

Correlation of Professional Antigen-Presenting Tbet⁺CD11c⁺ B Cells With Bone Destruction in Untreated Rheumatoid Arthritis

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Objective. Subsets of CD21^{-/low} memory B cells (MBCs), including double-negative (DN, CD27⁻IgD⁻) and Tbet⁺CD11c⁺ cells, are expanded in chronic inflammatory diseases. In rheumatoid arthritis (RA), CD21^{-/low} MBCs correlate with joint destruction. However, whether this is due to the Tbet⁺CD11c⁺ subset, its function and pathogenic contribution to RA are unknown. This study aims to investigate the association between CD21^{-/low}Tbet⁺CD11c⁺ MBCs and joint destruction as well as other clinical parameters and to elucidate their functional properties in patients with untreated RA (uRA).

Methods. Clinical observations were combined with flow cytometry ($n = 36$) and single-cell RNA sequencing (scRNA-seq) and V(D)J sequencing ($n = 4$) of peripheral blood (PB) MBCs from patients with uRA. The transcriptome of circulating Tbet⁺CD11c⁺ MBCs was compared with scRNA-seq data of synovial B cells. In vitro coculture of Tbet⁺CD11c⁺ B cells with T cells was used to assess costimulatory capacity.

Results. CD21^{-/low}Tbet⁺CD11c⁺ MBCs in PB correlated with bone destruction but no other clinical parameters analyzed. The Tbet⁺CD11c⁺ MBCs have undergone clonal expansion and express somatically mutated V genes. Gene expression analysis of these cells identified a unique signature of more than 150 up-regulated genes associated with antigen presentation functions, including B cell receptor activation and clathrin-mediated antigen internalization; regulation of actin filaments, endosomes, and lysosomes; antigen processing, loading, presentation, and costimulation; a transcriptome mirrored in their synovial tissue counterparts. In vitro, Tbet⁺CD11c⁺ B cells induced retinoic acid receptor-related orphan nuclear receptor γ T expression in CD4⁺ T cells, thereby polarizing to Th17 cells, a T cell subset critical for osteoclastogenesis and associated with bone destruction.

Conclusion. This study suggests that Tbet⁺CD11c⁺ MBCs contribute to the pathogenesis of RA by promoting bone destruction through antigen presentation, T cell activation, and Th17 polarization.

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation that often leads to cartilage and bone destruction and, if left untreated, results in varying degrees of disability.¹ Patients with the synovial lympho-myeloid immunohistochemistry pathotype, characterized by high lymphocyte infiltration and B cell dominance, are more likely to develop joint destruction compared to patients with other synovial pathotypes.² These patients have significant enrichment of osteoclast-associated genes in the synovium, indicating higher osteoclast-mediated bone erosion. A role for B cells in RA-associated bone erosion is further supported by the success of B cell depletion therapy in preventing joint destruction independent of other clinical improvements.^{3,4} However, the role of B cells in RA pathogenesis and erosive mechanisms is not fully understood. The erosive contribution may involve their role as antibody-secreting cells because autoantibodies to IgG (rheumatoid factor [RF]) and citrullinated proteins (anti-citrullinated protein antibodies [ACPAs]), both typical of RA, appear decades before disease onset^{5,6} and are associated with more destructive joint disease.⁷ However, the success of B cell depletion therapy is independent of a reduction in autoantibody levels,⁸ and the pathogenicity of ACPAs is unclear.^{9,10} This suggests that B cell functions beyond autoantibody production are critical in bone destruction and RA pathogenesis.

In recent decades, a CD21^{-/low} memory B cell (MBC) population has attracted attention because of its expansion with age and its involvement in chronic inflammatory diseases as well as viral infections.^{11–18} This is a heterogeneous cell population that includes CD27⁺ and double-negative (DN, CD27⁻IgD⁻) subsets as well as CD11c⁺ and/or Tbet⁺ subsets thereof. CD11c, also known as integrin αX, pairs with the integrin β₂ chain (CD18) and is widely expressed by myeloid-derived cells, such as dendritic cells, and is important for vascular adhesion and migration to inflamed tissues.¹⁹ CD11c and CD18 are expressed also in B cells and may therefore be involved in B cell migration.²⁰ Tbet is a transcription factor that was first found to be expressed in T cells, where it directs the development of the Th1 lineage and the production of interferon-γ (IFNγ).²¹ It was subsequently found to be expressed in B cells, where it mediates IFNγ-driven Ig class switching to IgG2a/c in mice.²²

CD21^{-/low} B cells and subsets thereof are also expanded in RA.^{23–30} We have found that the DN subset correlates with joint destruction in both established RA³¹ and untreated RA (uRA) at diagnosis,³² and others have found a CD11c⁺ subset associated with disease activity.^{29,33} Expansion of CD11c⁺ and/or Tbet⁺ B cell subsets has also been observed in inflamed synovial tissue and fluid.^{28,29,31,32,34} However, it is still unclear whether the CD21^{-/low} MBCs that correlate with joint destruction are Tbet⁺CD11c⁺ and the underlying mechanisms by which they contribute to bone destruction.

Here, we combined clinical data from patients with uRA with extensive flow cytometry analyses of peripheral blood (PB) to determine whether the CD21^{-/low} B cell population associated with joint destruction corresponds to Tbet⁺CD11c⁺ MBCs and used single-cell RNA sequencing (scRNA-seq) to identify the underlying B cell functions contributing to the joint destructive mechanisms in RA. We find that Tbet⁺CD11c⁺ MBCs, both DN and CD27⁺, are strongly associated with bone destruction in uRA. Together with the up-regulation of >150 differentially expressed genes (DEGs) in PB Tbet⁺CD11c⁺ MBCs associated with antigen processing and presentation, clathrin-mediated antigen uptake, and modulation of the actin cytoskeleton, with a mirrored signature in synovial tissue counterparts, this suggests that these cells function as professional antigen-presenting cells in the inflamed joint. We also show that Tbet⁺CD11c⁺ B cells are able to induce Th17 polarization of CD4⁺ T cells in vitro. Based on these results, we propose that Tbet⁺CD11c⁺ B cells play a role in the pathogenesis of RA by driving Th17 polarization and thereby bone destruction.

PATIENTS AND METHODS

PB samples were obtained from 36 patients with uRA at diagnosis and 11 healthy buffy coat donors. MBC phenotypes were characterized by flow cytometry of PB from 36 patients with uRA and, after sorting of MBCs from four of these patients, these were analyzed by scRNA-seq and V(D)J sequencing. The demographics and characteristics of individuals with uRA are detailed in Supplemental Table 1. Additional comparison of our PB MBC transcriptome was made with a publicly available data set of synovial B cells.³⁴ Complete details of the study's methodology can be found in the Supplemental Materials and Methods.

RESULTS

Elevation of PB CD21^{-/low}Tbet⁺CD11c⁺ MBCs and correlation with bone destruction in patients with RA at diagnosis. To determine whether the CD21^{-/low} MBCs that were associated with joint destruction in both patients with established RA and those with uRA^{31,32} were Tbet⁺ and/or CD11c⁺, we first analyzed CD19⁺CD20⁺ B cells from patients with uRA and healthy donors (HDs) for expression of these markers (Supplemental Tables 1–3). Tbet⁺CD11c⁺ cells were exclusive to the CD21^{-/low} population and are hereafter referred to as Tbet⁺CD11c⁺ (Figure 1A). Approximately 40% of the uRA CD21^{-/low} B cells were Tbet⁺CD11c⁺, significantly higher than in HDs (Figure 1B). When the CD21^{-/low} MBCs were divided into DN or CD27⁺ (Figure 1A), the proportion of Tbet⁺CD11c⁺ cells in the DN, but not in the CD27⁺ compartment, was significantly increased in uRA (Figure 1C and D). In the context of the total B cell compartment, Tbet⁺CD11c⁺ cells accounted for approximately 2.5% of all uRA B cells, significantly higher than in HDs

