Pathogenic Th17 cells in ZAP-70 mutant mice share T cell receptor features with Wild-type Tregs

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Abstract

Mutations in the ZAP-70 gene that cause moderate attenuation of TCR signaling, can result in autoimmune manifestations akin to rheumatoid arthritis (RA) in mice. Here, we characterized the single-cell gene expression profiles and T cell receptors (TCRs) repertoires of conventional (Tconv) and regulatory (Treg) $CD4^+$ T cell populations of arthritic (ZAC) and non-arthritic (SKG) ZAP-70 mutant as well as wild-type (WT) mice. Our analysis revealed two populations of Th17 cells in arthritic mice: Th17 *Il22*$^+$ and Th17 *Tnfsf11*$^+$. Notably, Th17 *Tnfsf11*$^+$ cells, are active in pathogenic transcriptional programs, uniquely localize in inflamed joints and are absent in WT or SKG mice. Additionally, their TCR repertoire is unique and convergent, and overlapped with the repertoires of WT Treg cells. Furthermore, the overall similarity between the Treg and Tconv repertoires was severely reduced in arthritic mice. Importantly, a group of cells with a transcriptional signature similar to that of Th17 *Tnfsf11*$^+$ was uniquely identified in synovial T cells from human RA joints. Our data suggests that the autoimmunity observed upon moderate reduction of TCR signaling strength results from T cells bearing self-reactive TCRs to evade negative selection, leading to the expression of TCRs typical of Tregs in Tconv cells as well as a depletion of specific receptors in the Treg compartment.

Introduction

The development of autoimmune diseases is influenced by various factors, with the presence of self-reactive conventional T cells (Tconvs) being a critical element[1]. During T cell

maturation, thymocytes expressing T cell receptors (TCRs) with high affinity to self-peptide-MHC complexes are typically eliminated from the repertoire via negative selection or diverted to the T regulatory (Treg) cell lineage[2,3]. Nonetheless, a subset of self-reactive cells persists in the periphery of healthy individuals, usually kept in check by the suppressive activity of Tregs[4,5].

Antigen recognition by the TCR is key for both Treg development in the thymus and for their function in peripheral tissues[6-8]; and it is generally accepted that the Treg repertoire is biased to self-recognition[9-12]. Therefore, disruption of the normal balance between Treg and Tconv repertoires can bias the immune system toward autoimmunity.

It has been previously described that specific mutations in the ZAP-70 gene that weaken downstream TCR signaling upon antigen recognition, and alter the Treg-Tconv repertoire balance, predisposes mice to an autoimmune disease closely resembling rheumatoid arthritis (RA)[13-15]. The SKG mouse model is characterized by a W163C mutation in the ZAP-70 gene that causes a fifteen-fold reduction in ZAP-70 affinity for phosphorylated CD3ζ[13] and necessitates secondary stimulation to induce the disease when mice are kept under specific pathogen-free (SPF) conditions. In contrast, ZAC mice, which carry an H165A mutation that causes only an eight-fold reduction, spontaneously develop chronic autoimmune arthritis, even under SPF conditions[15]. The underlying mechanism has been attributed to the disruption of the normal thymic selection process, by allowing T cells with moderately to strongly self-reactive TCRs to escape negative selection[14,15]; as a consequence, some T cells harboring

TCRs that would normally be observed in Treg repertoires would be expected to develop into self-reactive Tconvs.

In order to test this hypothesis, we examined the $CD4^+$ T cells of WT, non-arthritic SKG and arthritic ZAC mice at the single-cell level. Interestingly, we identified two Th17 cell subtypes in the joints of ZAC mice that were characterized by distinct transcriptional profiles and TCR repertoires, one of which exhibited a pathogenic signature and occurred exclusively in inflamed joints. Such a pathogenic signature was also uniquely detected in $CD4^+$ T cells obtained from inflamed joints of human RA patients. In addition, the TCR repertoire of pathogenic Th17 cells showed signs of convergence and was skewed toward that of WT Tregs. Furthermore, we found a reduced Treg-Tconv overlap within ZAC mice, suggesting that a depletion of critical components of the Treg TCR repertoire accompanies the expansion of Tconvs with a repertoire that is biased toward self-reactivity. Our results support of a model where, upon moderate ZAP-70-mediated signal weakening, T cells that would normally develop into Tregs instead develop into self-reactive Tconvs, resulting in a breakdown in self-tolerance and susceptibility to autoimmune arthritis.

Results

Arthritic ZAC mice harbor two groups of Th17 cells with distinct gene programs in their joints

To study the transcriptomes of self-reactive Tconv cells in-depth, we sorted cells from the joints, draining lymph nodes (dLN), and spleens of arthritic ZAC mice and performed RNA+V(D)J single-cell sequencing. Gene expression analysis revealed a considerable overlap in the transcriptional profiles of spleens and dLNs but a distinct profile for joint cells (Supplementary Fig. 1a). Unsupervised clustering delineated nine gene expression clusters with similar distribution in replicates across tissues (Supplementary Note 1, Fig. 1a-b, Supplementary Fig.1b). Two clusters (1 and 4) showed canonical Th17 markers (*Rorc*, *Il17a*, *Il17f*), but were distinct in their gene expression programs and tissue abundances (Fig. 1b-d, Supplementary Fig. 1b-d). Cluster 1 was abundant in joints, representing ~50% of cells obtained from this tissue, but was also detected in the spleen and dLNs at smaller but similar proportions (~15-17%). Relative to cluster 4, cells in cluster 1 differentially expressed *Klf2*, *S1pr1* and chemokine receptors such as *Ccr2*, *Ccr4* and *Ccr6*, suggesting a phenotype of migratory cells, possibly recently emigrated, or ready to emigrate from lymphoid tissues (Fig. 1d, Supplementary Table 4). Consistently, pathway analysis revealed an enrichment in the regulation of T cell migration (GO: 20000404) and cell chemotaxis (GO: 0060326) gene ontology (GO) Terms (Fig. 1e, Supplementary Table 5). In contrast, cluster 4 was almost exclusively observed in joints, representing approximately 27% of joint cells but less than 2% of dLN cells and less than 1% of Spleen cells (Fig. 1b). The top differentially expressed gene (DEG) in cluster 4 relative to cluster 1 was *Tnfsf11*, which codes for RANKL, a critical factor involved in osteoclast differentiation and activation that plays a central role in bone destruction in RA[16] (Fig. 1d). Two other osteoclastogenic genes (*Csf1*, *Anxa2*) were highly expressed in this cluster as well. Overall, we observed a significant enrichment in the

osteoclast differentiation (GO:0030316) GO term in cluster 4 as compared to cluster 1 (Supplementary Table 5). In addition, transcripts involved in the response to TNF stimuli (for example: *Tnfrsf18*, *Tnfrsf9*, *Tnfsf8*, *Tnfrsf4*, *Lta*, *Tnf*), and the cellular response to tumor necrosis factor (GO:0072356) GO terms were enriched in cluster 4. Moreover, this cluster uniquely expressed transcription factors associated with a pathogenic Th17 phenotype: *Rbpj*[17], *Crem*[18], *Atf3*[19], *Bhlhe40*[20], and *Igfbp7*[21] (Fig. 1d). Finally, expression of the transcription factor *Klf2*, a known inhibitor of NF-κB-dependent genes that also prevents Tfh differentiation and RORγt expression[22,23] was clearly absent from cluster 4 (Supplementary Fig. 1e). These results indicate that cluster 4 contains pathogenic Th17 cells with an increased potential to activate osteoclasts and thus promote bone damage. Interestingly, cluster 1 and cluster 4 could be clearly distinguished by their expression of *Il22* or *Tnfsf11* (Fig. 1f); thus, we annotated clusters 1 and 4 as Th17 *Il22*$^+$ and Th17 *Tnfsf11*$^+$, respectively. Finally, in order to explore the dynamics of the different Tconv cell states, we implemented Slingshot's pipeline for pseudotime and trajectory inference[24] with the naïve cluster of cells as the starting point. This resulted in the identification of the Th17 *Tnfsf11*$^+$ cluster as one of the endpoint lineage states (Fig. 1g), consistent with their effector phenotype. A population of pathogenic Th17 cells characterized by the expression of Th1 cell markers (*Ifng*; *Tbx21*, encoding for T-bet, and *Cxcr3;* Th17.1 cells)[25], and a group of osteoclastogenic Th17 cells derived from CD4$^+$ T cells previously expressing FoxP3 (exFoxP3) and characterized by the expression of *Sox4*, *Ccr6*, *Ccl20*, *Il23r* and *Tnfsf11*[26] have been previously reported to be enriched in arthritic joints; however, other than *Tnfsf11* we did not detect clear and specific

expression of these markers in cluster 4, suggesting that it represents a distinct group of cells (Supplementary Fig. 1f-g).

To contextualize our findings in arthritic ZAC mice tissues, we replicated the analyses in WT and non-arthritic SKG mice. For this analysis we sampled spleens and integrated ZAC spleen data in order to facilitate direct comparison of clusters (Supplementary Note 2). This clustering revealed that the abundance of Th17 cells increased progressively from WT (less than 1%) to SKG (2-9%) to ZAC (~36%) mice (Supplementary Fig. 2d). To compare the transcriptional programs of these Th17 cells with the ones from ZAC tissues, we calculated gene module scores for the signature genes from Th17 *Il22*$^{+}$and Th17 *Tnfsf11*$^{+}$ cells (Supplementary Table 6). Expression of the Th17 *Il22*$^{+}$ signature was clearly detected and gradually increased from WT, to SKG to ZAC splenic Th17 cells; however, the Th17 *Tnfsf11*$^{+}$ signature was largely undetected (Fig. 2a, Supplementary Fig. 2g). This result indicates that Th17 *Tnfsf11*$^{+}$ cells are uniquely present in the inflamed joints of arthritic mice.

In summary, we demonstrated the presence of two distinct groups of Th17 cells in arthritic ZAC mice: Th17 *Il22*$^{+}$ cells, characterized by a transcriptional profile indicative of migration and the expression of the transcription factor *Klf2*; and, Th17 *Tnfsf11*$^{+}$ cells, which exhibit a pathogenic transcriptome and uniquely localize within inflamed joints.

