES FROM BACTERIAL AND SELF PROTEINS**

3. **DISEASE-ENRICHED TCRS RECOGNISE SEQUENCE-RELATED HLA-B*27-BACTERIAL AND SELF PEPTIDES IN ANKYLOSING SPONDYLITIS AND ACUTE ANTERIOR UVEITIS**

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Reviewer Reports on the Second Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

In this second revision and rebuttal, the authors have attempted to assuage reviewers' concerns including the issue of whether the paper provides enough new evidence to support the hypothesis that cross-reacting microbial and self-antigens presented by HLA-B*27 could be important in the pathogenesis of HLA-B*27-associated diseases. Since the enriched TCR motifs that formed the basis for this work have been reported previously, the main novelty and strengths of this work are (1) the identification of self and bacterial peptides that can be recognized by these TCRs, (2) the structural work providing a putative motif that may be the basis of cross-reactivity, and (3) the data from AAU patients. The weaknesses continue to be the lack of direct evidence supporting the presence of autoreactive CD8+ T cells reacting to these peptides. Other weaknesses have emerged from the review process, including the tendency to cherry-pick supportive arguments.
Of the 5 paired eye and blood samples from AAU patients, only one patient had AS. AS and AAU overlap in their genetic predisposition and their appearance in affected patients pretty frequently, and they co-exist more often than the 20% suggested here. If these TCR motifs are so expanded in these individuals and they are important in the pathogenesis of HLA-B27-associated disease, why are these patients protected from AS? Perhaps there is sampling error given the small numbers. A similar question arises for the new evidence provided by co-authors of an increase in synovial fluid T cells (over matched peripheral blood) expressing the motif of interest in 2 subjects but not a third.
The authors continue to refer to an 'HLA-B*27:05' peptide library that was screened, when in fact the heavy chain has been mutated at several residues, including a particularly important residue for peptide binding. While some results were validated using non-mutated B*27:05, referring to the library a B*27:05 library is not accurate. In answering a previous query about why the peptides found here are not in previous datasets (for one of the most studied HLA class I alleles), the authors claim that most peptide elution studies have been done using transfected cells, which may differ from physiological APCs. This is not correct as considerable work has been done using cells from transgenic rats (Admon's group) where naturally occurring peptides are well represented.

Comments from Referee #1 on remaining concerns raised by Referee #2:

• AS versus AU – why do not all patients with AU develop AS? Given that these are complex diseases, the answer likely falls outside the scope of the question being studied here (the nature of peptides and TCRs related to AS)
• HLA-B*27:05 library. I do agree with the reviewer that the writing needs to be more accurate. The authors could refer to a HLA-B*27:05 library with several mutations that enabled yeast display.
• Previous peptide elution studies. The authors should cite all relevant papers. I would note that such mass spec studies are not comprehensive because a great diversity of peptides are presented by each HLA molecule. The fact that these peptides were not previously identified is not surprising.

Referee #3 (Remarks to the Author):

The authors have addressed well the main concerns I raised and I congratulate them on a fine study. Some minor follow up points:

In relation to the B27 self-immunopeptidome, I suggest the authors add a line or two in the paper to show how this has been considered as it's an obvious quesiton that will be queried by a reader.

As there are admitted issues with some of the structures due to unresolved domains reflecting in high Rfree values, the authors should mention this in the methods section as these Rfree values may raise eyebrows.

There are still some minor inconsistencies with the PDB reports and tables (eg Free values) - some PDB reports have repeated titles. I suggest putting in PDB accession codes in the suppl. crystallographic table so that the reader is clear what structure relates to a specific PDB report.

Author Rebuttals to Second Revision:

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Of the 5 paired eye and blood samples from AAU patients, only one patient had AS. AS and AAU overlap in their genetic predisposition and their appearance in affected patients pretty frequently, and they co-exist more often than the 20% suggested here. If these TCR motifs are so expanded in these individuals and they are important in the pathogenesis of HLA-B27-associated disease, why are these patients protected from AS? Perhaps there is sampling error given the small numbers. A similar question arises for the new evidence provided by co-authors of an increase in synovial fluid T cells (over matched peripheral blood) expressing the motif of interest in 2 subjects but not a third.

Our response: We agree that the rate of overlap in AS and AAU in our participants with HLA-BB*27+ AAU is likely not reflective of the rate of clinical overlap for the general AAU population given the sample size. The reviewer raises an important point that emphasizes the heterogeneity of complex diseases. These questions are raised throughout the field of rheumatology. For example, why do only some patients with rheumatoid arthritis (RA) develop interstitial lung disease (ILD) and many do not? The development of ILD in RA is influenced by both clinical parameters, such as obesity, CRP levels, functional status, and extensive smoking [1] as well as genetic variation in the MUC5B promotor region [2, 3].