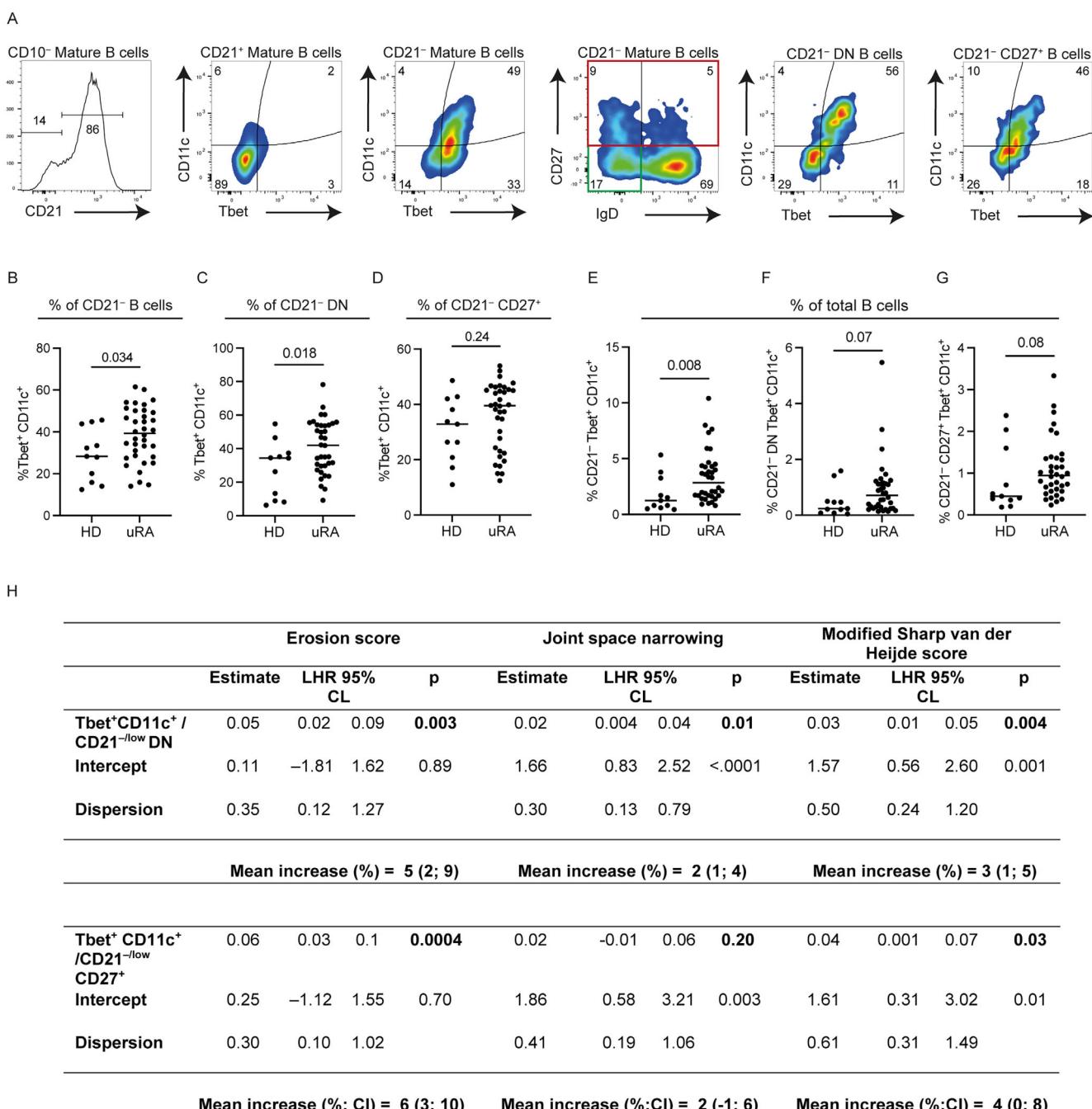


Figure 1. Expansion of Tbet⁺CD11c⁺ B cells and correlation with joint destruction in uRA. (A) Peripheral blood B cell gating strategy, CD21⁻CD27⁺ population framed in red, and DN cells framed in green. (B) Proportion of Tbet⁺CD11c⁺ cells within the total CD21^{-low} B cells in patients with uRA ($n = 36$) and HDs ($n = 11$). Mean indicated, unpaired *t*-test. Proportion of Tbet⁺CD11c⁺ cells within (C) CD21^{-low} DN B cells and (D) CD21⁻CD27⁺ B cells in patients with uRA and HDs. Mean indicated, unpaired *t*-test. (E) Proportion of CD21^{-low}Tbet⁺CD11c⁺ cells within the total B cells compartment in patients with uRA and HD. Median indicated, two-tailed Mann-Whitney test. Proportion of (F) CD21^{-low} DN Tbet⁺CD11c⁺ cells and (G) CD21^{-low} CD27⁺ Tbet⁺CD11c⁺ cells within the total B cells compartment in patients with uRA and HD. Median indicated, two-tailed Mann-Whitney test. (H) Parameter estimates with LHR 95% CLs for transformed ES (bone erosion), transformed joint space narrowing score (cartilage destruction), and transformed modified Sharp van der Heijde score according to zero-inflated negative binomial models. The parameter interpretation is presented as the mean increase (%) with 95% CI for a 1-unit (ie, 1%) increase in the B cell population ($n = 35$). CI, confidence interval; DN, double-negative; ES, erosion score; HD, healthy donor; LHR 95% CL, likelihood ratio 95% confidence limit; uRA, untreated rheumatoid arthritis.

(Figure 1E). Neither DN nor CD27⁺ Tbet⁺CD11c⁺ B cells showed significantly higher frequencies within the total B cell compartment, although an increasing trend was observed (Figure 1F and G).

Next, we investigated the association between DN and CD27⁺ Tbet⁺CD11c⁺ B cells and several clinical parameters, including joint destruction parameters, erosion score (ES, bone erosion), joint space narrowing score (JSN, cartilage destruction), and total modified Sharp van der Heijde score (mSHS), initially via a preliminary principal components analysis (Supplemental Figure 1A). Based on this, we further examined the associations between factors clustering close to Tbet⁺CD11c⁺ MBCs via Spearman correlations and statistical regression models (as described in this article) and only found associations with joint destruction outcomes (Supplemental Figure 1A and B). On inclusion, many patients do not present with radiologic destruction, and there is therefore an inherent inflation of zero values in the distribution of these radiologic scores (here approximately 27% for ES) (Supplemental Figure 1C). This type of data requires statistical models that account for zero inflation. However, there was the same percentage of “2” values for the ES. Because of the double inflation in ES, the values 0 to 2 were transformed into a zero category for statistical reasons, whereas the values 0 to 2 for JSN were transformed for consistency and will be referred to as transformed values. Furthermore, the mSHS was recalculated as the sum of the transformed ES and JSN, and the new 0 to 2 values were transformed into a zero category, rendering a transformed mSHS. As expected in patients with uRA at diagnosis, the degree of joint destruction was low. Nevertheless, both DN ($P = 0.003$) and CD27⁺ ($P = 0.0004$) Tbet⁺CD11c⁺ showed a significant positive correlation with transformed ES, and DN Tbet⁺CD11c⁺ also showed a significant positive correlation with transformed JSN ($P = 0.01$) (Figure 1H). Specifically, a 1% increase in DN Tbet⁺CD11c⁺ cells resulted in a 5% increase in ES and a 2% increase in JSN, and for CD27⁺ Tbet⁺CD11c⁺, the corresponding is a 6% increase in ES. In both populations, this resulted in significant correlations with the transformed mSHS. Age and presence or level of ACPAs and/or RF were not significant when introduced into the models. In conclusion, our results show that PB Tbet⁺CD11c⁺ MBCs are expanded in patients with uRA and that Tbet⁺CD11c⁺ MBCs, whether DN or CD27⁺, are correlated with ES.

Tbet⁺CD11c⁺ MBCs from PB and the distinct transcriptional signature.

To investigate potential mechanisms driving the association between Tbet⁺CD11c⁺ MBCs and bone destruction in uRA, we set out to analyze their transcriptome (Figure 2A). Because of the low frequency of MBCs, these were sorted to ensure sufficient Tbet⁺CD11c⁺ cells in the scRNA-seq data (Figure 2B). To assess marker expression and thereby identify the cells in scRNA-seq, we performed extensive phenotyping

by flow cytometry in eight patients, four of whom were also analyzed by 5' scRNA-seq and V(D)J sequencing (Figure 2A).

We first performed unsupervised clustering on the scRNA-seq data from patient 4, who had the highest frequency of Tbet⁺CD11c⁺ MBCs according to our flow cytometry. Unsupervised clustering grouped cells into five distinct clusters, clusters 0 to 4 (Figure 2C), with up-regulation of 68 to 245 genes per cluster (Figure 2D, Supplemental Table 4). Cluster 3 was uniquely CR2⁻ (CD21), contained in principle all TBX21⁺ (Tbet) and ITGAX⁺ (CD11c) cells, and was heterogeneous for CD27 expression (Figure 2E). Thereafter, markers previously used to identify subsets of the CD21^{-/low} B cell population^{17,18,35,36} were analyzed at the protein level by flow cytometry, comparing Tbet⁺CD11c⁺ with Tbet⁻CD11c⁻ MBCs, and at the RNA level in the scRNA-seq data. Tbet⁺CD11c⁺ MBCs, irrespective of DN or CD27⁺, showed increased expression of FcRL5, FcRL3, CD95, CXCR3, CD19, and CD20 (Figure 2F). These markers were also highly expressed in cluster 3 (Figure 2G). Both DN and CD27⁺ Tbet⁺CD11c⁺ cells showed reduced expression of CD62L, CD24, CD38, CXCR5, and CXCR4 (Figure 2H), with correspondingly low transcript levels in cluster 3 (Figure 2I). These expression patterns were consistent in patients with uRA (Figure 2J and K), as were the high levels of CD19 and CD20 (data not shown). Despite their overall phenotypic similarity, higher proportions of the CD27⁺ subset expressed CD95, CXCR3, CD62L, CD24, CD38, and CXCR5 (Figure 2L). We conclude that Tbet⁺CD11c⁺ MBCs are restricted to cluster 3 in the scRNA-seq data, which contains both CD27⁺ and DN subsets with subtle differences in marker expression.