The Th17 *Tnfsf11*$^{+}$ transcriptional signature is present in inflamed joints of human RA but not in other autoimmune diseases or non-autoimmune arthritis

To investigate the presence of cells resembling Th17 *Tnfsf11*$^{+}$ in human tissues, we calculated Th17 *Il22*$^{+}$ and Th17 *Tnfsf11*$^{+}$ gene signature module scores in publicly available single-cell RNAseq datasets. We analyzed data obtained from inflamed synovia[27] and PBMCs[28] of RA patients; and, for comparison, we also analyzed data from the synovia and infrapatellar fat pad (IPFP) of osteoarthritis (OA) patients[29] as well as peripheral CD4+ T cells from healthy controls and patients with other autoimmune diseases (myasthenia gravis, multiple sclerosis, and systemic lupus erythematosus)[30]. Detailed descriptions of the datasets are provided in Supplementary Table 7. Our analysis revealed that both Th17 signatures were detected in CD4^{+} T cells from the inflamed joints of RA patients and were non-overlapping, mirroring our observations in ZAC joints; in contrast, the expression of the Th17 *Tnfsf11*$^{+}$ signature was nearly undetectable in the circulating CD4^{+} T cells of RA patients (Fig. 2b-c). Notably, as in ZAC joints, the expression of Th17.1 or exFoxP3 markers was not specifically detected in the cluster of cells expressing Th17 *Tnfsf11*$^{+}$ in human RA joints (Supplementary Fig. 3a-d).

OA is the most common form of arthritis and is mainly provoked by the mechanical wear and tear of joints, and, even though inflammation is involved in its pathogenesis, it is not a disease of autoimmune origin[31]. In CD4^{+} T cells from inflamed synovia and IPFP of OA patients we detected mild expression levels of the Th17 *Il22*$^{+}$ signature, however, in contrast to RA synovia, the Th17 *Tnfsf11*$^{+}$ signature was undetected (Fig. 2d). Moreover, we observed

a similar result when analyzing circulating CD4$^+$ T cells from healthy controls and patients with other autoimmune diseases (Fig. 2e).

Taken together, our results indicate that, similar to the ZAC mouse model, Th17 *Tnfsf11*$^+$ cells are uniquely located in inflamed joints and are thus likely to be specifically involved in autoimmune arthritis.

Th17 *Tnfsf11*$^+$ cells are expanded, convergent, and contain public clones

Having identified two groups of potentially self-reactive Th17 cells with characteristic transcriptomic features, we next asked whether their repertoires are also distinct. First, we examined the degree of clonal expansion by calculating the proportion of a repertoire represented by each clone (clonal proportion) in each replicate, and classified clones as: small, medium or large (methods). We found that large and medium clones were abundant in both Th17 cell groups (Fig. 3a-b, Supplementary Fig. 4a). Next, we evaluated the repertoire similarity of both Th17 groups to other Tconv populations and between each other by using the Morisita-Horn index[32]. At the exact paired CDR3 amino acid sequence level, the repertoire of Th17 *Il22*$^+$ cells is largely similar to other Tconv repertoires from ZAC tissues. While there is some similarity between the Th17 *Tnfsf11*$^+$ repertoire and other Tconv cells, it is significantly reduced. Consistently, the similarity between the two Th17 cell repertoires was rather low (Fig. 3c). These results suggest that the Th17 *Tnfsf11*$^+$ cells possess a unique repertoire, presumably due to the targeting of different antigens.

Given the evidence of a unique repertoire in Th17 *Tnfsf11*$^{+}$ cells, we next evaluated TCR convergence. In order to evaluate TCR convergence independent of gene usage, we used TCRdist[33], which measures the distance between pairs of TCRs using CDR sequences. A bias in the distribution of the distances between TCRs from a repertoire to lower values indicates a higher similarity and therefore, can be interpreted as an indication of repertoire convergence. Therefore, we calculated TCR distances for the two Th17 clusters, using the naïve cluster as a reference. We found that the TCR distance distribution of the Th17 *Tnfsf11*$^{+}$ repertoire trended lower in paired and single chain analysis and was significantly lower in the alpha chain (Fig. 3d). This result indicates that the repertoire of Th17 *Tnfsf11*$^{+}$ is more convergent than that of Th17 *Il22*$^{+}$, especially in the alpha chain. Given their specific location in joints, and the evidence of similar cells occurring in human RA but not OA joints, our results suggest that Th17 *Tnfsf11*$^{+}$ may be responsible for the recognition of the self-antigens in the affected tissue and that the alpha chain may play a key role in such recognition.

Finally, we identified a total of 9 unique public clones (clones shared in two or more individuals) occurring in all tissues; most of them displayed medium clonal proportions in at least one replicate, were most commonly associated with Th17 *Il22*$^{+}$ phenotypes, but were also observed in Th17 *Tnfsf11*$^{+}$ (Fig. 3e, Supplementary Table 8). In particular, clones 5 and 6 were observed in Th17 *Tnfsf11*$^{+}$ but not in Th17 *Il22*$^{+}$ repertoires. Interestingly, clone 1 was largely public, as it occurred in 3 out of 4 replicates, exhibited medium to large clonal proportion, and occurred in the Th17 *Tnfsf11*$^{+}$ repertoire in at least one replicate. This

indicated that this clone is likely to be involved in the recognition of relevant arthritogenic self-antigens. Unexpectedly, among the public clones, we identified an exact match in the CDR3b sequence (CASSLGGQNTLYF; public clone 6) to a CD8 TCR annotated as recognizing a murine cytomegalovirus (MCMV) epitope (HGIRNASFI) by the VDJdb database of TCR sequences with known antigen specificities[34] (Supplementary Table 9).

Together, these results indicate that although the Th17 response in arthritic ZAC mice is polyclonal, the repertoire of tissue-specific Th17 *Tnfsf11*$^{+}$ cells is unique and convergent, suggesting the recognition of a limited set of self-antigens. Moreover, a number of highly expanded public clones was observed within the inflamed tissue.

Effector Treg cells are abundant in the joints of arthritic ZAC mice but potentially suppressed by cell crosstalk with self-reactive Tconvs

As discussed previously, the failure of Tregs to suppress self-reactive cells and inflammation is a key factor in the development of autoimmune diseases. To investigate the Treg landscape in ZAP-70 mutant mice, we conducted simultaneous sorting of Treg cells from previously described samples and performed single-cell RNA and V(D)J sequencing. In the tissues of ZAC mice, we identified seven gene expression clusters, including two groups of effector cells and one cluster of Trm cells which were notably expanded and abundant in joints (Supplementary Note 3, Fig. 4a, Supplementary Fig. 5d). Conversely, in WT and SKG Tregs, we observed limited effector and Trm cells, while naïve cells were abundant (Fig. 4b,

Supplementary Note 4). Remarkably, despite the evident expansion in the Treg compartment of ZAC mice, only one public clone could be identified (Supplementary Table 12). These findings suggest that Trm and effector Treg cells undergo expansion in the tissues of arthritic ZAC mice; however, clone sharing across subjects is much less frequent than in the Tconv compartment.

Despite the abundance of effector Treg cells in the sampled tissues, ZAC mice are overly autoreactive. To elucidate the disparities in cell activity among WT, SKG, and ZAC effector Tregs, we performed DEG analysis across mouse models. Our analysis revealed that while the transcriptional programs of WT and SKG cells are largely similar, several genes involved in TNF signaling are uniquely expressed in ZAC effector Tregs (Fig. 4c-d, Supplementary Table 13). While the exact effect of TNF signaling on Treg effector function remains unclear, studies have suggested its suppressive effects on Treg function in RA patients[35].

Various mechanisms for Treg suppression, including physical interactions with target cells or the secretion of soluble factors, have been documented in the literature[36,37]. Therefore, we sought to identify potentially significant cell-cell crosstalk events between effector Tregs and Th17 cells by predicting cell communication events using Cellphone DB[38]. Consistent with our previous findings, we observed an elevated number of cell communication events involving members of the TNF family from WT, SKG, and ZAC Th17 cells toward Treg cells (Fig. 4e). Thus, our findings suggest that despite their abundance, effector Treg function is impaired in arthritic ZAC mice, partly as a result of cell crosstalk with Th17 cells through members of the TNF signaling family.

TCR repertoires of ZAP-70 mutant Tconv, particularly Th17 *Tnfsf11*+, cells are more Treg- than Tconv-like

Because T cells harboring self-reactive TCRs would likely be deleted or develop into Tregs in WT mice, it was postulated that the appearance of such TCRs in the Tconv compartment in ZAP-70 mutant mice is a result of a shift from the deleted or Treg compartment toward the Tconv compartment as a consequence of a defective thymic selection[14,15]. A natural way of testing this hypothesis would be to compare the similarity of the repertoires of WT Tregs and SKG or ZAC Tconvs. If WT Tregs were more similar to ZAP-70 mutant Tconvs than to WT Tconvs, it would support the proposed repertoire shift hypothesis. To address this question, we first attempted to quantify the WT Treg-ZAC Tconv repertoire overlap within our dataset; however, the number of matches was too low to make a strong conclusion, presumably due to the low overall clonal overlap between WT and mutant mice. In order to expand the number of WT TCRs for comparison, we turned to previously published Tconv and Treg TCR reference datasets[39-42] (Supplementary Table 14). For the calculation, we treated each of our experimentally determined (WT Tconv, WT Treg, SKG Tconv, SKG Treg, ZAC Tconv, or ZAC Treg) TCRs as a "query" that was used to search in a reference Tconv or Treg dataset (Logunova[39], Lu[40], Ko[41], Wolf[42]). For each of these queries, we computed the number of reference Tconv and Treg matches and based on the number of matches, along with the total size of each reference dataset, we deemed a given query as "Treg-like", "Tconv-like" or "not significant" using a hypergeometric p-value cutoff of 0.001. We then quantified

the total number of matches in terms of the query repertoire size. In order to test our hypothesis, we normalized the results to those of the WT Tconv query group (Fig. 5a). Considering the largest (Logunova[39]) reference dataset, we found that the fraction of Treg-like TCRs within SKG and ZAC Tconv queries was approximately twice as high as the fraction in WT Tconvs; in addition, the fraction of Tconv-like TCRs was notably reduced (Fig. 5b). Similar trends were observed in the other reference datasets (Supplementary Fig. 7a). Furthermore, despite an increased fraction of Treg-like TCRs in the ZAC Th17 *Tnfsf11*$^{+}$ repertoire, this group consistently displayed the smallest fraction of Tconv-like TCRs, indicating that the TCRs in this cluster were largely absent from normal Tconv repertoires, but nevertheless similar to normal Treg repertoires (Fig. 5c, Supplementary Fig. 7b). These observations strongly support the repertoire shift hypothesis: ZAP-70 mutant Tconv repertoires are more similar to WT Treg repertoires than to WT Tconv repertoires, and this trend is most pronounced in the pathogenic Th17 *Tnfsf11*$^{+}$ cells.

