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Molecular mechanisms of autophagic memory in pathogenic T cells in human arthritis

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ABSTRACT

T-cell resilience is critical to the immune pathogenesis of human autoimmune arthritis. Autophagy is essential for memory T cell generation and associated with pathogenesis in rheumatoid arthritis (RA). Our aim here was to delineate the role and molecular mechanism of autophagy in resilience and persistence of pathogenic T cells from autoimmune arthritis.

We demonstrated "Autophagic memory" as elevated autophagy levels in $CD4^+$ memory T cells compared to $CD4^+$ naive T cells and in Jurkat Human T cell line trained with starvation stress. We then showed increased levels of autophagy in pathogenic $CD4^+$ T cells subsets from autoimmune arthritis patients. Using RNA-sequencing, transcription factor gene regulatory network and methylation analyses we identified MYC as a key regulator of autophagic memory. We validated MYC levels using qPCR and further demonstrated that inhibiting MYC increased autophagy. The present study proposes the novel concept of autophagic memory and suggests that autophagic memory confers metabolic advantage to pathogenic T cells from arthritis and supports its resilience and long term survival. Particularly, suppression of MYC imparted the heightened autophagy levels in pathogenic T cells. These studies have a direct translational valency as they identify autophagy and its metabolic controllers as a novel therapeutic target.

1. Introduction

Autoimmune rheumatic diseases (RA and JIA) are characterized by joint and tissue damage as a result of chronic inflammation and accumulation of autoreactive and inflammatory T cells in the synovium [1,2]. T cells — in particular resilient, auto-reactive, hyperactive T cells and ineffective T-cell regulatory mechanisms — are critical contributors to the pathophysiology of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) [3–8]. Thus understanding the mechanisms underlying long-term survival of these cells represents a key unmet scientific need and could identify new therapeutic targets to resolve inflammation.

During the first antigenic exposure, stimulated T cells rapidly proliferate and expand to mount an effector response [9]. Subsequently, the majority of effector T cells die due to apoptosis and aggregation of protein bodies, but some survive to become long-lived memory T cells [10]. The memory T cells can rapidly respond to encounters with a similar antigen. The T-cell memory response is important to quickly and effectively counteract secondary infection, but an abnormal memory response by memory T-cell subsets against self antigens is harmful and leads to persistent autoimmune disease [11,12]. Indeed, studies using tetramers against self peptides suggest that auto-reactive $CD4^+$ T cells from patients with RA have a memory T-cell phenotype [12,13]. We recently described a subset of $CD4^+$ T cells, known as circulating pathogenic-like lymphocytes (CPLs), which are significantly over-represented in patients with active inflammatory arthritis and resistant to biologic therapy [14]. CPLs are clonally expanded, pro-inflammatory, antigen-experienced cells that transit through the synovial microenvironment. Determining the competitive metabolic advantage of these pathogenic cells in autoimmune disorders is a fundamental, yet an

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unaddressed question. A deeper insight into this question could provide clues to the pathogenic mechanisms of the disease and potential targets for the therapy.

We have demonstrated a higher rate of autophagy (autophagic flux) in memory $CD4^+$ T cells compared to naive $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy patients [15]. We also observed a higher rate of autophagy in $CD4^+$ T cells from patients with RA compared to $CD4^+$ T cells from healthy controls [16]. Furthermore, studies in experimental mouse modal of arthritis showed better disease outcome when autophagy was inhibited using chloroquine when compared to diseased mouse injected with buffered saline. Studies on humans and mice suggest that higher rate of autophagy is required (an integral part of the mechanism) for long term survival of memory $CD4^+$ T cells or pathogenic $CD4^+$ T cells in rheumatic diseases.

Although autophagy is an essential process for generating and maintaining memory T cells [17,18], it is unknown whether autophagy itself is required for T-cell persistence and survival, and how autophagy is regulated in memory cells. Here, we propose the concept of "autophagic memory"— a persistence of epigenetic and genetic mechanisms that promote the elevated autophagy level and confer a competitive advantage for resilient, inflammatory T cells. We investigated how autophagy is modulated in naive and antigen-experienced $CD4^+$ T cells, dissected the molecular mechanisms of autophagic memory using model T-cell systems and finally evaluated autophagic memory in pathogenic $CD4^+$ T-cell subsets from patients with adult and juvenile forms of rheumatoid arthritis.

2. Methods

2.1. Patients and healthy donors

Blood samples from patients with RA, JIA and healthy controls were collected at Scripps Clinic, La Jolla, CA (USA), Gaslini Institute (Italy) and SingHealth-Duke NUS Academic Medical Centre (Singapore) respectively. PBMCs were prepared from EDTA-anticoagulated blood by Ficoll gradient (GE Healthcare, Piscataway, NJ) followed by washing with phosphate-buffered saline (PBS). Lymphocytes were suspended in freezing media (90% FCS, 10% DMSO) and stored in liquid nitrogen until further analysis. All protocols were approved by the Human Subjects Committees of the SingHealth Academic Medical Centre and Scripps Clinic. All samples were collected in compliance with the Helsinki Declaration. Informed consent was obtained from all participating subjects donating biological material.

2.2. Mouse spleenocytes

Spleens from 5 DBA/1 mice were harvested and single cells suspension was prepared. Spleenocytes were washed with PBS and resuspended in aliquots of 1 ml freezing media (90% FBS, 10% DMSO) and kept frozen in liquid nitrogen until further use. Spleenocytes were thawed and rested for 4 h at 37° C before CytoID staining for autophagy assessment.

2.3. Cell culture

The Jurkat T-cell line was obtained from American Type Culture Collection (ATCC TIB-152) and cultured in RPMI 1640 complete media (RPMI media plus 10% serum). Cells were maintained at 37 °C in a humidified incubator with 5% CO2. Cells were passaged 80–90% confluence for routine cell culture. For autophagy training Jurkat cells were cultured in low serum media (RPMI media plus 2% serum). For MYC inhibition 1058-F4(40 μM) or 10074-G5 (30 μM) inhibitors were used. DMSO was used for control.

2.4. Autophagy assesment

Autophagy was measured using the CytoID Autophagy detection kit (Enzo Lifescience). In brief, cells were stained with live/dead stain at room temperature for 20 min followed by CytoID dye staining along with antibodies for T cell subsets at 37 °C for 30 min. Autophagy levels are represented as mean fluorescent intensity (MFI) of CytoID staining.

2.5. Fluorescence activated cell sorting

Memory ($CD3^+CD4^+CD45RO+$) and naive ($CD3^+CD4^+CD45RO-$) T cells were sorted from thawed PBMCs using a FACS Aria III sorter (BD Biosciences). CPLs ($CD3^+$CD14-CD25-CD4 + HLA-DR +) and effector T cells ($CD3^+$CD14-CD25-CD4 + HLA-DR-) were also sorted from thawed PBMCs isolated from patients with JIA. Dead cells were excluded with Sytox Red (LifeTechnologies). Fluorochrome-conjugated antibodies were purchased from BioLegend. The sorted cells were washed with PBS and lysed in mirVana™ RNA lysis buffer (Thermo Fisher Scientific) and frozen at −80 °C until required for RNA extraction.