Subclustering and difference between DN and CD27⁺ Tbet⁺CD11c⁺ MBCs.

Cluster 3 included all Tbet⁺CD11c⁺ MBCs, but this cluster still showed heterogeneity in the expression of defining markers. Furthermore, cluster 3 represented 15% of all MBCs (Figure 2C), whereas by flow cytometry, Tbet⁺CD11c⁺ MBCs were 10% of all MBCs (data not shown). Taken together, this suggested that cluster 3 consisted of more than one subset and could be further subdivided. Unsupervised reclustering of cluster 3 resulted in three subclusters; 3.0, 3.1, and 3.2 (Figure 3A). Hierarchical clustering distinguished subcluster 3.0 from subclusters 3.1 and 3.2, with the latter two showing higher similarity (Figure 3B, Supplemental Table 5). Expression of the up-regulated markers Tbet, CD11c, FcRL5 and FcRL3, CD95, CD19, and CD20 was predominantly observed in subclusters 3.1 and 3.2 (Figure 3C and D), whereas CXCR3 and CD27 expression was mainly restricted to subclusters 3.2 and 3.0 (Figures 2E and G and 3C and E). Thus, based on the expression of key markers, Tbet⁺CD11c⁺ DN MBCs are in subcluster 3.1, and Tbet⁺CD11c⁺ CD27⁺ MBCs are in subcluster 3.2.

Ig isotypes were subsequently assessed both by identification of productive transcripts from the V(D)J sequencing data and by flow cytometry (Figure 3F–J). As expected, most DN

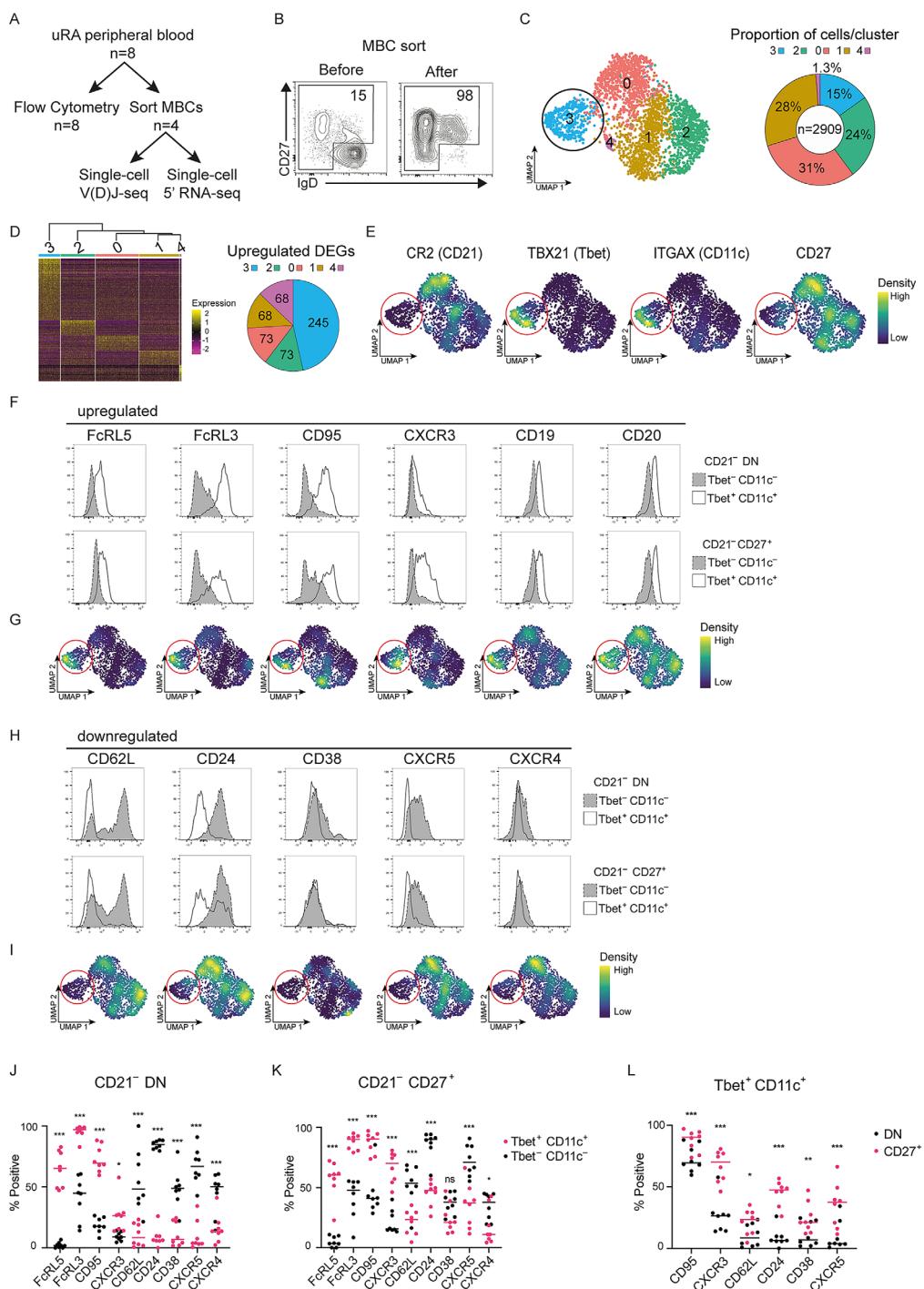


Figure 2. Single-cell transcriptome analysis locates peripheral blood Tbet⁺CD11c⁺ MBCs to a single cluster. (A) Peripheral blood MBCs from patients with uRA were isolated and analyzed for marker expression by flow cytometry ($n = 8$) and by single-cell 5' RNA and V(D)J sequencing ($n = 4$). (B) Purity of MBCs before and after sort (%). (C) Unsupervised clustering and UMAP projection of the sequenced transcriptome identified five clusters from patient 4. The panel shows a pie chart of the proportion of cells/cluster and the total number of cells in the center. (D) Heatmap of the relative expression of DEGs up-regulated/cluster and hierarchical clustering based on variable features. The panel shows a pie chart of the number of DEGs/cluster. (E) Density plots of CR2 (CD21), TBX21 (Tbet), ITGAX (CD11c), and CD27 expression. Histograms and density plots of indicated markers were analyzed by (F and H) flow cytometry and (G and I) single-cell RNA-seq and categorized as (F and G) up-regulated or (H and I) down-regulated in Tbet⁺CD11c⁺ MBCs. Proportion of cells positive for indicated markers in (J) CD21⁻DN Tbet⁺CD11c⁺ cells (pink) and CD21⁻DN Tbet⁻CD11c⁻ cells (black), (K) CD21⁻CD27⁺Tbet⁺CD11c⁺ cells (pink) and CD21⁻CD27⁺Tbet⁻CD11c⁻ cells (black), and (L) DN Tbet⁺CD11c⁺ cells (black) and CD27⁺Tbet⁺CD11c⁺ cells (pink). Lines indicate the median. Two-way analysis of variance with Sidak's multiple comparisons test was used. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n = 8 patients with uRA. DEG, differentially expressed gene; DN, double-negative; MBC, memory B cell; RNA-seq, RNA sequencing; UMAP, uniform manifold approximation and projection; uRA, untreated rheumatoid arthritis.