Because the query-reference matching procedure described above requires a number of ad hoc normalization steps, we next sought a simpler procedure that could be used as an independent check. To this end, we took advantage of a machine learning tool that enables the quantification of the regulatory potential of a TCR (TCR-intrinsic regulatory potential, TiRP) based on TCR beta chain features[43]. As expected, our experimentally determined Treg datasets exhibited higher TiRP scores than WT Tconv cells, and their respective Tconv compartments (Supplementary Fig. 7c-d). Importantly, the ZAP-70 mutant Tconvs also exhibited significantly higher TiRP scores than WT Tconvs (Fig. 5d-e). Furthermore, when

considering the ZAC Th17 phenotypes, we found that Th17 *Tnfsf11*$^{+}$ TiRP scores were significantly higher compared to those of WT Tconvs, indicating a bias toward a Treg-like repertoire (Fig. 5f-g). These results agree with those of the query-reference matching procedure, and together, support the repertoire shift model as an explanation for how ZAP-70 mutation-mediated TCR signal weakening leads to the emergence of self-reactive Th17 cells that develop a pathogenic phenotype and accumulate in joints upon activation.

Evidence of missing TCR specificities in ZAP-70 mutant WT Treg repertoires

A second consequence of the repertoire shift hypothesis is that a weakening of TCR signaling impacts the Treg compartment by generating TCR deficiencies. It has been reported that missing specificities in the Treg repertoire have a large impact on the suppressive function against self-reactive T cells[8,44]. This raises the possibility that an absence of Treg specificities within ZAP-70 mutant mice repertoires contributes to a loss in tolerance toward specific self-antigens. To explore this hypothesis, we analyzed the Treg-Tconv repertoire similarity within each mouse and compared it across groups (WT, SKG, ZAC). The Morisita-Horn index for exact CDR3 similarity showed a notably reduced Treg-Tconv similarity in ZAC mice relative to WT or SKG, especially at the single chain level (Fig. 6a). The Treg-Tconv overlap was generally distributed across effector phenotypes (Tconv: intermediate, Th17 and Tfh. Treg: intermediate and effector) in non-arthritic mice, suggesting a suppressive potential for shared Tregs towards activated Tconvs; however, such enrichment was not observed in ZAC mice (Supplementary Fig. 8a-h). To further test these observations, we again employed TCRdist[33]

and calculated repertoire-repertoire correlations for pooled WT, SKG and ZAC repertoires: Here, a pair of similar repertoires exhibit high correlation coefficients (represented in yellow). We found that WT Treg and WT Tconv repertoires were well correlated, whereas SKG and ZAC Tconv repertoires exhibited lower correlation with Treg repertoires. Indeed, the ZAC Treg repertoire was poorly correlated with other groups in general, indicating a reduced similarity to any other tested repertoires (Fig. 6b). Together, these results are consistent with a scenario wherein ZAP-70 mutant repertoires are depleted in certain Treg TCR specificities; which, along with a more self-reactive Th17 population, contributes to a loss of tolerance and the development of autoimmune arthritis.

Discussion

In this study, we provide a comprehensive characterization of the Treg and Tconv phenotypes and repertoires of WT, non-arthritic SKG and arthritic ZAC mice, at the single cell level. We identified two transcriptionally distinct populations of Th17 cells within tissues of arthritic ZAC mice: Th17 *Il22*$^{+}$ with a migratory phenotype, and Th17 *Tnfsf11*$^{+}$ with a pathogenic gene program that is almost exclusively detected in joints. The latter pathogenic group may target a restricted set of antigens, as these cells harbor unique and convergent TCRs, with a greater similarity to the TCRs of WT Treg cells, which are broadly acknowledged to be biased to self-reactivity[9-12]. Furthermore, the fact that cells with a gene signature consistent with the Th17 *Tnfsf11*$^{+}$ group were observed only within CD4^{+} T cells from inflamed RA synovia in humans suggests a potential role in RA pathogenesis.

Pathogenic Th17 cells expressing markers of Th1 cells (Th17.1)[25] or derived from FoxP3^{+}CD4^{+} T cells (exFoxP3)[26] have been previously reported to play a role in RA; however, our results suggest that the full phenotype of the Th17 *Tnfsf11*$^{+}$ cells identified in this study is different from these pathogenic populations, as no clear or exclusive expression of Th17.1 or exFoxP3 marker genes were detected in Th17 *Tnfsf11*$^{+}$ cells.

Investigations into the contribution of high-affinity versus lower-affinity T cell specificities to autoimmune disease establishment and progression, as observed in experimental autoimmune encephalomyelitis (EAE), have revealed a critical role of high-affinity autoreactive cells in initiating disease that can be maintained by lower-affinity cells once established[45]. In our study, it is possible that the Th17 *Tnfsf11*$^{+}$ population comprises cells critical for the establishment of disease, given their unique repertoire and tissue location; while Th17 *Il22*$^{+}$ cells may contribute to disease progression and maintenance and represent a previous differentiation state for cells that later acquire a Th17 *Tnfsf11*$^{+}$ phenotype. Such dynamics would be in line with increased diversity and reduced clonal dominance in established disease stages[46,47].

Consistently, our trajectory prediction suggests that Th17 *Tnfsf11*$^{+}$ cells represent a terminal differentiation state, preceded by the Th17 *Il22*$^{+}$ phenotype. This observation also aligns with previous research showing that in EAE Th17 cells progress from a self-renewing cellular state primarily enriched in lymph nodes, to an effector pre-Th1-like phenotype found in both lymph nodes and the central nervous system (CNS) and characterized by the expression of

chemokine receptors, to a Th17/Th1-like effector phenotype that is concentrated in the CNS and exhibits a pathogenic signature with high expression of *Tnfsf11*[48].

In addition to these observations on the pathogenic phenotype of ZAC Th17 cells, we carried out the first systematic investigation at single cell resolution of the repertoire shift of conventional T cells upon TCR signal weakening. Our results are consistent with the observation that T cells infiltrating prostatic autoimmune lesions in Aire$^{-/-}$ mice express receptors preferentially found in Tregs from Aire$^{+/+}$ mice[49]. In addition, it was shown that exFoxP3 cells display a higher osteoclastogenic potential as compared to Th17 derived from naïve CD4^{+} T cells, presumably due to their higher affinity to self-antigens[26]. Together with our findings, these reports point to a key role of TCRs normally occurring in the Treg compartment in the recognition of self-antigens, and suggests that aberrant expression of such TCRs in Tconv cells may drive autoimmune disease. The unique identification of CD4^{+} T cells expressing the Th17 *Tnfsf11*$^{+}$ signature in inflamed synovia from RA patients highlights the potential involvement of such cells in autoimmune arthritic inflammation, and motivates further identification of their tissue-specific self-antigens.

Remarkably, we observed a number of public clones within ZAC Tconv cells, particularly concentrated within both Th17 phenotypes, and notably expanded. The significance of public clones, in the development of autoimmune conditions, has been previously documented[50,51]. However, despite our analysis, only one of the CDR3 sequences within public clones was

found in a database with annotated peptide-MHC specificity. Consequently, the identity of any potential epitopes or amino acid motifs remains an open problem.

As Th17 *Il22*$^{+}$ and Th17 *Tnfsf11*$^{+}$ cells were predicted to be related both by trajectory analysis and by the existence of a limited number of shared clones, we speculate that the Th17 *Tnfsf11*$^{+}$ phenotype is only acquired within joints and only by cells bearing self-reactive TCRs. The data supports a model wherein these cells are critical in triggering and establishing disease in target tissues, whereas Th17 *Il22*$^{+}$ cells play a supporting inflammatory role, but are not strongly self-reactive.

Evidence suggests that the affinity of the TCR not only influences the selection of Tregs but also shapes their phenotypes and functional properties in the periphery[52,53]. In our study, we identified a population of Treg effector cells enriched in joint tissue of ZAC mice, and largely absent in WT or SKG mice. However, it has been clearly established that Tregs from ZAC mice are severely affected by the mutation in ZAP-70 resulting in an impaired regulatory ability[15]. Furthermore, our prediction of cell-cell crosstalk events between Treg and Tconv cells in ZAC mice identified communication through the TNF family as a potential mechanism of Treg suppression mediated by pathogenic Th17 cells. Signaling through TNF family members such as *Tnfsf4-Tnfsfr4* (OX40L-OX40)[54] or *Tnfsf14* (LIGHT)- *Tnfrsf14*[55] has been shown to impair the suppressive ability of Treg cells. While Treg-Tconv cell contact appears to be necessary for efficient suppression of Tconv proliferation *in vitro*[56]. In the

context of ZAC mice, this interaction could potentially favor counter-regulation of Tregs by pathogenic Th17 cells, exacerbating their baseline Treg impaired activity.

Finally, the repertoire shift hypothesis raises the prospect of loss of certain TCR specificities within the Treg compartment. Considering that antigen recognition by the Treg TCR appears to be less degenerate than in Tconv cells[8,57,58], the loss of certain Treg specificities could profoundly impact tolerance towards specific self-antigens[44]. While it is generally acknowledged that Treg and Tconv repertoires are distinct, a small proportion of their repertoires typically overlap[47,59,60]. In this study, we observed a reduction in the similarity between ZAC Treg and Tconv repertoires, specially within activated Tconv cell groups, suggesting a divergence in the Treg-Tconv repertoires that could result in the loss of critical Treg function. This result is consistent with the observation of a significantly reduced proportion of potentially self-reactive receptors in the Treg repertoires of autoimmune Aire$^{-/-}$ mice[49]. Therefore, it is possible to speculate that moderate TCR weakening by ZAP-70 mutation may not only shift the Tconv repertoire but also deplete the Treg repertoire of relevant TCRs. The functional identity of such critical TCRs remains unknown, but worthy of future study as a starting point for development of TCR-transgenic Treg immunotherapy for the treatment of autoimmune diseases.

Taken together, the consequences of weakened TCR signaling in the development of autoimmune disease are multilayered. Firstly, it allows cells with self-reactive TCRs to evade negative selection or deflection into the Treg repertoire by differentiation into self-reactive

Tconvs. At the same time, Tregs are affected in at least two ways: on the one hand, the Treg repertoire may become deficient in some normally-occurring and potentially critical TCRs, resulting in not a shift, but also a bifurcation of the repertoire. On the other hand, Treg effector activity upon TCR weakening is significantly disrupted[15], rendering Tregs insufficient for the suppression of self-reactive Tconvs (Fig. 7).

It should be noted that our study has several limitations, such as the limited sample size and repertoire coverage that can be practically analyzed by current single cell sequencing approaches. In addition, the pathogenic potential of Th17 *Tnfsf11*$^{+}$, their self-reactivity, as well as their potential to suppress Treg function have not been further experimentally assessed. Moreover, the antigen specificity of public clones could only be predicted, which yielded only a single match to a functionally annotated TCR. This TCR was described as being specific to a viral epitope presented via MHC class I. Future *in vitro* and *in vivo* experiments will be required to elucidate the specific role and antigen specificities of Th17 cell populations in disease establishment and progression. In spite of these limitations, our research expands the cellular and molecular level understanding of the breakdown in tolerance during autoimmune disease and proposes a straightforward path to discovering specific TCR-antigen interactions involved in RA.

