

Senescent-like Activated Naive B Cells Contribute to the Progression to Clinical Rheumatoid Arthritis

Corresponding Author: Professor William Robinson

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

A. Summary of the key results

B. Originality and significance:

The manuscript by Wu et al. seeks to understand how shifts in the early emigrants of the B cell compartment are associated with the progression of a seropositive (asymptomatic preclinical) adult females with circulating autoantibodies to citrullinated proteins is followed about 400 days later by a clinical state meeting criteria for the diagnosis of rheumatoid arthritis. From a large consortium of investigators, the investigators identify only five clinical converters, and findings from two timepoints are compared with those of five seropositive individuals who do not progress to a RA diagnosis within a similar time period. It is a controversial topic, and the manuscript may be too ambitious as it seeks to embrace too broad of a view. Additional cohorts of unrelated individuals are examined, but it is unclear if these are appropriate and well-matched for gender, ethnicity, and race. After RA diagnosis, do these subjects still lack exposure to therapeutic agents that are serious potential confounders.

There is a challenge of precise nomenclature and B-cell subset assignment. As for defining relevant B cell subsets, earlier schema exclusively used surface phenotype by flow cytometry, while the current study uses transcriptomics with ADT.

Mixing in concepts and references from murine transgenic mice may not be helpful.

One especially notable issue is the usage of "transiting" and "transiting B cells", which are a stage of differentiation that follows after late activated naïve B cells in a patient with an abnormal immune system. The authors do not seem to be referring to the well-accepted transitional B cells that are the B lineage cells that newly emerges from the bone marrow.

The authors refer to "atypical" and "ABC", while the latter can refer to Aging-associated B cells. These are now confusing terms that come from very different studies. These B cell subsets may correspond to the CD19+ CD27- IgD- double-negative B cells that may be referred to as a DN1 subset, although this is controversial. Others have referred to an expansion of the DN2 subset in RA patients, but have "transiting B cells" been previously described (see below? Arguably, this manuscript might have greater clarity if the phenotype (or transcriptomic pattern) is first used to name each subset, and this descriptor is then used in the text. The commentary recently reported in Immunity might be relevant to consider this controversial area (Eisenbarth et al. Immunity November 2024).

On this topic, in the title "transition" may be mixing clinical with the name of a B cell subset and further obscuring the intent, which in the title might better be described as "disease progression".

The authors use accepted criteria that a preclinical state to an overt clinical condition can be met if there is >1 swollen joint. Yet do these five individual Converter subjects develop this polyarthritis disease? How many swollen and tender joints does each develop?

C. Data & methodology: validity of approach, quality of data, quality of presentation

A key area of uncertainty is shown in the representative UMAP in Figure 1b, which shows subsets for B cells (are these all assigned based on the CD19 transcript?). "In Converters, aNAVs constituted 43.01±5.91% of B cells at the pre-clinical RA stage and 38.55±7.60% post onset." What was the representation of this subset in healthy controls? Three healthy controls seem inadequate to distinguish progressions in the disease state from what might truly be inter-individual variation. These values seem too high for all B lineage CD19+ cells in the blood, even after negative B cell selection, as shown in UMAP in Figure 1b. If this is true, a supplemental figure should show 5 individuals at pre- and post-conversion timepoints, as well as show the five non-converters, and also show the variation between healthy controls.

In Figure 1f, what are each of these bars?

A large number of individual transcripts are mentioned, and it is uncertain which have been validated in the literature for

identifying lymphocyte subsets in health and disease.

If this becomes a central finding, the text might better focus on Kruppel-like factors (KLF) transcript expression, and the biological context.

As “senescence” is key, how can this assignment be solely based on β -galactosidase expression? Don't healthy adults have late-activated naïve B cells, and how is this different from the notion that clones of naïve B cells arise from low-level “tickling” of BCR, without sufficient co-stimulation/T cell help/cytokine stimulation?

D. Appropriate use of statistics and treatment of uncertainties

Methods are appropriate however there is a concern, as many central findings are based on trends rather than statistically significant correlations.

E. Conclusions: robustness, validity, reliability

The authors state that they closely examine the samples from two timepoints for each subject, which are ~400 days apart. Did the authors intentionally ignore the samples they have accumulated from other time points? And many other subjects. Is this manuscript an exercise in cherry-picking?

CAR T cell therapy does not need to be revisited three or more times throughout the manuscript. The adoption of CAR T cells from cancer therapy does not provide an approach that builds on findings that are novel in the current manuscript. The first paragraph of the discussion might better solely focus on what is novel here.

The somewhat tautologic presentation on BCR binding specificity, poly-specificity, and broad reactivity vs. definable fine specificity. Arguably, much of this reactivity here involves recognition of citrulline-modified de-iminated polypeptides. Net positive charge in HCDR3 may correlate with polyreactivity, but certainly there must be other contact residues in these paratopes.

The repertoire analysis is intriguing, but it is unclear whether these patterns of difference are meaningful.

F. Suggested improvements: experiments, data for possible revision

As referred to above.

G. References: appropriate credit to previous work?

Too many reviews and commentaries from the authors.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

As mentioned above.

Reviewer #2

(Remarks to the Author)

Review of Wu et al.

From the TIP-RA project the authors compared B cell subsets and their properties in 10 individuals, all of whom had anti-citrullinated protein antibodies (ACPA+), but only 5 progressed to clinical RA over a 2-year period whereas the other 5 did not. Additionally, they had 3 healthy controls that were ACPA- and remained asymptomatic. The study design, thereby, enabled identification of markers that could be used to distinguish between progressors and non-progressors. Applying single cell RNA-seq to peripheral blood B cells they identified a subset of CXCR5⁺ CD38^{lo} cells that were selectively increased in ACPA+ progressors but not in non-progressors (Fig. 1). This subset (named aNAVXCR5⁺) was elevated for BCR-proximal components, had down-regulated select signaling molecules (Fig. 2) and were enriched for markers of cellular senescence (Fig. 3). VDJ-seq showed increased prevalence of somatically unmutated IgM+ cells in RA converters (80–81%), compared to non-converters (~60%) and healthy controls (70–72%) (Fig. 4). Monoclonal antibodies derived from converter clones were polyreactive against a diverse antigen panel (Fig. 4). Following clonally related receptors they found that H/L pairs in converters did not progress to class switched, memory phenotypes (Fig. 5). aNAVXCR5⁺ and ‘Transit’ cells were enriched in RA compared to osteoarthritis (OA) synovial fluid (Fig. 6). Addressing the origin of aNAV cells they found that resting naïve B cells (rNAV) from CCP+ RA donors converted to an aNAVXCR5⁺ phenotype over 2-day activation with anti-IgM, citrullinated protein antigens and CpG (Fig. 7h, i), suggesting that aNAVXCR5⁺ cells resulted from stimulation by self-antigens in vivo. Chronic stimulation ex vivo of aNAVXCR5⁺ from RA patients led to increased senescence as measured by β -galactosidase expression (Fig. 7 m, n). aNAVXCR5⁺ cells were proposed to function by presenting antigens to CD4⁺ and CD8⁺ T cells (Fig. 8).

The unique cohort design of this study (despite small numbers) is a major strength of this manuscript which, together with an array of state-of-the-art genomic analysis to query differences between ACPA+ progressors and non-progressors, makes for an interesting story. Identification of increased aNAVXCR5⁺ B cells well before clinical RA is novel, as is the potential connection of these cells to B cell senescence. I am more circumspect, however, about the impact of these observations on the field of immune aging. In other words, is Nature Aging the appropriate journal for dissemination of these observations? There are a few lines in the Discussion about immune aging, autoimmunity etc., but most of the conclusions have little to do with aging.

Beyond this editorial point, several scientific questions need clarification.

1. Are aNAVXCR5⁺ cells CD69⁺ (cell surface)? They have CD69 in the CITE-seq panel, and the corresponding flow cytometry pattern should be shown. Identification of this population by flow cytometry would also permit screening of ACPA+ individuals for progression. Some flow analysis is shown in Fig. 7, but in this example the CD69⁺ aNAVXCR5⁺ population is not visible.

2. One of their key findings from the scRNA-Seq is that BCR proximal genes are up-regulated while signaling genes are

down-regulated (Fig. 2). But there is more going on that makes this conclusion somewhat facile. First, while BCR proximal genes are certainly up regulated in aNAVXCR5+ cells from converters compared to non-converters (last 2 columns in Fig. 2d), it is also evident that rNAVs from converters have especially high expression of the same genes (column 3, Fig. 2d). Relative to this population, BCR-proximal genes are down-regulated in converter aNAVXCR5+. The authors do not discuss at all the high levels of BCR-proximal genes in rNAVs exclusively from converters and the implications of this observation to their conclusions.

3. A senescence-like state of converters in aNAVXCR5+ is presented in Fig. 3, in part based on cell cycle regulators Cdkn1a and SASP components TGFβ1 and IL6. However, these genes are robustly (maximally?) expressed in non-converters (column 3, Fig. 3) and barely at all in converters (column 5, Fig. 3). What am I missing?

4. Figures 4 and 5 convey a lot of information about immunoglobulin expression and will be a useful resource for future studies. Additional clarification on two points will be valuable for a reader. First, I don't follow how only aNAVs from converters express IgM. All subsets shown in Fig. 4a and b are naïve B cells and should express IgM. Second, while the authors highlight PCA differences in heavy chain repertoire between converters and non-converters, there appear (to the eye) bigger differences in the light chain repertoire (Fig. 4g right). This is never discussed.

5. Figures 7 and 8 examine the origin of aNAVXCR5+ cells and their function, respectively. They show that rNAVs from RA patients convert to an aNAVXCR5+ phenotype over 2-day treatment with anti-IgM, citrullinated protein antigens (Cit-Ags) or CpG. I find it surprising that comparable numbers of rNAV cells convert in response to either pan activators (such as anti-IgM or CpG) and specific activators such as Cit-Ags. This implies that virtually all BCR-responsive cells bind and are activated by Cit-Ags. Is this possible? It is important to know whether this is also true of rNAVs isolated from healthy donors or is a special property of rNAVs in individuals with RA.

6. The same is true for senescence conversion in Fig. 7m. Do most BCR-responsive cells recognize Cit-Ags? Is this true for aNAVXCR5+ from non-RA patients? Since senescence is closely connected to cell division, it is important to know the cell division status of aNAVXCR5+ cells stimulated under these conditions for 7 days.

In summary, this data heavy manuscript describes an interesting subset of human B cells that correlate with progression of ACPA+ individuals to RA. However, much of the paper is descriptive and contributes little to understanding mechanisms that drive RA in vivo. Some attempts towards this end in Figures 7 and 8 are compromised by lack of healthy or non-RA controls. A substantially revised manuscript that cuts through data noise to focus on the most salient features that provide insights into disease mechanisms would be of value.

Reviewer #3

(Remarks to the Author)

A. Summary of the key results

The authors compare B cell populations prior to and following conversion to RA using single cell sequencing as their primary method. The authors identify differences in the activated Naïve B cell compartment when comparing aNAV from converters to non-converters, even prior to conversion. Notable differences include a number of metabolic and BCR signaling pathways, and perhaps cellular stress and cell cycle which the authors speculate to indicate senescence, however these pathways overlap considerably with pathways that are active in plasma cell differentiation and could instead indicate the cells are prone to differentiate (more comments below). The authors also include VDJ analysis and surprisingly find that most of the cells are singletons and not clonally expanded, even from the converters. This brings up a concern about lack of depth discussed at length below, but is nonetheless potentially interesting. The authors then note a difference in HLA-DR gene expression in joints, and perform elegant in vitro culture experiments demonstrating that aNAV cells can present antigen. While the authors implicate aNAV cells as being robust antigen presenting cells, there is not actually a direct comparison to rNAV cells as it stands. The data quality and breadth of analysis is very high for human studies, and these are rare human samples. I am quite positive about this study overall, but think that some additional analysis is required to support rather bold claims about cellular senescence versus competing alternative conclusions.

B. Originality and significance: if not novel, please include reference

I am not aware of any publications that reduce the novelty.

C. Data & methodology: validity of approach, quality of data, quality of presentation

Overall this is a well written manuscript, and generally easy to follow. One exception is that I do not understand what Figure 5f is a measurement of. How are "new influx" "arrested" and "differentiated" defined/determined? Please expand.

D. Appropriate use of statistics and treatment of uncertainties

Authors appropriately discuss the limitations of human studies with only roughly n=5 samples, but this is inherent to the depth of sequencing analysis being performed.

E. Conclusions: robustness, validity, reliability

Comments for this section and the next are intertwined, presented below.

F. Suggested improvements: experiments, data for possible revision

The authors suggest that the gene signature of the converters aNAV populations display anergy-like transcriptional profiles. However, the cells are activated and therefore are not anergic in the classical sense. Can the authors include a comparison to published data of bona fide anergic B cells?

An alternative explanation for cells to stop dividing would be that they've begun differentiating into plasma cells (PC), which would be consistent with upregulation of CD38 in the aNAVCD38hi population. The authors should rule out PC differentiation as an alternative conclusion. J-chain is not sufficient, it is up even in GC B cells.

Lack of clonal expansion in Figure 5 is potentially really interesting, as it suggests a type of EF response that is continuously recruiting newly activated cells as opposed to expansion. The later hypothesis would also be consistent with Fig. 4e,f. However, this could alternatively be explained by insufficient sequencing depth. Now that the authors have identified the

aNAV compartment as the cells of interest, it would be very interesting to go back and purify only those cells to achieve more depth per patient (or pool data from existing data sets), and see whether the observation that most cells are singletons holds true. In contrast, in Fig. 5e, many aNAVCXCR5+ clones continued to be aNAVCXCR5+ cells in the post sample, suggesting the opposite, that those clones continued to expand and potentially of high importance that this was a unique feature of the converters. The authors conclude that this is a feature consistent with senescence-associated developmental arrest, but it could be just the opposite. Further, Figure 5i demonstrates somatic hypermutation indicating that clone had to undergo several divisions, since cells undergo roughly one point mutation per division. Since this is a main conclusion of the paper, and highly relevant to generating a model of how B cell tolerance is broken, it is crucial to resolve this difference.

While Figure 8 is an elegant demonstration of B cell antigen presentation to T cells, there is not a huge difference between aNAV, rNAV, and CD27+ cells. The way the data are presented is always a single B cell population with different conditions, then the authors claim that aNAVs robustly activated while rNAV did not activate. The authors do not directly compare aNAV vs rNAV for the same condition and readout, and from the data spread I expect that if they did they would not find a significant difference of aNAV vs rNAV in most readouts (although %IFN γ may be an exception with a mean of ~1% aNAV and ~0.5% rNAV, which would add interest). This comparison should be performed, and while the results are interesting either way, the authors should discuss this and whether it is a potential limitation of the interpretations.

Line 164-166, authors conclude that CXCR5+ and CD38hi distinguish earlier and later stage aNAV cells, respectively. It is very difficult to tell whether this is the case from the pseudotime trajectory in Fig 1e, where it actually looks like green precedes light blue. It may be that there is a very small light blue population between orange and green, but with the colors and the relative abundance it is difficult to see. If that is the aNAV population, please change the colors to be easier to see, if not, please explain how the authors conclude the aNAVCD38hi population is the later stage and provide justification for that conclusion.

G. References: appropriate credit to previous work?

Another group has recently reported the expansion of aNAV cells in pre-RA patients and should be cited: PMID 39931747 "Rheumatoid Arthritis Related B-cell changes are found already in the risk-RA phase" de Vries et al 2025 EJI

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

There is not sufficient evidence that aNAVs were "senescent" and certainly not that they "served as potent antigen presenting cells to activate both anti-citrullinated antigen CD4+ and CD8+ T cells" (abstract lines 42-44) given that nowhere did the authors demonstrate the specificity of the T cells, and more importantly that the aNAV were not superior to other B cell types examined (rNAV and CD27+). The authors must tone down such conclusions or provide additional analysis to support such claims. Additional claims that are too forceful are:

lines 409-419

436 – 437 nothing to demonstrate they are "functionally stalled", rather the opposite

438-439 "prolonged circulation, impaired differentiation, and persistent presence in peripheral blood" was not tested and no citations are included

440-441 "stuck phenotype is driven by dysregulated B cell signaling shaped by chronic antigenic stimulation, anergy-like functional tuning" no actual comparisons to anergic B cells were included, dysregulated BCR signaling was not demonstrated

504 "anti-citrullinated antigen CD4 and CD8 T cell responses" these cells were not demonstrated to be citrullinated antigen-specific

573-574 "Our findings identify a pathogenic naïve B cell subset – senescent polyreactive" authors did not directly demonstrate that these are pathogenic, nor convincingly senescent. Tone it down to "potentially pathogenic".

It would be helpful if the authors put the concept of senescence (cessation of cell division) as it relates to progressive long term autoimmune disease into context. On its face, I would predict senescence to limit autoimmune disease since the cells would stop dividing and thereby limit the expansion of the population. And would exposure to citrullinated proteins then push cells out of senescence to enhance their proliferation?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The responses and revisions in the most recent version are substantial, effectively addressing the primary concerns of the reviewers.

Reviewer #3

(Remarks to the Author)

The authors have addressed all my concerns, and the new data and analyses strengthened the findings. That the singleton data held up to further enquiry is indeed intriguing. I thank the authors for their efforts. I will just point out that there is a duplication in the abstract; the line "A significant fraction..." is duplicated about 4 lines below.

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Author Rebuttals to Initial Comments:

Point-by-Point Response to Reviewers' Comments

June 01, 2026

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Reviewer #1 (Remarks to the Author):

A. Summary of the key results

B. Originality and significance:

The manuscript by Wu et al. seeks to understand how shifts in the early emigrants of the B cell compartment are associated with the progression of a seropositive (asymptomatic preclinical) adult females with circulating autoantibodies to citrullinated proteins is followed about 400 days later by a clinical state meeting criteria for the diagnosis of rheumatoid arthritis. From a large consortium of investigators, the investigators identify only five clinical converters, and findings from two timepoints are compared with those of five seropositive individuals who do not progress to a RA diagnosis within a similar time period. It is a controversial topic, and the manuscript may be too ambitious as it seeks to embrace too broad of a view. Additional cohorts of unrelated individuals are examined, but it is unclear if these are appropriate and well-matched for gender, ethnicity, and race. After RA diagnosis, do these subjects still lack exposure to therapeutic agents that are serious potential confounders.

Response: We thank Reviewer #1 for these thoughtful and constructive comments. In this revision, we have addressed these concerns by providing additional clinical information, refining the B cell subtype annotations, and substantially expanding the validation analyses.

Most importantly, we have now acquired new preclinical samples from our clinical collaborators and validated the elevation of CXCR5⁺ CD69⁺ activated naive B cells and their senescence-like phenotype in Converters, including both male and female subjects, by flow cytometry (**new Figure 7a-h**). In addition, we have further validated our major findings using a large, recently published scRNA-seq dataset from an independent preclinical RA cohort [1], including 67 ACPA⁺ Converters sampled before clinical RA onset and 35 ACPA⁺ Nonconverters (**new Figure 7i-r**). Together, these additional data provide strong support for our major conclusions and substantially reduce the concern that the original findings were driven by a small discovery cohort.

Regarding the Reviewer's concern about whether samples obtained after RA diagnosis could be confounded by therapeutic exposure, we have clarified this point in the Methods section (page 22, lines 862-863). At the time of post-onset sampling, four of the five Converters were not receiving RA-directed medication, whereas one participant had been prescribed leflunomide. Thus, the majority of post-onset Converter samples were collected before exposure to RA-directed systemic therapy. As a result, treatment exposure is unlikely to confound the conclusions drawn from these analyses.