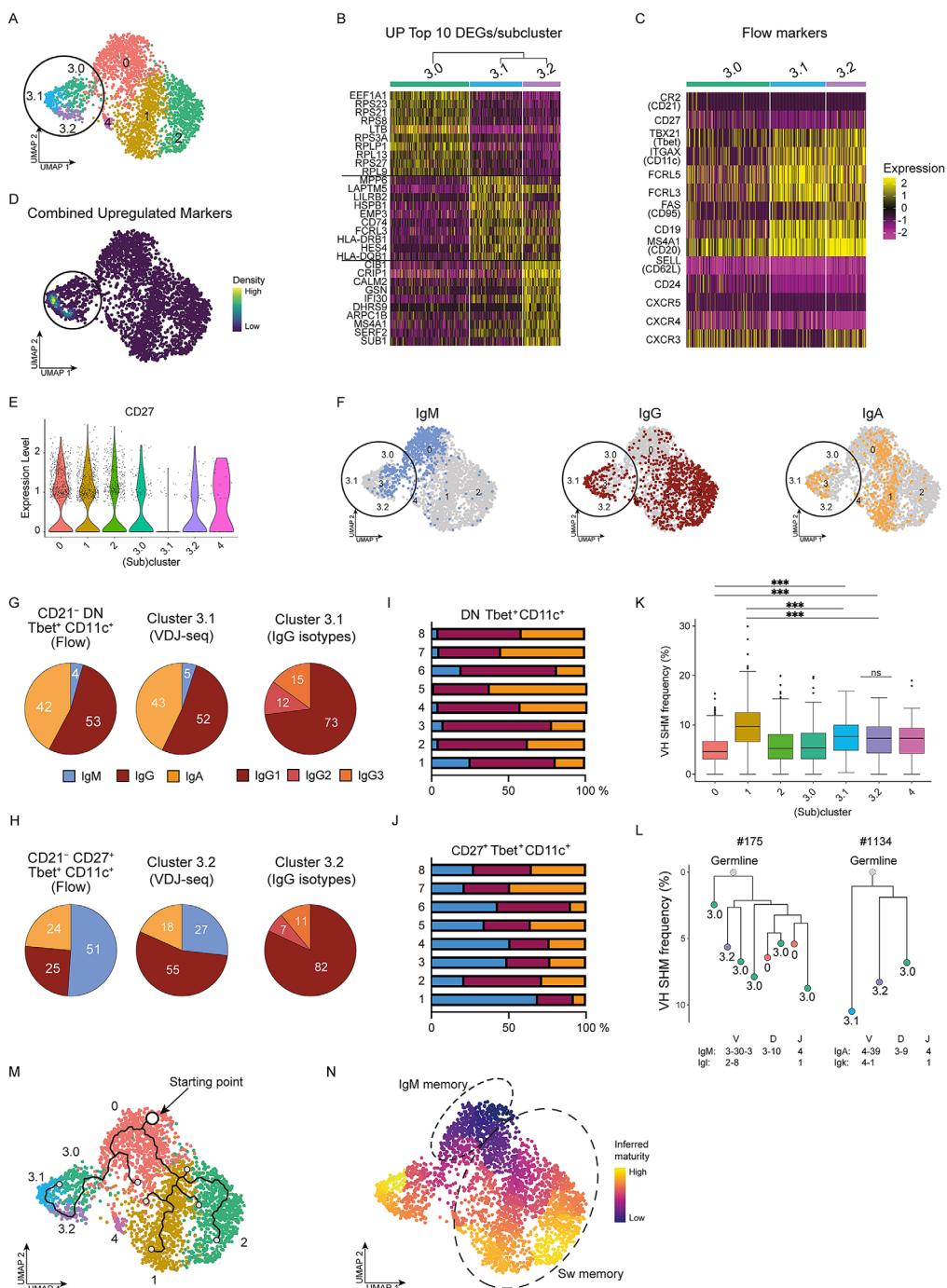


Figure 3. Subclusters 3.1 and 3.2 are composed of peripheral blood Tbet⁺CD11c⁺ MBCs, which have undergone somatic hypermutation and clonal expansion. (A) Unsupervised subclustering of cluster 3. (B) Relative expression of top 10 up-regulated DEGs in subclusters and hierarchical clustering based on variable features. (C) Relative expression of genes defined by flow cytometry as up-regulated and down-regulated. (D) Density plot of combined expression of up-regulated markers. (E) Scaled expression of CD27 across (sub)clusters. (F) Productively recombined IgH isotypes determined by V(D)J sequencing. Proportion of Ig isotypes, inferred by flow cytometry (left), V(D)J sequencing (middle), and V(D)J sequencing-determined IgG subclasses (right), in (G) DN Tbet⁺CD11c⁺ and subcluster 3.1 and (H) CD27⁺Tbet⁺CD11c⁺ and subcluster 3.2. Ig isotype distribution in (I) DN Tbet⁺CD11c⁺ and (J) CD27⁺Tbet⁺CD11c⁺ ($n = 8$ patients with uRA). (K) Frequency of SHM in Ig heavy-chain V genes. Analysis of variance with the post hoc Tukey test was used. *** $P < 0.001$. (L) Dendograms display cells assigned to the indicated clonotype, with designated V(D)J genes. The length of branches represents the degree of SHM relative to the inferred germline. (M) Dimension plot colored by cluster assignment, with Monocle3-based developmental trajectory superimposed. The arrow indicates the origin of the trajectory. (N) Dimension plot colored by inferred maturity from pseudotemporal analysis. Dashed circles indicate IgM⁺ and switched cells. Data from patient 4 are shown throughout the figure. DEG, differentially expressed gene; DN, double-negative; MBC, memory B cell; ns, not significant; SHM, somatic hypermutation; UMAP, uniform manifold approximation and projection; uRA, untreated rheumatoid arthritis; VH, heavy chain V gene.

Tbet⁺CD11c⁺ MBCs were switched to either IgG or IgA, with 73% of IgGs being IgG1 (Figure 3G). The distribution of Ig isotypes by flow cytometry in CD27⁺ Tbet⁺CD11c⁺ MBCs showed some discrepancy with subcluster 3.2 (Figure 3H). However, IgG1 also dominated the IgG isotype in subcluster 3.2, consistent with Tbet-mediated class switching to its murine equivalent, IgG2a/c.²² The distribution of Ig isotypes remained relatively consistent across patients with uRA, with DN switching predominantly to IgA or IgG, although the IgA:IgG ratio varied between donors (Figure 3I). In contrast, CD27⁺ Tbet⁺CD11c⁺ MBCs consistently showed a higher use of the IgM isotype (Figure 3J). In conclusion, subclusters 3.1 and 3.2 represent DN and CD27⁺ Tbet⁺CD11c⁺ MBCs, respectively, with the DN population largely class switched and the CD27⁺ population containing more IgM⁺ MBCs.

Somatic hypermutation of Tbet⁺CD11c⁺ MBCs and clonal expansion. Previous studies in other diseases have observed somatic hypermutation (SHM) in CD21^{-low} and Tbet⁺CD11c⁺ B cells.¹⁸ Here, we investigated whether the B cell receptors (BCRs), Ig heavy and light chains, of Tbet⁺CD11c⁺ MBCs in uRA showed signs of SHM by examining V(D)J sequencing data. The frequency of SHM in VH sequences was comparable between subclusters 3.1 (DN) and 3.2 (CD27⁺) and was higher than that in cluster 0 but lower than that in cluster 1 (Figure 3K).

In diseases such as malaria, CD11c⁺ B cells have been shown to undergo proliferative expansion,¹⁴ with similar observations in murine equivalent age-associated B cells (ABCs).³⁷ As a proxy for clonal expansion, we assessed the presence of clonotypes, defined as sequences with identical V and J usage and equal CDR3 length with ≥85% nucleotide identity, in both IgH and IgL. This analysis resulted in 19 clonotypes involving subclusters 3.1 (DN) and/or 3.2 (CD27⁺; Supplemental Table 6). Most clonotypes consisted of two to four members, with one exception, clonotype 175, which consisted of eight members of the IgM isotype. This clone was shared between subclusters 3.2 (CD27⁺) and 3.0 and cluster 0 (Figure 3L, Supplemental Table 6). Another example with three members, clonotype 1134 of the IgA isotype, was shared between subclusters 3.0, 3.1 (DN), and 3.2 (CD27⁺). All heavy chain V gene (VH) sequences in the 19 clonotypes showed signs of SHM (ie, no germline sequences), and all members within a clonotype expressed either Igκ or Igλ only, whereas in some clonotypes, the IgH isotype varied between members (eg, one member expressed IgM and another expressed IgG1; Supplemental Table 6). Nevertheless, given the relatively low number of cells in subclusters 3.1 (DN) and 3.2 (CD27⁺) and the relatively high number of clonotypes, this suggests substantial clonal expansion of Tbet⁺CD11c⁺ cells in uRA.

The fact that clonotypes were shared between subclusters 3.1 (DN) and 3.2 (CD27⁺) and other clusters prompted us to infer a developmental pathway. Using cluster 0 with the most IgM-

expressing cells as a starting point resulted in multiple end points (Figure 3M), suggesting a developmental pathway from cluster 0 to subcluster 3.0 and from there to subcluster 3.2 (CD27⁺) followed by 3.1 (DN). Furthermore, pseudotemporal analysis to estimate maturation supported the trajectory results in that the most mature MBCs were in cluster 2, a cluster of predominantly IgG-expressing cells, and in subcluster 3.1 (DN) with predominantly switched cells (Figure 3N). Thus, both subsets of Tbet⁺CD11c⁺ MBCs have undergone clonal expansion, express BCRs that have undergone SHM, and appear to be able to differentiate from CD27⁺ to CD27⁻ (DN).