2.6. RNA extraction and qPCR

RNA was extracted from cells using the mirVana Total RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was converted to cDNA using SuperScript IV Reverse Transcriptase (Invitrogen). Real time quantitative PCR (RT-qPCR) was performed using SYBR™ Green Master Mix (Invitrogen) on a LightCycler 480 thermocycler system (Roche). GAPDH was used as an internal control for normalization of gene expression by delta CT method. List of primers are included in supplementary table 3.

2.7. RNA-sequencing and methylation analysis

RNA from sorted CPLs and effector T cells, and RNA and genomic DNA from Jurkat T cells was extracted using an AllPrep DNA/RNA/miRNA Universal Isolation Kit (Qiagen). The RNA sequencing library was prepared using the Nextera Smart-Seq® Kit (Clontech). Paired-end RNA sequencing was performed on an Illumina HI-Seq platform (Illumina). DNA methylation was analyzed using an Infinium HumanMethylation450 BeadChip (Illumina). RNA isolated from Jurkat cells and Teff cells from human PBMCs had RNA integrity number (RIN) greater than 7. RIN number was not obtained for CPLs due to limited amount of RNA obtained from sorted cells. However all the cDNA sample were evaluated after library prep using bioanalyser, sequencing read data was evaluated using FastQC tool. All the libraries passed the FastQC test.

2.8. RNA-sequencing and methylation array data processing

Raw RNA-sequencing reads were mapped to the human genome (Hg19) using STAR aligner [19] with default parameter. The counts of reads mapped over gene features were obtained using the featureCounts method of the Subread package [20]. Fold change was calculated in the R statistical programming software, exactTest built in the edgeR package and recommended for two group test was used for differential gene expression analysis. Genes with FDR of less than 0.05 were considered significantly differentially expressed. STEM software [21] was used for clustering temporal gene expression. For DNA methylation, data were analyzed according to the recommended pipeline of the Bioconductor minfi [22] package. Data were background-subtracted and normalized in GenomeStudio (Illumina), then further processed with subset-quantile within the array normalization, which normalizes Infinium type I and II probes together. We excluded probes targeting sex chromosomes sites, and we imposed a detection *P*-value threshold of 0.01. The detection P value was calculated in GenomeStudio by comparing the signal intensity (methylated probe

intensity + unmethylated probe intensity) of a given CpG-targeting probe with the signal intensity of negative control probes. The methylation levels (β), which ranged from 0 (totally unmethylated) to 1 (completely methylated), were calculated for each CpG site R minfi package.

2.9. Network analysis

A global network of transcription factors and their target genes was created using the Encode ChIP-seq data, as previously described [23]. Using the list of differentially expressed genes (log2 fold-change > 1), a sub-network of the global TF–gene network was created and centrality scores for nodes were calculated using both the PageRank and betweeness centrality algorithms in the Python NetworkX library. The network was visualized using Cytoscape software [24] and betweeness centrality scores were used to define the radius of the nodes (represented as circles in the network).

2.10. Statistical data analysis

Statistical analyses of qPCR and CytoID data were performed using GraphPad Prism software. Data are presented as the means ± standard error of the mean. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Naive and memory CD4$^+$ T cells from patients with RA exhibit heightened autophagy

T cells that have experienced activation exhibit higher autophagic flux compared to naive T cells [15]. We hypothesized that higher levels of autophagy activation could be particularly relevant in T cells experiencing chronic pro-inflammatory stimuli, present in autoimmune diseases, as compared to T cells from healthy individuals which are not exposed to a chronic inflammatory environment. We evaluated the effects of T-cell receptor (TCR) activation in memory and naive CD4$^+$ T cells, using T cells isolated from patients with RA and healthy subjects. Fluorescence-activated cell-sorted T cells were activated with CD3$^+$ CD28$^+$ beads and autophagy was measured 5 days later. Consistent with our hypothesis, autophagy levels were significantly higher in both naive and memory CD4$^+$ T cells from patients with RA compared to respective cells from healthy subjects, indicating an increased baseline level of autophagy in patients. In addition, autophagy was even higher in memory T cells compared to naive T cells from patients with RA and healthy subjects (Fig. 1A). Furthermore, no statistical difference in autophagy levels were observed between the naive and memory CD4$^+$ T cells from healthy individuals in absence of TCR stimulus or in presence of suboptimal (3 cells: 1 bead ratio) TCR stimulus (Sup Fig. 1). These data suggest that higher autophagy is required to sustain the effector response generated by antigen experienced memory CD4$^+$ T cells when they encounter secondary TCR stimulus.

Pathogenic subsets of memory T cells can be identified in patients with RA. If autophagy is a method used by pathogenic cells to survive, the level of autophagy in these cells should be higher when compared to total effector T cells. We have observed a population of memory, activated, antigen experienced, pro-inflammatory T cells (CPLs), that correlate with disease activity, in patients with active arthritis who are resistant to therapy. Autophagy may be a key mechanism for resilience and survival of CPLs. Indeed, we found that CPLs from patients with arthritis showed significantly higher autophagic rate compared to effector CD4+T cells isolated from the same patients (Fig. 1B). These data further substantiate the relevance of autophagic memory in pathogenic CD4$^+$ T-cell subsets.

We next evaluated whether this phenomenon of elevated autophagic rate in human memory CD4$^+$ T cells is also present in mice by measuring autophagy levels in memory and naive CD4$^+$ T cells from healthy mice. CD4$^+$ memory T cells from mice spleen showed higher autophagic rate compared to naive cells (Fig. 1C). Similar to observations in humans, CD4$^+$ memory T cells from mice spleenocytes showed