Methods

Mice

Animal experiments were approved by the institutional review board of Osaka University, and animals were treated in accordance with the institutional guidelines of the Immunology Frontier Research Center, Osaka University. BALB/c and SKG mice were acquired from CLEA Japan. SKG[13], ZAC[15] and FIG (Foxp3-IRES-GFP knock-in[61]) mice were previously described. In this study, FIG BALB/c WT, FIG SKG and FIG ZAC mice were used and generated as previously reported [15]. All animals were maintained in Specific pathogen-free (SPF) facilities.

Droplet-based single cell RNA sequencing.

$CD4^{+}Foxp3^{-}$ or $Foxp3^{+}$ cells from FIG BALB/c WT, FIG SKG or FIG ZAC mice were sorted by FACSAria SORP (BD Biosciences). For FIG BALB/c WT and FIG SKG mice, cells were obtained from spleens, processed with the Chromium Single Cell 5' Library & Gel bead kit (10x Genomics), loaded onto a Chromium Single Cell G Chip, and encapsulated in a Chromium single cell controller (10x Genomics) to generate single-cell gel beads in the emulsion (GEMs) following manufacturer's protocol. After encapsulation, reverse transcription and cDNA amplification were performed, followed by target enrichment of V(D)J segments and the independent construction of V(D)J and Gene expression libraries as recommended by the manufacturer's instructions.

For FIG ZAC mice, cells from inflamed joint, draining popliteal lymph node (dLN) and spleen from each replicate were barcoded with TotalSeq hashtag antibodies (BioLegend) and

then pooled by tissue and cell type (i.e., Joint Tconv, Joint Treg, etc.) before the generation of GEMs. Next, RNA and V(D)J libraries were produced as described above and an additional library for the barcoded antibodies was prepared. HiSeq3000 and NovaSeq6000 were used for sequencing.

Single-cell RNAseq data processing

Raw reads were aligned to the mouse reference genome (GRCm38 and GRCm38 VDJ v5.0.0, from 10× Genomics), filtered, and demultiplexed to create gene expression matrices by Cell Ranger (v.5.0). The resulting data was analyzed in R software (v 4.1.2) with Seurat v4.0 package[62,63].

Where necessary, hashtag demultiplexing was performed by the *HTODemux* function from Seurat and only data from cells classified as singlets was kept for further steps. Next, low-quality data was removed from all datasets, this means cells with unique feature counts less than 200 or over 2500, and cells in which mitochondrial reads represented >5% of reads. Only cells annotated to a single alpha and a single beta chain in the V(D)J result were retained for further analysis. Normalization was performed by the "LogNormalize" method from Seurat and the top 2,000 variable genes were identified in each dataset independently.

Batch correction and data integration were performed in combinations of data as follows: 1) Tconvs from ZAC tissues; 2) Tregs from ZAC tissues; 3) Tconvs from spleen (including WT, SKG and ZAC samples); 4) Tregs from spleen (including WT, SKG and ZAC samples).

Batch correction was performed with the Canek R package[64] using the intersecting variable genes for the datasets to be integrated in each run.

After integration and QC, the final datasets for analysis consisted of: 6,509 cells for ZAC Tconv, 5,306 cells for ZAC Treg, 24,743 cells for Spleen Tconv and 25,227 cells for Spleen Treg (Supplementary Table 1). The uniform manifold approximation and projection (UMAP) were used to visualize gene expression profiles and clusters from the principal components (PCs) representation of the integrated datasets; the number of PCs were independently selected on each dataset as the elbow suggested in variance plot of PCs. Public single-cell RNAseq data was pre-processed accordingly.

Single cell gene expression analysis

Clustering and cell-type annotation

The identification of cell clusters based on gene expression data was performed in the PCs representation of integrated datasets with the *FindClusters* function from Seurat using a resolution parameter of 0.5. Clusters were annotated by the examination of expression of canonical Treg or Tconv markers together with the inspection of markers defining each cluster and enriched GO Biological Process Terms (Supplementary Notes 1-4). Additional exclusion of artifactual populations was performed after clustering: cells lacking expression of *Cd3d*, *Cd3e*, and *Cd3g*, invariant NKT (iNKT) cells, and clusters with high expression of Hemoglobin genes (*Hba-a1, Hba-a2, Hbb-bt, Hbb-bs*).

DEG and gene set enrichment analysis

Identification of cluster marker genes was performed with *FindAllMarkers* function in Seurat, and significant markers were considered if fold change (FC) ≥1.2 and adjusted p value ≤0.05 and where used for gene set enrichment analysis.

When the comparison of two populations was required, DEGs were identified with the *FindMarkers* function with the min.pct parameter set at 0.25. Genes with adjusted p value ≤0.05 where considered significantly changed and were classified as upregulated if FC ≥1.5 or downregulated if FC ≤0.67.

Gene set enrichment analysis was performed with enrichR (v3.1) package[65] using the *GO_Biological_Process_2021* database as reference.

For the generation of of ZAC Th17 *Tnfsf11*$^{+}$ and Th17 *Il22*$^{+}$ gene signatures for module score calculation, significant markers were considered if fold change (FC) ≥2 and adjusted p value ≤0.05.

Trajectory analysis

Trajectory analyses were performed by using the Slingshot Bioconductor package (v2.10.0)[24] with the PCs representation of integrated datasets and their respective cluster labels as inputs. We set the parameter *start.clus='naive'* to use the cluster of Naive cells as the starting point of the inferred trajectories.

Module score calculation

Calculation of expression levels from Th17 ZAC programs in spleen and public human scRNAseq datasets was performed by the *AddModuleScore* function from Seurat using the specified gene lists or their corresponding human orthologs (Supplementary Table 6) and default parameters.

For public scRNAseq datasets, clustering was performed as described above. Where it was possible to annotate Th17 cells, these were separated in an independent Seurat object and processed for module score calculation and visualization. If Th17 cells were not clearly identified, the whole $CD4^+$ T cell clusters were separated in independent Seurat objects for module score calculation.

Cell-cell crosstalk prediction

We used Cellphone DB[38] to predict cell-cell communication. After converting mouse gene IDs to human orthologues, we ran CellPhoneDB v4.0.0 in statistical analysis mode, for the subsets of effector Treg together with Intermediate or Th17 Tconvs. Results were visualized by the ktplots R package. We manually searched for all significant interactions in each mouse model. Only statistically significant interactions with a reasonable biological meaning are shown in detail in the results.

Single TCR V(D)J sequencing analysis

Clonal expansion and repertoire overlap

Clonal expansion was assessed by the calculation of clonal proportions, then, clones were classified by their size as: small (clonal proportion $<1x10^{-3}$), medium (clonal proportion from $1x10^{-3}$ – $1x10^{-2}$) and large (clonal proportion $>1x10^{-2}$). Given that only a small sample size could be recovered for some replicates, which inflates clonal proportions of lowly abundant clones, we labeled singletons (clone size=1) separately.

The Morisita-Horn index is a typical metric used in ecology to calculate the similitude of two ecosystems in terms of species distribution and abundance, and it has been introduced as a metric for repertoire overlap analysis; its values go from 0 to1, where 1 represents a perfect overlap[32]. The Morisita-Horn index was calculated between repertoires of each individual replicate, at the pared or single CDR3 amino acid level by the Immunarch (v0.9.0) R package and median Morisita-Horn indices were compared by a Wilcoxon test using a significance level of 0.05. In addition, clonotype tracking across Treg and Tconv phenotypes was performed by Immunarch.

Clonal publicity

Public clones were defined as TCRs sharing paired gene usage and CDR3 amino acid sequences and occurring in more than subject.

TCR distances and repertoire-repertoire correlation

In order to evaluate if the overall features of selected repertoires show convergence, we calculated receptor distances with TCRdist. TCRdist allows for the calculation of a distance measure between TCRs which considers pMHC binding structural information[33], then, a

neighbor distance metric consisting of the weighted average of the distance of a given TCR to the nearest 25th percentile of the other TCRs in the repertoire can be calculated (weighted neighbor distance). Then, distance distributions of selected repertoires were compared and distributions biased toward lower distances were interpreted as an indication of repertoire convergence. In order to restrict the convergence analysis to the qualitative composition of the repertoires, we used unique clone sequences i.e., clonal counts where not considered. Median distances of Naïve, Th17 *Il22*$^{+}$ and Th17 *Tnfsf11*$^{+}$ cells were compared by a Wilcoxon test with a significance level of 0.05. Finally, the epitope-epitope (or repertoire-repertoire) neighbor distance score correlation metric from the TCRdist algorithm was computed for assessment of intra Treg-Tconv repertoire similitude. Briefly, a score is assigned to each TCR in one repertoire which is the average distance to the nearest TCRs in a reference repertoire. Then, the sets of scores obtained by two different repertoires can be compared over the entire merged set of TCRs and a correlation score is calculated, in principle, two similar repertoires will be well correlated.

TiRP score calculation

Lagattuta et al proposed a Treg-propensity scoring system for the TCR to quantify the likelihood of Treg cells based on the TCR feature on Treg fate (TiRP score)[43]. The source code was obtained from https://github.com/immunogenomics/TiRP. The TiRP score of this study was calculated using the default parameter. For each TCR sequence, the v-gene and amino acid of CDR3 were utilized as input information. Median TiRP scores were compared across groups by a Wilcoxon test with a significance level of 0.05.

Published sequence preparation

The published mouse Treg and Tconv TCR beta chain sequence data were obtained from four studies[39-42]. The detailed accession ID and references were summarized in Supplementary Table 14. The annotation of raw sequences was performed by mixcr (3.0.13)[66] with align, assemble, assembleContigs, and exportClones process. The sequences that have complete sequences of frameworks and CDRs regions were utilized in this study.

Sequence searching and comparison

We implemented the TCR sequence searching using Interclone method[67]. The source code was deposited in https://gitlab.com/sysimm/interclone and the searching parameter using the default setting. In brief, InterClone regards the CDR1,2,3 of one TRB seq as a pseudo sequence and considers one pair of hitting using the following CDR similarity and coverage thresholds: CDR1 ≥ 90%, CDR2 ≥ 90%, CDR3 ≥ 80%, and Coverage ≥ 90%. We first searched Tconv and Treg TCR templates from the same reference using identical query inputs. Then we compare the corresponding hitting number with Tconv and Treg templates for each query and calculate the significant Tconv or Treg-biased query sequence using a hypergeometric p-value cutoff of 0.001. We then quantified the total number of matches in terms of the query repertoire size and normalized the results to the matches in the query WT Tconvs (Fig. 5a).