Gene signature expression of Tbet⁺CD11c⁺ PB MBCs as professional antigen-presenting cells. We next sought to investigate the potential functions of Tbet⁺CD11c⁺ MBCs and the similarities and/or differences between DN and CD27⁺ subtypes in uRA. Because cells in subcluster 3.0 were transcriptionally distinct from subclusters 3.1 (DN) and 3.2 (CD27⁺), we first compared subcluster 3.1 (DN) with subcluster 3.0 and identified 94 up-regulated DEGs (Figure 4A, Supplemental Table 7). The same comparison was performed between subcluster 3.2 (CD27⁺) and subcluster 3.0, identifying 186 up-regulated DEGs (Figure 4B, Supplemental Table 8). Within these up-regulated gene signatures, 51 were shared by both subclusters (Supplemental Table 9), whereas 43 were unique to 3.1 (DN) and 135 were unique to 3.2 (CD27⁺) (Figure 4C, Supplemental Tables 10 and 11), demonstrating considerable overlap in gene expression profiles.

To identify potential functions of DN and CD27⁺ Tbet⁺CD11c⁺ MBCs, we performed functional enrichment for Gene Ontology (GO) terms (ie, molecular functions, biologic processes, and cellular components). Several GO terms were identified from up-regulated DEGs in subcluster 3.1 (DN) (Figure 4D, Supplemental Table 12), with the overarching terms including “antigen processing and presentation via the major histocompatibility complex (MHC),” “antigen receptor signaling pathway,” “regulation of proliferation,” and “granules.” The same overarching terms were also observed in subcluster 3.2 (CD27⁺) (Figure 4E, Supplemental Table 13). The 51 shared DEGs showed the same overarching terms as cluster 3.1 (Figure 4F, Supplemental Table 14), whereas additional GO terms were observed when comparing subclusters 3.0 and 3.2 (Figure 4E), including “cell activation,” “clathrin-coated vesicles” (as part of “antigen processing and presentation via the MHC”), “regulation of actin filament organization,” and “leading-edge membrane,” which was unique to subcluster 3.2 (Figure 4G, Supplemental Table 15). No GO terms were enriched in the 43 DEGs unique to subcluster 3.1.

The strong association with antigen processing and presentation prompted examination of all MHC genes as well as CD74 and B2M. These genes were highly expressed in subclusters 3.1 (DN) and 3.2 (CD27⁺), followed by relatively high expression of

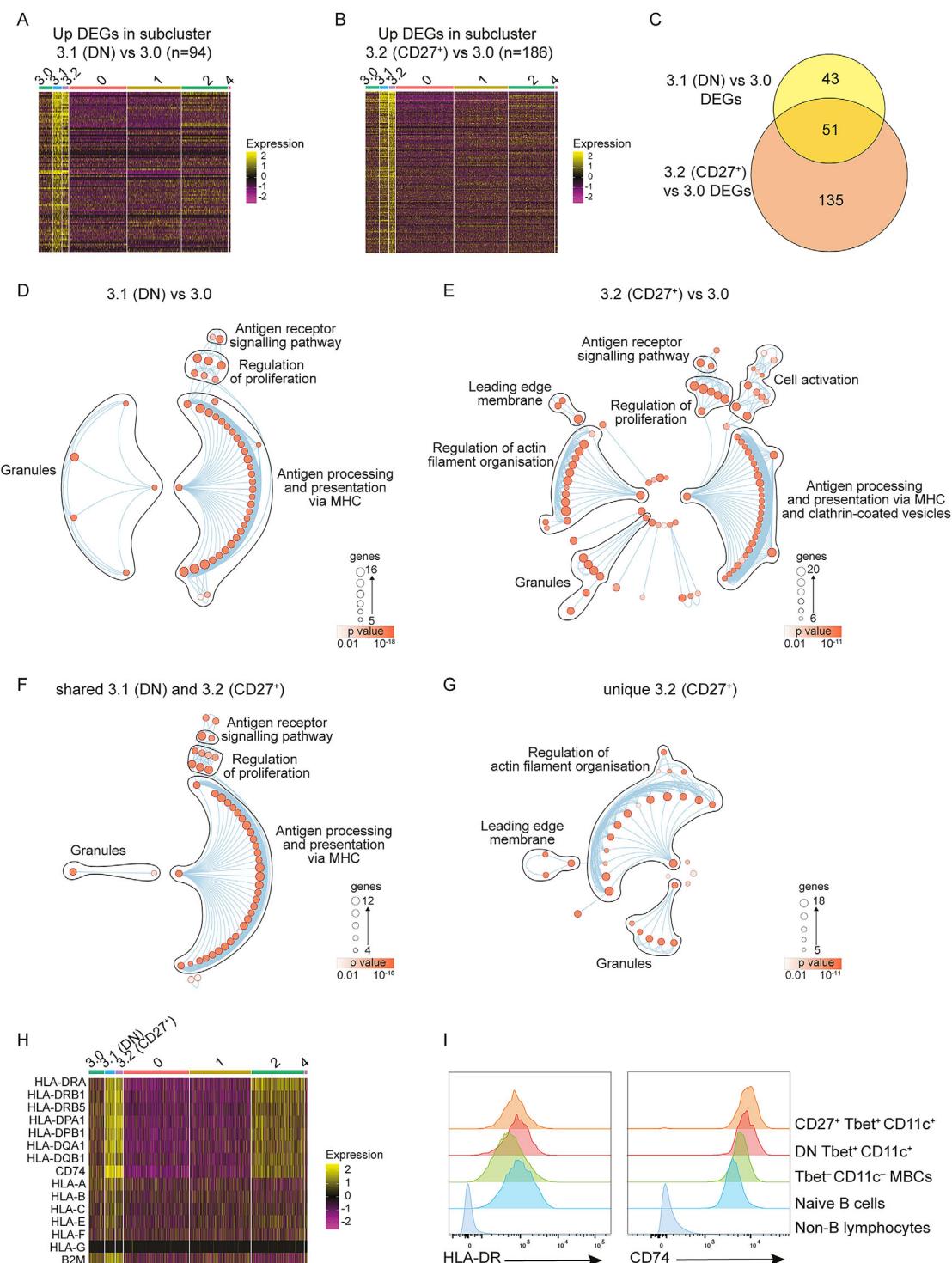


Figure 4. Peripheral blood DN and CD27⁺ Tbet⁺CD11c⁺ MBCs express an antigen-processing and antigen-presenting signature. Heatmaps show relative expression of up-regulated DEGs for (A) subcluster 3.1 compared to subcluster 3.0 and (B) subcluster 3.2 compared to subcluster 3.0. (C) Venn diagram shows the number of unique and shared DEGs for subclusters 3.1 and 3.2 compared to subcluster 3.0. Network graph shows clustered Gene Ontology terms (nodes) from functional enrichment analysis of up-regulated DEGs for (D) subcluster 3.1 compared to subcluster 3.0 and (E) subcluster 3.2 compared to subcluster 3.0, up-regulated DEGs shared between (F) subclusters 3.1 and 3.2, and up-regulated DEGs unique to (G) subcluster 3.2 (excluding shared DEGs with subcluster 3.1). The size of nodes indicates the number of DEGs in the terms. (H) Heatmap shows relative expression of MHC classes I and II and associated genes. (I) Histograms show the protein expression levels (detected by flow cytometry) of HLA-DR (left) and CD74 (right) in CD21^{-low}CD27⁺ Tbet⁺CD11c⁺ MBCs, CD21^{-low} DN Tbet⁺CD11c⁺ MBCs, Tbet⁻CD11c⁻ MBCs, and non-B lymphocytes. DEG, differentially expressed gene; DN, double-negative; MBC, memory B cell; MHC, major histocompatibility complex.

MHC class II and CD74 genes in cluster 2 (Figure 4H). In addition, protein expression levels of HLA-DR and CD74 were high in both DN and CD27⁺ Tbet⁺CD11c⁺ MBCs (Figure 4I). Taken together, subclusters 3.1 (DN) and 3.2 (CD27⁺) have very similar gene expression signatures, enriched for antigen-processing and antigen-presentation functions. They also have subtle differences, with subcluster 3.2 (CD27⁺) expressing higher levels of genes regulating clathrin-mediated endocytosis and the actin cytoskeleton.