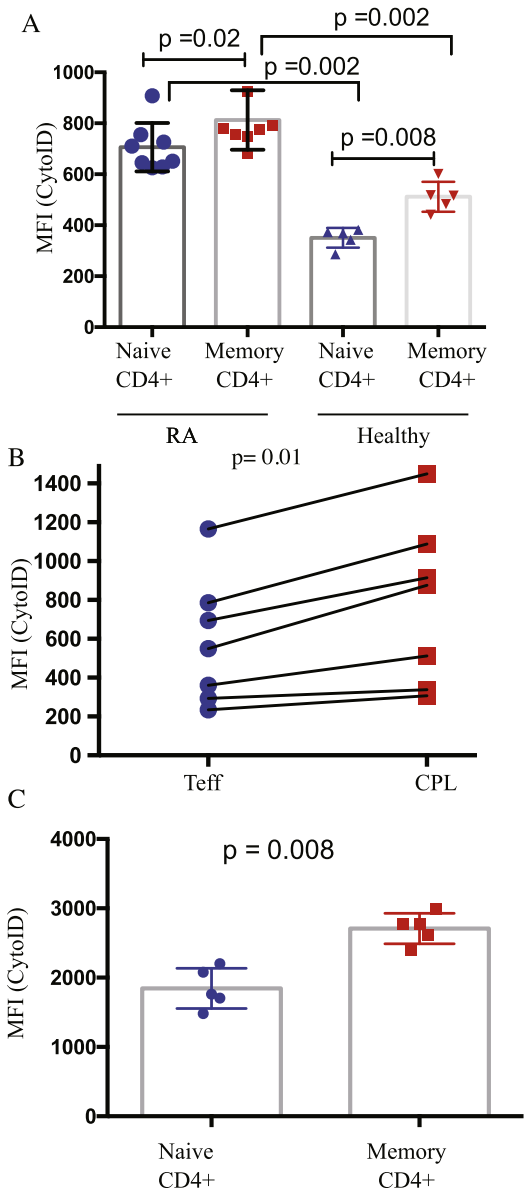


Fig. 1. Autophagy levels in T-cell subsets. A) Peripheral blood mononuclear cells (PBMCs) from patients with RA (n = 8) and healthy subjects (n = 5) were stimulated with CD3/CD28-coated beads to activate T cells. Autophagy levels were measured after 5 days by flow cytometry. The MFI of CytoID dye in memory (red; CD3$^+$CD4$^+$CD45RO+) and naive (blue; CD3$^+$CD4$^+$CD45RO-) cells represents as the level of autophagy. A Mann-Whitney test was used to compare the means of the two groups. **B)** PBMCs from patients with juvenile idiopathic arthritis patients (n = 7) were stained and autophagy was quantitated as CytoID MFI levels. The MFI of CytoID dye in pathogenic CPLs (red; CD3$^+$CD4$^+$CD14-CD25-HLA-DR+) and Teff (blue; CD3$^+$CD4$^+$CD14-CD25-HLA-DR-) cells represents the level of autophagy. **C)** Spleenocytes from healthy control mice (n = 5) were stained to measure autophagy in CD4$^+$ memory and naive T cells. The MFI of CytoID dye in memory (CD3$^+$CD4$^+$CD44$^+$) and naive (CD3$^+$CD4$^+$CD44-CD62L-) represents the level of autophagy. A Mann-Whitney test was used to compare the means of the two groups. CPL, Circulating Pathogenic-like Lymphocytes; MFI, mean fluorescence intensity; RA, rheumatoid arthritis; Teff, effector T cell. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher autophagic rate compared to naive cells, further substantiating autophagic memory as means of memory T cell persistence across species.

3.2. MYC is a key regulatory gene of autophagic memory in human T cells

We next hypothesized that the increased levels of autophagy in persistent antigen experienced, memory T-cell subsets is a part of a competitive and long-lasting metabolic advantage, which we termed "autophagic memory". We used Jurkat cells as a model human T-cell line to study the molecular mechanisms underlying autophagic memory. Eliciting starvation-induced autophagy in this cell line allowed us to evaluate the mechanisms of autophagic modulation and the subsequent persistence of these cells over several generations. We starved the cells in low serum conditions for 5 days and then re-cultured these cells in complete media for an additional 5 days. These cells represent the "trained" cell group. These cells were then exposed to a second starvation stimulus by incubating them in Earle's balanced salt solution (EBSS) for 3 h. We found that pre-starved cells exposed to repeat starvation stimulus exhibited "autophagic memory", as a significantly higher level of autophagy was elicited upon a secondary exposure to starvation conditions compared to non-starved control cells exposed only to starvation when cultured in EBSS (Fig. 2A).

Pre-starved Jurkat T cells were then cultured in complete media for an additional 100 days. As a Control, non-starved cells were cultured in complete media for 100 days alongside pre-starved cells. Considering one day of cellular growth and division as one generation, the cells at generation 5, 30, 70 and 100 (days after 5 days pre-starvation training) were harvested for transcriptome and methylome analysis by next-generation RNA-sequencing and methylation array. Fold changes in gene expression were calculated for pre-starved cells compared to control cells at days 5, 30, 70 and 100. We hypothesized that some genes contributing to autophagic memory in trained, pre-starved cells would have their expression modulated during the learning phase and would retain their expression pattern over several generations to affect autophagic memory mechanisms.

We first filtered the gene list for temporal expression analysis on the basis of FDR and fold change (FDR < 0.05 and abs(log2_fold_change) > 1) at day 5 and then clustered the filtered genes to find those with a similar expression pattern. After filtering, 533 genes were examined for expression pattern using Short Term Expression Miner (STEM) software [21]. The STEM algorithm matches temporal expression of each gene with a pre-defined set of profiles to find significantly enriched patterns. This temporal gene expression analysis found gene enrichment in 8 out of 50 predefined profiles (Fig. 2B).

The gene expression profiles 47, 45, 5 and 11 showed initial modulation followed by stability until the 30th generation (Fig. 2B) suggesting retention of the autophagic memory at the molecular level. WIPI1, the human homologue of yeast gene ATG18, is a biomarker of autophagosome formation. WIPI1 mRNA levels mimic autophagy levels as measured by immunofluorescence imaging and puncta formation [25]. In line with expression pattern of profile 47, 45, 5 and 11, we found that WIPI mRNA expression was also stably upregulated until the 30th generation in pre-starved Jurkat cells (Sup Fig. 1A). Conversely, profiles 16, 40 and 27 exhibited a more cyclical pattern, which is indicative of genes involved in cell division.

We next performed gene set enrichment analysis using the GSEA web tool to understand the function of the genes enriched in profiles 47, 45, 11 and 5. The enrichment for gene ontology (GO) biological process found genes involved in the cellular stress response, signal transduction, lipid biosynthesis and lipid metabolic processes and the immune response (Table S1). These findings suggest that metabolic reprogramming and modulation of the immune response are central to autophagic memory.

To identify the key regulatory molecules driving autophagic memory, a global transcription factor (TF) gene regulatory network (TF-GRN) analysis was performed. The global networks of TFs and non-regulatory genes were created from previously published data [23]. Briefly, experimentally validated targets for 329 TFs were obtained from the ENCODE and HTRIdb databases to create the global TF-GRN. This global TF-GRN comprised 21,940 genes including 329 TFs with target information. The autophagic memory TF-GRN was created by subsetting the global network with genes upregulated or downregulated in pre-starved cells compared to control cells at day 5 post starvation. After imposing the fold-change cutoff, a total of 533 genes were used to create the TF-GRNs. Key regulatory genes were identified using centrality measures within the autophagic memory network (Fig. 2C). Betweenness centrality is a key centrality measure that reflects the interactions between a node with all other nodes of the network, and identifies the nodes that have the highest control over the network [26]. In our dataset, the betweenness centrality score was highest for MYC followed by EGR1 and JUND.

We also calculated the "PageRank" score for all nodes in the network. PageRank is another centrality measure used by the Google search engine to rank a given web page [27]. The PageRank algorithm uses the degree of connection to and from a node to rank the node. Again, MYC followed by EGR1 and JUND achieved the highest PageRank score (Fig. 2D), confirming a possible regulatory role for these genes. From these network analyses, we propose that MYC is a potential hub and key driver of the autophagic memory network. Concordant results using two methods to measure centrality substantiate the finding that MYC is central regulator of autophagic memory.

Progressive downregulation of MYC mRNA expression in Jurkat T cells was observed until the 30th generation, even after placing the cells in normal, serum-containing media compared to non-starved control cells (Fig. 2E). This finding led us to investigate whether MYC transcriptional suppression is mediated at the epigenetic level. Consequently, we cultured pre-starved Jurkat T cells in normal media for 100 generations along with control cells and harvested them at days 5, 30, 70 and 100 for DNA methylation analysis.