Figure legends

Figure 1. Two transcriptionally different Th17 cell populations exist in inflamed joints of arthritic ZAC mice. a) UMAP plot of Tconv cells obtained from joints, draining lymph node (dLN) and spleen of arthritic ZAC mice colored by cluster. Unsupervised clustering was applied by the Louvain algorithm and clusters were annotated as described in the text. **b)** Proportion of annotated Tconv cell populations in joints, dLN and spleen of arthritic ZAC mice. **c)** Relative expression of canonical Tconv markers across gene expression clusters. Circle color and intensity represent the average expression whereas circle size represents the percentage of cells from each cluster expressing the gene. **d)** Volcano plot of the differentially expressed genes (DEG) among cluster 1 (Th17 *Il22*$^{+}$) and cluster 4 (Th17 *Tnfsf11*$^{+}$). **e)** Top 5 gene Ontology (GO) Terms enriched in DEGs from cluster 1 (Th17 *Il22*$^{+}$) vs cluster 4 (Th17 *Tnfsf11*$^{+}$). The color intensity of bars represents the odds ratio (OR) of enrichment. **f)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice, cells are colored according to their expression of *Il22* or *Tnfsf11*. **g)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice, with cells colored according to their inferred pseudotime values; clusters are connected according to trajectory prediction by Slingshot's pipeline

Figure 2. The Th17 *Tnfsfs11*$^{+}$ gene signature is uniquely detected in CD4^{+} T cells from inflamed synovia of rheumatoid arthritis (RA) patients. Gene signatures of Th17 *Il22*$^{+}$ and Th17 *Tnfsf11*$^{+}$ cells were obtained and used for calculation of module scores in scRNAseq datasets of **a)** Th17 cells obtained from spleens of WT, SKG and ZAC mice (n=2 each), **b)** CD4^{+} T cells from inflamed synovia of RA patients (n=3)[27], **c)** CD4^{+} T cells from

peripheral blood of RA patients (n=1)[28], **d)** CD4$^+$ T cells from synovia and infrapatellar fat pad (IPFP) tissue of osteoarthritis (OA) patients (n=3)[29], **e)** Circulating Th17 cells from healthy donors (n=3), and patients with autoimmune diseases other than RA: myasthenia gravis (MG) (n=3), multiple sclerosis (MS) (n=4), systemic lupus erythematosus (SLE) (n=3)[30].

Figure 3. The TCR repertoire of Th17 *Tnfsf11*$^+$ cells is unique and convergent, and contain public clones. a) UMAP plot of Tconv cells from tissues of arthritic ZAC mice, cells are colored according to clonal proportion classification. **b)** Distribution of clonal proportion classes across ZAC Tconv gene expression clusters. **c)** Morisita-Horn index of the paired CDR3aa similitude between the Th17 *Il22*$^+$ and Th17 *Tnfsf11*$^+$ repertoires (gray bar); and between both Th17 populations *vs* the other Tconv phenotypes in ZAC tissues (brown and green bars). **d)** Distribution of neighbor distances to the nearest 25th percentile (methods) in the repertoires of Naïve, Th17 *Il22*$^+$ and Th17 *Tnfsf11*$^+$cells. Paired (left) and single alpha (middle) and beta (right) TCR chain distributions are shown. **e)** Public clones and their clonal proportion classification and distribution across Tconv phenotypes in each mouse replicate. *: $p<0.05$, **$p<0.01$; Wilcoxon Test

Figure 4. Effector Treg cells are abundant in inflamed joints of ZAC mice but potentially suppressed by cell-cell crosstalk with Th17 *Tnfsf11*+ cells. a) Proportion of annotated Treg cell populations in joints, dLN and spleen of arthritic ZAC mice (n=4). **b)** Proportion of annotated Treg cell populations in spleen of WT (n=2), SKG (n=2) and arthritic

ZAC (n=2) mice. **c)** Heatmap of differentially expressed genes (DEG) between effector Treg cells from WT, SKG and arthritic ZAC mice. **d)** Heatmap of the top 3 gene Ontology (GO) Terms enriched in DEGs from WT, SKG and arthritic ZAC mice. Color scale represents -log10 of adjusted p values. **e)** Circos plot of predicted ligand-receptor interactions between selected Tconv and Treg cell clusters in WT, SKG and ZAC mice. Cluster labels are indicated in blue for Treg and magenta for Tconv cells. Each interaction is represented by a different color and their direction goes from the arrow start (provide the ligand) to the arrow point (provide the receptor).

Figure 5. TCR repertoires of ZAP-70 mutant Tconv cells, especially of pathogenic Th17 cells, are more Treg-like than Tconv-like. a) Description of the method employed for the assessment of repertoire similitude to reference WT Treg and WT Tconv repertoires. Each of our experimentally determined query TCRs were used to search CDR matches in a reference Tconv or Treg dataset[39-42]. We next computed the number of reference Tconv and reference Treg matches and deemed the query TCR "Treg-like", "Tconv-like" or "not significant" using a hypergeometric p-value cutoff of 0.001. The total number of matches was calculated in terms of the query repertoire size and results were normalized to the matches in the query WT Tconvs. Normalized fraction of query WT, SKG and ZAC Tconv repertoires **(b)** or ZAC Naïve, Th17 Il22+ and Th17 Tnfsf11+ repertoires **(c)** matching with reference WT Tconv (magenta section, negative values) or WT Treg repertoires (blue section, positive values) obtained from a representative reference dataset [39]. **d)** Distribution of TCR-intrinsic regulatory potential (TiRP) scores for Tconv repertoires of WT, SKG and ZAC mice.

e) Median TiRP score change of SKG and ZAC Tconv repertoires relative to the WT Tconv repertoire. **f)** Distribution of TiRP scores for WT Tconv, and ZAC Th17 *Il22*$^{+}$ and Th17 *Tnfsf11*$^{+}$ repertoires. **g)** Median TiRP score change of ZAC Th17 *Il22*+ and Th17 *Tnfsf11*+ repertoires relative to WT Tconv. **p<0.01, ****: p<0.0001; Wilcoxon Test

Figure 6. Intra-Treg-Tconv similitude in WT, SKG and ZAC repertoires. a) Morisita-Horn index calculated at the exact CDR3 amino acid level between the Tconv and Treg compartments of WT, SKG and ZAC mice. Left panel shows the analysis for single alpha chains, middle panel shows single beta chains and right panel depicts paired chains. **b)** Heatmap of weighted neighbor distance correlations between WT, SKG and ZAC Treg and Tconv repertoires as calculated by TCRdist (methods). Color scale ranges from blue (low correlation) to yellow (high correlation), and higher correlation scores indicate closer repertoires.

Figure 7. Consequences of weakened TCR signaling in the shaping of Treg and Tconv repertoires and autoimmunity. The SKG (W163C) and ZAC (H165A) mutations in the ZAP-70 gene cause a reduction in its affinity for phosphorylated CD3ζ within a range that results in the development of autoimmune disease. he consequences of a weakened TCR signaling in the development of autoimmune disease are multilayered. On one hand, cells with self-reactive TCRs are be aberrantly selected into the Tconv compartment instead of the Treg compartment (repertoire shift). This defective selection also deprives the Treg repertoires of specific receptors potentially critical for the proper suppression of autoimmune

responses and this is evidenced by a reduced similarity between Treg and Tconv repertoires within an individual (repertoire bifurcation). In addition, Treg effector activity upon TCR weakening is significantly disrupted, rendering Tregs insufficient for the suppression of self-reactive Tconvs. These events together, favor the activation of self-reactive T cells, their differentiation into pathogenic phenotypes and their accumulation in target tissues.

Supplementary Figure 1. Single-cell transcriptomic analysis of Tconv cells obtained from tissues of arthritic ZAC mice. a) UMAP plot of integrated Tconv cells obtained from joints, draining lymph node (dLN) and spleen of arthritic ZAC mice. Each panel is colored according to cell source. **b)** Proportion of annotated Tconv cell populations in joints, dLN and spleen of each arthritic ZAC mice included in the study. **c)** Expression of top 5 gene markers defining each cluster; clusters were subsampled to 100 cells for visualization. **d)** Heatmap of the top 5 enriched GO Terms in each cluster, color scale represents -log10 of adjusted p values. **e)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice colored according to their expression of *Klf2*. **f)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice colored according to their expression of markers of Th17.1 cells: *Ifng*, *Tbx21* and *Cxcr3*. **g)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice colored according to their expression of markers of exFoxP3 cells: *Sox4*, *Ccr6*, *Ccl20*, *Il23r*, and *Il17re*.

Supplementary Figure 2. Single-cell transcriptomic and repertoire analysis of Tconv cells obtained from spleens of WT, SKG and ZAC mice. a) UMAP plot of integrated Tconv cells obtained from spleens of WT (n=2), SKG (n=2) and arthritic ZAC mice (n=2).

Each panel is colored according to cell source. **b)** UMAP plot of integrated Tconv cells obtained from spleens of WT, SKG and arthritic ZAC mice, colored by cluster identity. Unsupervised clustering by the Louvain algorithm was applied, and clusters were annotated as described in the Supplementary Notes. **c)** Relative expression of canonical Tconv markers across gene expression clusters. Circle color and intensity represents average gene expression whereas circle size represents the percentage of cells from each cluster expressing the gene. **d)** Proportion of annotated Tconv cell populations in spleens of WT, SKG, and arthritic ZAC mice. **e)** Expression of top 5 gene markers defining each cluster. Clusters were subsampled to 100 cells for visualization. Significant enriched GO Terms in each cluster are depicted to the right, if no GO Terms were significantly enriched, top GO Term is shown and font color is grayed out. **f)** Heatmap of overlap between cell annotations in the ZAC Tconv dataset (columns) and the WT, SKG, ZAC spleen (Spleen Tconv) dataset (rows). Cell colors represent the proportion of ZAC Tconv annotations within Spleen Tconv annotations (i.e., each column sums up to 1). **g)** Violin plot of module scores for the gene signatures of Th17 *Il22+* (left) and Th17 *Tnfsf11+* (right) cells in Th17 cells from spleens of WT, SKG and arthritic ZAC mice. **h)** Distribution of clonal proportion classes across gene expression clusters in spleens of WT, SKG and ZAC Tconv cells.

Supplementary Figure 3. CD4$^+$ T cells from RA synovia expressing the Th17 *Tnfsfs11*$^+$ gene signature are different from Th17.1 or exFoxP3 cells. UMAP plot of CD4$^+$ T cells from inflamed synovia of RA patients (n=3) [27], cells are colored according to the density of

expression of individual markers of Th17.1 (*Ifng*, *Tbx21* and *Cxcr3*) **(a)** and exFoxP3 cells (*Sox4*, *Ccr6*, *Ccl20*, *Il23r*, and *Il17re*) **(c)**; or as module scores **(b, d)**.

Supplementary Figure 4. Repertoire analysis of Tconv cells obtained from tissues of arthritic ZAC mice. a) Distribution of clonal proportion classes across gene expression cluster separated by mouse replicate.