There is a challenge of precise nomenclature and B-cell subset assignment. As for defining relevant B cell subsets, earlier schema exclusively used surface phenotype by flow cytometry, while the current study uses transcriptomics with ADT. Mixing in concepts and references from murine transgenic mice may not be helpful.

Response: We agree that classical flow cytometry has provided the foundation for defining human B cell subsets based on surface marker expression. However, conventional flow cytometry is limited by the number of markers that can be measured simultaneously and may not fully capture the transcriptional heterogeneity of the B cell compartment. In contrast, single-cell RNA-seq combined with CITE-seq enables broader assessment of transcriptional states and protein-level surface marker expression, while paired VDJ-seq allows integration of transcriptomic profiles with in-depth BCR repertoire profiling. This combined approach has been increasingly used to characterize B cell subsets and BCR landscapes in healthy and disease conditions [2-6]. Therefore, our study is not the first to use transcriptional features together with CITE-seq ADT-based surface markers to annotate B cell subsets. Nonetheless, this approach allowed us to identify distinct circulating B cell subpopulations, ranging from CD27⁻ IgM⁺ IgD⁺ naive B cell clusters to CD27⁺ memory B cell clusters.

We agree with the Reviewer that precise nomenclature and careful B cell subset assignment are important, especially when integrating transcriptomic, antibody-derived tag (ADT), and classical immunophenotyping information, with the goal of being able to compare results across studies. We have therefore refined our B cell annotation and nomenclature throughout the revised manuscript to avoid ambiguity and to ensure that each subset is defined using well-established human B cell markers. Naive B cell clusters were defined as CD19⁺ CD27⁻ IgM⁺ IgD⁺ TCL1A⁺. Within the naive compartment, resting naive and activated naive B cell clusters were distinguished by CD69 expression together with a well-established activation-associated transcriptional program, including NFKB1, NFKB2, JUN, CD83, FOS, and related genes, as shown in **new Figure 2a**. CD27⁺ B cell clusters were annotated as memory B cell subsets and further classified into three unswitched memory B cell clusters and one switched memory B cell cluster based on IGHM/IgM and IGHD/IgD expression measured by scRNA-seq and CITE-seq, together with BCR class-switch status determined by VDJ-seq. We also identified one atypical B cell cluster with a clear CD27⁻ IgD^{low} TBX21⁺ ITGAX⁺ CD21^{low} profile.

These annotations are based on well-established principles of human B cell immunophenotyping and are supported by our CITE-seq surface marker data. They do not rely on murine transgenic mouse concepts for subset definition. We have revised

the text accordingly and removed or minimized potentially confusing terminology, so that the updated annotation more clearly reflects human B cell biology in the context of this study. Please refer to the updated **Figure 1** and revised text on page 5, lines 143-161.

One especially notable issue is the usage of “transiting” and “transiting B cells”, which are a stage of differentiation that follows after late activated naïve B cells in a patient with an abnormal immune system. The authors do not seem to be referring to the well-accepted transitional B cells that are the B lineage cells that newly emerges from the bone marrow.

Response: We agree with the Reviewer that the term “transitional B cells” is conventionally used to refer to recent bone marrow emigrant B cells, and that our previous use of “transitioning B cells” could cause confusion. To avoid this ambiguity, we have removed the term “transitioning B cells” from the revised manuscript.

We have now re-annotated this cluster as unswitched memory B cell cluster 1 (UnswMem1), because these cells express CD27 and IgM, consistent with an unswitched memory B cell phenotype. In the revised annotation, we identify three unswitched memory B cell clusters that can be distinguished by their IgM and IgD expression patterns (**new Figure 1c, d**). UnswMem1 cells are CD27⁺ with high IgM and IgD expression; UnswMem2 cells are CD27⁺ with high IgM but reduced IgD expression; and UnswMem3 cells are CD27⁺ with reduced expression of both IgM and IgD.

We hope that this revised annotation clarifies that these cells are not intended to represent classical transitional B cells newly emerging from the bone marrow, but rather a subset within the CD27⁺ unswitched memory B cell compartment. We have updated the relevant text (page 5, lines 143-161) and figures accordingly.

The authors refer to “atypical” and “ABC”, while the latter can refer to Aging-associated B cells. These are now confusing terms that come from very different studies. These B cell subsets may correspond to the CD19⁺ CD27⁻ IgD⁻ double-negative B cells that may be referred to as a DN1 subset, although this is controversial. Others have referred to an expansion of the DN2 subset in RA patients, but have “transiting B cells” been previously described (see below? Arguably, this manuscript might have greater clarity if the phenotype (or transcriptomic pattern) is first used to name each subset, and this descriptor is then used in the text. The commentary recently reported in Immunity might be relevant to consider this controversial area (Eisenbarth et al. Immunity November 2024).

Response: We thank the Reviewer for this constructive suggestion. We agree that the terminology used to describe atypical B cells, ABCs, and double-negative B cell subsets remains controversial and can cause confusion, particularly because these terms have been used differently across studies and disease contexts. As discussed in a recent review by Professor Iñaki Sanz [7], even the term “atypical B cells” remains debated, as accumulating evidence indicates that these cells represent a normal component of human immune responses rather than a purely abnormal or disease-specific population.

Now we have avoided using “ABC” to refer to atypical B cells in the revised manuscript, because “ABC” may be interpreted as age-associated B cells, activated B cells, or atypical B cells depending on the context. Instead, we now use the more descriptive term “atypical B cells” and define this cluster based on its observed transcriptomic and surface marker profile.

The atypical B cell cluster identified in our study shows a clear CD27⁻ IgD^{low} TBX21⁺ ITGAX⁺ CD21^{low} profile, consistent with commonly used markers of human atypical B cells. Although these cells had downregulated CXCR5 expression, we did not annotate them as double-negative 2 (DN2) B cells because these cells retain low-level IGHD/IgD expression (**new Figure 1c, d**). Therefore, this cluster may be heterogeneous and may include DN2-like cells together with some recently activated naive-like B cells. For this reason, we chose a conservative annotation based on the marker profile observed in our own data.

Defining the full biological identity of atypical B cells is not the major aim of the current study. Our goal was to identify B cell-related differences between ACPA⁺ Converters and Nonconverters at the preclinical at-risk stage. In this analysis, regardless of whether this CD27⁻ IgD^{low} TBX21⁺ ITGAX⁺ CD21^{low} cluster is annotated as atypical B cells or another related DN-like subset, it was not significantly altered at the ACPA⁺ at-risk stage. In contrast, the major disease-associated signal was observed in the IgM⁺ IgD⁺ activated naive B cell compartment.

We have updated the relevant text on page 5, lines 143-161, revised the figures accordingly, and cited both the Sanz review and the Reviewer-suggested Eisenbarth et al. review.

On this topic, in the title “transition” may be mixing clinical with the name of a B cell subset and further obscuring the intent, which in the title might better be described as “disease progression”.

Response: We thank the Reviewer for this helpful suggestion. We agree that the term “transition” in the title could be confusing, as it may be interpreted as referring either to clinical disease transition or to a transitional B cell state. To avoid this ambiguity, we have revised the title to:

“Senescence-like Activated Naive B Cells Contribute to the Progression to Clinical Rheumatoid Arthritis.”

This revised title more clearly emphasizes disease progression rather than B cell subset transition. It also more accurately reflects the scope of our findings. First, we use “senescence-like” because these activated naive B cells show a clear senescence-associated program, but we cannot conclude that they are fully senescent or irreversibly cell-cycle arrested. Second, we use “contribute to the progression to clinical RA” rather than “promote citrullinated antigen-specific T cell responses” because, although our B-T cell co-culture experiments support enhanced T cell activation, we did not directly test

TCR specificity in these assays. We believe the revised title is more precise, conservative, and aligned with the data presented in the manuscript.

The authors use accepted criteria that a preclinical state to an overt clinical condition can be met if there is >1 swollen joint. Yet do these five individual Converter subjects develop this polyarthritis disease? How many swollen and tender joints does each develop?

Response: We thank the Reviewer for raising this important clinical point. We would like to clarify that our criterion for clinical RA conversion was not based on >1 swollen joint, but rather on clinical evaluation showing ≥ 1 swollen joint consistent with inflammatory arthritis (IA), as assessed by a rheumatologist or trained study nurse.

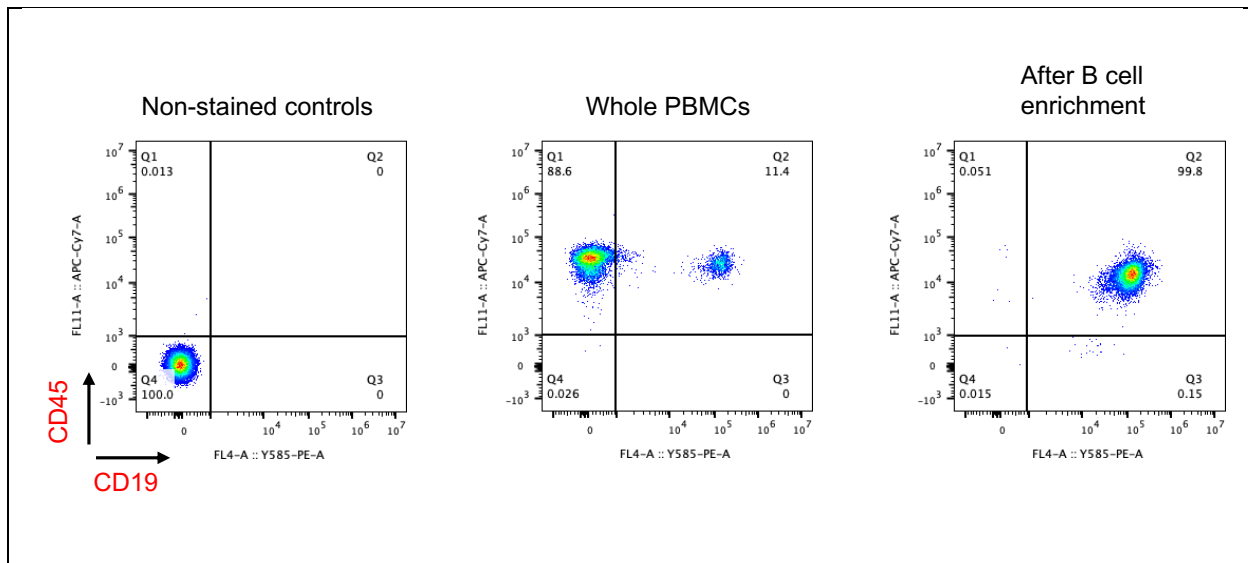
We have now provided the numbers of swollen and tender joints for each Converter in the initial exploratory cohort in Supplementary Table 1. All five Converter subjects met the clinical RA conversion criteria used in this study. We have also clarified this point in the revised Methods section to avoid confusion (page 22, lines 873-875).

C. Data & methodology: validity of approach, quality of data, quality of presentation

A key area of uncertainty is shown in the representative UMAP in Figure 1b, which shows subsets for B cells (are these all assigned based on the CD19 transcript?). ”

Response: Regarding the Reviewer’s question about Figure 1b, specifically whether the B cell subsets were assigned solely based on CD19 transcript expression, we would like to clarify that the cells shown in Figure 1b were generated from B cell-enriched samples. Before single-cell RNA sequencing, we used a commercial magnetic bead-based B cell enrichment kit (STEMCELL Technologies, Cat#100-0971) to enrich B cells from PBMCs. This strategy was chosen because B cells account for only a minority of total PBMCs, and enrichment allowed us to obtain sufficient sequencing depth for both B cell transcriptomic and BCR repertoire analyses.

Therefore, the cells shown in Figure 1b were not assigned as B cells solely based on CD19 transcript expression. Before sequencing, we evaluated the efficiency of the B cell enrichment procedure by flow cytometry (**Response Figure 1**). As shown in the figure below, after enrichment, the majority of isolated cells showed strong surface CD19 protein expression. During initial single-cell data processing, a very small number of contaminating non-B cells, including T cells and monocytes, were detected based on strong expression of lineage markers such as CD3 or CD14 together with low or absent CD19 expression. These contaminating cells were removed during quality control before downstream B cell subset annotation and analysis.



Response Figure 1. Validation of the human B cell enrichment strategy.

One PBMC sample from a healthy control was used to test the efficiency of the human B cell enrichment kit based on negative selection. Cells were stained for CD45 and CD19, and an unstained control was used to establish the baseline signal. After B cell enrichment, most enriched cells were CD45+CD19+, confirming the efficiency of this approach.

In Converters, aNAVs constituted 43.01+/- 5.91% of B cells at the pre-clinical RA stage and 38.55%+/-7.60% post onset.” What was the representation of this subset in healthy controls? Three healthy controls seem inadequate to distinguish progressions in the disease state from what might truly be inter-individual variation. These values seem too high for all B lineage CD19+ cells in the blood, even after negative B cell selection, as shown in UMAP in Figure 1b. If this is true, a supplemental figure should show 5 individuals at pre- and post-conversion timepoints, as well as show the five non-converters, and also show the variation between healthy controls.

Response: We have now added a description of the percentage of the aNAV^{CXCR5+} cluster in healthy control samples (page 5, lines 170-172):

“In healthy controls, aNAV^{CXCR5+} cells constituted $7.68 \pm 4.38\%$ of B cells at the first timepoint and $8.38 \pm 9.49\%$ at the second timepoint.”

These values were significantly lower than those observed in Converters ($43.01 \pm 5.91\%$ at pre and $38.55 \pm 7.60\%$ at post) and were comparable to those observed in Nonconverters ($11.34 \pm 5.10\%$ at pre and $10.68 \pm 9.09\%$ at post).

We would also like to clarify that the original Fig. 1f already shows intra-group variation, as each dot represents one participant. We agree that three healthy controls represent a relatively small sample size for comparison. However, this analysis is based on single-cell RNA sequencing, in which thousands of B cells were analyzed from each sample, rather than a single bulk measurement per participant. In addition, each participant was sampled at two independent timepoints. The frequency of each B cell cluster, including the aNAV^{CXCR5+} cluster, was relatively stable and comparable across both timepoints in

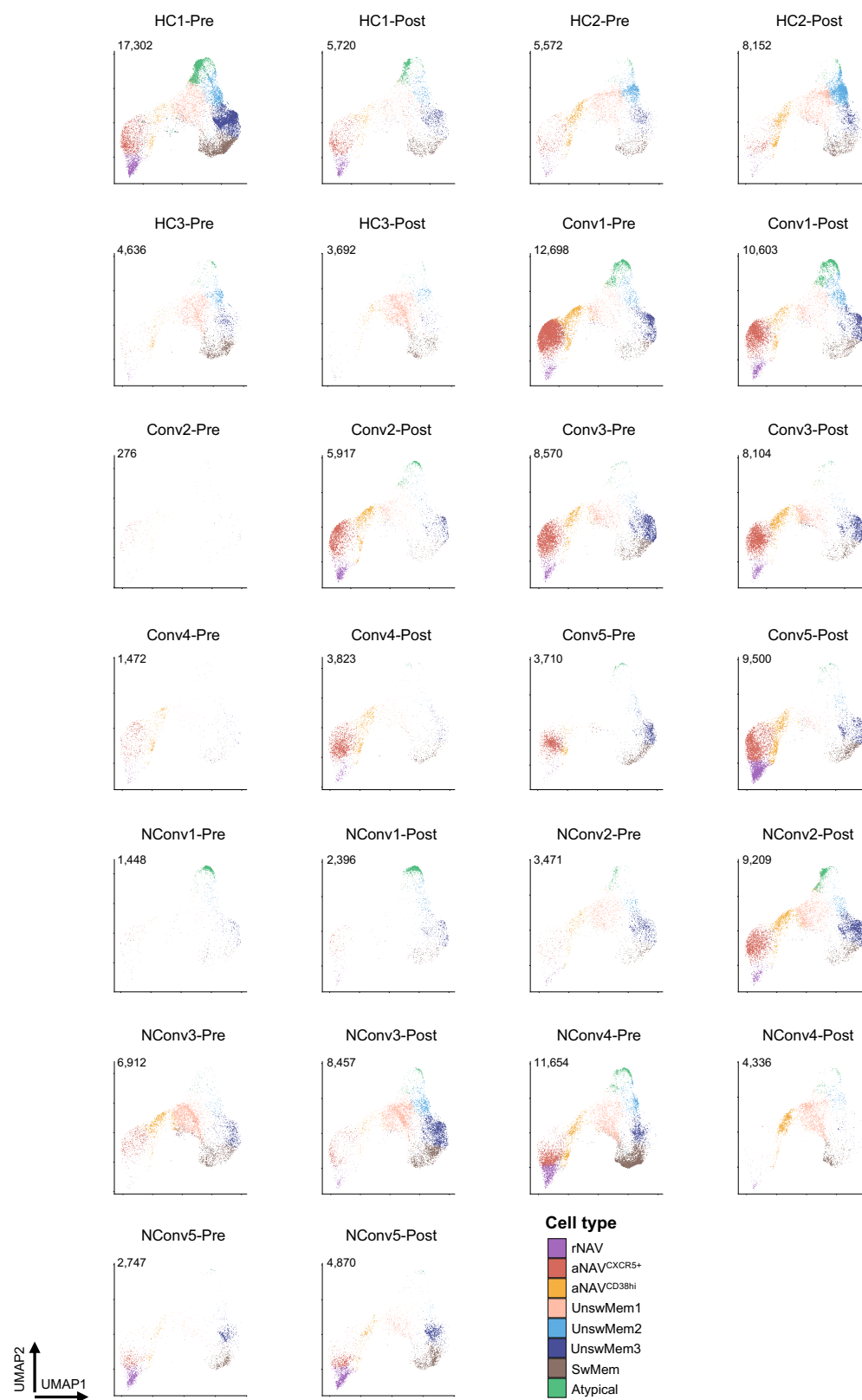
healthy controls and Nonconverters. Therefore, although there were three healthy control participants, the baseline frequency of the aNAV^{CXCR5+} cluster is supported by six independently sequenced healthy control samples across two timepoints, as well as ten independently sequenced Nonconverter samples across two timepoints. This supports that the marked expansion of aNAV^{CXCR5+} cells identified in Converters is specific rather than simply reflecting general inter-individual variation.

Regarding the reviewer's concern that "these values seem too high for all B lineage CD19⁺ cells in the blood, even after negative B cell selection," we would like to clarify that the increased percentage of aNAV^{CXCR5+} cells in Converters was defined primarily by their transcriptional states. Therefore, the absolute percentage may differ from values obtained by previous flow cytometry analysis using surface protein markers. Indeed, in our new validation dataset, flow cytometry confirmed that the percentage of CXCR5⁺ CD69⁺ naive B cells was significantly increased in Converters at the preclinical stage. However, the magnitude of increase detected by flow cytometry (~ one-fold increase) was more modest than that observed in the initial scRNA-seq analysis (~ 2-3 folds increase). This difference may reflect differences between mRNA-level transcriptional clustering and protein-level surface marker detection. Thus, the percentages derived from scRNA-seq clustering should not be directly compared with those from conventional flow cytometry-based analyses.

We have also added a new supplemental figure showing the UMAP and B cell number for each individual sample. Please refer to the **new Extended Data Fig. 1**. We note that, in scRNA-seq experiments, the total number of recovered cells per sample can vary due to differences in the initial PBMC input, B cell isolation efficiency, and technical factors during 10x loading and library preparation. For example, during processing of the Conv2-Pre sample, a clog occurred in the 10x scRNA-seq instrument. Although the sample was reloaded, a substantial number of B cells were lost. Importantly, this technical issue affected the total number of recovered B cells, but should not affect the B cell composition or per-cell transcriptomic information of the recovered cells. Moreover, in our scRNA-seq analysis, we performed standard quality control and batch correction to reduce technical variation and ensure that samples could be integrated and compared appropriately.