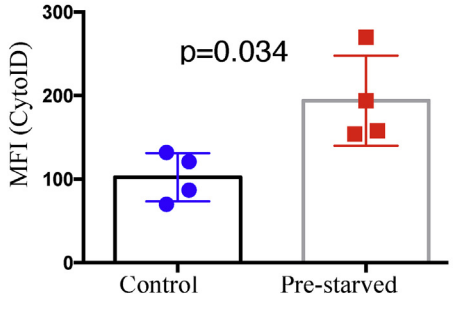
We specifically looked for DNA methylation at MYC CpG regulatory sites and found hypermethylation in 5/7 sites (Fig. 2F). Three of these sites remained hypermethylated until the 30th generation and were negatively correlated with the MYC RNA level (Fig. 2F). DNA methylation in genetic regulatory regions is associated with transcriptional inactivation [28–30]. These data suggest that MYC is epigenetically suppressed in cells that maintain autophagic memory. This epigenetic modification may contribute to the molecular mechanisms of autophagic memory in pre-starved, trained Jurkat cells to the 30th generation.

3.3. MYC helps retain autophagic memory and resilience of pathogenic human $CD4^+$ memory T cells (CPL) in autoimmune arthritis

To extend and validate the observation that MYC is a key regulator of autophagic memory, we analyzed the transcriptome of CPLs and Teff $CD4^+$ T cell subsets sorted from JIA patients (n = 8) PBMCs. RNA-sequencing data from CPLs were compared with the total effector T-cell population from the same patients. Differential gene expression analysis found 2210 genes that were significantly modulated in a CPL versus effector T-cell comparison. A total of 623 differentially expressed genes (absolute (logFold change > 2) were used to construct the TF-GRN of CPLs, as described for the Jurkat T-cell TF-GRN network. As before, betweenness centrality and PageRank scores for the CPL TF-GRN were calculated to identify key regulators of the network. MYC received the highest PageRank and the betweenness centrality score (Fig. 3B). Similar to the autophagic memory network of Jurkat T cells, the CPL TF-GRN is driven by the suppression of MYC (Fig. 3A).

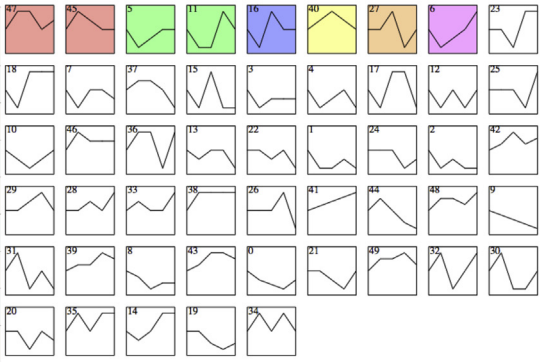
MYC levels were further analyzed in sorted $CD4^+$ memory and naive T cells isolated from healthy controls (n = 5) and patients with RA (n = 11) and cells sorted from mouse spleenocytes. Consistent with the data derived from serum starved Jurkat T cells and pathogenic CPLs

A



B

Profile	Number of genes
47	128
45	53
5	62
11	39
16	53
40	41
27	25
6	22



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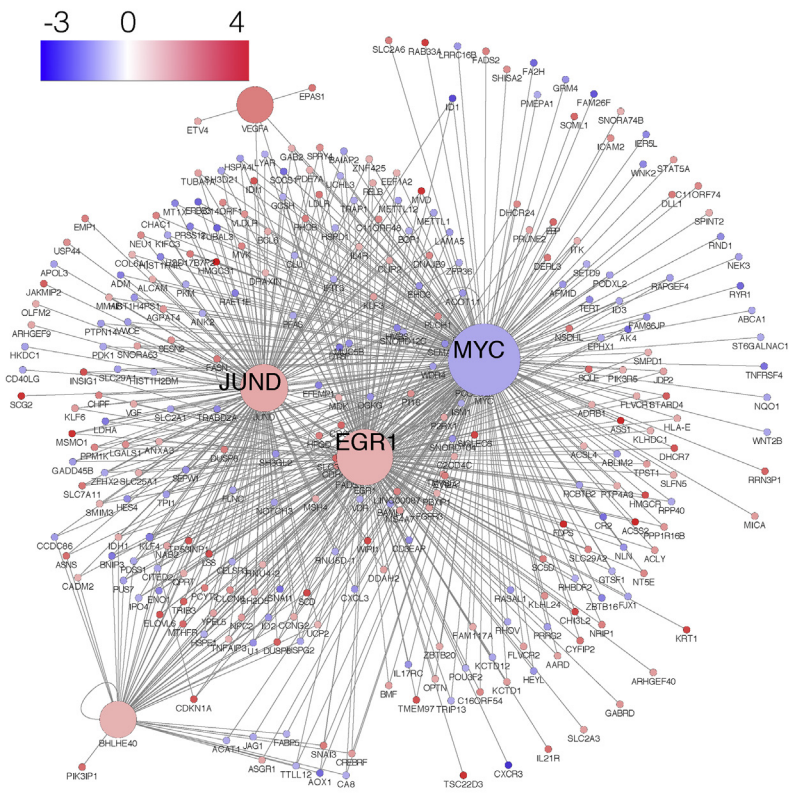
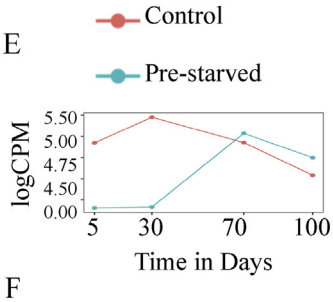


D

Centrality scores for TOP 5 network regulator

Gene Name	betweenness centrality	PageRank score
MYC	0.3	0.175
EGR1	0.169	0.134
JUND	0.099	0.101
BHLHE40	0.008	0.028
VEGFA	0.007	0.004

E



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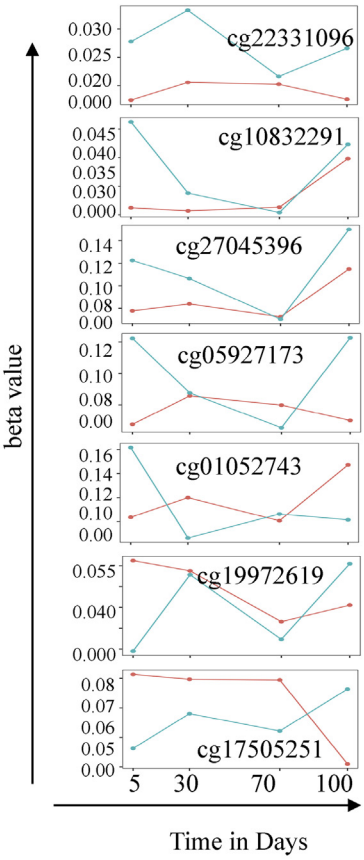