To determine whether this transcriptome was consistent across patient samples, we analyzed the data from all four uRA scRNA-seq samples after integration (Supplemental Figure 2). Unsupervised clustering of the integrated sample, with a significantly increased number of cells, resulted in more clusters compared to our analysis of patient 4 alone. Nevertheless, CD21⁻Tbet⁺CD11c⁺ cells were predominantly located in one cluster (labeled cluster 3 for simplicity). Unsupervised subclustering of cluster 3 resulted in four subclusters instead of the three observed with patient 4. DN and CD27⁺ still clustered as two subclusters labeled 3.1 and 3.2, whereas subcluster 3.0 split into two (3.0a and 3.0b). This pattern was observed in all patient samples despite the different frequencies of Tbet⁺CD11c⁺ cells. In addition, subcluster 3.2 but not subcluster 3.1 expressed CD27, and the expression pattern of flow markers, as well as Ig isotype expression, including a dominance of IgG1 among the IgGs, was as expected. Furthermore, the DN and CD27⁺ signatures of patient 4 were observed in subclusters 3.1 and 3.2 with some overlap. Based on these data, we conclude that the Tbet⁺CD11c⁺ gene signatures are common to all four patients with uRA. Taken together, these results support an ability of Tbet⁺CD11c⁺ MBCs to act as professional antigen-presenting cells in RA.

Gene expression profile of synovial Tbet⁺CD11c⁺ B cells reminiscent of their circulating counterparts.

That Tbet⁺CD11c⁺ MBCs expressed a gene signature suggestive of professional antigen-presenting cells was unforeseen because the cells were obtained from PB. However, such a signature might be expected in synovia, where tertiary lymphoid structures with T and B cells have been described.^{2,38–42} We hypothesized that Tbet⁺CD11c⁺ MBCs circulate to and/or from inflamed synovial tissue, where they process and present antigen to T cells. To test this hypothesis, we analyzed scRNA-seq data generated from RA synovial tissue³⁴ containing synovial naive B cells (SC-B1), classical MBCs (SC-B2), and a small but distinct population of autoimmune-associated B cells expressing Tbet and CD11c (SC-B3), here referred to as synovial Tbet⁺CD11c⁺ B cells.

Synovial Tbet⁺CD11c⁺ B cells shared key phenotypic markers with those in PB: low levels of CR2 and high levels of TBX21, ITGAX, FCRL5, FAS, CD19, MS4A1, and CXCR3 but not FCRL3 (Figure 5A). High expression of CD27 was observed

in a fraction of synovial Tbet⁺CD11c⁺ B cells pertaining to a combination of CD27⁺ and DN cells. We then evaluated both the 3.1 (DN) and 3.2 (CD27⁺) gene expression signatures in synovial B cells to determine their similarity to their PB counterparts. Both signatures were in principle mirrored in synovial Tbet⁺CD11c⁺ B cells (Figure 5B and C), indicating that synovial Tbet⁺CD11c⁺ B cells represent a mixture of CD27⁺ and DN. Only 5% of the signature genes were expressed at low levels in synovial Tbet⁺CD11c⁺ B cells, including CD72, CD79A, HLA-DRB5, MT-ND5, NR4A1, P2RX5, PLAC8, RHOB, TXNDC5, and the aforementioned FCRL3.

Synovial Tbet⁺CD11c⁺ B cells showed the same up-regulation of genes associated with inflammatory migration (CXCR3); the antigen receptor signaling pathway, cell activation, or proliferation (eg, CD19, MS4A1, CSK, FGR, NFATC2, PLCG2, POU2F2, PTPN6, RAC1, RAC2, RFTN1; Figure 5B and C); antigen processing and loading (eg, CD74, HLA-DMA and B, DOA and B, B2M); presentation (eg, HLA-DRA and B1, DPA and B, DQA and B); and costimulation (eg, CD80 and 86; Figure 5D). In addition, they also showed up-regulation of genes important for clathrin-mediated antigen uptake by the BCR (eg, AP2M1, SNX9)^{43,44}; endosomes, lysosomes, and granules (eg, LAPT5, STX7, VAMP8)^{45,46}; and genes that regulate actin filament polymerization and depolymerization (eg, ACTB, ARPC, DBNL, FLNA, MTSS1, TLN1, TMSB4X, VASP)^{47,48} (Figure 5B and C).

CD19⁺CD11c⁺ cells have been previously observed in RA synovial tissue,³⁴ but CD19⁺Tbet⁺ B cells have not, nor has their location in relation to T cells. We therefore performed imaging of inflamed synovial tissue from patients with established RA to investigate the colocalization of CD19⁺Tbet⁺ B cells and CD3⁺ T cells. Tbet⁺ B cells were present in lymphocyte-rich areas adjacent to T cells (Figure 5E). In conclusion, PB and synovial Tbet⁺CD11c⁺ B cells share a gene signature indicating a potential role in presenting antigen and activating neighboring T cells within the synovial tissue.

Tbet⁺CD11c⁺ B cells and differentiation of CD4⁺ T cells into Th17 cells.

To test our hypothesis that Tbet⁺CD11c⁺ B cells can drive T cell responses, we generated Tbet⁺CD11c⁺ B cells from HDs in vitro and cocultured them with autologous T cells with plate-bound anti-CD3 because the antigen is unknown (Figure 6A). Several combinations of stimuli can drive Tbet⁺CD11c⁺ B cells, with Toll-like receptor 7/9 (TLR-7/9) ligands and anti-Ig being consistent elements, whereas cytokine contributions vary.^{49–51} Here, PB B cells were activated with either anti-Ig in combination with interleukin-2 (IL-2 stimulated) or anti-Ig and TLR-9 ligand (CpG) in combination with IL-2 and IFN γ (IFN γ stimulated).

Two days after activation, approximately 90% of IFN γ -stimulated B cells were Tbet⁺CD11c⁺ compared to only 20% of IL-2-stimulated B cells (Figure 6B), and a higher proportion of IFN γ -stimulated B cells were HLA-DR^{high}CD86^{high}CD19^{high}