In the individual UMAP plots, although the total number of recovered B cells varied across samples, the aNAV^{CXCR5+} cluster was consistently more frequent in Converter samples, including Conv2-Pre.

Extended Data Fig. 1



New Extended Data Fig. 1. UMAP visualization of B cells from individual samples. UMAP plots of B cells are shown for each sequenced sample, including both pre- and post-onset timepoints. Pre indicates the first sampling timepoint, and post indicates the second sampling timepoint. The total number of B cells included for each sample is shown in the upper-left corner of each UMAP. HC, healthy control; Conv, Converters; NConv, Nonconverters.

In Figure 1f, what are each of these bars?

Response: As explained above, Fig. 1f shows the composition of each B cell subcluster within each sample. The percentage was calculated by dividing the number of cells in each cluster by the total number of B cells from the same sample. Each dot represents one participant, and each bar represents one condition at one timepoint. Thus, this analysis shows both inter-individual variation within each group and the consistency of the B cell composition across two independently sequenced timepoints.

For example, in the rNAV panel, the first bar labeled HC-pre indicates the percentage of rNAV cells among total B cells in each healthy control at the first time point. Similarly, the other bars represent the corresponding percentages for each condition and time point, including Converters and Nonconverters at their baseline and follow-up visits.

We have revised the Methods section (page 24, lines 949-953) and Figure 1 legend (page 32, lines 1195-1198) to clarify how these percentages were calculated and what each bar represents. We hope this clarification makes the figure easier for readers to interpret.

A large number of individual transcripts are mentioned, and it is uncertain which have been validated in the literature for identifying lymphocyte subsets in health and disease.

If this becomes a central finding, the text might better focus on Kruppel-like factors (KLF) transcript expression, and the biological context.

Response: We thank the Reviewer for this helpful suggestion. In this study, we report the preclinical expansion and activation of activated naive B cells (aNAV) in ACPA⁺ Converters, but not in ACPA⁺ Nonconverters. We identified several key features of this disease-associated aNAV^{CXCR5⁺} population based on its distinct transcriptional profile.

First, we found that aNAV^{CXCR5⁺} cells from Converters were hyperactivated at the preclinical stage, as indicated by significantly increased expression of genes associated with inflammatory and activation programs, including FOS, JUN, NFKB1, CD69, and related genes. Second, we found that aNAV cells from Converters acquired a senescence-like transcriptional program at the preclinical stage, with increased expression of genes associated with cellular senescence, stress responses, and cell-cycle suppression, including CDKN1A, BTG2, YPEL5, and PLK3. These genes have established roles in immune activation, cell-cycle regulation, and senescence-related

pathways. For example, FOS, JUN, NFKB1, and CD69 are classic immune activation genes. CDKN1A and BTG2 are well-established cell-cycle suppressors [8, 9], YPEL5 has been implicated in the p53-associated senescence pathway [10], and PLK3 has been included in a reported core senescence-associated transcriptomic signature and linked to DNA damage checkpoint and cell-cycle regulatory pathways [11]. We have added additional information and references regarding these senescence-related genes in the revised text on page 7, lines 239-243.

We would like to clarify that these transcripts were not used as primary markers to define lymphocyte subsets in this study. Instead, B cell subsets were annotated using well-established human B cell markers, as described above. The individual transcripts highlighted in the Results section were used to interpret the functional state of the disease-associated aNAV^{CXCR5+} population, rather than to assign B cell subset identity.

We agree with the Reviewer that the Krüppel-like factor (KLF) family provides important biological context and may help interpret this senescence-associated transcriptional program. We have now included the expression level of KLF4 for new Figure 3h, showing increased expression in Converters compared with Nonconverters at the preclinical stage (0.408 ± 0.173 versus 0.160 ± 0.120 , $P < 0.05$). We also confirmed a significantly elevated frequency of KLF2+ aNAV cells in preclinical Converters compared with Nonconverters and healthy controls in our new validation samples ($7.39 \pm 5.00\%$ in preclinical Converters versus $1.42 \pm 2.15\%$ in Nonconverters and $1.02 \pm 1.30\%$ in healthy controls, $P < 0.05$), as indicated by flow cytometry in **new Figure 7h**.

The increased expression of KLF family genes in converter aNAV cells is intriguing because several KLF factors have been implicated in cell-cycle regulation, B cell differentiation, and maintenance of specific B cell states. First, multiple KLF family members have been reported to function as tumor suppressors, suggesting potential roles in regulating cell proliferation [12-18]. Second, previous studies have shown that KLF2 helps maintain a circulation-competent pre-GC B cell phenotype by repressing germinal center entry [19, 20]. This may help explain why aNAV^{CXCR5+} cells, despite expressing CXCR5 (a marker associated with follicular positioning and germinal center entry), remain accumulated in the circulation. KLF4 has also been reported to act as a brake on naive B cell proliferation [21-23], its sustained expression may reinforce B cell senescence. KLF6, although less studied in B cells, has been reported to be upregulated in activation-prone B cells in celiac disease and co-expressed with CD69, FOS, and JUN, a transcriptional signature also enriched in converter aNAV cells [24]. These functions are consistent with the senescence-like phenotype observed in converter aNAV cells.

At this stage, we do not have direct functional evidence demonstrating that these KLF genes play a causal role in the pathogenic accumulation of aNAV cells in ACPA⁺ Converters. We have therefore discussed the biological context of KLF family genes more cautiously and highlighted the need for future studies to determine whether KLF-mediated transcriptional regulation contributes to altered B cell homeostasis and disease progression (page 15, lines 590-604).

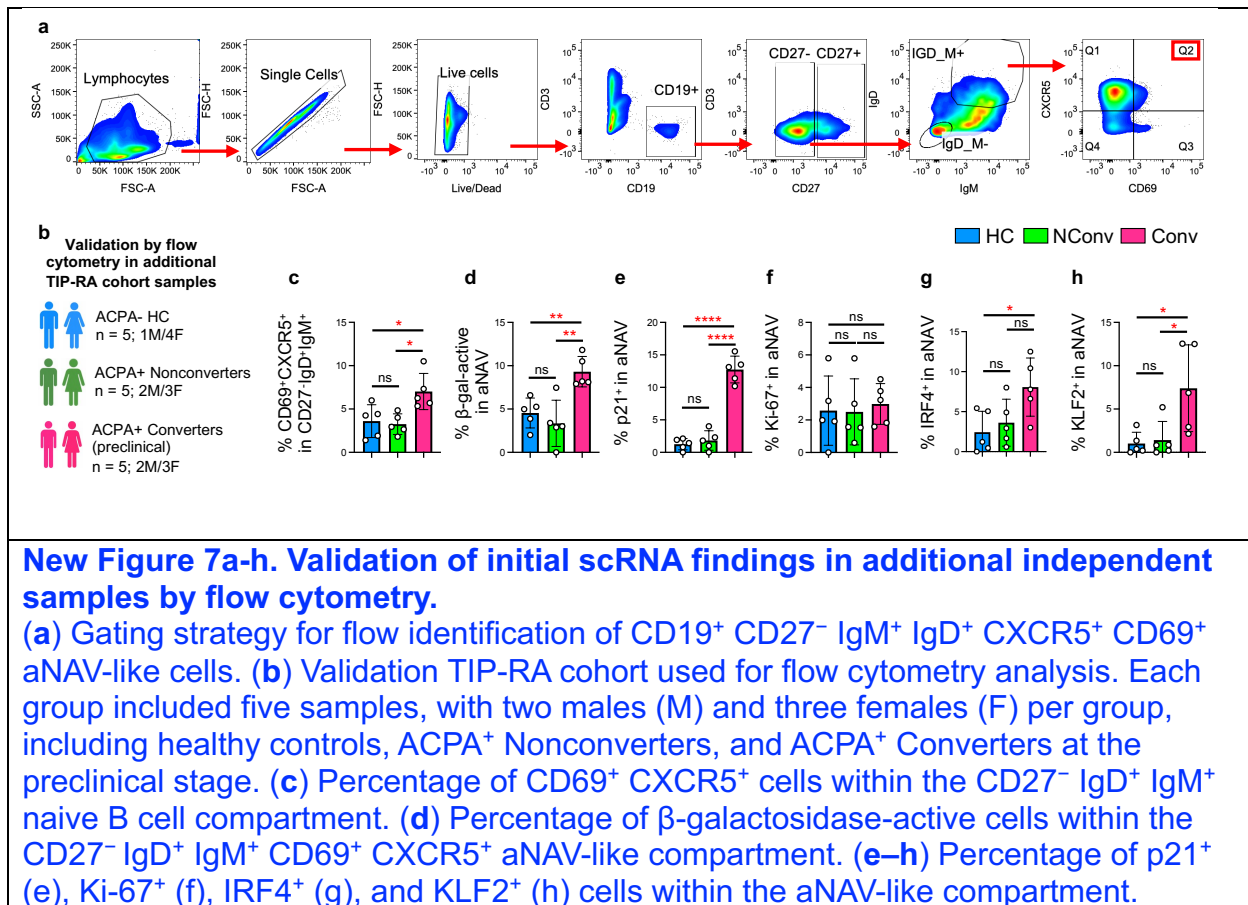
As “senescence” is key, how can this assignment be solely based on β -galactosidase expression?

Response: We appreciate the Reviewer’s concern and would like to clarify that the senescence-like phenotype described in this study was not based solely on β -galactosidase expression. In the exploratory cohort, we identified a senescence-like phenotype in disease-associated aNAV^{CXCR5+} cells based on multiple lines of evidence.

First, Converter aNAV^{CXCR5} cells showed broad downregulation of energy metabolism-related pathways, a feature commonly associated with cellular senescence (original Figure 3a). Second, aNAV cells from Converters showed significant enrichment of transcriptional programs related to cellular senescence and cell-cycle arrest (original Figure 3b, c). Third, aNAV cells expressed increased levels of senescence-associated secretory phenotype (SASP)-related genes, including IL6 and TGFB1 (original Figure 3b). Forth, several key senescence-related regulatory genes, particularly CDKN1A and BTG2, were consistently elevated in all five Converters compared with Nonconverters at the preclinical stage (original Figure 3d–h).

Because access to the preclinical samples was limited, in the original manuscript we further evaluated whether aNAV cells from clinically established ACPA⁺ RA patients also showed a senescence-like phenotype using a commercial flow cytometry-based senescence assay (Invitrogen, #C10840). This kit measures β -galactosidase activity, a classic marker of cellular senescence. Using this assay, we found that ACPA⁺ RA patients had an increased frequency of β -galactosidase-active cells within the aNAV population (original Figure 7k, l), consistent with the transcriptomic findings from preclinical RA samples. Together, these data suggest that aNAV B cells may acquire a senescence-like phenotype during progression to clinical RA.

During this revision, we obtained additional preclinical samples from our clinical collaborators, including both male and female subjects, and further validated the expansion of aNAV-like cells and their senescence-like phenotype by flow cytometry (**new Figure 7a, b**). These new data confirmed that, CD19⁺ CD27[−] IgM⁺ IgD⁺ CXCR5⁺ CD69⁺ aNAV-like cells can be detected by flow cytometry, and were significantly elevated in preclinical ACPA⁺ Converters compared with Nonconverters and healthy controls (**new Figure 7c**). β -galactosidase-active aNAV cells were significantly increased in Converters compared with Nonconverters and healthy controls (**new Figure 7d**). More importantly, p21/CDKN1A expression was dramatically higher in Converter aNAV cells than in the corresponding populations from Nonconverters and healthy controls (**new Figure 7e**).



New Figure 7a-h. Validation of initial scRNA findings in additional independent samples by flow cytometry.

(a) Gating strategy for flow identification of CD19⁺ CD27⁻ IgM⁺ IgD⁺ CXCR5⁺ CD69⁺ aNAV-like cells. (b) Validation TIP-RA cohort used for flow cytometry analysis. Each group included five samples, with two males (M) and three females (F) per group, including healthy controls, ACPA⁺ Nonconverters, and ACPA⁺ Converters at the preclinical stage. (c) Percentage of CD69⁺ CXCR5⁺ cells within the CD27⁻ IgD⁺ IgM⁺ naive B cell compartment. (d) Percentage of β -galactosidase-active cells within the CD27⁻ IgD⁺ IgM⁺ CD69⁺ CXCR5⁺ aNAV-like compartment. (e–h) Percentage of p21⁺ (e), Ki-67⁺ (f), IRF4⁺ (g), and KLF2⁺ (h) cells within the aNAV-like compartment.

Taken together, the transcriptomic, metabolic, SASP-related, β -galactosidase, p21 protein, and additional sample validation data provide strong evidence that aNAV cells can acquire a senescence-like program at the preclinical and onset stages of RA. We have revised the manuscript to use the term “senescence-like” more precisely and to clarify that this phenotype may contribute to progression toward clinical RA.

Don't healthy adults have late-activated naïve B cells, and how is this different from the notion that clones of naïve B cells arise from low-level “tickling” of BCR, without sufficient co-stimulation/T cell help/cytokine stimulation?

Response: We agree with the Reviewer that late-activated naïve B cells can be present in healthy adults and that low-level BCR “tickling,” in the absence of sufficient co-stimulation, T cell help, or cytokine stimulation, may contribute to basal activation within the naïve B cell compartment. This is an important point, and it is one reason why we included healthy controls as a baseline reference group in our analysis.

Indeed, the aNAV^{CXCR5⁺} cluster can be detected in healthy controls and Nonconverters, with a frequency of approximately 5–10% of the total B cell compartment based on transcriptional clustering. This frequency was relatively consistent across healthy controls and Nonconverters, likely reflecting a basal level of naïve B cell activation. However, our data suggest that the aNAV^{CXCR5⁺} population observed in ACPA⁺

Converters is distinct from this physiological low-level activation state. Specifically, the transcriptional profile of aNAV cells from Nonconverters was similar to that of healthy controls, whereas aNAV cells from Converters showed a distinct disease-associated transcriptional program (**new Figure 2b-d**). Converter aNAV^{CXCR5⁺} cells expressed significantly higher levels of immediate early response genes, including FOS and JUN, together with downstream immune activation genes such as NFKB1 and NFKB2, as well as a series of stress-response genes, such as DNAJA1/DNAJB1 (**new Fig. 2d, e**). This degree of coordinated immune activation and stress-response gene expression is unlikely to reflect only normal low-level tonic BCR signaling.

Therefore, we do not interpret the presence of aNAV cells as inherently pathological. Rather, our findings suggest that, in ACPA⁺ Converters, this normally present activated naive-like compartment becomes quantitatively expanded and transcriptionally reprogrammed, acquiring a stronger inflammatory and senescence-like state that distinguishes it from the corresponding population in Nonconverters and healthy controls. We have added a new discussion of this point in the revised manuscript (page 17, lines 642-652).

D. Appropriate use of statistics and treatment of uncertainties

Methods are appropriate however there is a concern, as many central findings are based on trends rather than statistically significant correlations.

Response: We acknowledge the Reviewer's concern. Some findings, such as the increased BCR diversity and higher percentage of unique clones in Converter aNAV cells at the preclinical stage, were observed as trends but did not reach statistical significance in this exploratory cohort. We agree that these results should be interpreted cautiously.

We believe that the lack of statistical significance is likely related to the limited sample size of the discovery cohort. We have also acknowledged this limitation in the Discussion (page 20, lines 791-796) and emphasized that larger independent cohorts will be needed to determine whether these trends are statistically significant and biologically reproducible.

E. Conclusions: robustness, validity, reliability

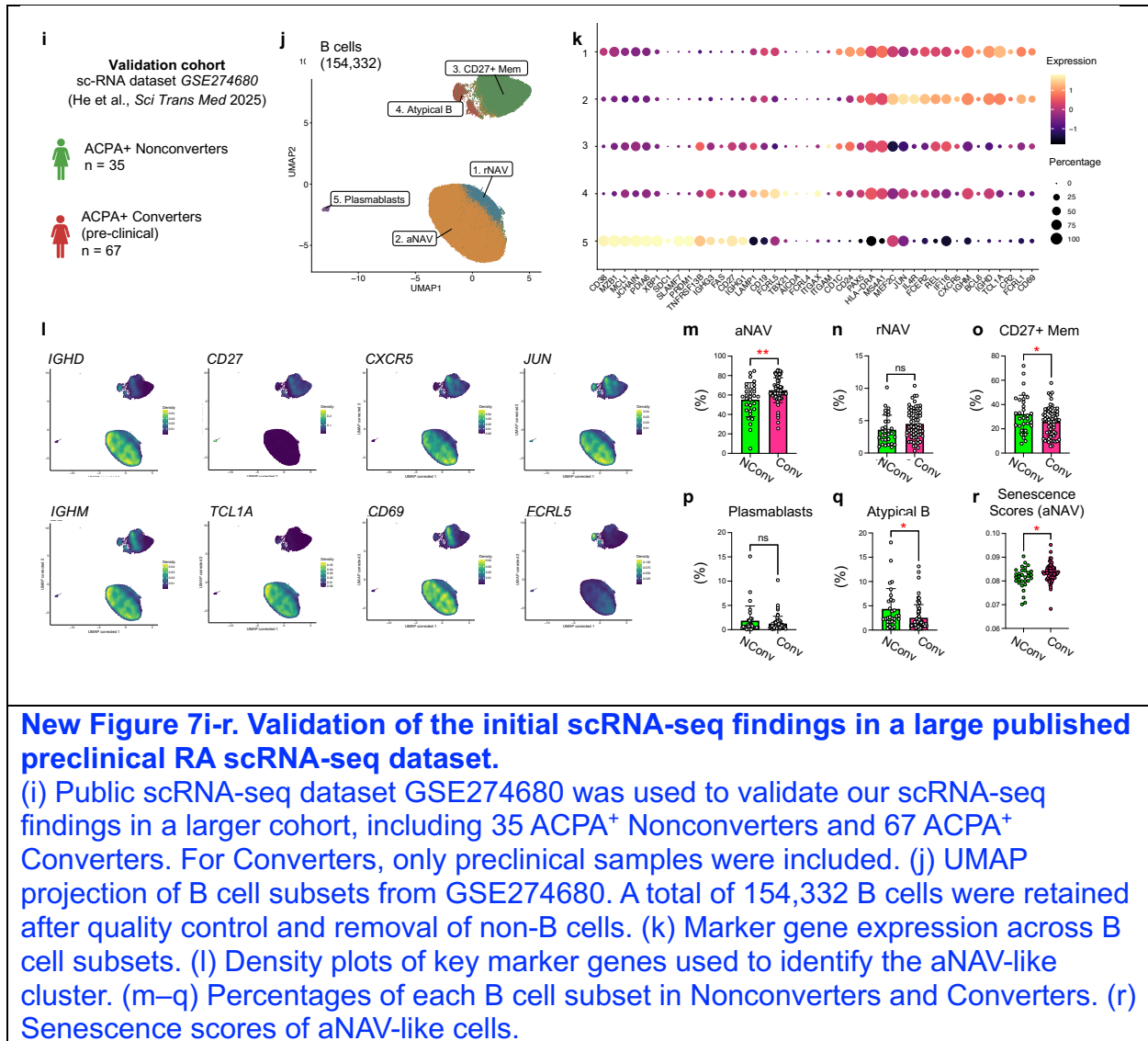
The authors state that they closely examine the samples from two timepoints for each subject, which are ~400 days apart. Did the authors intentionally ignore the samples they have accumulated from other time points? And many other subjects. Is this manuscript an exercise in cherry-picking?

Response: We respectfully disagree with the characterization of this study as cherry-picking. Cherry-picking implies that all available data were known in advance and that only a selective subset was reported to support a predetermined conclusion. This was certainly not the case in our study.