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Fig. 2. MYC is a key regulator of autophagic memory in Jurkat T cells. A) Autophagy levels were measured by CytoID staining in Jurkat T cells pre-starved for 5 days (2% serum in RPMI media) or control Jurkat T cells (10% serum in RPMI media), then cultured for 5 days in complete media (10% serum in RPMI media) and starved a second time by culturing the cells in EBSS for 3 h. Four replicate experiments were performed. The data represent the means ± standard deviation. **B**) STEM analysis of differentially expressed genes in pre-starved versus control Jurkat T cells clustered the genes with similar temporal expression profiles. The time courses for the significantly enriched profiles (based on permutation test built in the STEM software) are shown in the colored boxes along the top row (right), with the number in the box corresponding to the profile number. The table (left) shows the number of genes clustered in each respective profile. **C**) Network of transcription factors and their target genes that were differentially expressed in pre-starved Jurkat T cells compared to control Jurkat T cells. The betweenness centrality score is represented by the size of the nodes. The color of the node represents the logfold change in gene expression. The sizes of the nodes (or hubs) indicate the relative importance of the individual transcription factors within the whole gene network. **D**) Top five genes ranked by betweenness centrality and PageRank score. **E**) mRNA expression of MYC over 100 generations in pre-starved and non starved Jurkat T cells cultured in complete media (RPMI media, 10% serum). The logCPM values for MYC are plotted as a time-series graph. Cyan represents Jurkat T cells pre-starved and subsequently cultured in normal, complete media; red represents control Jurkat T cells cultured in normal, complete media. **F**) 5 of the 7 MYC regulatory regions demonstrated increased DNA methylation at 5 days when starved samples were compared to the controls. Three of these MYC regulatory regions remained hypermethylated until the 30th generation in pre-starved Jurkat T cells. Beta values for the methylation levels at various MYC CpG sites are plotted as a time series 1.2 fold change in beta value is considered differentially methylated, average beta values from two replicates are plotted as time series graph. MFI, mean fluorescence intensity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

from JIA patients, we found MYC expressed at lower levels in human $CD4^+$ memory T cells compared to naive $CD4^+$ T cells from healthy subjects (Fig. 3D) and patients with RA (Fig. 3E). $CD4^+$ memory cells from mice also had lower MYC expression compared to naive $CD4^+$ T cells (Fig. 3C) further emphasising the role of MYC as a key regulator in retaining autophagic memory.

3.4. MYC confers the metabolic advantage to pathogenic and stress induced T cells by regulating the glycolysis and fatty acid synthesis pathway genes

MYC is known to modulate the metabolic programming in T cells (36). We analyzed gene of glycolysis and fatty acid synthesis pathway in stress induced trained Jurkat cells and CPLs from patients with JIA. The starvation-induced trained Jurkat T cells showed suppressed MYC expression, and suppression of genes associated with glycolysis (Fig. S2 B); conversely, genes associated with fatty-acid synthesis and cholesterol metabolism were up-regulated (Fig. S3). RNA-sequencing data from CPLs also showed up regulation of fatty acid synthase (FASN) and carnitine palmitoyltransferase 1A (CPT1A), two key genes of fatty acid synthesis pathway (Fig. S4 A). However, key genes of the glycolysis pathways, including HK1, GLUT1, PFKFB3 and ENO1 did not show any change in expression in CPLs compared to the effector $CD4^+$ T-cell population (Fig. S4 B). RNA-sequencing results were also validated by qPCR for key genes in glycolysis and fatty acid synthesis pathway including the MYC gene expression from jurkat cells (Fig. S5). Out of 12 genes validated, expression of 9 genes (MYC, ALDOA, GPI, HK2, PFKP, CPT1A, ACSL4, FASN, PLA2G6) were significantly ($p < 0.05$) different between the pre-starved and control jurkat cells while 3 genes (SCD, ACACA, PGK1) did not pass the statistical significance.

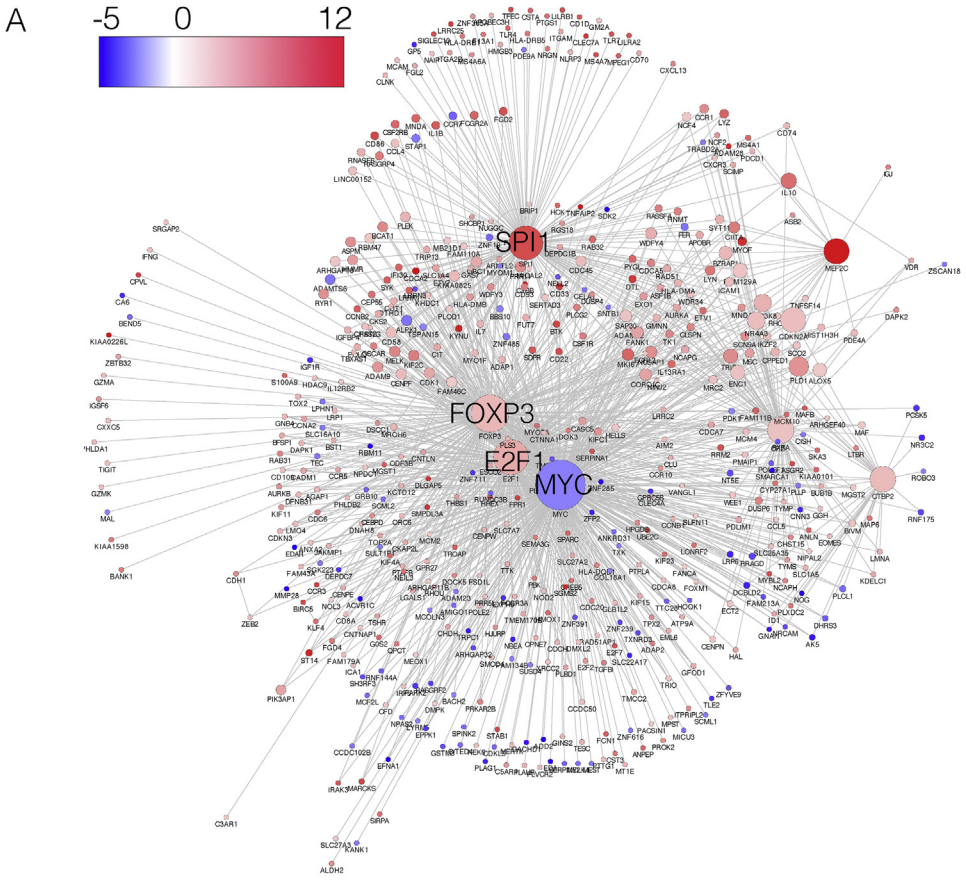
3.5. MYC inhibition increases autophagy

Our data from Jurkat T cells and primary $CD4^+$ memory T cells identify MYC as a central regulator of autophagic memory, suggesting the hypothesis that MYC suppression leads to higher autophagy to support memory-cell survival and persistence of pathogenic $CD4^+$ T cells. To confirm our findings we tested two MYC inhibitors, 10058-F4 and 10074-G5 [31], for their ability to increase autophagy levels. Human Jurkat T cells were cultured for 18 h in the presence or absence of these inhibitors and autophagy was measured by CytoID staining and flow cytometry. Cells cultured in the presence of either MYC inhibitor showed, albeit at different levels, significantly increased autophagy levels compared to control cells (Fig. 4A). These experiments clearly demonstrated that MYC suppression is a mechanism of enhancing autophagy. We further studied whether inhibiting MYC alone mimics the training imparted by serum starvation. We cultured the cells in presence and absence of MYC inhibitor (10074-G5) in control media (10% serum) and 2% serum media for 5 days and further cultured these cells for another 5 days in 10% serum containing media. After 5 days of normalization of pretreated and control cells, we measured autophagy levels in response to secondary stress stimulus by culturing the cells in EBSS for 3 h. Similar to serum starved trained cells autophagy levels were higher in cells pretreated with MYC inhibitor (Fig. 4B). These results further substantiate the role of MYC in regulating autophagic memory.