Supplementary Figure 5. Single-cell transcriptomic and repertoire analysis of Treg cells obtained from tissues of arthritic ZAC mice. UMAP plot of Treg cells obtained from joints, draining lymph node (dLN) and spleen of arthritic ZAC mice (n=4) colored according to cell source **(a)**, and cluster identity **(b)**. Unsupervised clustering by the Louvain algorithm was applied, and clusters were annotated as described in the Supplementary Notes. **c)** Relative expression of canonical Treg markers across gene expression clusters. Circle color and intensity represents average gene expression whereas circle size represents the percentage of cells from each cluster expressing the gene. **d)** Proportion of annotated Treg cell populations in joints, dLN and spleen of each arthritic ZAC mice. **e)** UMAP plot of Tconv cells from tissues of arthritic ZAC mice, colored according to clonal proportion classification. **f)** Distribution of clonal proportion classes across ZAC Treg cell clusters. Morisita-Horn index (y axis) between ZAC Treg gene expression clusters (plot subtitles and x axis) **(g)**, and between ZAC Tconv (x axis) and ZAC Treg cell clusters (plot subtitles) **(h)**.

Supplementary Figure 6. Single-cell transcriptomic and repertoire analysis of Treg cells obtained from spleens of WT, SKG and ZAC mice. UMAP plot of integrated Treg cells obtained from spleens of WT (n=2), SKG (n=2) and arthritic ZAC mice (n=2) colored according to cell source **(a)** and cluster identity **(b)**. Unsupervised clustering by the Louvain algorithm was applied, and clusters were annotated as described in the Supplementary Notes. **c)** Relative expression of canonical Treg markers across gene expression clusters. Circle color and intensity represents average gene expression whereas circle size represents the percentage of cells from each cluster expressing the gene. **d)** Heatmap of overlap between cell annotations in the ZAC Treg dataset (columns) and the WT, SKG, ZAC spleen (Spleen Treg) dataset (rows). Cell colors represent the proportion of ZAC Treg annotations within Spleen Treg annotations (i.e., each column sums up to 1). **e)** Expression of top 5 gene markers defining each cluster. Clusters were subsampled to 100 cells for visualization. Significant enriched GO Terms in each cluster are depicted to the right, if no GO Terms were significantly enriched, top GO Term is shown and font color is grayed out. **f)** Distribution of clonal proportion classes across gene expression clusters in spleens of WT, SKG and ZAC Treg cells.

Supplementary Figure 7. Similitude of TCR repertoires of ZAP-70 mutant Tconv cells, to reference WT Treg and WT Tconv repertoires. Normalized fraction of query WT, SKG and ZAC Tconv repertoires **(a)** or ZAC Naïve, Th17 Il22+ and Th17 Tnfsf11+ repertoires **(b)** matching with reference WT Tconv (magenta section, negative values) or WT Treg repertoires (blue section, positive values) obtained from reference datasets ([40], [41], [42]). **c)**

Distribution of TCR-intrinsic regulatory potential (TiRP) scores for Treg repertoires of WT, SKG and ZAC mice. Distribution of TiRP scores of WT Tconv cells is shown as reference. d**)** Median TiRP score change of SKG and ZAC Treg repertoires relative to the WT Tconv repertoire.

Supplementary Figure 8. Intra-Treg-Tconv similitude in WT, SKG and ZAC repertoires. a) Morisita-Horn index calculated at the exact CDR3 amino acid level between the Tconv and Treg compartments of WT, SKG and ZAC mice, split by Tconv phenotype (x axis). Clonotype tracking plots of intra-Treg-Tconv shared clones across Treg and Tconv gene expression clusters for WT **(b-c)**, SKG **(d-e)**, and ZAC **(f-h)** mice. No Treg-Tconv sharing was detected in ZAC Replicate 3.

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Figure 1

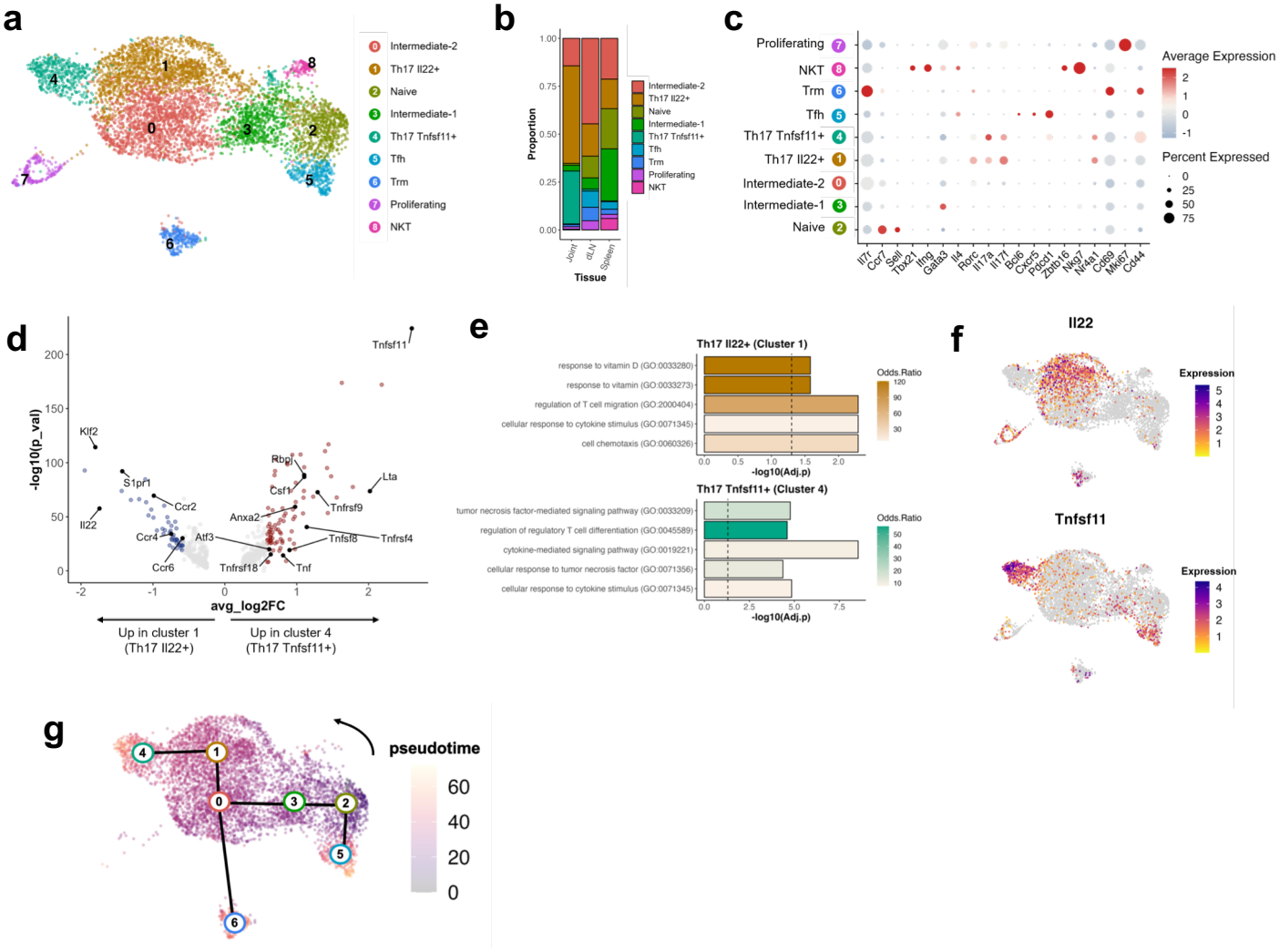

a
0 Intermediate-2
1 Th17 Il22+
2 Naive
3 Intermediate-1
4 Th17 Tnfsf11+
5 Tfh
6 Trm
7 Proliferating
8 NKT
b
Proportion
Tissue
Joint
dLN
Spleen
c
Average Expression
Percent Expressed
Il7r
Ccr7
Sell
Tbx21
Ifng
Gata3
Il4
Rorc
Il17a
Il17f
Bcl6
Cxcr5
Pdcd1
Zbtb16
Nkg7
Nr4a1
Cd69
Mki67
Cd44
d
-log10(p_val)
avg_log2FC
Up in cluster 1 (Th17 Il22+)
Up in cluster 4 (Th17 Tnfsf11+)
e
Th17 Il22+ (Cluster 1)
response to vitamin D (GO:0033280)
response to vitamin (GO:0033273)
regulation of T cell migration (GO:2000404)
cellular response to cytokine stimulus (GO:0071345)
cell chemotaxis (GO:0060326)
Th17 Tnfsf11+ (Cluster 4)
tumor necrosis factor-mediated signaling pathway (GO:0033209)
regulation of regulatory T cell differentiation (GO:0045589)
cytokine-mediated signaling pathway (GO:0019221)
cellular response to tumor necrosis factor (GO:0071356)
cellular response to cytokine stimulus (GO:0071345)
Odds.Ratio
-log10(Adj.p)
f
Il22
Tnfsf11
Expression
g
pseudotime