Our original study was designed prospectively as an exploratory single-cell RNA-seq analysis to identify B cell-related differences between ACPA⁺ Converters and ACPA⁺ Nonconverters, or between ACPA⁺ RA pre-clinical and post-onset stage. At the time of study design and sample selection, we did not know what biological differences would emerge from the sequencing data. We selected two time points and three comparison groups to enable assessment of both disease progression over time and differences between individuals who did or did not progress to clinical RA. In fact, before conducting the study, we expected that the major differences might occur between the preclinical and post-onset time points within Converters. The data instead revealed that the dominant differences had already emerged between Converters and Nonconverters at the preclinical stage.

We did not intentionally ignore additional time points or subjects. Because our integral single-cell immune profiling (scRNA + CITE-seq + BCR sequencing) are very costly and resource-intensive, we were only able to sequence a limited number of samples in this exploratory study. We therefore selected a carefully matched cohort using predefined criteria, including age, sex, ACPA positivity, absence of inflammatory arthritis at baseline, subsequent progression status, timing relative to RA onset, and availability of paired samples. These selection criteria were established before sequencing and analysis.

A recent *Science Translational Medicine* study by He et al. [1] reported preclinical activation of naive B cells in ACPA⁺ Converters, supporting the major findings of our study. This independent dataset included transcriptomic profiling (without VDJ-seq) of a substantially larger cohort. During this revision, we validated our major findings by re-analyzing this published dataset, which contains single-cell RNA-seq data from B cells of 67 ACPA⁺ Converters and 35 ACPA⁺ Nonconverters (**new Figure 7i**).



Consistent with our discovery cohort, the percentage of aNAV cells was significantly higher in Converters than in Nonconverters at the preclinical stage, whereas CD27⁺ memory B cells and atypical B cells were lower (**new Figure 7j–q**). More importantly, in this larger cohort, senescence scores calculated using the same senescence-related gene set as in our exploratory study were also significantly higher in Converter aNAV cells than in their counterparts from Nonconverters (**new Figure 7r**), a finding not reported in the original *Science Translational Medicine* study. Thus, our study not only validate the preclinical activation of naive B cells reported by He et al., but also add new insight by showing that disease-associated B cells may acquire a senescence-like transcriptional program during progression toward clinical RA.

In addition, in this revision, when we obtained the new independent preclinical samples from our clinical collaborators, the group information was blinded until the flow cytometry validation was completed. These validation data confirmed that the Converter group could be clearly separated from the Nonconverter and healthy control groups

based on the increased frequency and senescence-like phenotype of aNAV^{CXCR5+} cells. We hope these clarifications help address the reviewer's concern.

CAR T cell therapy does not need to be revisited three or more times throughout the manuscript. The adoption of CAR T cells from cancer therapy does not provide an approach that builds on findings that are novel in the current manuscript. The first paragraph of the discussion might better solely focus on what is novel here.

Response: We agree with the Reviewer that repeated discussion of CAR-T cell therapy distracted from the main findings of the manuscript and did not directly build on the novel observations reported here. We have therefore substantially reduced the discussion of CAR-T cell therapy in the revised manuscript and refocused the Discussion on the novel findings of this study, including the preclinical expansion of aNAV cells, their senescence-like phenotype, and their potential contribution to progression toward clinical RA.

The somewhat tautologic presentation on BCR binding specificity, poly-specificity, and broad reactivity vs. definable fine specificity. Arguably, much of this reactivity here involves recognition of citrulline-modified de-aminated polypeptides. Net positive charge in HCDR3 may correlate with polyreactivity, but certainly there must be other contact residues in these paratopes.

Response: We agree with the Reviewer that the description of original Figure 4 in the Results section was somewhat redundant, particularly because both original Figure 4h and original Extended Data Figure 4 address the broader and stronger antigen reactivity of antibodies derived from Converter aNAV IgM sequences compared with those from Nonconverters.

We would like to clarify that original Figure 4h represents a broad ELISA screening performed at a single antibody concentration across the antigen panel, whereas original Extended Data Figure 4 shows antibody titration assays performed for selected representative antibodies based on the screening results from Figure 4h. Thus, the extended data provide additional validation of binding strength rather than an independent repeated analysis of the same result. We have revised the Results section to reduce redundancy (page 8, line 305).

Regarding the Reviewer's comment that "much of this reactivity here involves recognition of citrulline-modified deaminated polypeptides" and that "there must be other contact residues in these paratopes," we agree that the net charge of HCDR3 alone is unlikely to fully explain the broad polyreactivity observed in these antibodies. We have therefore revised the Discussion (page 18, lines 704-708) to clarify this point. Specifically, we now state:

"However, CDR3 charge alone is unlikely to fully explain the broad polyreactivity observed in these antibodies. Other structural and sequence features, including CDR3 length, conformational flexibility, germline V and J gene usage, light-chain pairing, and

additional contact residues within the antibody paratope, are also likely to contribute to antigen binding across both native and citrullinated antigens.”

We hope this revision better acknowledges that polyreactivity and RA-associated antigen recognition likely depend not only on the net charge of HCDR3, but also on additional structural features of the antibody paratope.

The repertoire analysis is intriguing, but it is unclear whether these patterns of difference are meaningful.

Response: We acknowledge the Reviewer’s comment that the repertoire analysis is intriguing, and we agree that the biological meaning of these patterns should be interpreted carefully.

By performing paired BCR repertoire analysis of matched preclinical and post-onset samples from ACPA⁺ Converters and Nonconverters, we aimed to examine how aNAV-associated BCR lineages evolve over time in these two distinct at-risk groups. Specifically, we subset IgM⁺ aNAV BCRs at the first time point and then tracked related BCRs at the second time point based on similar heavy-chain sequences.

This analysis revealed different longitudinal patterns between Converters and Nonconverters. In Nonconverters, most IgM⁺ aNAV-related BCRs identified at the first time point were later detected in more differentiated B cell states at the second time point. In contrast, in Converters, IgM⁺ aNAV-related BCRs were more frequently retained within the aNAV compartment over time. These findings suggest that Converter aNAV BCR lineages may undergo limited progression into later B cell states during this time interval, consistent with the senescence-like and differentiation-restrained phenotype observed in our transcriptomic analyses. However, we acknowledge that these data do not directly demonstrate developmental arrest or anergy.

We have revised the manuscript to present these repertoire findings more cautiously and to clarify that they provide supportive evidence for altered BCR lineage dynamics in Converters, rather than definitive proof of developmental arrest or anergy tuning. We also acknowledge that in vitro B cell differentiation assays will be needed to directly determine whether pathogenic aNAV cells exhibit true anergy-like functional tuning or developmental arrest (page 15, lines 566-579).

F. Suggested improvements: experiments, data for possible revision

As referred to above.

G. References: appropriate credit to previous work?

Too many reviews and commentaries from the authors.

Response: We have reduced the citations of review and commentaries from the authors.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

As mentioned above.

Reviewer #2 (Remarks to the Author):

Review of Wu et al.

From the TIP-RA project the authors compared B cell subsets and their properties in 10 individuals, all of whom had anti-citrullinated protein antibodies (ACPA+), but only 5 progressed to clinical RA over a 2-year period whereas the other 5 did not. Additionally, they had 3 healthy controls that were ACPA- and remained asymptomatic. The study design, thereby, enabled identification of markers that could be used to distinguish between progressors and non-progressors. Applying single cell RNA-seq to peripheral blood B cells they identified a subset of CXCR5⁺ CD38^{lo} cells that were selectively increased in ACPA+ progressors but not in non-progressors (Fig. 1). This subset (named aNAVCXCR5⁺) was elevated for BCR-proximal components, had down-regulated select signaling molecules (Fig. 2) and were enriched for markers of cellular senescence (Fig. 3). VDJ-seq showed increased prevalence of somatically unmutated IgM⁺ cells in RA converters (80–81%), compared to non-converters (~60%) and healthy controls (70–72%) (Fig. 4). Monoclonal antibodies derived from converter clones were polyreactive against a diverse antigen panel (Fig. 4). Following clonally related receptors they found that H/L pairs in converters did not progress to class switched, memory phenotypes (Fig. 5). aNAVCXCR5⁺ and ‘Transit’ cells were enriched in RA compared to osteoarthritis (OA) synovial fluid (Fig. 6). Addressing the origin of aNAV cells they found that resting naïve B cells (rNAV) from CCP+ RA donors converted to an aNAVCXCR5⁺ phenotype over 2-day activation with anti-IgM, citrullinated protein antigens and CpG (Fig. 7h, i), suggesting that aNAVCXCR5⁺ cells resulted from stimulation by self-antigens in vivo. Chronic stimulation ex vivo of aNAVCXCR5⁺ from RA patients led to increased senescence as measured by β -galactosidase expression (Fig. 7 m, n). aNAVCXCR5⁺ cells were proposed to function by presenting antigens to CD4⁺ and CD8⁺ T cells (Fig. 8).

The unique cohort design of this study (despite small numbers) is a major strength of this manuscript which, together with an array of state-of-the-art genomic analysis to query differences between ACPA+ progressors and non-progressors, makes for an interesting story. Identification of increased aNAVCXCR5⁺ B cells well before clinical RA is novel, as is the potential connection of these cells to B cell senescence. I am more circumspect, however, about the impact of these observations on the field of immune aging. In other words, is Nature Aging the appropriate journal for dissemination of these observations? There are a few lines in the Discussion about immune aging, autoimmunity etc., but most of the conclusions have little to do with aging.

Response: We sincerely thank Reviewer #2 for her/his careful review and constructive comments. We also appreciate the Reviewer’s recognition that “the unique cohort design of this study is a major strength” and that our work uses “an array of state-of-the-art genomic analyses.”

In this revision, we addressed Reviewer #2's concerns by validating our initial scRNA-seq findings using two independent approaches: flow cytometry in a new validation cohort and analysis of a large, recently published pre-RA scRNA-seq dataset. These new data provide additional evidence supporting the preclinical expansion and senescence-like phenotype of aNAV cells. Together with recent findings from other groups, our revised analyses further highlight a potential role for naive B cell activation and senescence-like programming in progression toward clinical RA.

Regarding the question of “whether *Nature Aging* is the appropriate journal for disseminating these observations”, we respectfully believe that our study is of potential interest to *Nature Aging* readers, as cellular senescence is a major area within the journal's scope (<https://www.nature.com/nataging/aims>).

Although our study does not focus on chronological aging per se, increasing evidence indicates that senescence is not limited to natural aging, but also participates in development, tissue remodeling, chronic inflammation, and disease progression [25-27]. In this study, we report that B cells, a critical component of the immune system, can acquire a strong senescence-like program during the preclinical stage of RA. This phenotype has now been further validated by flow cytometry in this revision.

This finding challenges the conventional view that pathogenic immune activation is primarily driven by the proliferative expansion of immune cells. Instead, our data suggest that disease-associated activated naive B cells may aberrantly accumulate, at least in part, through a senescence-like state rather than through active proliferation. We believe this represents a novel mechanism linking immunosenescence to autoimmunity and preclinical disease progression. Therefore, we believe these findings are highly relevant to the aging, senescence, and immunology research community and are appropriate for publication in *Nature Aging*.

Beyond this editorial point, several scientific questions need clarification.

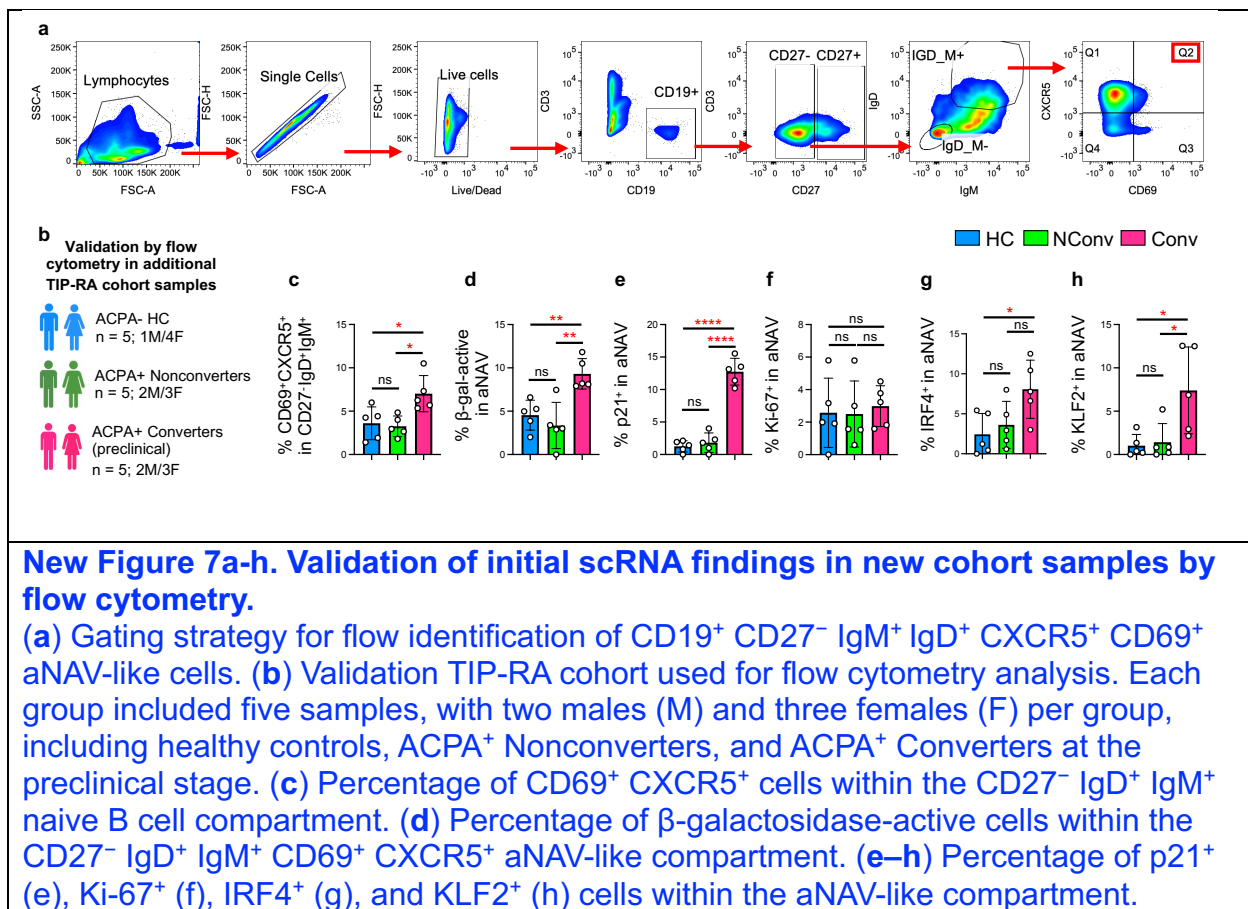
1. Are aNAV CXCR5⁺ cells CD69⁺ (cell surface)? They have CD69 in the CITE-seq panel, and the corresponding flow cytometry pattern should be shown. Identification of this population by flow cytometry would also permit screening of ACPA⁺ individuals for progression. Some flow analysis is shown in Fig. 7, but in this example the CD69⁺ aNAV CXCR5⁺ population is not visible.

Response: We agree with the Reviewer that surface protein validation is important for confirming the phenotypic identity of aNAV cells and for evaluating their potential use in screening ACPA⁺ individuals for progression risk.

In our initial CITE-seq analysis, the CD69 ADT signal was not sufficiently specific, as broad staining was observed across all cell populations. Therefore, we did not emphasize or display the CD69 CITE-seq data in the original manuscript to avoid potential confusion. However, we agree that flow cytometry is a more appropriate approach to validate the surface phenotype of this population.

To determine whether CXCR5⁺ CD69⁺ aNAV-like cells can be detected by flow cytometry in preclinical RA samples, we obtained additional preclinical samples from our clinical collaborators, including both male and female subjects. In this revision, we performed flow cytometry to validate both the expansion of aNAV-like cells and their senescence-like phenotype (**new Figure 7a, b**). The group information was blinded until the flow cytometry validation was completed

These new data confirmed that CD19⁺ CD27⁻ IgM⁺ IgD⁺ CXCR5⁺ CD69⁺ aNAV-like cells can be detected in all three groups and are significantly elevated in ACPA⁺ Converters at the preclinical stage compared with Nonconverters and healthy controls (**new Figure 7c**). We acknowledge that the magnitude of aNAV elevation observed by flow cytometry was less dramatic than that observed in our initial scRNA-seq analysis. This difference may reflect technical and biological differences between transcript-level clustering and protein-level flow cytometric detection, as well as differences in assay sensitivity and marker resolution between the two platforms.



New Figure 7a-h. Validation of initial scRNA findings in new cohort samples by flow cytometry.

(a) Gating strategy for flow identification of CD19⁺ CD27⁻ IgM⁺ IgD⁺ CXCR5⁺ CD69⁺ aNAV-like cells. (b) Validation TIP-RA cohort used for flow cytometry analysis. Each group included five samples, with two males (M) and three females (F) per group, including healthy controls, ACPA⁺ Nonconverters, and ACPA⁺ Converters at the preclinical stage. (c) Percentage of CD69⁺ CXCR5⁺ cells within the CD27⁻ IgD⁺ IgM⁺ naive B cell compartment. (d) Percentage of β -galactosidase-active cells within the CD27⁻ IgD⁺ IgM⁺ CD69⁺ CXCR5⁺ aNAV-like compartment. (e–h) Percentage of p21⁺ (e), Ki-67⁺ (f), IRF4⁺ (g), and KLF2⁺ (h) cells within the aNAV-like compartment.

In addition, β -galactosidase-active aNAV-like cells, as measured using a flow cytometry-based β -galactosidase activity assay kit (Invitrogen, C10841), were significantly increased in Converters compared with Nonconverters and healthy controls (**new Figure 7d**). More importantly, p21/CDKN1A expression was markedly higher in Converter aNAV-like cells than in the corresponding populations from Nonconverters

and healthy controls (**new Figure 7e**). Converter aNAV-like cells also expressed higher levels of IRF4 and KLF2, while Ki-67 expression remained unchanged (**new Figure 7f–h**). Together, these new flow cytometry data validate the major features of aNAV-like cells observed in our initial scRNA-seq analysis and support their hyperactivated and senescence-like state in preclinical RA Converters.

2. One of their key findings from the scRNA-Seq is that BCR proximal genes are up-regulated while signaling genes are down-regulated (Fig. 2). But there is more going on that makes this conclusion somewhat facile. First, while BCR proximal genes are certainly up regulated in aNAVCXCR5⁺ cells from converters compared to non-converters (last 2 columns in Fig. 2d), it is also evident that rNAVs from converters have especially high expression of the same genes (column 3, Fig. 2d). Relative to this population, BCR-proximal genes are down-regulated in converter aNAVCXCR5⁺. The authors do not discuss at all the high levels of BCR-proximal genes in rNAVs exclusively from converters and the implications of this observation to their conclusions.