4. Discussion

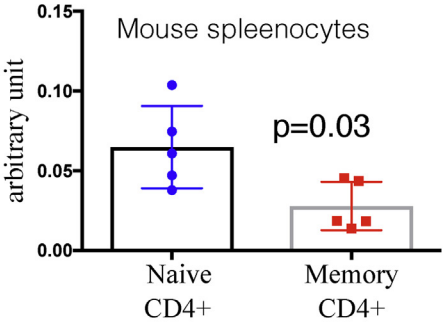
In the present study, we propose the concept of “autophagic memory”, which we define as the ability of memory T cells to respond to a second stimulus with a higher autophagy levels compared to the naive cells. Higher autophagy was also observed in murine $CD4^+$ memory T cells (Fig. 1C), which substantiates in different species the concept that autophagic memory is a means of pathogenic T-cell survival and persistence. We also demonstrate higher autophagy levels in $CD4^+$ T cell subsets from patients with RA and JIA. Specifically, CPLs from patients with JIA, as well as the naive and memory compartment of $CD4^+$ T cells from patients with RA, exhibited higher levels of autophagy compared to Teff from JIA, naive and memory cells from healthy control subjects (Fig. 1A and B). This increase in autophagy probably contributes to the persistence and survival of inflammatory pathogenic cells in affected patients. Previous study, using TCR sequence genotyping, have shown that CPLs are identical to synovial T cells from inflamed joints of the patients and they associate with disease activity [14]. Significantly higher autophagy in CPLs suggests that heighten autophagy is required not only for circulating pro inflammatory cells but also for persistent cells, more specific to site of inflammation. Classical $CD4^+$ memory T cells are primarily defined by their ability to produce cytokines and expand rapidly in response to recall antigen exposure. Our findings suggest that autophagic memory can be added to the functional repertoire of memory T cells. Memory T and memory B cells shares the transcriptional programme with long term hematopoetic stem cells [32]. Various mouse and human studies, focused on $CD8^+$ T cells, have shown the essential role of autophagy in memory T cell generation and maintenance [17,18]. The autophagic memory could be a mechanism of survival for other cells including memory $CD8^+$ T cell subsets, memory B cells and the immune subsets that tend to persist in other chronic inflammatory disease. However, further investigation is required to evaluate autophagic memory in other longer lasting cells or persistent inflammatory cells in other chronic inflammatory diseases.

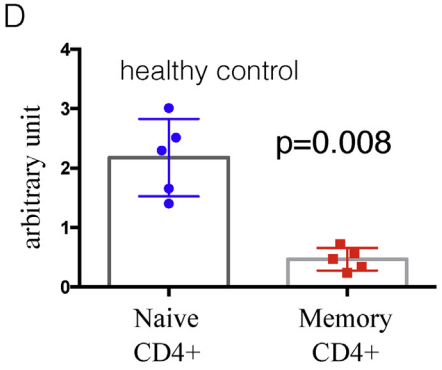
Previous studies have shown persistence of pathogenic $CD4^+$ T cells subsets in RA [33,34] but the molecular mechanisms underlying the resilience of these pathogenic subsets are unknown. The complexity of functional subtypes within the $CD4^+$ T cell compartment [35] has likely affected data interpretation to understand these molecular mechanisms. Nevertheless, studies on memory T-cell development have suggested that epigenetic and transcriptional changes are associated



B Centrality scores for TOP 5 network regulator

Gene Name	betweenness centrality	PageRank score
MYC	0.3	0.145
E2F1	0.179	0.093
FOXP3	0.144	0.082
SPI1	0.129	0.069
RXRA	0.019	0.034





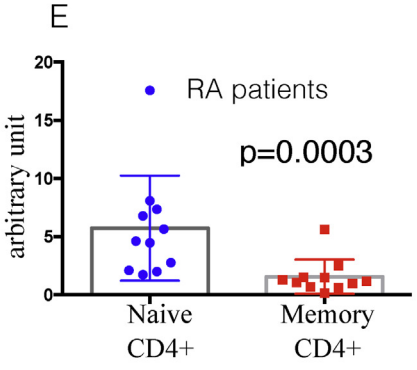


Fig. 3. MYC is a key regulator in pathogenic cells from human arthritis. A) A network of transcription factors and their targets genes that are differentially expressed in pathogenic circulating pathogenic-like lymphocytes compared to effector T cells was generated. Betweenness centrality score is represented as the size of the nodes. The color of the node represents the logfold change in gene expression. B) Top five genes ranked by betweenness centrality and PageRank score. **C**) Memory and naive CD4$^+$ T cells were sorted from mice spleenocytes (n = 5). A SYBR™ green-based quantitative PCR assay was used to calculate MYC gene expression in sorted cells. MYC expression was normalized to GAPDH and the relative expression values are represented as arbitrary units. A Mann-Whitney test was used to compare the means of the two groups. The bar graph shows MYC expression in naive and memory CD4$^+$ T cells from mice spleenocytes. **D**) MYC expression in naive and memory CD4$^+$ T cells sorted from peripheral blood mononuclear cells isolated from healthy human individuals (n = 5). **E**) MYC expression in naive and memory CD4$^+$ T cells sorted from peripheral blood mononuclear cells isolated from patients with RA (n = 11). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

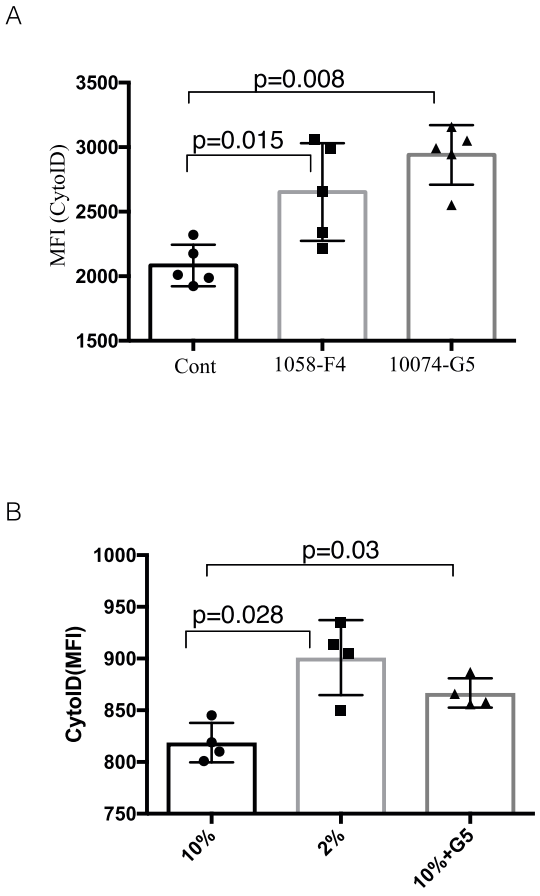


Fig. 4. MYC inhibition increases autophagy. A) Human Jurkat T cells were cultured in the presence of the MYC inhibitor 1058-F4 (40 μM) or 10074-G5 (30 μM) or DMSO (cont) for 24 h. After 24 h, cells were harvested and stained by CytoID staining for autophagy quantification. The MFI of CytoID dye in control and treated cells represents the autophagy level. B) Autophagy levels were measured by CytoID staining in Jurkat T cells pre-starved for 5 days (2% serum in RPMI media) and cells cultured in presence of MYC inhibitor (10074-G5) or control Jurkat T cells (10% serum in RPMI media). The pre-starved cells, cells cultured in presence of MYC inhibitor and control cells were further cultured for 5 days in complete media (10% serum in RPMI media) and starved a second time by culturing the cells in EBSS for 3 h. The data represent the means ± standard deviation from 4 biological replicates. A Mann-Whitney test was used to compare the mean of the two groups.