The data in this manuscript propose a core antigen specificity of disease-associated TCRs in AS and AAU, however, it does not rule out additional factors (such as genetics, environmental exposures, or changes to the intestinal microbiota) that are likely critical in the development of clinical manifestations of disease for either the joint/spine in AS or the eye in AAU. Large genomic studies have suggested both a large overlap of genetic risk alleles for AS an AAU as well as distinct genetic factors that may confer risk for AS or AAU [4, 5]. We hypothesize that tissue-specific mechanisms in the eye, spine, or joint (that may vary from one individual to the next) may either facilitate an inflammatory response or protect against disease. These questions will be important to address in future studies, outside the scope of this current manuscript.

In regard to Extended Figure 2B, where we saw joint-specific enrichment of the BV9-YSTDT-J2-3 motif in 2 of 3 patients, we would note that, given our sample size, we are unable to adjust for all the potential clinical and biological confounders that may influence the frequency of putative pathogenic T cells in the blood and joint, e.g., disease duration, during of a flare, concurrent medications, etc. Future studies may be able to clarify this biology.

The authors continue to refer to an 'HLA-B*27:05' peptide library that was screened, when in fact the heavy chain has been mutated at several residues, including a particularly important residue for peptide

binding. While some results were validated using non-mutated B*27:05, referring to the library a B*27:05 library is not accurate.

Our response: We accept that our reference to the HLA-B*27:05 library construct is (unintentionally) misleading and we apologize for this. In the finalized version of the manuscript, we outline that the yeast display version is a triple-mutated HLA-B*27:05 heavy chain variant and we now refer to this yeast display construct as HLA-B*27:05^{3mut} throughout the manuscript (blue highlighted).

In answering a previous query about why the peptides found here are not in previous datasets (for one [i]of the most studied HLA class I alleles), the authors claim that most peptide elution studies have been done using transfected cells, which may differ from physiological APCs. This is not correct as considerable work has been done using cells from transgenic rats (Admon's group) where naturally occurring peptides are well represented.

Our response: In our rebuttal, we specifically focused on immunopeptidomic data generated from human cells to ascertain if our predicted peptides were represented in peptide datasets derived from human cellular samples. We did not intentionally overlook the important work from Arne Admon, Joel Taurog, José A. López de Castro and colleagues regarding immunopeptidomics studies in the transgenic rat model ([6, 7]. However, we do accept the Reviewer's point regarding the importance of such work. We have since searched datasets from Admon and Taurog's 2017 publication [6], but we have not identified rat homologs of peptides such as GPER 1, PRPF3 and other peptides reported in our manuscript. We now make reference to these rat transgenic models in addition to the human transfected cells in the revised manuscript as sources of HLA-B*27:05 immunopeptidomic data in the revised manuscript (page 12, blue highlighted).

Comments from Referee #1 on remaining concerns raised by Referee #2:

AS versus AU – why do not all patients with AU develop AS? Given that these are complex diseases, the answer likely falls outside the scope of the question being studied here (the nature of peptides and TCRs related to AS) HLA-B*27:05 library. I do agree with the reviewer that the writing needs to be more accurate. The authors could refer to a HLA-B*27:05 library with several mutations that enabled yeast display.

Our response: As requested by both reviewers, we now clearly state that our HLA-B*27:05 yeast display construct is a triple amino acid mutated heavy chain, namely HLA-B*27:05^{3mut}, in the revised manuscript (blue highlighted).

Previous peptide elution studies. The authors should cite all relevant papers. I would note that such mass spec studies are not comprehensive because a great diversity of peptides are presented by each HLA molecule. The fact that these peptides were not previously identified is not surprising.

Our response: As requested by Reviewers 1 and 2, we now acknowledge published literature relating to HLA-B*27:05 immunopeptidomics in the revised manuscript (page 12, blue highlighted)

Referee #3 (Remarks to the Author):

The authors have addressed well the main concerns I raised and I congratulate them on a fine study. Some minor follow up points:

In relation to the B27 self-immunopeptidome, I suggest the authors add a line or two in the paper to show how this has been considered as it's an obvious question that will be queried by a reader.

Our response: We now briefly mention and acknowledge published literature relating to HLA-B*27:05 immunopeptidomics in the revised manuscript (page 12, blue highlighted)

As there are admitted issues with some of the structures due to unresolved domains reflecting in high Rfree values, the authors should mention this in the methods section as these Rfree values may raise eyebrows.

There are still some minor inconsistencies with the PDB reports and tables (eg Free values) - some PDB reports have repeated titles. I suggest putting in PDB accession codes in the suppl. crystallographic table so that the reader is clear what structure relates to a specific PDB report.

Our response: We solved a second AS4.3-RNASEH2B-B*27 structure to replace the previous dataset which suffered radiation damage during data collection. This structure is deposited into Protein Data Bank under the same accession code. We also edited the Extended Data Table. 6 as the reviewer suggested so the table is consistent with PDB validation report.

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