Response: We sincerely thank the Reviewer for this constructive suggestion. We agree that the elevated expression of BCR component and BCR-proximal signaling genes in Converter rNAV cells is an important observation that was not sufficiently discussed in the original manuscript. We have now described this finding in the Results section and expanded the Discussion to address its biological implications (page 15, lines 550-565). Specifically, we now state:

“Interestingly, we observed distinct BCR signaling patterns across the rNAV and aNAV^{CXCR5+} compartments in Converters. At the preclinical stage, Converter rNAV cells showed significant upregulation of BCR component genes and BCR-proximal signaling genes, suggesting enhanced early BCR activation potential. In contrast, Converter aNAV^{CXCR5+} cells retained elevated expression of BCR component genes but showed reduced expression of BCR-proximal signaling genes. This pattern suggests that early B cell activation may occur in Converters at the rNAV stage, followed by potentially reduced BCR responsiveness at the aNAV^{CXCR5+} stage, consistent with possible chronic low-level antigen engagement or potential anergy-like tuning [28, 29]. However, although Converter rNAV cells showed evidence of early activation potential, our differential gene expression analysis indicated that the major transcriptional differences between Converters and Nonconverters emerged primarily at the aNAV^{CXCR5+} stage rather than the rNAV stage. Based on these findings, we propose a model in which rNAV cells in Converters have increased activation potential, followed by aberrant amplification within the aNAV^{CXCR5+} compartment, where aNAV cells acquire a highly stressed, senescence-like, and potentially differentiation-restrained state.”

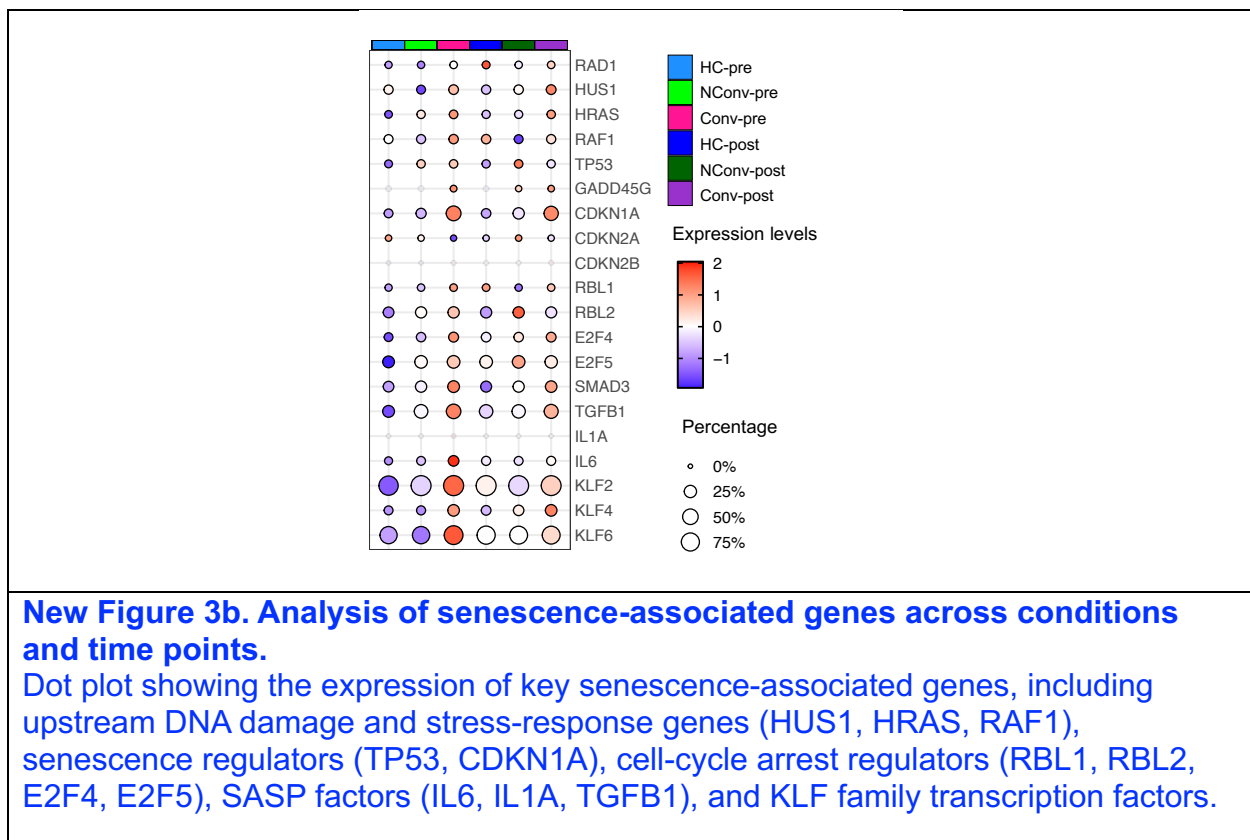
We believe this revised interpretation more accurately reflects the data in Figure 2 and addresses the Reviewer’s point that the BCR-related transcriptional changes may begin in Converter rNAV cells but become more disease-defining in the aNAV^{CXCR5+} compartment.

3. A senescence-like state of converters in aNAVCXCR5⁺ is presented in Fig. 3, in part based on cell cycle regulators Cdkn1a and SASP components TGFβ1 and IL6.

However, these genes are robustly (maximally?) expressed in non-converters (column 3, Fig. 3) and barely at all in converters (column 5, Fig. 3). What am I missing?

Response: We sincerely thank the Reviewer for this careful observation. After rechecking the figure and underlying data, we found that the original heatmap contained a color-labeling error, which caused the expression pattern to be displayed incorrectly. We apologize for this mistake.

We have now corrected the heatmap and carefully rechecked the underlying expression values. The corrected figure confirms that Converter aNAV^{CXCR5+} cells express higher levels of senescence-associated genes at the preclinical stage, including cell-cycle regulatory genes and SASP-related genes (**new Figure 3b**).



4. Figures 4 and 5 convey a lot of information about immunoglobulin expression and will be a useful resource for future studies. Additional clarification on two points will be valuable for a reader. First, I don't follow how only aNAVs from converters express IgM. All subsets shown in Fig. 4a and b are naïve B cells and should express IgM. Second, while the authors highlight PCA differences in heavy chain repertoire between converters and non-converters, there appear (to the eye) bigger differences in the light chain repertoire (Fig. 4g right). This is never discussed.

Response: We thank the Reviewer for this helpful suggestion. We apologize for the confusion caused by the original description in the Results section.

Figure 4a shows the isotype composition of the overall BCR repertoire across groups, whereas Figure 4b analyzes the cell type composition of each isotype. Specifically, for Figure 4b, all IgM-expressing, IgA-expressing, or IgG-expressing B cells were each set as 100%, and we then calculated the relative contribution of each B cell subset to each isotype-expressing compartment. Thus, our statement did not mean that only aNAV^{CXCR5+} cells from Converters express IgM. Rather, IgM was expressed across most B cell subsets, as expected for naive and unswitched B cells.

We have revised the Results section to clarify this point as follows:

“We analyzed the cell type composition of B cells expressing IgM, IgA, or IgG. IgM was expressed across most B cell subsets but was reduced in SwMem and atypical B cells, whereas IgA and IgG expression was more concentrated in UnswMem3 and SwMem cells (Fig. 4b). In Converters, IgM expression was predominantly contributed by aNAV^{CXCR5+} cells, whereas in Nonconverters and healthy controls, IgM expression was contributed by both naive and unswitched memory B cell subsets (Fig. 4b).”

Regarding the more pronounced differences in the light-chain repertoire, we agree that this is a potentially meaningful observation. However, we do not currently have sufficient quantitative evidence to conclude that light-chain V–J recombination is more variable than heavy-chain V–J recombination. Therefore, in the revised manuscript, we have added a new discussion of this observation (page 18, lines 688-699) and highlighted the need for future investigation:

“Notably, both heavy- and light-chain V–J patterns differed in Converter aNAV cells compared with their counterparts from Nonconverters and healthy controls, with more pronounced differences observed in the light-chain repertoire. This suggests that light-chain variation may also contribute to the reprogramming of aNAV BCRs. At the same time, because heavy-chain CDR3 is a major determinant of BCR clonotype structure and polyreactivity, heavy-chain sequences remain useful for tracking aNAV-related BCR lineages over time. These skewed V–J recombination patterns may increase BCR diversity, enabling Converter aNAV-derived IgM to recognize a broader range of self-antigens and thereby generating more frequent polyreactive BCRs. Future studies will be needed to determine whether the pronounced light-chain rearrangement observed in Converter aNAV cells directly contributes to polyreactivity or antigen recognition.”

We hope these revisions clarify the interpretation of Figure 4 and more appropriately address the potential importance of light-chain repertoire differences.

5. Figures 7 and 8 examine the origin of aNAV^{CXCR5+} cells and their function, respectively. They show that rNAVs from RA patients convert to an aNAV^{CXCR5+} phenotype over 2-day treatment with anti-IgM, citrullinated protein antigens (Cit-Ags) or CpG. I find it surprising that comparable numbers of rNAV cells convert in response to either pan activators (such as anti-IgM or CpG) and specific activators such as Cit-Ags. This implies that virtually all BCR-responsive cells bind and are activated by Cit-Ags. Is this possible? It is important to know whether this is also true of rNAVs isolated from healthy donors or is a special property of rNAVs in individuals with RA.

Response: We thank the Reviewer for this important comment. Naive B cell activation can result from complex and integrated signals, and this short-term in vitro assay was designed to test whether different stimulation conditions could promote acquisition of an aNAV-like phenotype, rather than to define citrullinated antigen-specific BCR binding. Anti-IgM was included as a positive control for BCR crosslinking, but it does not necessarily reproduce full physiological BCR activation that occurs in vivo. In addition, we observed a basal level of naive B cell activation in the untreated condition, which was approximately half or less of that seen in the anti-IgM-treated group. Therefore, although the quantitative levels of aNAV^{CXCR5+} induction after anti-IgM and Cit-Ag treatment appeared comparable, this does not necessarily indicate that the two conditions activate naive B cells through the same BCR-dependent mechanism.

In this revision, we repeated the antigen stimulation assay using naive B cells from healthy controls. These results provide interesting new insight into how RA B cells respond differently to different antigenic and innate stimuli compared with healthy control B cells. In RA B cells, both Cit-Ags and CpG induced resting naive B cell activation after 2 days of culture and promoted an aNAV senescence-like phenotype after 7 days of culture (**new Figure 8b, c, and i**). In contrast, in healthy control B cells, only CpG induced aNAV differentiation and senescence-like features, whereas Cit-Ags did not show a comparable effect (**new Figure 8d, e and j**).

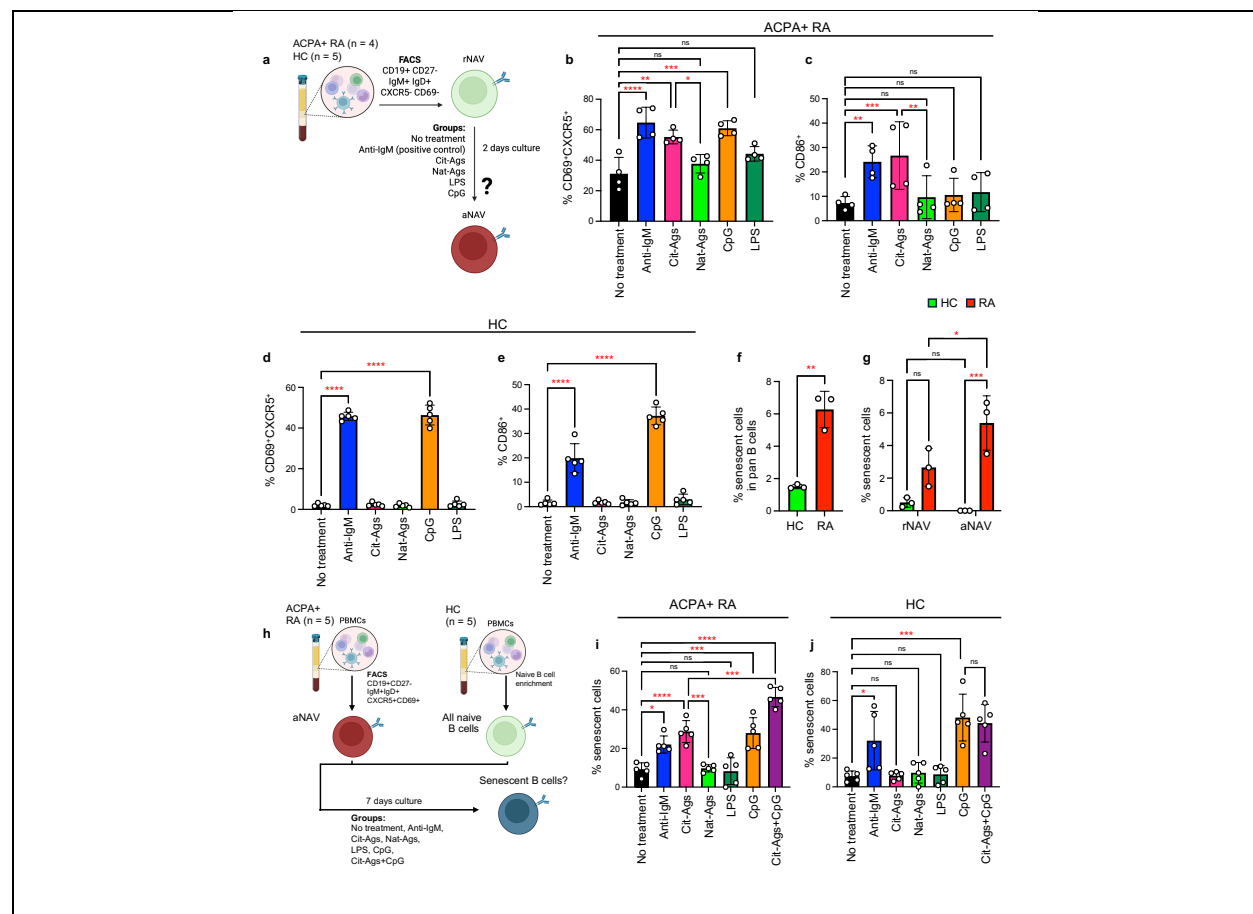
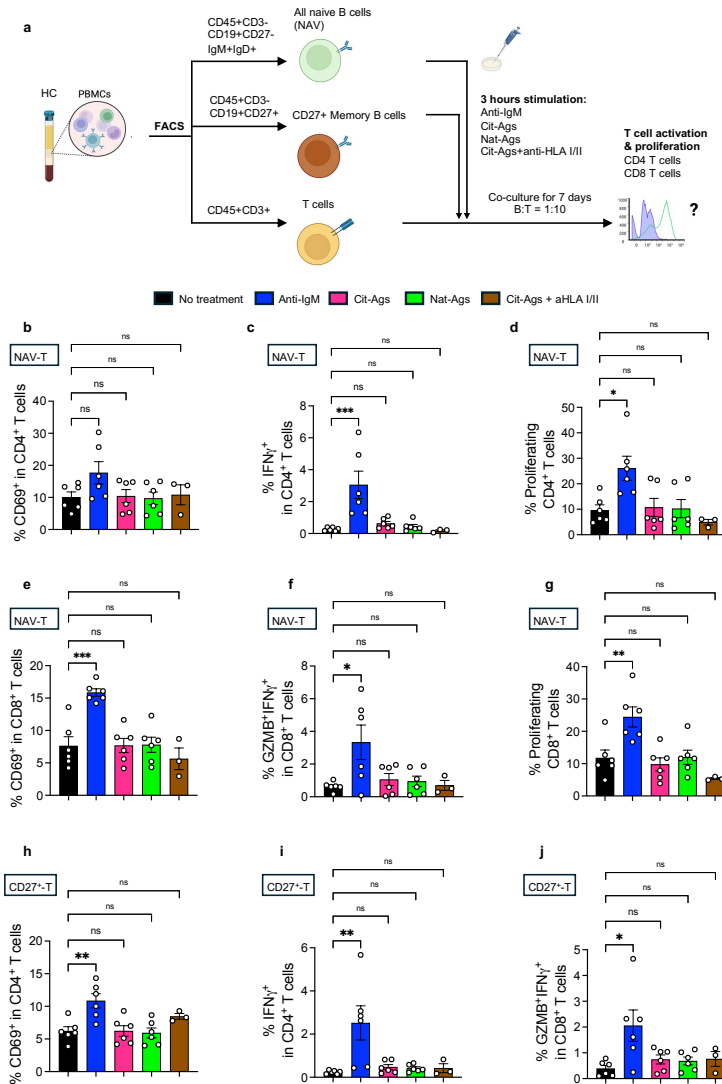


Figure 8. Distinct effects of antigen exposure on naive B cell activation and senescence in RA and healthy controls.

(a) Experimental design for antigen screening to identify potential triggers of naive B cell activation. rNAV cells were FACS-sorted from ACPA⁺ RA patients (n = 4) or healthy controls (n = 5) and cultured for 2 days with anti-IgM, citrullinated antigens (Cit-Ags), native antigens (Nat-Ags), CpG, or LPS. (b) Percentages of CD69⁺ CXCR5⁺ cells in RA samples after stimulation. (c) Percentages of CD86⁺ cells in RA samples after stimulation. (d) Percentages of CD69⁺ CXCR5⁺ cells in healthy control samples after stimulation. (e) Percentages of CD86⁺ cells in healthy control samples after stimulation. (f) Frequencies of β -galactosidase-active senescence-like cells among total B cells from ACPA⁺ RA patients and healthy controls. n = 3 per group. (g) Frequencies of β -galactosidase-active senescence-like cells in rNAV and aNAV subsets from ACPA⁺ RA patients and healthy controls. n = 3 per group. (h) Experimental design for antigen screening to identify potential inducers of naive B cell senescence. For RA samples, aNAV cells were FACS-sorted and cultured for 7 days with anti-IgM, Cit-Ags, Nat-Ags, CpG, LPS, or Cit-Ags + CpG. For healthy controls, because aNAV cells were too rare to sort reliably, total naive B cells were isolated using a naive B cell enrichment kit and cultured with the same stimulation panel for 7 days. (i, j) Percentages of β -galactosidase-active senescence-like cells after stimulation in RA and healthy control samples. n = 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by Student's t test (b–g), one-way ANOVA (i,j), or two-way ANOVA where indicated; ns, not significant.

We would also like to clarify that, because aNAV cells are too rare to be reliably FACS-sorted from healthy controls, we used a naive B cell enrichment kit (STEMCELL Technologies, Cat#100-0971) to isolate total naive B cells for the senescence-induction and assessed whether increased β -galactosidase activity could be detected within the aNAV-like compartment after stimulation (**new Figure 8h**). Despite this methodological difference, the treatment concentrations, culture duration, and readout strategy were kept comparable between the RA and healthy control experiments. These new data support the conclusion that the effect of Cit-Ags in promoting naive B cell activation and senescence-like features is preferentially observed in RA patient-derived naive B cells. They also further support the idea that Cit-Ags and CpG act through functionally distinct mechanisms, which may explain their synergistic effects.

We also repeated our B-T co-culture experiments using healthy control samples. In this experiment, naive B cells and CD27⁺ memory-like B cells were FACS-sorted and co-cultured with autologous CD3⁺ T cells under the same experimental conditions used in our previous RA assays. The results showed that Cit-Ag-loaded B cells failed to induce T cell activation in healthy control samples (**new Extended Data Figure 8**). This finding further supports the idea that RA-derived B cells are uniquely primed to respond to citrullinated antigens and promote downstream T cell activation.



New Extended Data Fig. 9. Citrullinated antigen-stimulated B cells fail to activate T cells in healthy control samples.

(a) Experimental design to assess whether antigen-stimulated B cells activate T cells. Total naive B cells and CD27⁺ memory B cells were FACS-sorted from healthy controls using the indicated markers, stimulated with antigens for 3 h, and then co-cultured with CD3⁺ T cells for 7 days. T cell activation and proliferation were analyzed by flow cytometry. (b–d) Percentages of CD69⁺, IFN γ ⁺, and proliferating CD4⁺ T cells co-cultured with naive B cells. n = 3–6 per group. (e–g) Percentages of CD69⁺, GZMB⁺ IFN γ ⁺, and proliferating CD8⁺ T cells co-cultured with naive B cells. n = 3–6 per group. (h, i) Percentages of CD69⁺ and IFN γ ⁺ cells among CD4⁺ T cells co-cultured with CD27⁺ memory B cells. n = 3–6 per group. (j) Percentage of GZMB⁺ IFN γ ⁺ cells among CD8⁺ T cells co-cultured with CD27⁺ memory B cells. n = 3–6 per group. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA; ns, not significant. NAV: naive B cells.