with memory T-cell persistence [36–38]. Here, we used a human T-cell line (Jurkat T cell) as a model system to investigate molecular mechanism of autophagic memory. By performing a temporal follow-up of transcriptome of "trained" cells (pre-exposed to a starvation stimulus), we were able to identify a transcriptional profile specific to autophagic memory.

By integrating the transcriptional profile of our model T-cells (533 genes differentially regulated in pre-starved vs non-starved Jurkat cells) with ENCODE CHIP-sequencing data, we identified MYC as a potential key regulator of autophagic memory. Transcriptome analysis of pathogenic CPL from JIA confirmed MYC as key regulator gene that contributes to heighten autophagic levels. MYC was suppressed at transcriptional level in both the trained Jurkat T cell line and in pathogenic CPLs. Furthermore, we also validated that MYC is suppressed in $CD4^+$ memory T cells from RA and healthy individuals (Fig. 3B and C). MYC is a known oncogene that stimulates proliferation and cellular growth and is essential for memory T cell generation. In contrast, we found that suppression of MYC is required for persistence and long term survival of pathogenic and memory $CD4^+$ T cells. Importantly, we show here that inhibition of MYC transcriptional activity increases autophagy (Fig. 4), which further substantiated our finding that MYC suppression is central to autophagic memory and long term persistence. Our data suggest that MYC might be regulated differently and specifically during generation, contraction and maintenance phase of memory T cell life-cycle.

MYC regulates metabolic reprogramming during T-cell activation [39] and functions as both a transcriptional activator and repressor [40]. Upregulation of fatty acid synthesis genes in pathogenic T cells from JIA and pre-starved modal T-cell line suggest that perhaps pathway pertaining to fatty acid synthesis are more relevant to autophagic memory and long term persistence. The genes of the fatty acid synthesis pathway could be prioritised as therapeutic targets for manipulating the autophagic process in pathogenic T cells from inflammatory rheumatic diseases.

A mechanism of direct transcriptional activation of glycolytic genes by MYC has been demonstrated in previous reports [39,41]. By contrast, how and whether MYC (alone or in coordination with other factors) controls fatty-acid synthesis genes needs to be investigated. MYC is also a well-known oncogene and is deregulated in many human cancers. MYC induces cellular proliferation and growth, causing the tumorigenesis [42] and thus MYC inhibitors have been well-studied and are reported to have anti-tumor effects [43,44]. Given the oncogenic role of MYC, direct MYC activators might not be suitable for clinical utility in RA, due to adverse cancerous effects, but genes regulated by MYC could be potential targets. Inhibiting genes in the fatty acid synthesis pathway could also be a therapeutic strategy to inhibit autophagic memory in persistent, pathogenic T cells.

5. Conclusions

In summary, we have demonstrated the phenomenon of autophagic memory in $CD4^+$ memory T cells in RA and JIA. We propose that autophagic memory is a mechanism employed by pathogenic $CD4^+$ T cell subsets (CPLs and memory T cells) to increase resilience. Using the Jurkat T-cell line to monitor autophagic learning, we identified MYC as a key regulator of the autophagic memory and we validated these data in human $CD4^+$ T-cell subsets. Collectively, our data suggest that autophagic memory is a key process mediating long-term persistence of memory cells, in particular arthritis-specific $CD4^+$ T cells and T-cell subsets. We believe that these findings have direct translational implications, as therapeutics directly targeting autophagy or indirectly manipulating MYC could improve treatment outcomes for patients with rheumatoid arthritis and juvenile rheumatoid arthritis.

Conflicts of interest disclosure

The authors declare no conflicts of interest.

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Author contributions

P.K performed the experiments, bioinformatics, analyzed the data and wrote the manuscript. J.Y, J.L.V, B.P, S.S and C.C performed the experiments. M.G, A.C,A.M, K.P recruited the patients and obtained the relevant blood samples. T.A, M.L participated in study design and

manuscript preparation. S.A conceived the study, analyzed the data and wrote the manuscript.

Data and materials availability

Raw RNA-sequencing data will be provided upon request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2018.07.014.