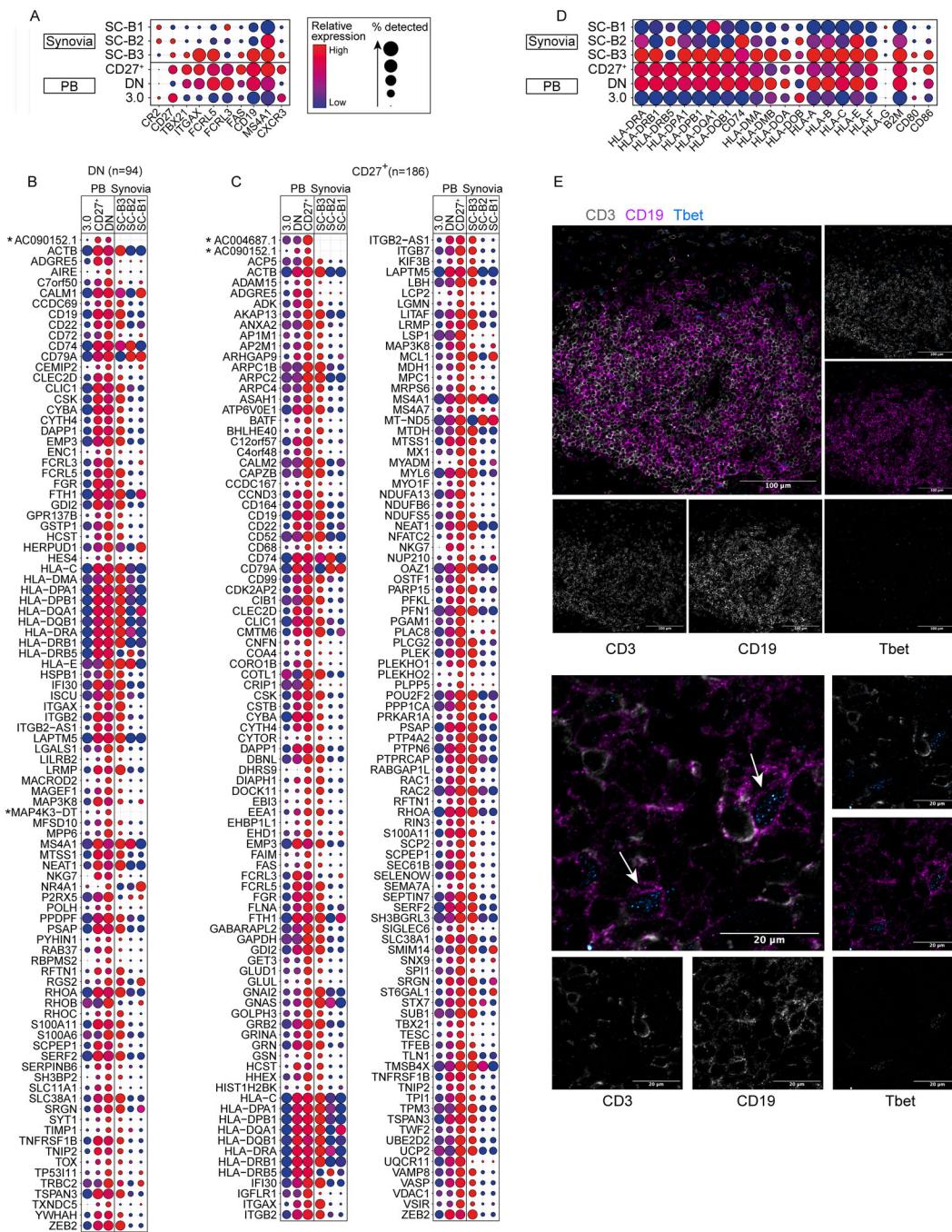


Figure 5. Synovial autoimmune-associated B cells express an antigen-processing and antigen-presenting signature mirroring that of circulating Tbet⁺CD11c⁺ MBCs. Dot plots show relative expression within individual tissue types of (A) select marker genes defined by flow cytometry, (B) up-regulated DEGs ($n = 94$) from subcluster 3.1 (DN), (C) up-regulated DEGs ($n = 186$) in subcluster 3.2 (CD27⁺), and (D) MHC class I and II and associated genes in PB subclusters and in synovial naive (SC-B1), MBC (SC-B2), and Tbet⁺CD11c⁺ (SC-B3) B cell populations. The size of dots corresponds to the percentage of cells expressing the indicated gene. (E) Immunofluorescence of inflamed synovium from a patient with RA. Synovium stained with CD19 (magenta), CD3 (gray), and Tbet (cyan) displaying merged overlay of all channels, paired merging of CD3 and Tbet and CD19 and Tbet, and individual channels in gray. Imaged at $\times 20$ (top panels) and $\times 63$ (lower panels). Arrows indicate some of the CD19⁺Tbet⁺ B cells. Representative of synovia of two patients with RA. DEG, differentially expressed gene; DN, double-negative; MBC, memory B cell; MHC, major histocompatibility complex; PB, peripheral blood; RA, rheumatoid arthritis.

and lacked CD27 (Figure 6C–E). After coculture of B and T cells for five days, the majority of CD4⁺ T cells expressed CD25, regardless of the type of B cell stimulation (Figure 6F). Although

more CD4⁺ T cells responded to IL-2-stimulated B cells, high levels of proliferation were found after coculture with both IFNy- and IL-2-stimulated B cells (Figure 6G). CD4⁺ T cell

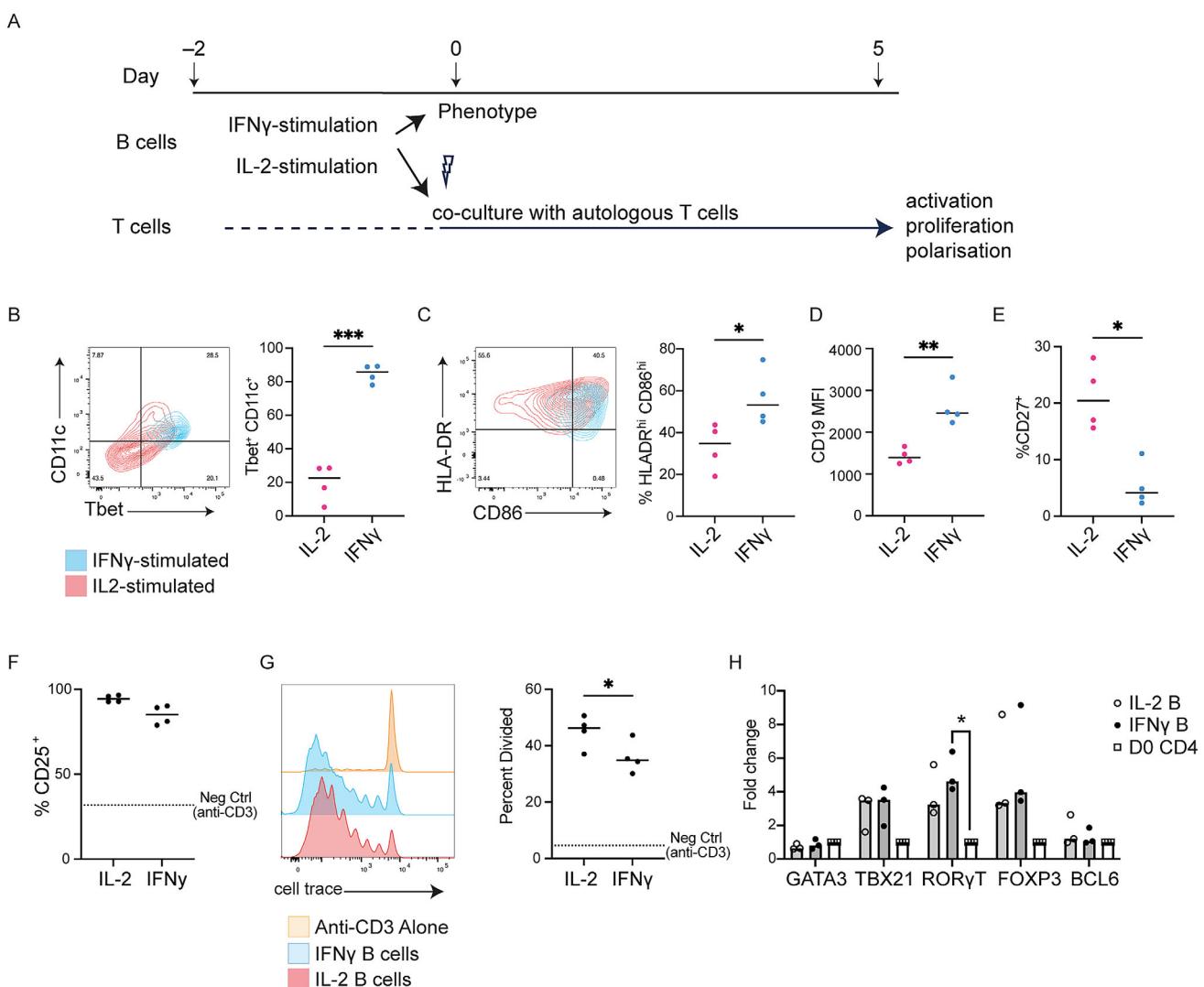


Figure 6. In vitro derived Tbet⁺CD11c⁺ B cells stimulate CD4⁺ T cell activation, proliferation, and RORyT expression. (A) B cells from healthy donors ($n = 4$) were stimulated with anti-Ig and IL-2 (IL-2 stimulated) or anti-Ig, IL-2, CpG B ODN 2006 (TLR-9), and IFN γ (IFN γ stimulated) for 48 hours. B cells were harvested and irradiated to prevent proliferation and cocultured with autologous T cells (1:2) for five days. Cultures were then harvested, and CD4⁺ T cells were analyzed for activation markers, proliferation (cell trace), and mRNA expression of polarizing transcription factors. After 48 hours of stimulation, B cells were analyzed for protein expression of (B) CD11c and Tbet, (C) HLA-DR and CD86, (D) CD19, and (E) CD27. After five days of coculture with IL-2-stimulated or IFN γ -stimulated B cells, CD4⁺ T cells were analyzed for (F) expression of CD25 and (G) proliferation by loss of cell trace, quantified as the percentage of cells that have divided. (H) CD4⁺ T cells were simultaneously sorted by FACS, and mRNA was extracted. Quantitative reverse transcription polymerase chain reaction (RT-PCR), was conducted for polarizing T helper cell transcription factors. Data were normalized to day 0 CD4⁺ T cells, and fold change was calculated. Comparison of the IFN γ -stimulated B cell condition and day 0 CD4⁺ T cells was conducted using the Wilcoxon paired *t*-test. **P* < 0.05. FACS, fluorescence-activated cell sorting; IFN, interferon; IL, interleukin; mRNA, messenger RNA; ODN, oligonucleotide; RORyT, retinoic acid receptor-related orphan nuclear receptor γ T; RT-PCR, real-time polymerase chain reaction; TLR, Toll-like receptor.

polarization was analyzed by quantitative polymerase chain reaction using unique transcription factors as a readout. Significantly higher levels of *RORC* (retinoic acid receptor-related orphan nuclear receptor γ T [RORyT]), the master regulator of Th17 polarization, were observed after coculture with IFN γ -stimulated B cells (Figure 6H). Although these data suggest that Tbet⁺CD11c⁺ B cells do not drive elevated T cell activation or proliferation over conventionally activated B cells, they can promote the polarization of CD4⁺ T cells into Th17 cells. In the context of RA and our

observations that Tbet⁺CD11c⁺ B cells are associated with bone destruction, the differentiation of CD4⁺ T cells into Th17 cells is of particular interest because such T cells have been shown to promote osteoclastogenesis and thereby mediate bone destruction.⁵²

DISCUSSION

Our investigation of Tbet⁺CD11c⁺ MBCs in patients with uRA using clinical data, flow cytometry, scRNA-seq, and in vitro

modeling of T cell activation has provided new insights to the function of these cells as bone erosive mediators in RA. Two subsets of $Tbet^+CD11c^+$ MBCs, DN and CD27⁺, were associated with bone destruction. Transcriptionally, they shared gene expression profiles associated with antigen processing and presentation, with more pronounced up-regulation of genes involved in clathrin-mediated BCR endocytosis and cytoskeletal changes in the CD27⁺ subset. Most strikingly, synovial $Tbet^+CD11c^+$ B cells shared gene expression profiles with circulating DN and CD27⁺ $Tbet^+CD11c^+$ MBCs. A role in antigen presentation in the synovium is supported by CD19⁺ $Tbet^+$ B cells spatially localized adjacent to T cells in lymphocyte clusters. Furthermore, coculture of $Tbet^+CD11c^+$ B cells and autologous T cells induced the expression of the transcription factor ROR γ T, crucial for the polarization of Th17 cells⁵³ and known to contribute to bone destruction by promoting osteoclast differentiation.