Figure 2

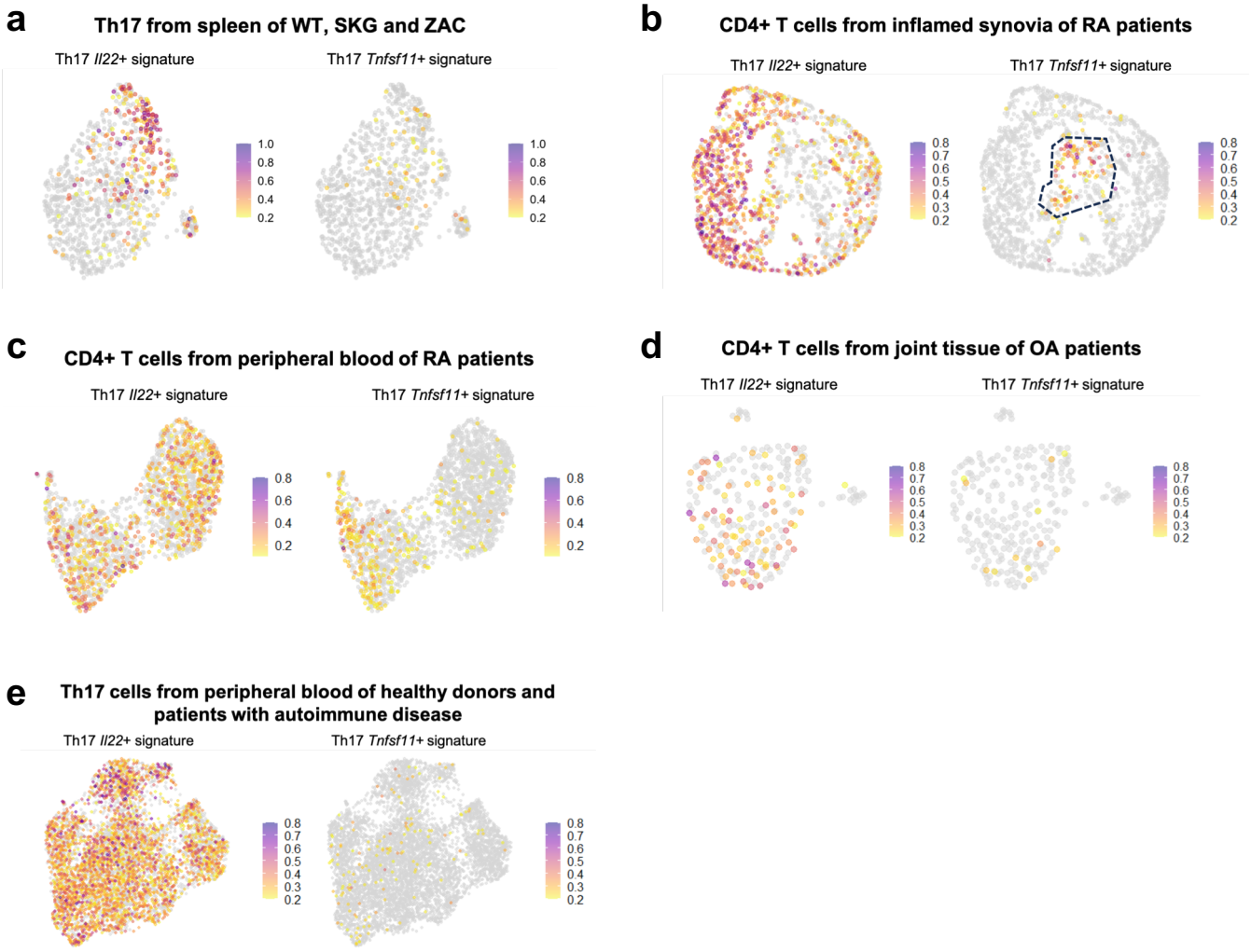

a
Th17 from spleen of WT, SKG and ZAC
Th17 Il22+ signature
Th17 Tnfsf11+ signature
1.0
0.8
0.6
0.4
0.2
b
CD4+ T cells from inflamed synovia of RA patients
Th17 Il22+ signature
Th17 Tnfsf11+ signature
0.8
0.7
0.6
0.5
0.4
0.3
0.2
c
CD4+ T cells from peripheral blood of RA patients
Th17 Il22+ signature
Th17 Tnfsf11+ signature
0.8
0.6
0.4
0.2
d
CD4+ T cells from joint tissue of OA patients
Th17 Il22+ signature
Th17 Tnfsf11+ signature
0.8
0.7
0.6
0.5
0.4
0.3
0.2
e
Th17 cells from peripheral blood of healthy donors and patients with autoimmune disease
Th17 Il22+ signature
Th17 Tnfsf11+ signature
0.8
0.7
0.6
0.5
0.4
0.3
0.2

Figure 3

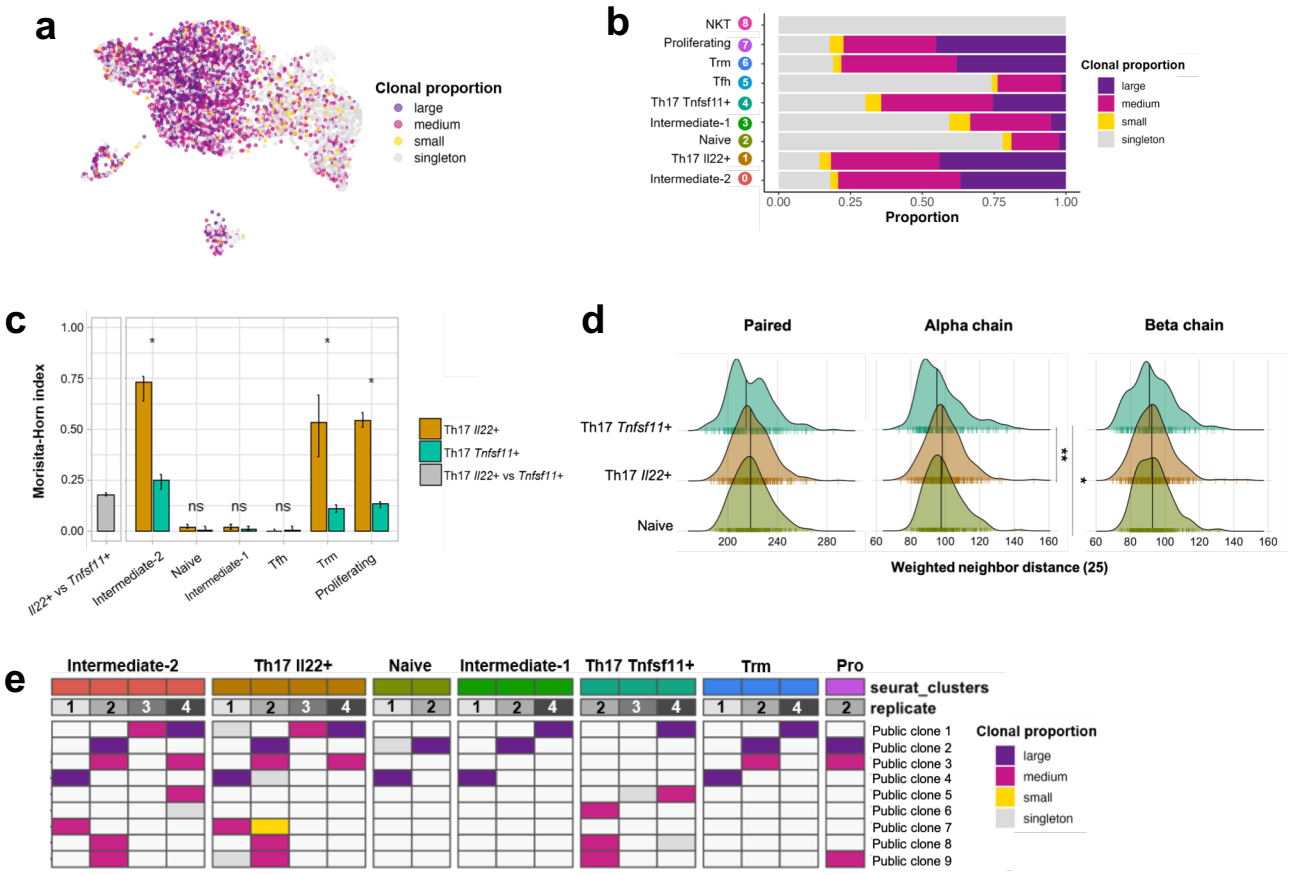

a
Clonal proportion
large
medium
small
singleton
b
NKT
Proliferating
Trm
Tfh
Th17 Tnfsf11+
Intermediate-1
Naive
Th17 Il22+
Intermediate-2
0.00
0.25
0.50
0.75
1.00
Proportion
Clonal proportion
large
medium
small
singleton
c
Morisita-Horn index
1.00
0.75
0.50
0.25
0.00
ns
ns
ns
Il22+ vs Tnfsf11+
Intermediate-2
Naive
Intermediate-1
Tfh
Trm
Proliferating
Th17 Il22+
Th17 Tnfsf11+
Th17 Il22+ vs Tnfsf11+
d
Paired
Alpha chain
Beta chain
Th17 Tnfsf11+
Th17 Il22+
Naive
**
*
200
240
280
60
80
100
120
140
160
Weighted neighbor distance (25)
e
Intermediate-2
Th17 Il22+
Naive
Intermediate-1
Th17 Tnfsf11+
Trm
Pro
seurat_clusters
replicate
Public clone 1
Public clone 2
Public clone 3
Public clone 4
Public clone 5
Public clone 6
Public clone 7
Public clone 8
Public clone 9
Clonal proportion
large
medium
small
singleton

Figure 4

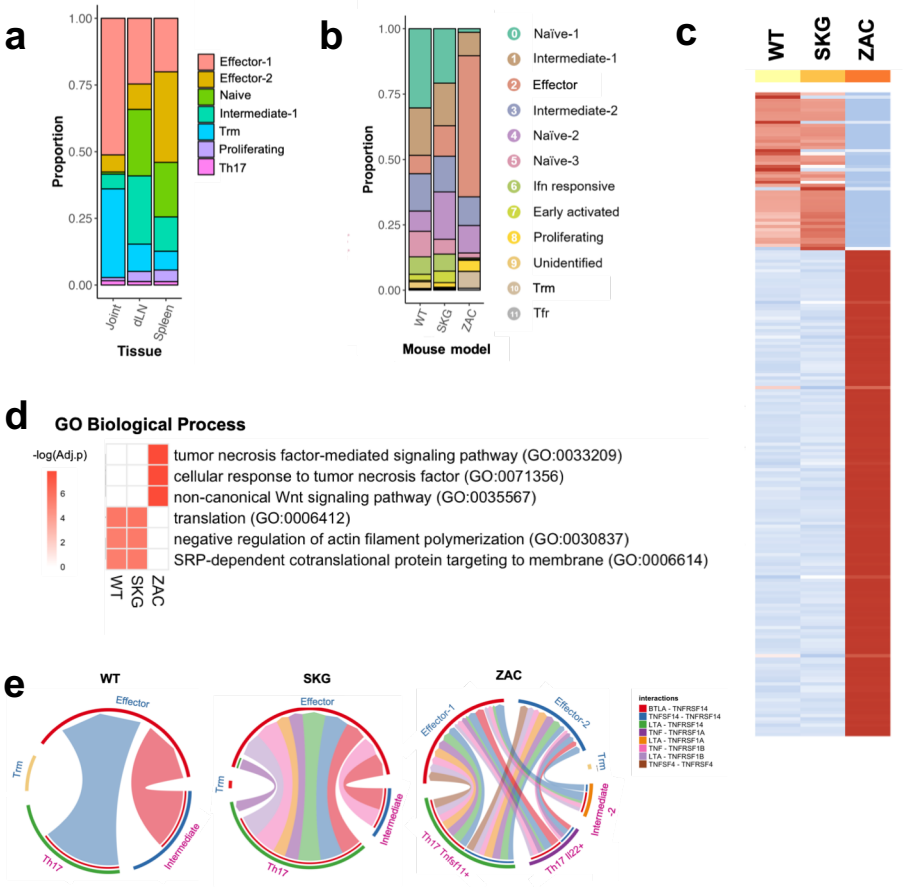

a
Proportion
1.00
0.75
0.50
0.25
0.00
Joint
dLN
Spleen
Tissue
Effector-1
Effector-2
Naive
Intermediate-1
Trm
Proliferating
Th17
b
Proportion
WT
SKG
ZAC
Mouse model
Naïve-1
Intermediate-1
Effector
Intermediate-2
Naïve-2
Naïve-3
Ifn responsive
Early activated
Proliferating
Unidentified
Trm
Tfr
c
WT
SKG
ZAC
d
GO Biological Process
-log(Adj.p)
6
4
2
0
tumor necrosis factor-mediated signaling pathway (GO:0033209)
cellular response to tumor necrosis factor (GO:0071356)
non-canonical Wnt signaling pathway (GO:0035567)
translation (GO:0006412)
negative regulation of actin filament polymerization (GO:0030837)
SRP-dependent cotranslational protein targeting to membrane (GO:0006614)
WT
SKG
ZAC
e
WT
SKG
ZAC
Effector
Trm
Th17
Intermediate
Effector-1
Effector-2
Intermediate -2
Th17 Tnfsf11+
Th17 Il22+
interactions
BTLA - TNFRSF14
TNFSF14 - TNFRSF14
LTA - TNFRSF14
TNF - TNFRSF1A
LTA - TNFRSF1A
TNF - TNFRSF1B
LTA - TNFRSF1B
TNFSF4 - TNFRSF4