References

[1] S. Albani, E.C. Koffeman, B. Prakken, Induction of immune tolerance in the treatment of rheumatoid arthritis, Nat. Rev. Rheumatol. 7 (2011) 272–281.
[2] T. Kobezda, S. Ghassemi-Nejad, K. Mikecz, T.T. Glant, Z. Szekanecz, Of mice and men: how animal models advance our understanding of T-cell function in RA, Nat. Rev. Rheumatol. 10 (2014) 160–170.
[3] B.J. Prakken, R. Samodal, T.D. Le, F. Giannoni, G.P. Yung, J. Scavulli, et al., Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 4228–4233.
[4] S. Kamphuis, W. Kuis, W. de Jager, G. Teklenburg, M. Massa, G. Gordon, et al., Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis, Lancet 366 (2005) 50–56.
[5] B. Prakken, S. Albani, A. Martini, Juvenile idiopathic arthritis, Lancet 377 (2011) 2138–2149.
[6] R. Spreafico, M. Rossetti, J.W. Whitaker, W. Wang, D.J. Lovell, S. Albani, Epipolymorphisms associated with the clinical outcome of autoimmune arthritis affect CD4+ T cell activation pathways, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 13845–13850.
[7] Z. Yang, Y. Shen, H. Oishi, E.L. Matteson, L. Tian, J.J. Goronzy, et al., Restoring oxidant signaling suppresses proarthritogenic T cell effector functions in rheumatoid arthritis, Sci. Transl. Med. 8 (2016) 331ra38.
[8] M. Rossetti, R. Spreafico, A. Consolaro, J.Y. Leong, C. Chua, M. Massa, et al., TCR repertoire sequencing identifies synovial Treg cell clonotypes in the bloodstream during active inflammation in human arthritis, Ann. Rheum. Dis. 76 (2017) 435–441.
[9] R.W. Dutton, L.M. Bradley, S.L. Swain, T cell memory, Annu. Rev. Immunol. 16 (1998) 201–223.
[10] M. Lenardo, K.M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, et al., Mature T lymphocyte apoptosis–immune regulation in a dynamic and unpredictable antigenic environment, Annu. Rev. Immunol. 17 (1999) 221–253.
[11] M.M. Martinic, C. Huber, K. Coppieters, J.E. Oldham, A.L. Gavin, M.G. von Herrath, Expression level of a pancreatic neo-antigen in beta cells determines degree of diabetes pathogenesis, J. Autoimmun. 35 (2010) 404–413.
[12] E.A. James, M. Rieck, J. Pieper, J.A. Gebe, B.B. Yue, M. Tatum, et al., Citrulline-specific Th1 cells are increased in rheumatoid arthritis and their frequency is influenced by disease duration and therapy, Arthritis Rheum. 66 (2014) 1712–1722.
[13] S.C. Law, S. Street, C.H. Yu, C. Capini, S. Ramnoruth, H.J. Nel, et al., T-cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles, Arthritis Res. Ther. 14 (2012) R118.
[14] R. Spreafico, M. Rossetti, J. van Loosdregt, C.A. Wallace, M. Massa, S. Magni-Manzoni, et al., A circulating reservoir of pathogenic-like CD4+ T cells shares a genetic and phenotypic signature with the inflamed synovial micro-environment, Ann. Rheum. Dis. 75 (2016) 459–465.
[15] J. van Loosdregt, R. Spreafico, M. Rossetti, B.J. Prakken, M. Lotz, S. Albani, Hydroxychloroquine preferentially induces apoptosis of CD45RO+ effector T cells by inhibiting autophagy: a possible mechanism for therapeutic modulation of T cells, J. Allergy Clin. Immunol. 131 (2013) 1443–1446 e1.
[16] J. van Loosdregt, M. Rossetti, R. Spreafico, M. Moshref, M. Olmer, G.W. Williams, et al., Increased autophagy in CD4+ T cells of rheumatoid arthritis patients results in T-cell hyperactivation and apoptosis resistance, Eur. J. Immunol. 46 (2016) 2862–2870.
[17] D.J. Puleston, H. Zhang, T.J. Powell, E. Lipina, S. Sims, I. Panse, et al., Autophagy is a critical regulator of memory CD8(+) T cell formation, Elife (2014) 3.
[18] X. Xu, K. Araki, S. Li, J.H. Han, L. Ye, W.G. Tan, et al., Autophagy is essential for effector CD8(+) T cell survival and memory formation, Nat. Immunol. 15 (2014) 1152–1161.
[19] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, et al., STAR: ultrafast universal RNA-seq aligner, Bioinformatics 29 (2013) 15–21.
[20] Y. Liao, G.K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features, Bioinformatics 30 (2014) 923–930.
[21] J. Ernst, Z.S.T.E.M. Bar-Joseph, A tool for the analysis of short time series gene expression data, BMC Bioinf. 7 (2006) 191.
[22] M.J. Aryee, A.E. Jaffe, H. Corrada-Bravo, C. Ladd-Acosta, A.P. Feinberg, K.D. Hansen, et al., Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays, Bioinformatics 30 (2014) 1363–1369.
[23] V. Narang, M.A. Ramli, A. Singhal, P. Kumar, G. de Libero, M. Poidinger, et al., Automated identification of core regulatory genes in human gene regulatory networks, PLoS Comput. Biol. 11 (2015) e1004504.
[24] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, et al., Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504.
[25] T. Proikas-Cezanne, Z. Takacs, P. Donnes, O. Kohlbacher, WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome, J. Cell Sci. 128 (2015) 207–217.
[26] L F, A set of measures of centrality based on betweenness, Sociometry 40 (1977) 35–41.
[27] L.B.S. Page, R. Motwani, T. Winograd, The PageRank Citation Ranking: Bringing Order to the Web, Stanford Digital Library Working Paper SIDL-WP-1999-0120 Stanford University (1999).
[28] I. Keshet, J. Lieman-Hurwitz, H. Cedar, DNA methylation affects the formation of active chromatin, Cell 44 (1986) 535–543.
[29] S.U. Kass, N. Landsberger, A.P. Wolffe, DNA methylation directs a time-dependent repression of transcription initiation, Curr. Biol. 7 (1997) 157–165.
[30] K.K. Farh, A. Marson, J. Zhu, M. Kleinewietfeld, W.J. Housley, S. Beik, et al., Genetic and epigenetic fine mapping of causal autoimmune disease variants, Nature 518 (2015) 337–343.
[31] M.J. Huang, Y.C. Cheng, C.R. Liu, S. Lin, H.E. Liu, A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia, Exp. Hematol. 34 (2006) 1480–1489.
[32] C.J. Luckey, D. Bhattacharya, A.W. Goldrath, I.L. Weissman, C. Benoist, D. Mathis, Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 3304–3309.
[33] M. Londei, C.M. Savill, A. Verhoef, F. Brennan, Z.A. Leech, V. Duance, et al., Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 636–640.
[34] M. Schirmer, A.N. Vallejo, C.M. Weyand, J.J. Goronzy, Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28- T cells from rheumatoid arthritis patients, J. Immunol. 161 (1998) 1018–1025.
[35] M.A. Kunicki, L.C. Amaya Hernandez, K.L. Davis, R. Bacchetta, M.G. Roncarolo, Identity and diversity of human peripheral Th and T Regulatory cells defined by single-cell mass cytometry, J. Immunol. 200 (2018) 336–346.
[36] B. Youngblood, J.S. Hale, R. Ahmed, T-cell memory differentiation: insights from transcriptional signatures and epigenetics, Immunology 139 (2013) 277–284.
[37] H.K. Komori, T. Hart, S.A. LaMere, P.V. Chew, D.R. Salomon, Defining CD4 T cell memory by the epigenetic landscape of CpG DNA methylation, J. Immunol. 194 (2015) 1565–1579.
[38] V.S. Patil, A. Madrigal, B.J. Schmiedel, J. Clarke, P. O'Rourke, A.D. de Silva, et al., Precursors of human CD4(+) cytotoxic T lymphocytes identified by single-cell transcriptome analysis, Sci Immunol (2018) 3.
[39] R. Wang, C.P. Dillon, L.Z. Shi, S. Milasta, R. Carter, D. Finkelstein, et al., The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation, Immunity 35 (2011) 871–882.
[40] M. Wanzel, S. Herold, M. Eilers, Transcriptional repression by myc, Trends Cell Biol. 13 (2003) 146–150.
[41] Z. Nie, G. Hu, G. Wei, K. Cui, A. Yamane, W. Resch, et al., c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells, Cell 151 (2012) 68–79.
[42] R.M. Kortlever, N.M. Sodir, C.H. Wilson, D.L. Burkhart, L. Pellegrinet, L. Brown Swigart, et al., Myc cooperates with ras by programming inflammation and immune suppression, Cell 171 (2017) 1301–1315 e14.
[43] D.R. Carter, J. Murray, B.B. Cheung, L. Gamble, J. Koach, J. Tsang, et al., Therapeutic targeting of the MYC signal by inhibition of histone chaperone FACT in neuroblastoma, Sci. Transl. Med. 7 (2015) 312ra176.
[44] R. Su, L. Dong, C. Li, S. Nachtergaele, M. Wunderlich, Y. Qing, et al., R-2HG exhibits anti-tumor activity by targeting FTO/m(6)A/MYC/CEBPA signaling, Cell 172 (2018) 90–105 e23.