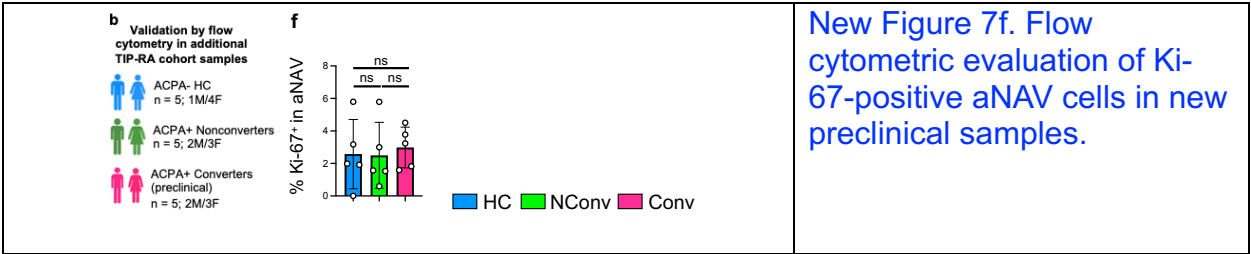
We acknowledge that, at this stage, we do not fully understand why Cit-Ags have such broad effects in inducing naive B cell activation and senescence-like features in RA samples, or why the native forms of the same antigens have minimal effects in this assay. We speculate that this effect may reflect a broader citrullination-dependent signal in RA B cells, rather than specific recognition of individual citrullinated antigens by naive BCRs. It may also represent a polyclonal recall-like response reflecting prior exposure to citrullinated antigens in ACPA⁺ individuals. We believe this is an interesting question for future study, but it is beyond the scope of the current work.

We have added these new data to the revised manuscript as **new Figure 8** and **new Figure 9** and discussed the findings in the Results (page 12, lines 467-477; page 13, lines 499-503) and Discussion sections (page 17, lines 653-670). We hope these additional experiments address the Reviewer’s concern and clarify that the Cit-Ag response is not a general property of normal activated naive B cells, but instead appears to reflect a disease-associated feature of RA B cells.

6. The same is true for senescence conversion in Fig. 7m. Do most BCR-responsive cells recognize Cit-Ags? Is this true for aNAVCXCR5⁺ from non-RA patients? Since senescence is closely connected to cell division, it is important to know the cell division status of aNAVCXCR5⁺ cells stimulated under these conditions for 7 days.

Response: We thank the Reviewer for this constructive suggestion. As discussed above, in this revision we provide new data showing that the senescence-like phenotype of aNAV cells can be strongly induced by either Cit-Ags or CpG in RA samples, whereas in non-RA healthy control samples, only CpG induced this effect (**new Figure 8**). These findings suggest that RA-derived naive B cells may have a specific response to citrullinated antigens compared with healthy control B cells.

Regarding the cell division status, since in this revision we were able to analyze additional validation samples from preclinical RA subjects by flow cytometry, we decided to assess Ki-67 expression in the aNAV compartment. The data showed that the percentage of Ki-67-positive cells was low in aNAV cells, generally less than 5%, and was comparable among healthy controls, Nonconverters, and Converters at the preclinical stage (**new Figure 7f**). These results suggest that aNAV cells are not actively proliferating in either healthy controls or preclinical RA subjects. Together with the marked elevation of β -galactosidase activity and p21/CDKN1A expression in the aNAV compartment of preclinical RA Converters, these new flow cytometry data support the presence of a clear senescence-like program in aNAV cells before RA onset, which may contribute to disease progression.



We agree with the Reviewer that the classical concept of “senescence” is closely linked to cell-cycle arrest and that cell division status is important for interpreting these data. Because we do not yet have direct evidence that disease-associated aNAV cells are irreversibly cell-cycle arrested, which would require longitudinal assessment across multiple time points, we have revised the manuscript to use the term “senescence-like” rather than definitive senescence. We have updated the relevant text throughout the manuscript accordingly.

In summary, this data heavy manuscript describes an interesting subset of human B cells that correlate with progression of ACPA+ individuals to RA. However, much of the paper is descriptive and contributes little to understanding mechanisms that drive RA in vivo. Some attempts towards this end in Figures 7 and 8 are compromised by lack of healthy or non-RA controls. A substantially revised manuscript that cuts through data noise to focus on the most salient features that provide insights into disease mechanisms would be of value.

Response: We thank the Reviewer for this constructive summary and helpful guidance. During this revision, we have substantially revised the manuscript to focus more clearly on the most salient findings and their relevance to disease progression.

To address the concern that the original manuscript was largely descriptive, we added several new validation and functional analyses. First, we validated the expansion of CXCR5⁺ CD69⁺ activated naive B cells and their senescence-like phenotype by flow cytometry using additional preclinical samples, including both Converters, Nonconverters, and healthy controls. Second, we added new healthy control data to strengthen the interpretation of the functional assays in new Figures 8 and 9. Third, we re-analyzed a large recently published scRNA-seq dataset from an independent preclinical RA cohort, which independently validated the preclinical expansion of aNAV cells and further supported their senescence-like transcriptional program.

We acknowledge that, at this point, it is difficult to test the causal relationship between aNAV expansion and RA progression in vivo, as there is currently no suitable animal model that faithfully mimics the ACPA+ at-risk stage in humans. Historically, the ACPA+ at-risk stage has often been considered a single preclinical phase. However, through efforts from our group and others, it is now increasingly clear that ACPA+ at-risk individuals are heterogeneous, and only a subset progress to clinical RA. In this context, our study provides valuable information by identifying a disease-associated aNAV population that distinguishes ACPA+ Converters from Nonconverters before clinical onset, particularly through its senescence-like program. Therefore, although the Reviewer considers some of these findings descriptive, we believe they provide an important foundation for future genetic, functional, and mechanistic studies aimed at defining how naive B cell activation and senescence-like programming may contribute to RA progression.

Reviewer #3 (Remarks to the Author):

A. Summary of the key results

The authors compare B cell populations prior to and following conversion to RA using single cell sequencing as their primary method. The authors identify differences in the activated Naïve B cell compartment when comparing aNAV from converters to non-converters, even prior to conversion. Notable differences include a number of metabolic and BCR signaling pathways, and perhaps cellular stress and cell cycle which the authors speculate to indicate senescence, however these pathways overlap considerably with pathways that are active in plasma cell differentiation and could instead indicate the cells are prone to differentiate (more comments below). The authors also include VDJ analysis and surprisingly find that most of the cells are singletons and not clonally expanded, even from the converters. This brings up a concern about lack of depth discussed at length below, but is nonetheless potentially interesting. The authors then not a difference in HLA-DR gene expression in joints, and perform elegant in vitro culture experiments demonstrating that aNAV cells can present antigen. While the authors implicate aNAV cells as being robust antigen presenting cells, there is not actually a direct comparison to rNAV cells as it stands. The data quality and breadth of analysis is very high for human studies, and these are rare human samples. I am quite positive about this study overall, but think that some additional analysis is required to support rather bold claims about cellular senescence versus competing alternative conclusions.

Response: We appreciate the Reviewer's positive assessment of our study, particularly the recognition that "the data quality and breadth of analysis is very high for human studies" and that "these are rare human samples." We also appreciate the thoughtful concerns regarding the interpretation of the aNAV transcriptional state, the depth of VDJ analysis, and the need to more carefully distinguish senescence-like features from alternative explanations, such as plasma cell differentiation.

In this revision, we have substantially expanded the validation analyses and revised the manuscript to make our conclusions more precise. First, we obtained new samples from our clinical collaborators and validated the elevation of CXCR5⁺ CD69⁺ aNAV-like naive B cells and their senescence-like phenotype in preclinical RA Converters by flow cytometry, including both male and female subjects (**new Figure 7a–h**). These data confirmed increased aNAV frequency, increased β -galactosidase activity (a classic senescence marker), and markedly elevated p21/CDKN1A expression in Converter aNAV cells. We also assessed Ki-67 and found that Ki-67-positive cells were generally rare in the aNAV compartment and were not markedly increased in Converters, supporting the interpretation that these cells are not actively proliferating.

Second, we validated our major findings using a large, recently published scRNA-seq dataset from an independent preclinical RA cohort [1], including 67 ACPA⁺ Converters sampled before clinical RA onset and 35 ACPA⁺ Nonconverters (**new Figure 7i–r**). This

independent re-analysis confirmed the preclinical expansion of aNAV cells in Converters and further showed increased senescence-related scores in Converter aNAV cells. Importantly, this independent dataset also showed that the plasmablast compartment was not expanded in preclinical RA, supporting our interpretation that plasmablast differentiation is not a major event in the circulation during the preclinical RA stage.

Third, we generated new sequencing data from enriched naive B cells to further increase BCR sequencing depth. This analysis confirmed that the majority of aNAV cells in pre-RA Converters, clinically established RA patients, and healthy controls were singletons (**new Extended Data Fig. 6**).

Fourth, we repeated the antigen stimulation and B-T co-culture assays using healthy control samples. These experiments revealed a distinct response to citrullinated antigens (Cit-Ags) between RA and healthy control B cells. In RA samples, both Cit-Ags and CpG induced resting naive B cell activation after 2 days of culture and promoted an aNAV senescence-like phenotype after 7 days of culture (**new Figure 8b, c, and i**). In contrast, in healthy control samples, only CpG induced aNAV differentiation and senescence-like features, whereas Cit-Ags had minimal effects (**new Figure 8d, e, and j**). In the B-T co-culture experiments, Cit-Ag-stimulated B cells induced T cell responses in RA samples but not in healthy control samples (**new Figure 9 and Extended Data Figure 9**).

Fifth, we tested plasmablast/plasma cell differentiation capacity in naive B cells treated with our antigen panel using RA and healthy control samples. Consistent with our previous observations, Cit-Ags and CpG induced aNAV expansion in RA samples, whereas only CpG induced aNAV expansion in healthy control samples. Importantly, none of these antigen conditions induced plasmablast or plasma cell differentiation in the absence of additional differentiation signals (**new Extended Data Figure 10**).

We have also revised the manuscript to tone down several statements and avoid overinterpretation.

Together, these new analyses and textual revisions strengthen the major conclusions while addressing the Reviewer's concern that the original claims should be more carefully supported and more cautiously interpreted. Please refer to each individual response below.

B. Originality and significance: if not novel, please include reference

I am not aware of any publications that reduce the novelty.

C. Data & methodology: validity of approach, quality of data, quality of presentation

Overall this is a well written manuscript, and generally easy to follow. One exception is that I do not understand what Figure 5f is a measurement of. How are "new influx" "arrested" and "differentiated" defined/determined? Please expand.

Response: We thank the Reviewer for pointing out that the definition of Figure 5f was unclear. In this analysis, we first subset IgM+ aNAV^{CXCR5+} BCR sequences from the first time point. We then searched for related BCR sequences in the second time point repertoire, defined by shared V and J gene usage, identical CDR3 length, and $\geq 75\%$ CDR3 sequence similarity. After identifying these related BCRs at the second time point, we examined the B cell subset identity of the cells carrying these related BCRs.

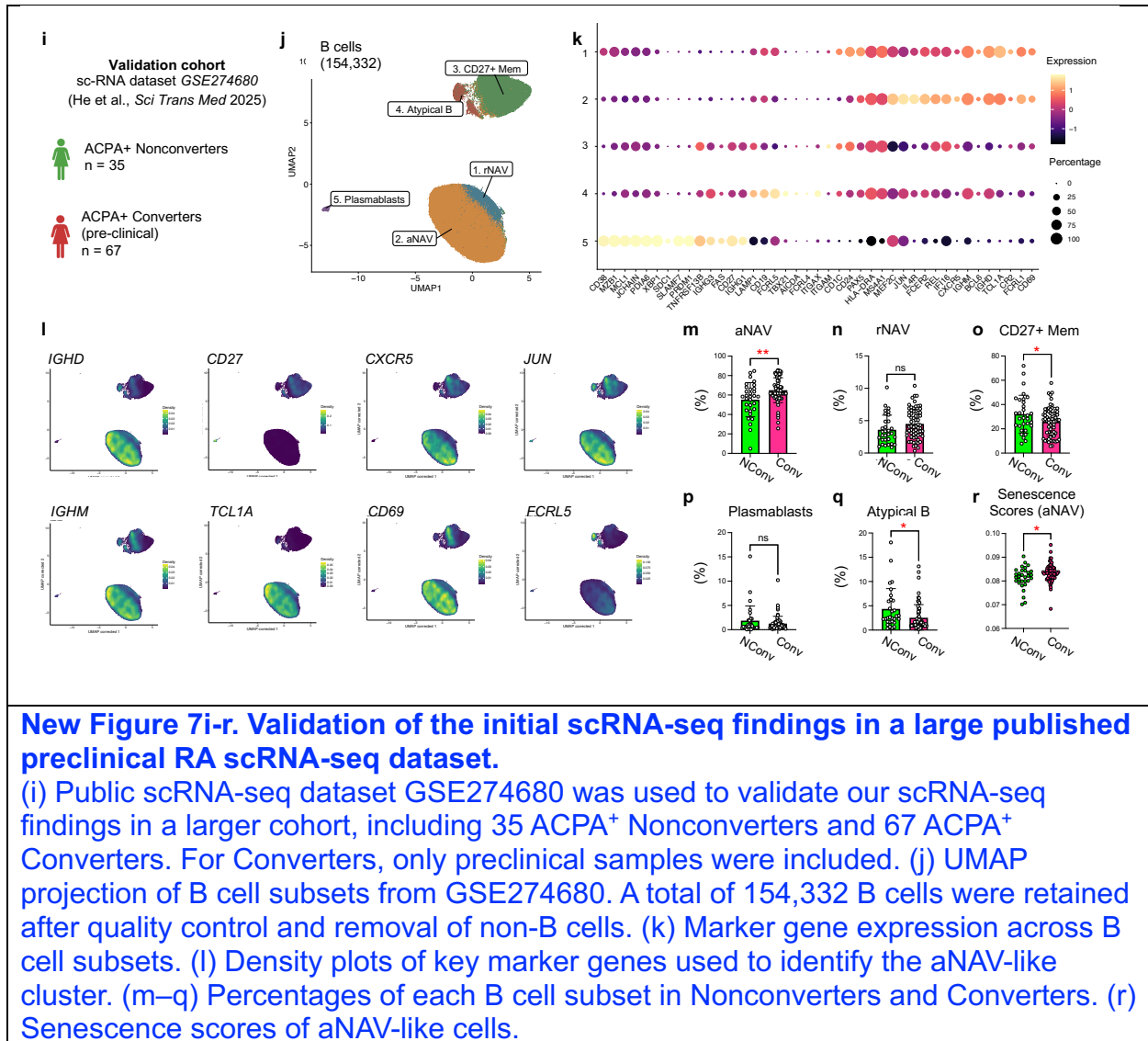
We classified these second-time-point aNAV-related BCRs into three categories based on their cell state. aNAV-related BCRs detected again in the aNAV^{CXCR5+} compartment were defined as “arrested,” because these BCR lineages remained in the same cell state. aNAV-related BCRs detected in resting naive B cells were defined as “new influx,” reflecting newly detected resting naive cells related to the initial aNAV^{CXCR5+} repertoire. aNAV-related BCRs detected in later B cell states, including aNAV^{CD38^{hi}}, memory, and atypical B cell compartments, were defined as “differentiated.” We have revised the Results (page 9, lines 330-336) and figure legend (page 40, lines 1282-1288) to better explain how these categories were defined.

D. Appropriate use of statistics and treatment of uncertainties

Authors appropriately discuss the limitations of human studies with only roughly n=5 samples, but this is inherent to the depth of sequencing analysis being performed.

Response: We thank the Reviewer for understanding the cost and practical limitations of deep single-cell sequencing studies, especially when using rare longitudinal human samples from the preclinical RA stage.

After our original manuscript was submitted, a recent *Science Translational Medicine* study by He et al. [1] reported preclinical activation of naive B cells in ACPA⁺ Converters, supporting the major findings of our study. This independent study included transcriptomic profiling of a substantially larger cohort. During this revision, we validated our major findings by re-analyzing this published dataset, which contains single-cell RNA-seq data from B cells of 67 ACPA⁺ Converters and 35 ACPA⁺ Nonconverters (**new Figure 7i–r**). This independent validation strengthens the conclusions from our original discovery cohort and helps address concerns related to sample size.



Consistent with our discovery cohort, the percentage of aNAV cells was significantly higher in Converters than in Nonconverters at the preclinical stage, whereas CD27⁺ memory B cells and atypical B cells were lower (**new Figure 7j–q**). More importantly, in this larger cohort, senescence scores calculated using the same senescence-related gene set as in our exploratory study were also significantly higher in Converter aNAV cells than in their counterparts from Nonconverters (**new Figure 7r**), a finding not reported in the original *Science Translational Medicine* study. Thus, our study not only validate the preclinical activation of naive B cells reported by He et al., but also add new insight by showing that disease-associated B cells can acquire a senescence-like transcriptional program during progression toward clinical RA.

E. Conclusions: robustness, validity, reliability

Comments for this section and the next are intertwined, presented below.

F. Suggested improvements: experiments, data for possible revision

The authors suggest that the gene signature of the converters aNAV populations display anergy-like transcriptional profiles. However, the cells are activated and therefore are not anergic in the classical sense. Can the authors include a comparison to published data of bona fide anergic B cells?

Response: Anergy-like tuning refers to a state shaped by chronic antigen engagement, in which B cells retain surface BCR expression and antigen-binding capacity but exhibit dampened downstream BCR signaling [28]. These cells may also have developmental arrest [28]. Importantly, anergic B cells can remain transcriptionally active; therefore, anergy does not imply that the cells are completely nonfunctional.

We agree with the Reviewer that our current evidence is not sufficient to define Converter aNAV cells as classical anergic B cells. We have revised the manuscript to avoid implying that these cells are bona fide anergic B cells. Instead, we now use the more cautious term “potential anergy-like tuning” to discuss a partial transcriptional pattern in Converter aNAV cells that may reflect altered BCR responsiveness, potentially shaped by chronic antigenic stress.

At present, we are not aware of a suitable published single-cell RNA-seq dataset of bona fide human anergic B cells that would allow direct mapping or rigorous comparison with the pathogenic aNAV population identified in our study. Therefore, we have reduced the emphasis on anergy and clarified that this interpretation remains hypothesis-generating and requires future functional validation.

Our suggestion of “potential anergy-like tuning” is based on several observations. First, we observed distinct BCR signaling patterns across the rNAV and aNAV^{CXCR5+} compartments in Converters. Converter rNAV cells showed significant upregulation of BCR component genes and BCR-proximal signaling genes, suggesting enhanced early BCR activation. In contrast, Converter aNAV^{CXCR5+} cells retained elevated expression of BCR component genes but showed reduced expression of BCR-proximal signaling genes. This pattern is consistent with a potential anergy-like tuning state, in which chronic or endogenous antigen engagement may preserve antigen-binding capacity while dampening downstream BCR responsiveness and limiting later B cell differentiation [28, 29].

Second, our BCR trajectory analysis revealed distinct longitudinal patterns of aNAV-associated BCR lineages between Converters and Nonconverters. In Nonconverters, most IgM⁺ aNAV-related BCRs were later detected in more differentiated B cell states, suggesting progression beyond the aNAV compartment over time. In contrast, in Converters, IgM⁺ aNAV-related BCRs were more frequently retained within the aNAV compartment at the second time point. This pattern is consistent with limited progression of Converter aNAV BCR lineages into later B cell states and supports a senescence-like and differentiation-restrained model.