Figure 5

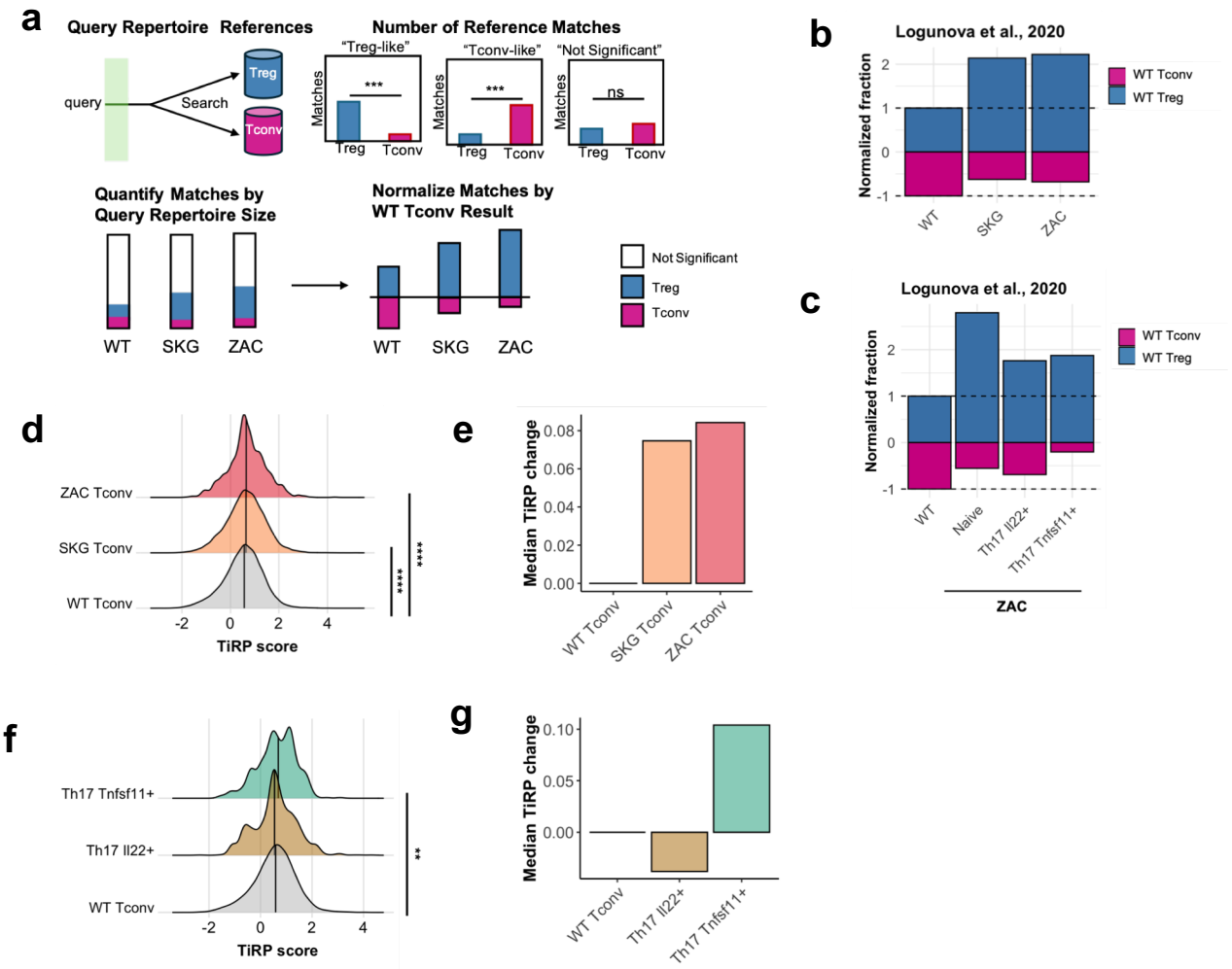

a
Query Repertoire
References
query
Search
Treg
Tconv
Number of Reference Matches
"Treg-like"
"Tconv-like"
"Not Significant"
Matches
***
***
ns
Treg
Tconv
Quantify Matches by Query Repertoire Size
Normalize Matches by WT Tconv Result
WT
SKG
ZAC
Not Significant
Treg
Tconv
b
Logunova et al., 2020
Normalized fraction
WT Tconv
WT Treg
c
Logunova et al., 2020
Naive
Th17 Il22+
Th17 Tnfsf11+
ZAC
d
ZAC Tconv
SKG Tconv
WT Tconv
****
****
TiRP score
e
Median TiRP change
f
Th17 Tnfsf11+
Th17 Il22+
WT Tconv
**
TiRP score
g
Median TiRP change

Figure 6

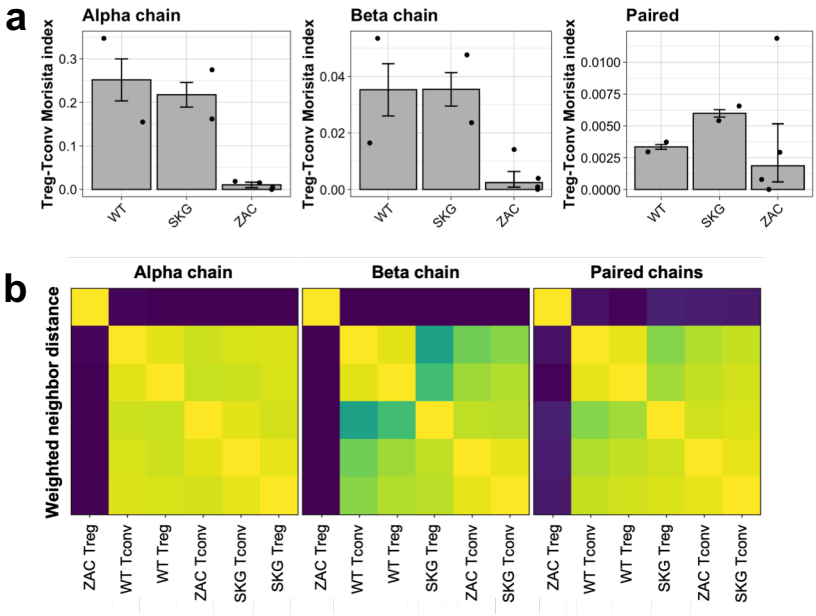
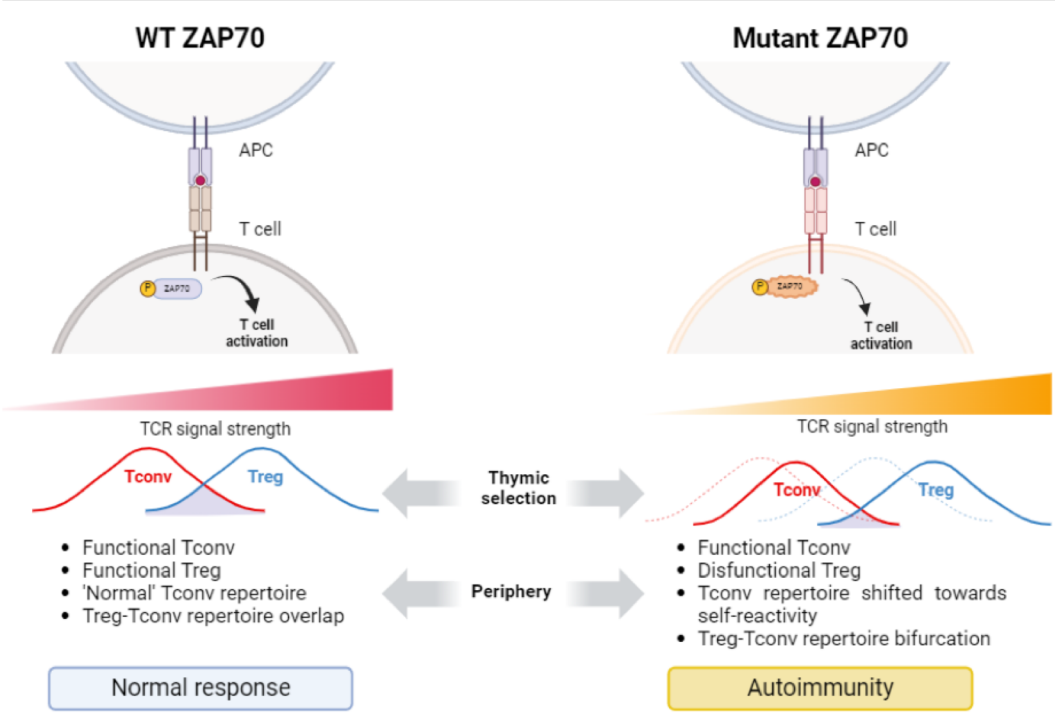

a
Alpha chain
Beta chain
Paired
Treg-Tconv Morisita index
0.3
0.2
0.1
0.0
0.04
0.02
0.00
0.0100
0.0075
0.0050
0.0025
0.0000
WT
SKG
ZAC
b
Alpha chain
Beta chain
Paired chains
Weighted neighbor distance
ZAC Treg
WT Tconv
WT Treg
ZAC Tconv
SKG Tconv
SKG Treg

Figure 7


WT ZAP70
Mutant ZAP70
APC
T cell
ZAP70
T cell activation
TCR signal strength
Tconv
Treg
Thymic selection
Periphery
Functional Tconv
Functional Treg
'Normal' Tconv repertoire
Treg-Tconv repertoire overlap
Normal response
Disfunctional Treg
Tconv repertoire shifted towards self-reactivity
Treg-Tconv repertoire bifurcation
Autoimmunity