In line with our own and work by others reporting expansion of CD21⁻DN CD24⁻ or CD21⁻CD11c⁺ subsets in RA,^{29,30} we find expansion of $Tbet^+CD11c^+$ B cells in our cohort of patients with uRA. Moreover, both DN and CD27⁺ $Tbet^+CD11c^+$ MBCs in uRA largely share cell surface phenotypic markers observed in other disease settings (eg, HIV and systemic lupus erythematosus [SLE]) and in health^{17,18} (eg, FcRL3, FcRL5, and high levels of CD19 and CD20). They also express CXCR3, especially the CD27⁺ subset, but low levels of CXCR4 and CXCR5, suggestive of inflammatory tissue migration (eg, in RA to the inflamed joints) rather than secondary lymphoid organs.⁵⁴ In addition, our trajectory modeling and BCR analysis indicate that $Tbet^+CD11c^+$ MBC maturation follows directly from IgM memory, independent from conventional switched MBCs. Here, $Tbet^+CD11c^+$ CD27⁺ cells mature to DN cells, where up-regulation of genes associated with BCR signaling and internalization in the CD27⁺ compartment is indicative of more recent activation. Overall, our data demonstrate elevated levels of circulating clonally expanded $Tbet^+CD11c^+$ MBCs in uRA differentiating from CD27⁺ to DN cells.

Despite shared phenotypic qualities, our results support the idea of different functional roles of $Tbet^+CD11c^+$ MBCs in different disease settings. In SLE, $Tbet^+CD11c^+$ cells display a plasma cell fate.^{55,56} They correlate with autoantibody levels and plasma cell frequencies and express associated genes (eg, *IRF4*, *PRDM1* (Blimp1), *XBP1*, and *IL6R*). However, $Tbet^+CD11c^+$ MBCs in uRA do not appear to be plasma cell precursors, reinforced by low levels of the aforementioned genes and IRF4 protein (Supplemental Figure 3). Our results are also supported by others, in which neither *PRDM1* nor *XBP1* was detected in DN or CD27^{+/−} $Tbet^+CD11c^+$ B cells,^{28,29} and they lack association with plasmablasts.³³ This does not exclude the potential for $Tbet^+CD11c^+$ MBCs to differentiate into plasma cells in the synovium, as previously indicated by shared clonotypes between DN2 and plasma cells within the RA synovium.³⁰ However, our results highlight that correlations between bone destruction outcomes

in uRA and $Tbet^+CD11c^+$ MBCs are independent of autoantibodies. Instead, our data indicate that in RA, $Tbet^+CD11c^+$ MBCs can act as professional antigen-presenting cells, supported by our observation of specific up-regulation of >150 DEGs that encode components of biologic processes required to fulfill this role. Previous work has shown that T cell activation in models of autoimmunity, including humanized RA models, is dependent on B cell-mediated antigen presentation.⁵⁷ Based on our results, the expansion of $Tbet^+CD11c^+$ MBCs in RA may well be related to disease pathogenesis rather than an epiphenomenon of inflammation because we did not observe an association between these cells and clinical parameters such as C-reactive protein, erythrocyte sedimentation rate, or disease activity. The correlation of $Tbet^+CD11c^+$ MBCs with joint destructive outcomes, together with their antigen-presenting phenotype, supports a model whereby $Tbet^+CD11c^+$ MBCs present antigen to T cells, ultimately promoting destructive mechanisms.

Analyses of patients with inborn errors of immunity have shown that the expansion of $CD21^{low}Tbet^{high}$ is T cell dependent and that $Tbet$ itself is essential for the development of the $CD21^{low}CD11c^{high}Tbet^{high}$ subset in vitro and in vivo.^{18,58,59} $Tbet$ not only is involved in IgG subclass switching but also appears to regulate epigenetic changes at a number of genetic loci. Because different combinations of stimuli, such as BCR and TLR agonists, together with cytokines (eg, IFN γ or IL-27) induce differentiation into $Tbet^+$ B cells in vitro, part of the transcriptional landscape is similar whereas other parts are not.⁵⁹ Differential chromatin accessibility may explain why $Tbet^+CD11c^+$ MBCs in RA express genes consistent with a role as antigen-processing and antigen-presenting cells, whereas these cells in SLE express genes consistent with a role as plasma blast precursors. This suggests that a milieu with different cytokines and antigens would influence the gene expression profile of $Tbet^+$ cells and their function. The nature of the antigen may also be important because $Tbet^+$ B cells, at least in malaria, respond well to membrane-associated, but not soluble, antigens.⁶⁰

Our results not only demonstrate professional antigen-presenting capacity of $Tbet^+CD11c^+$ MBCs but also demonstrate that they favor polarization of helper T cells to Th17. These results are supported by prior observations in murine counterpart ABCs, which stimulate IL-17 production in T helper cells in vitro.⁶¹ Th17 cells are present in early treatment-naïve RA synovia,⁶² and functionally they are associated with and actively contribute to bone erosive mechanisms through promotion of osteoclastogenesis. Pathogenic bone erosion is dependent on the excessive formation and activation of osteoclasts, centrally driven by RANKL, which binds to its receptor RANK on osteoclast precursors.⁵² Synovial fibroblast production of RANKL is stimulated,⁶³ for example, by IL-17. The primary sources of IL-17 are Th17 cells and proinflammatory Th17-converted Treg cells ($ROR\gamma T^+FoxP3^+$), two highly osteoclastogenic helper subsets found in the RA joint.^{64–66} In addition to fibroblast activation, Th17 cells are known to promote

ectopic lymphoid follicle formation, a phenomenon that is associated with bone erosion outcomes. In the RA synovium, IL-23 activity, essential for Th17 maintenance,⁶⁷ is associated with ectopic lymphoid follicles.⁶⁸

One limitation of our study is the early stage of disease, which is characterized by a low degree of joint destruction, a challenge for conventional radiographic assessment. Additionally, synovial tissue data were obtained from patients with established RA and thus may have been potentially influenced by treatment, limiting our analysis of the Tbet⁺CD11c⁺ MBCs in early disease stages in the synovium. Yet one more limitation is the sequencing depth of the scRNA-seq data, as observed for CD21 and CD27, for example. Nevertheless, the strength of this study is the drug-naïve nature of our RA cohort, recruited at diagnosis before treatment initiation because this eliminates the influence of immunomodulatory therapies or long-term chronicity of the disease.

Overall, this work significantly advances our understanding of the function of Tbet⁺CD11c⁺ B cells in RA. Our results suggest that circulating DN Tbet⁺CD11c⁺ and CD27⁺ Tbet⁺CD11c⁺ MBCs in uRA are poised to act as professional antigen-presenting cells in synovial tissue. Their presence in both PB and synovia indicates circulation between the two, acting repeatedly as antigen-presenting cells and thus perpetuating T cell activation. Together with the strong correlation between these cells and bone destruction, this argues for a pathogenic role for these cells in RA and suggests a model in which the Tbet⁺CD11c⁺ MBCs drive the differentiation of CD4⁺ T cells into Th17 cells, thereby potentially contributing to ectopic lymphoid neogenesis and bone destruction in RA. The elevation of Tbet⁺CD11c⁺ MBCs in individuals with RA may be indicative of aggressive disease and higher risk of structural joint damage and, with further investigation, may have utility as a clinical biomarker for erosive disease.

AUTHOR CONTRIBUTIONS

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

Study conception and design. McGrath, Grimstad, Thorarinsdottir, Tilevik, Gjertsson, Mårtensson.

Acquisition of data. McGrath, Thorarinsdottir, Aranburu, Jonsson, Camponeschi, Ekwall.

Analysis and interpretation of data. McGrath, Grimstad, Thorarinsdottir, Forslind, Glinatsi, Agelii, Aranburu, Sundell, Tilevik, Gjertsson, Mårtensson.

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