However, we acknowledge that these transcriptomic and trajectory analyses do not directly demonstrate developmental arrest or anergy. Future in vitro B cell differentiation and functional signaling assays will be needed to determine whether pathogenic aNAV cells exhibit true anergy-like functional tuning, impaired differentiation capacity, or both. We have added a new discussion regarding this limitation in our revised manuscript (page 15, lines 576-579).

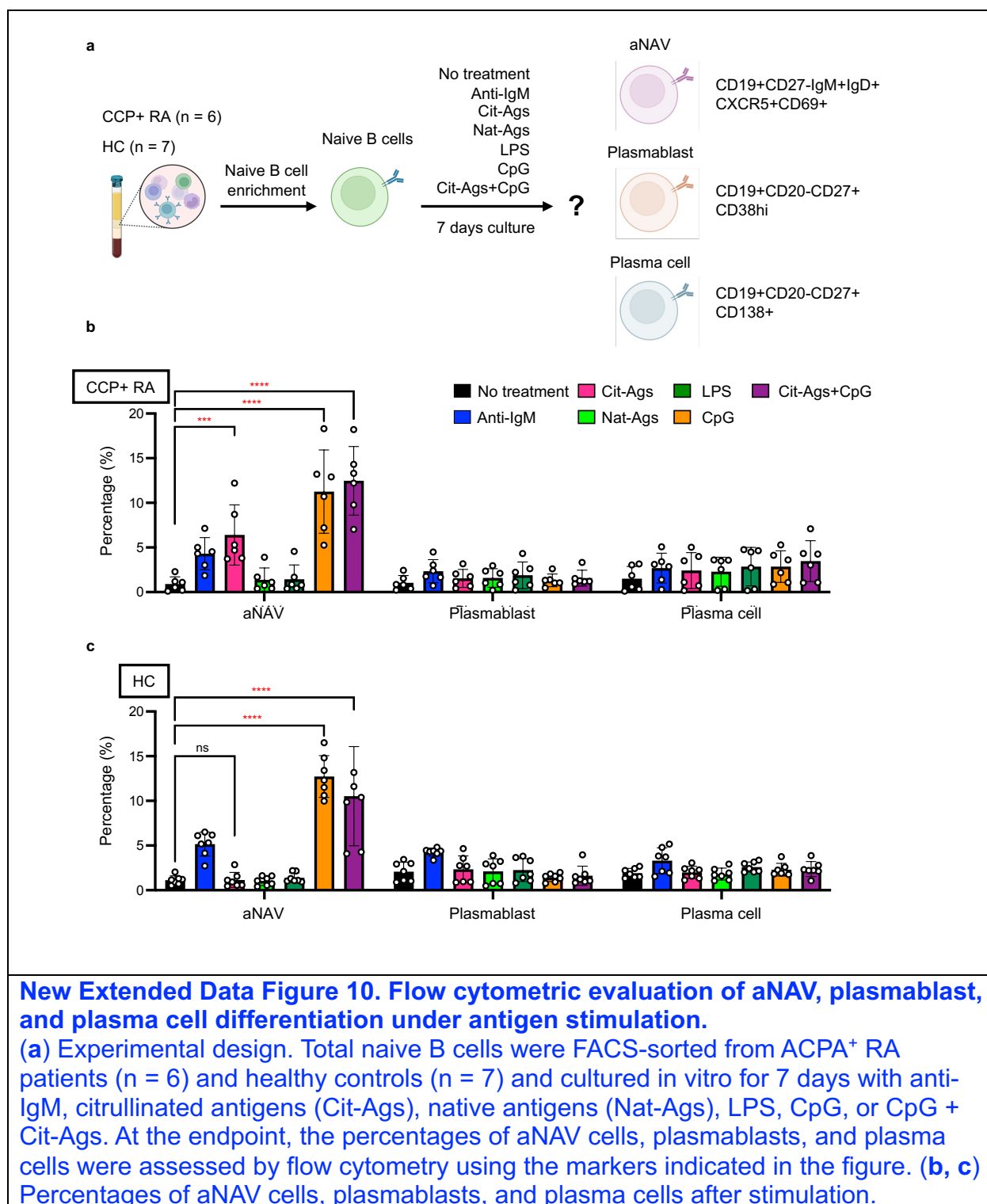
An alternative explanation for cells to stop dividing would be that they've begun differentiating into plasma cells (PC), which would be consistent with upregulation of CD38 in the aNAVCD38^{hi} population. The authors should rule out PC differentiation as an alternative conclusion. J-chain is not sufficient, it is up even in GC B cells.

Response: We agree with the Reviewer that plasma cell differentiation is an important alternative explanation that should be carefully evaluated. We have therefore performed additional analyses and added new validation data to address this concern.

First, in the large cohort validation analysis described above, the plasmablast compartment was not significantly expanded in ACPA⁺ Converters compared with Nonconverters, whereas the aNAV compartment was significantly increased, consistent with our discovery cohort (**new Figure 7m–q**). This suggests that plasmablast expansion is not a major feature of circulating B cell alterations at the preclinical RA stage.

Second, we tested the plasmablast/plasma cell differentiation capacity of naive B cells treated with our antigen panel using both RA and healthy control samples (**new Extended Data Figure 8**). Consistent with our previous observations, Cit-Ags and CpG induced aNAV expansion in RA samples, whereas only CpG induced aNAV expansion in healthy control samples. Importantly, none of these antigen conditions induced plasmablast or plasma cell differentiation in the absence of additional differentiation signals. In our previous experiments, we showed that these same stimulation conditions induced naive B cell activation and senescence-like features in RA samples (**new Figure 8**). Taken together, these data suggest that the expansion of senescence-like aNAV population is unlikely to be accompanied by increased differentiation toward the plasmablast or plasma cell lineage.

However, we do not exclude the possibility that a subset of aNAV cells retains plasmablast/plasma or memory B cell differentiation potential. Our scRNA-seq data indicate that the aNAV compartment is heterogeneous, with approximately 50% of cells showing a strong senescence-like program (**new Figure 3b**). On the other hand, our BCR trajectory analysis showed that a fraction of aNAV-related BCRs can still progress into later B cell states (**new Figure 5e, f**). Thus, we interpret the aNAV compartment as a heterogeneous population in which some cells acquire a senescence-like and differentiation-restrained state, whereas others may retain the capacity to progress toward downstream B cell fates. We have revised the manuscript to clarify this interpretation (page 15, lines 580-589) and avoid implying that all aNAV cells are irreversibly arrested or incapable of differentiation.



Lack of clonal expansion in Figure 5 is potentially really interesting, as it suggests a type of EF response that is continuously recruiting newly activated cells as opposed to expansion. The later hypothesis would also be consistent with Fig. 4e,f. However, this

could alternatively be explained by insufficient sequencing depth. Now that the authors have identified the aNAV compartment as the cells of interest, it would be very interesting to go back and purify only those cells to achieve more depth per patient (or pool data from existing data sets), and see whether the observation that most cells are singletons holds true. In contrast, in Fig. 5e, many aNAVCXCR5⁺ clones continued to be aNAVCXCR5⁺ cells in the post sample, suggesting the opposite, that those clones continued to expand and potentially of high importance that this was a unique feature of the converters. The authors conclude that this is a feature consistent with senescence-associated developmental arrest, but it could be just the opposite.

Response: We thank the Reviewer for this constructive suggestion. We agree that the lack of large clonal expansion in the aNAV compartment is potentially interesting, but that it could also be influenced by sequencing depth. We have therefore performed several additional analyses and generated new validation data to address this concern.

First, we would like to clarify the original Figure 5 analysis. In that analysis, we subset IgM⁺ aNAV^{CXCR5⁺} BCR sequences at the preclinical time point and then traced related BCRs across the whole BCR repertoire from the same patient. aNAV^{CXCR5⁺}-related BCRs could be detected not only within the aNAV^{CXCR5⁺} compartment, but also in other non-aNAV B cell subsets. As mentioned above, the aNAV^{CXCR5⁺} IgM-related BCRs at the second time point were then analyzed in Figure 5e–h to assess their longitudinal cell-state and isotype trajectories.

Second, to test whether increasing the number of aNAV and aNAV-related BCRs would reveal stronger clonal expansion, we performed several new analyses. In this revision, we added analysis of the post-onset time point, as well as a combined analysis of preclinical and post-onset samples. This included 16,814 BCRs at the preclinical time point (**new Figure 5a**), 18,068 BCRs at the post-onset time point (**new Extended Data Figure 6a**), and 33,496 BCRs after merging both time points (**new Extended Data Figure 6b**). Across individual time points and the merged dataset, the majority of aNAV and aNAV-related BCRs remained singletons, and the percentage of clonal BCRs was comparable across conditions and time points (**new Extended Data Figure 11c–e**).

Third, we also analyzed clonality only within the aNAV cell cluster, without including related BCRs from other B cell subsets. This analysis again showed that most aNAV IgM BCRs were singletons. A small fraction of clonal aNAV BCRs was detected, but this fraction was comparable among Converters, Nonconverters, and healthy controls (**new Extended Data Figure 6f–h**).

Fourth, although the percentage of clonal BCRs within the aNAV and aNAV-related BCR compartments was comparable across groups, our analysis in Fig. 5d showed that clonal BCRs from Converter aNAV^{CXCR5⁺}-related clones contributed a significantly higher proportion of the total clonal BCR repertoire than those in Nonconverters and healthy controls. This indicates that aNAV-derived clonal BCRs may still contribute substantially to overall BCR clonal expansion in Converters. Therefore, it would be inappropriate to conclude that aNAV cells “lack clonal expansion.”

Finally, in this revision, we performed additional scRNA-seq and VDJ-seq using RA and healthy control B cells. We enriched the naive B cells using a commercial naive B cell enrichment kit (STEMCELL Technologies, Cat#100-0971) to increase sequencing depth within the naive B cell compartment. We chose this strategy because resting naive B cells need to be retained in the dataset to distinguish resting naive and activated naive B cell states by scRNA-seq. In this new dataset, 29,738 cells (three samples) were included, and we identified two resting naive-like clusters and two activated naive-like clusters (**new Extended Data Figure 6i–k**). Consistent with the original dataset, these naive and activated naive-like B cells were mostly singletons, with very low levels of clonality (**new Extended Data Figure 6l**).

Taken together, these analyses suggest that the singleton-dominant pattern of aNAV and related BCRs is unlikely to be explained solely by insufficient sequencing depth. Rather, the data support a model in which the aNAV compartment is continuously replenished by newly recruited singleton B cells, rather than being dominated by large clonal expansions. At the same time, we agree with the Reviewer that the persistence of singleton BCRs in the aNAV compartment in Converters could be interpreted in more than one way. We have therefore revised the manuscript to describe this pattern more cautiously as a retained or differentiation-restrained aNAV-associated BCR trajectory, rather than definitive evidence of senescence-associated developmental arrest. Larger longitudinal studies with deeper purified aNAV sequencing will be needed to fully resolve the clonal dynamics of this population.

Further, Figure 5i demonstrates somatic hypermutation indicating that clone had to undergo several divisions, since cells undergo roughly one point mutation per division. Since this is a main conclusion of the paper, and highly relevant to generating a model of how B cell tolerance is broken, it is crucial to resolve this difference.

Response: We thank the Reviewer for pointing this out. We agree that the presence of somatic hypermutation indicates that at least some aNAV-related BCR lineages have undergone cell division and diversification. We would like to clarify that the BCRs shown in the original Figure 5l were selected from aNAV-related cells that had progressed into non-aNAV^{CXCR5+} compartments, including switched memory B cells (aNAV1R1, aNAV2R1) and atypical B cells (aNAV1R2). Therefore, these examples represent a subset of aNAV-related BCR lineages that differentiated into later B cell states and acquired somatic mutations.

Our interpretation is that the aNAV compartment in preclinical RA Converters is heterogeneous. Although a substantial fraction of aNAV cells displays a senescence-like and potentially differentiation-restrained phenotype, a subset of aNAV IgM BCRs can still progress into later B cell states and acquire somatic hypermutation. Thus, the presence of somatic hypermutation in selected aNAV-related progeny does not contradict the observation that many aNAV cells/BCRs remain retained in the aNAV state in circulation. Rather, it suggests that only a limited fraction of aNAV-related BCR lineages enters downstream differentiation pathways, while a large fraction remains in the aNAV state.

In the revised Discussion (page 15, lines 562-565), we now propose a more nuanced model in which newly emerged singleton rNAV cells in Converters show increased activation potential, followed by potential antigenic stress and proinflammatory amplification within the aNAV^{CXCR5+} compartment, ultimately contributing to systemic autoimmune activation. Within this heterogeneous aNAV compartment, a substantial portion of cells acquire a senescence-like and potentially differentiation-restrained state, whereas others may retain the capacity to divide and differentiate into later B cell states.

We also highlight the complexity of these events, in which chronic antigenic stress, dysregulated BCR signaling, and metabolic reprogramming may all contribute to aNAV accumulation. Therefore, aberrant aNAV accumulation is unlikely to be explained by proliferative clonal expansion or tolerance breakdown alone. We have revised the manuscript to better clarify this heterogeneity, avoid overgeneralizing developmental arrest to all aNAV cells, and highlight the need for future studies to determine the mechanisms controlling these divergent trajectories.

While Figure 8 is an elegant demonstration of B cell antigen presentation to T cells, there is not a huge difference between aNAV, rNAV, and CD27⁺ cells. The way the data are presented is always a single B cell population with different conditions, then the authors claim that aNAVs robustly activated while rNAV did not activate. The authors do not directly compare aNAV vs rNAV for the same condition and readout, and from the data spread I expect that if they did they would not find a significant difference of aNAV vs rNAV in most readouts (although %IFN γ may be an exception with a mean of ~1% aNAV and ~0.5% rNAV, which would add interest). This comparison should be performed, and while the results are interesting either way, the authors should discuss this and whether it is a potential limitation of the interpretations.

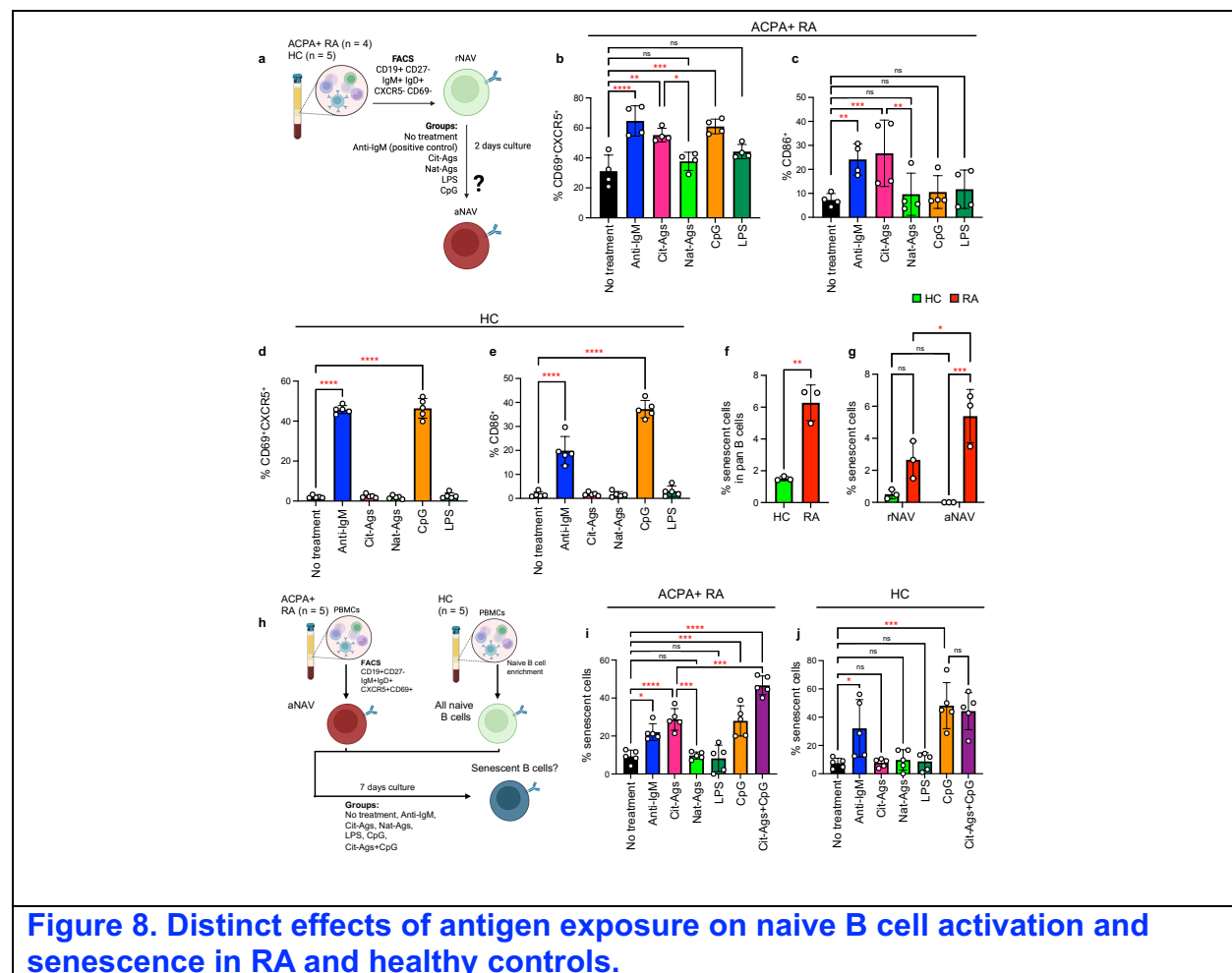
Response: We thank the Reviewer for this careful observation. We agree that antigen-stimulated rNAV cells also showed the capacity to activate T cell responses in Figure 8, and that some readouts were close to those observed with aNAV cells. We have revised the manuscript to describe this rNAV trend more clearly (page 13, lines 489-491) and to avoid overinterpreting the data as evidence that aNAV cells have superior antigen-presenting capacity compared with rNAV or CD27⁺ B cells.

We have also described in the revised text a potential difference between rNAV and aNAV cells under citrullinated antigen stimulation. Specifically, aNAV cells induced a significantly higher frequency of IFN- γ ⁺ CD4⁺ T cells than rNAV cells under citrullinated antigen stimulation (aNAV, 1.06 $\pm$ 0.54% versus rNAV, 0.23 $\pm$ 0.23%; $P < 0.05$) (page 13, lines 490-493). We present this as a specific functional difference observed in this assay, while acknowledging that rNAV cells can also contribute to T cell activation.

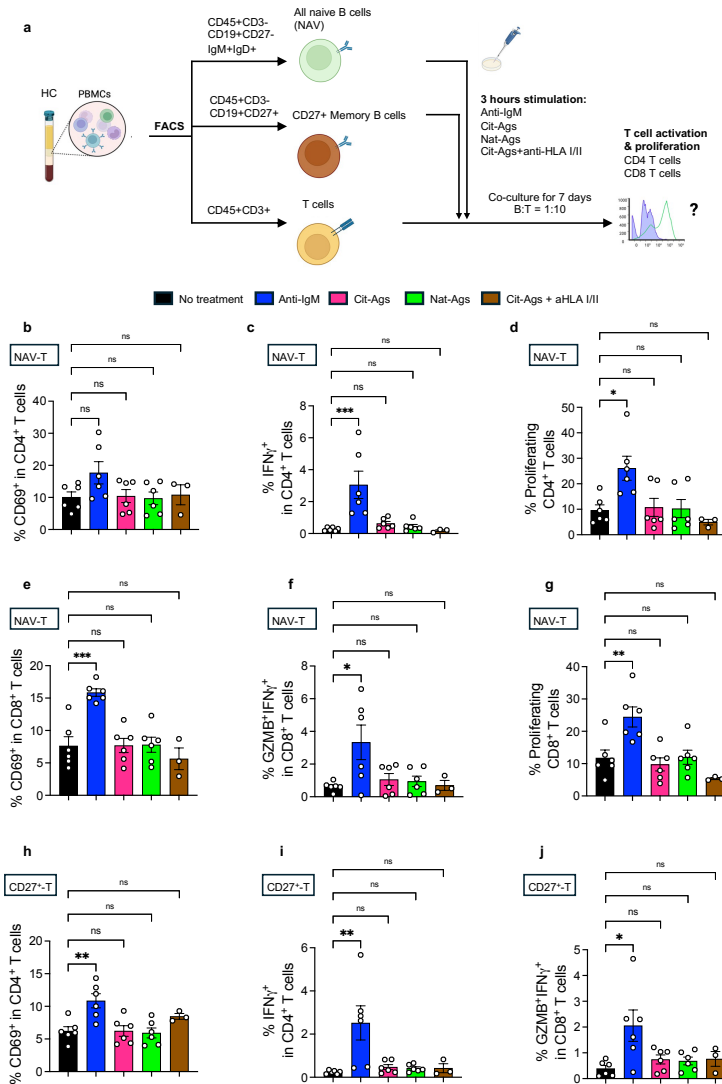
We would like to clarify that the primary goal of this experiment was to test whether aNAV cells can function as antigen-presenting B cells and activate T cell responses, particularly in response to citrullinated antigens. Our results showed that aNAV cells induced T cell activation after citrullinated antigen stimulation, and that this response was reduced by anti-HLA class I/II blockade, supporting an antigen presentation-dependent mechanism.

It should also be noted that our rNAV activation assay showed that rNAV cells can acquire an aNAV-like phenotype after 2 days of culture. Therefore, in the 7-day B cell–T cell co-culture assay, a fraction of rNAV cells may have differentiated into an aNAV-like state during culture. Because we did not longitudinally track B cell phenotypes throughout the co-culture period, this assay is not ideal for definitively comparing the intrinsic APC capacity of true rNAV versus aNAV cells. Thus, in this experiment, “rNAV” and “aNAV” refer to the initial B cell states before co-culture, rather than necessarily reflecting stable B cell states throughout the entire 7-day assay.

In this revision, we also repeated the antigen stimulation (**new Figure 8**) and B cell–T cell co-culture assays (**new Extended Data Figure 9**) using healthy control samples. In contrast to RA B cells, citrullinated antigens did not induce comparable aNAV differentiation, senescence-like features, or T cell activation in healthy control samples, whereas CpG remained effective. These new findings, together with our previous observation that native antigens failed to induce naive B cell activation, senescence and later T cell response, further support the idea that RA-derived B cells, especially aNAV cells, are uniquely primed to respond to citrullinated antigens, and promote downstream T cell activation.



(a) Experimental design for antigen screening to identify potential triggers of naive B cell activation. rNAV cells were FACS-sorted from ACPA⁺ RA patients (n = 4) or healthy controls (n = 5) and cultured for 2 days with anti-IgM, citrullinated antigens (Cit-Ags), native antigens (Nat-Ags), CpG, or LPS. (b) Percentages of CD69⁺ CXCR5⁺ cells in RA samples after stimulation. (c) Percentages of CD86⁺ cells in RA samples after stimulation. (d) Percentages of CD69⁺ CXCR5⁺ cells in healthy control samples after stimulation. (e) Percentages of CD86⁺ cells in healthy control samples after stimulation. (f) Frequencies of β -galactosidase-active senescence-like cells among total B cells from ACPA⁺ RA patients and healthy controls. n = 3 per group. (g) Frequencies of β -galactosidase-active senescence-like cells in rNAV and aNAV subsets from ACPA⁺ RA patients and healthy controls. n = 3 per group. (h) Experimental design for antigen screening to identify potential inducers of naive B cell senescence. For RA samples, aNAV cells were FACS-sorted and cultured for 7 days with anti-IgM, Cit-Ags, Nat-Ags, CpG, LPS, or Cit-Ags + CpG. For healthy controls, because aNAV cells were too rare to sort reliably, total naive B cells were isolated using a naive B cell enrichment kit and cultured with the same stimulation panel for 7 days. (i, j) Percentages of β -galactosidase-active senescence-like cells after stimulation in RA and healthy control samples. n = 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by Student's t test (b–g), one-way ANOVA (i,j), or two-way ANOVA where indicated; ns, not significant.

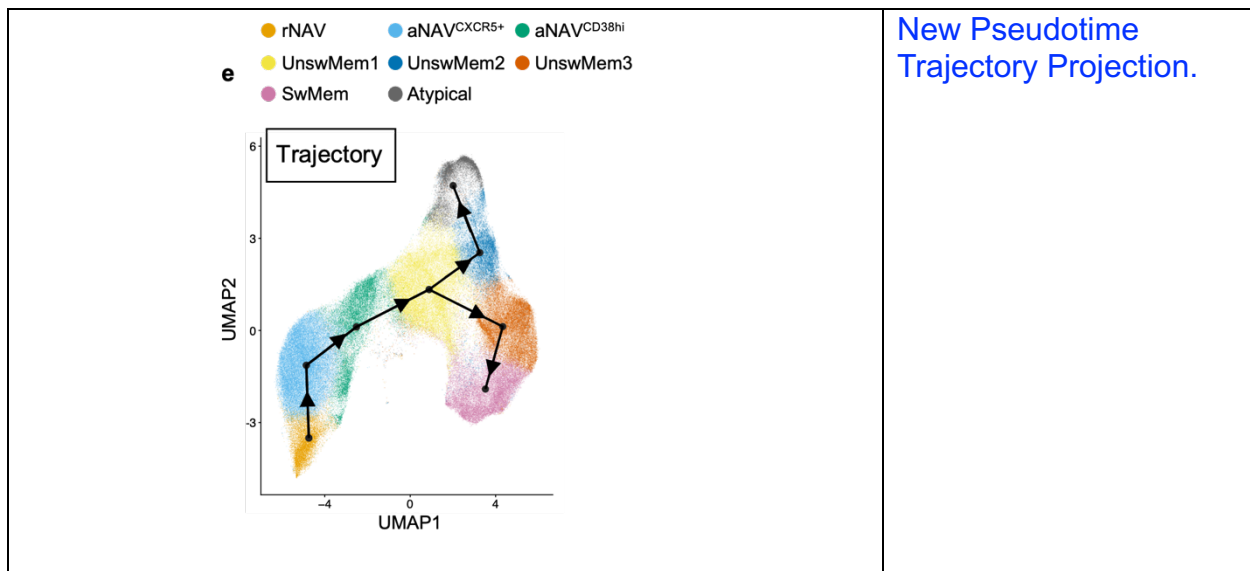


New Extended Data Fig. 9. Citrullinated antigen-stimulated B cells fail to activate T cells in healthy control samples.

(a) Experimental design to assess whether antigen-stimulated B cells activate T cells. Total naive B cells and CD27⁺ memory B cells were FACS-sorted from healthy controls using the indicated markers, stimulated with antigens for 3 h, and then co-cultured with CD3⁺ T cells for 7 days. T cell activation and proliferation were analyzed by flow cytometry. (b–d) Percentages of CD69⁺, IFN γ ⁺, and proliferating CD4⁺ T cells co-cultured with naive B cells. n = 3–6 per group. (e–g) Percentages of CD69⁺, GZMB⁺ IFN γ ⁺, and proliferating CD8⁺ T cells co-cultured with naive B cells. n = 3–6 per group. (h, i) Percentages of CD69⁺ and IFN γ ⁺ cells among CD4⁺ T cells co-cultured with CD27⁺ memory B cells. n = 3–6 per group. (j) Percentage of GZMB⁺ IFN γ ⁺ cells among CD8⁺ T cells co-cultured with CD27⁺ memory B cells. n = 3–6 per group. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA; ns, not significant. NAV: naive B cells.

Line 164-166, authors conclude that CXCR5⁺ and CD38^{hi} distinguish earlier and later stage aNAV cells, respectively. It is very difficult to tell whether this is the case from the pseudotime trajectory in Fig 1e, where it actually looks like green precedes light blue. It may be that there is a very small light blue population between orange and green, but with the colors and the relative abundance it is difficult to see. If that is the aNAV population, please change the colors to be easier to see, if not, please explain how the authors conclude the aNAVCD38^{hi} population is the later stage and provide justification for that conclusion.

Response: We thank the Reviewer for this comment. We believe this confusion was partly caused by the previous visualization of the pseudotime trajectory analysis. In this revision, we have re-performed the trajectory analysis and projected the trajectory directly onto the UMAP (**new Figure 1e**). This updated analysis more clearly suggests a transcriptional relationship from rNAV to aNAV^{CXCR5⁺} and then toward aNAV^{CD38^{hi}} cells.



We acknowledge that some aNAV^{CD38^{hi}} cells are positioned close to rNAV cells on the UMAP, suggesting that these cells may not always pass through a strict aNAV^{CXCR5⁺} intermediate state. We have therefore revised the manuscript to avoid implying that these B cell states represent a definitive continuous linear differentiation lineage. Instead, we now describe them more cautiously as transcriptionally related activation states that may reflect dynamic activation or recirculation states in the circulation.

We have revised the text as follows:

“Pseudotime analysis suggested that rNAV, aNAV, and downstream memory-associated B cell populations are transcriptionally related, with aNAV cells positioned between rNAV and later B cell states. This analysis further showed a branching structure toward SwMem or atypical B cell populations (Fig. 1e).”

In addition, our gene expression analysis in the new Figure 2a showed that B cell activation markers gradually increased from rNAV to aNAV^{CXCR5+} and then to aNAV^{CD38^{hi}} cells. The aNAV^{CD38^{hi}} population expressed the highest levels of CD38, as well as BCR signaling-related genes such as LYN and SYK. These data support the interpretation that aNAV^{CD38^{hi}} cells represent a more activated state than aNAV^{CXCR5+} cells.

However, to avoid overstatement, we have removed the terms “early activated naive” and “late activated naive” from the manuscript. Instead, we now refer to both populations as activated naive B cell states and use CXCR5 and CD69 to distinguish them from rNAV cells and CD38 to distinguish these two aNAV subsets.

G. References: appropriate credit to previous work?

Another group has recently reported the expansion of aNAV cells in pre-RA patients and should be cited: PMID 39931747 “Rheumatoid Arthritis Related B-cell changes are found already in the risk-RA phase” de Vries et al 2025 EJI

Response: We thank the Reviewer for this suggestion. We have now cited this recent study by de Vries et al. in the revised manuscript (page 19, lines 758-767).

We agree that this study is highly relevant to our findings. de Vries et al. reported profound changes in the circulating B cell compartment in the preclinical RA-risk phase, including an expansion of activated naive B cells in individuals who later developed RA. This observation is consistent with our finding that activated naive B cells are expanded in ACPA⁺ Converters before clinical RA onset.

We have also clarified the relationship between the two studies in the revised manuscript. Specifically, de Vries et al. used multiparameter spectral flow cytometry, which provides important information at the cell-surface protein level. In contrast, our study uses longitudinal single-cell multi-omics, allowing us to further define the transcriptional landscape, BCR repertoire features, clonal persistence, and functional heterogeneity of the B cell compartment during progression from the preclinical at-risk stage to clinical RA. Thus, the findings from de Vries et al. provide complementary support for the expansion of activated naive B cells in preclinical RA, while our study extends these observations by identifying their molecular, repertoire, and functional features.

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

There is not sufficient evidence that aNAVs were “senescent” and certainly not that they “served as potent antigen presenting cells to activate both anti-citrullinated antigen CD4⁺ and CD8⁺ T cells” (abstract lines 42-44) given that nowhere did the authors demonstrate the specificity of the T cells, and more importantly that the aNAV were not superior to other B cell types examined (rNAV and CD27⁺). The authors must tone down such conclusions or provide additional analysis to support such claims. Additional claims that are too forceful are:

Response: We thank the Reviewer for this constructive suggestion. We agree that some of our original statements were too strong, and we have now toned down these conclusions in the Abstract, Introduction, Results, and Discussion.

In Abstract, regarding the description of aNAVs as “senescent,” we acknowledge that, although our new validation data showed that aNAVs from Converters contained significantly higher frequencies of β -galactosidase-active cells and p21-positive cells, these data do not definitively demonstrate irreversible cell-cycle arrest. We therefore revised the wording throughout the manuscript and now describe this phenotype as a “senescence-like program” or “senescence-like phenotype” rather than definitive cellular senescence.

Regarding the concern about the statement that aNAVs “served as potent antigen-presenting cells to activate both anti-citrullinated antigen CD4⁺ and CD8⁺ T cells,” we have revised this statement to more accurately reflect our experimental observations. The revised Abstract now states: “aNAVs were detectable in RA synovium and displayed antigen-presentation-associated features. In vitro, aNAVs promoted T cell activation under citrullinated antigen stimulation in an HLA-dependent manner.” We removed the term “potent APCs” to avoid implying that aNAVs are superior antigen-presenting cells compared with other B cell subsets, such as rNAV or CD27⁺ B cells. We also removed wording that directly labels the responding T cells as anti-citrullinated antigen-specific T cells, since T cell specificity was not directly demonstrated.

In addition, we revised statements related to developmental arrest. We no longer claim that aNAVs represent a definitively developmentally arrested population. Instead, we now state that a significant fraction of aNAV IgM BCRs was retained in the aNAV state from the preclinical to post-onset stage, while a subset progressed into later B cell states and underwent class switching and somatic hypermutation.

We also clarified that the expansion, activation, and senescence-like phenotype of aNAVs were validated in multiple additional independent RA cohorts. Together, these revisions more accurately reflect the strength of our data and avoid overinterpreting the functional and senescence-associated findings.

lines 409-419

Response: We have removed the statement that aNAVs were “developmentally arrested.” In the revised manuscript, we use more cautious wording and state that a subset of Converter aNAV cells may acquire a “potentially differentiation-restrained state”.

436 – 437 nothing to demonstrate they are “functionally stalled”, rather the opposite

Response: We have removed the statement that aNAVs were “functionally stalled.” We now use more cautious wording: “Results from this study showed that aNAV cells are not actively proliferating B cells. This suggests that, despite their high CXCR5 expression, the increased frequency of CXCR5⁺ aNAV cells in ACPA⁺ Converters may

not be driven mainly by active proliferative expansion. Instead, these cells may be retained or accumulate in circulation at the preclinical RA stage.”

438-439 “prolonged circulation, impaired differentiation, and persistent presence in peripheral blood” was not tested and no citations are included

Response: We have removed the statement regarding “prolonged circulation, impaired differentiation, and persistent presence in peripheral blood,” as these possibilities were not directly tested. We now state: “Future in vitro B cell differentiation assays and functional BCR signaling assays will be needed to determine whether pathogenic aNAV cells exhibit true anergy-like functional tuning, impaired differentiation capacity, or both.”

440-441 “stuck phenotype is driven by dysregulated B cell signaling shaped by chronic antigenic stimulation, anergy-like functional tuning” no actual comparisons to anergic B cells were included, dysregulated BCR signaling was not demonstrated

Response: We have removed the statement that the “stuck” phenotype is driven by dysregulated B cell signaling or anergy-like functional tuning, since these mechanisms were not directly demonstrated. We revised this section to discuss these possibilities more cautiously as hypotheses requiring future testing.

504 “anti-citrullinated antigen CD4 and CD8 T cell responses” these cells were not demonstrated to be citrullinated antigen-specific

Response: We have removed the phrase “anti-citrullinated antigen CD4⁺ and CD8⁺ T cell responses,” since T cell specificity was not directly demonstrated.

573-574 “Our findings identify a pathogenic naïve B cell subset – senescent polyreactive” authors did not directly demonstrate that these are pathogenic, nor convincingly senescent. Tone it down to “potentially pathogenic”.

Response: We have toned down the conclusion that aNAVs represent a definitively “pathogenic” B cell subset. We now describe these cells as showing aberrant activation and a senescence-like phenotype. When discussing potential disease relevance, we use “potentially pathogenic” rather than definitive pathogenicity.

It would be helpful if the authors put the concept of senescence (cessation of cell division) as it relates to progressive long term autoimmune disease into context. On it's face, I would predict senescence to limit autoimmune disease since the cells would stop dividing and thereby limit the expansion of the population. And would exposure to citrullinated proteins then push cells out of senescence to enhance their proliferation?

Response: We agree that, based on the classical definition of senescence as stable cell-cycle arrest, senescence could be predicted to limit autoimmune progression by restricting the expansion of pathogenic cell populations. This concept is particularly relevant for highly proliferative pathogenic cell types, such as synovial fibroblasts in RA. Indeed, a previous study showed that selective activation of melanocortin receptor MC1 induced a senescence-like phenotype in synovial fibroblasts, characterized by arrested

proliferation and activation of p53/p16/p21-related pathways, and this therapeutic induction of senescence limited inflammatory arthritis progression [30].

However, our interpretation is that the functional consequence of senescence or senescence-like programming in autoimmunity is highly dependent on cell type, disease stage, and tissue context. In the above-mentioned study, synovial fibroblasts are highly proliferative effector cells in established inflammatory arthritis; therefore, inducing senescence in these cells may exert an anti-inflammatory and tissue-protective effect by limiting their pathogenic expansion. In contrast, a recent preprint by a UCLA team [31] reported a senescence-like T cell subset expanded in patients with chronic inflammatory demyelinating polyneuropathy (CIDP). These cells showed increased SA- β -gal activity, elevated CDKN1A expression, and expression of SASP factors, and demonstrated enhanced capacity to induce neuropathy in a CIDP mouse model. These findings support the concept that senescence-like programs can have divergent functional consequences in autoimmune diseases depending on cellular and disease context.

In our study, we focused on circulating naive B cells at the preclinical stage of RA. Our data suggest that maintaining a non-stressed, non-senescence-like state may be important for preserving naive B cell homeostasis and normal BCR trajectory, particularly in ACPA⁺ at-risk individuals. We found that aNAV cells are generally not actively proliferating. However, in ACPA⁺ Converters, these cells acquire a senescence-like and potentially differentiation-restrained state, likely associated with chronic antigenic or metabolic stress. Consistent with this model, treatment with citrullinated antigens and CpG synergistically induced aNAV-like activation and senescence-like features in vitro. Thus, our findings do not support the idea that exposure to citrullinated proteins pushes these cells out of senescence to enhance proliferation. Rather, our data suggest that citrullinated antigen exposure imposes stress on naive B cells, promotes their transition into an aberrantly activated state, and contributes to acquisition of a senescence-like, proinflammatory phenotype.

Importantly, although these senescence-like aNAV cells are not actively proliferating, they may still contribute to autoimmune progression through non-proliferative mechanisms. They exhibit a hyperactivated and proinflammatory transcriptional program, can be detected in RA synovium, produce inflammatory cytokine, and present antigens to activate T cell responses. In this model, continuous emergence of new rNAV cells/BCRs from the bone marrow (**new Figure 5e, f**), followed by antigen-driven activation and stress-induced accumulation of aNAV cells, may progressively reshape the B cell transcriptome and repertoire, ultimately contributing to systemic immune activation and progression toward clinical RA. Nonetheless, we acknowledge that the complex roles of senescence across different cell types and tissue contexts during RA progression require further characterization (page 16, lines 629-641).

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