SUPPLEMENTARY MATERIAL

TNF-induced Inflammatory Genes Escape Repression in Fibroblast-like Synoviocytes: Transcriptomic and Epigenomic Analysis

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This work was funded by grants from NIH AI046712, AR046713 and AR050401 (LBI) and the Feldstein Medical Foundation (GDK).

Supplementary Methods

Patients

Synovial tissues were obtained from RA patients who underwent, as part of standard medical care, total knee replacement or elbow synovectomy, using a protocol approved by the Hospital for Special Surgery Institutional Review Board that adheres to NIH guidelines and regulations. The specimens used in this study, that otherwise would have been discarded, were deidentified and obtained under a waiver of consent. The diagnosis of RA was based on the 1987 American College of Rheumatology criteria. The results described in the manuscript have been verified in several independent experiments with FLS derived from more than 10 different RA patients. Since the specimens were deidentified any information other than the confirmed diagnosis of RA was not available.

Cell purification and culture

Synovial tissue fragments were incubated with Liberase and fibroblast-like synoviocytes (FLS) cells were allowed to adhere to tissue culture dishes and passaged every 3-5 days (4-5 passages). $CD14^+$ cells were purified from healthy donors with the use of anti-CD14 magnetic beads (Miltenyi Biotec) from peripheral blood mononuclear cells purchased from the New York Blood Center. Cells were cultured as previously described[1 2] in the presence or absence of TNF (10 ng/ml; Pepro Tech). In experiments using I-BET, DMSO was used as a vehicle control.

MTT Cell survival assay

Cell Proliferation Kit I (Roche) was used to measure FLS viability through MTT based colorimetric assay following manufacturer's protocol.

Sequencing (RNA-seq, ChIP-seq, ATAC-seq)

RNAseq data for FLS was from dbGAP: phs001371.v1.p1 and for macrophages from previous data[3] plus newly generated two replicates of the TNF stimulation time course. Macrophage ChIP-seq and ATAC-seq data were from GSE100383 and FLS ChIP-seq and ATAC-seq data were obtained following standard protocols[3 4] (detailed protocols available upon request). Sequencing libraries were generated using TruSeq (RNA-seq, ChIP-seq) or Nextera (ATAC-seq) kits (Illumina). Single end sequencing was performed (Illumina HiSeq 2500, 50 bp read lengths) at the Weill Cornell Medicine Epigenomics Core Facility. Reads were aligned to the human reference hg19 and quality control of aligned reads was performed using FastQC. Additional information about RNAseq, ChIPseq and ATACseq data and concordance between replicates is provided in supplementary figures S1C, S3A and S3B.

Differential gene expression and pathway analysis

Gene count quantification for RNA-seq was performed using HTSeq and Counts Per Million (CPM) quantification using R. Count tables were processed and filtered based on expression in TNF treated conditions (> 100 counts per gene). Differential testing was performed between TNF treated libraries and the control libraries using DESeq2. DESeq2 significance thresholds were set to adjusted p-value < 0.05. Significant differentially expressed gene lists were generated and used as input for Ingenuity Pathway Analysis (IPA).

Peak identification and gene ontology analysis

We used HOMER (http://homer.ucsd.edu/homer/) to identify peaks of ChIP-seq and ATAC-seq. A false-discovery rate threshold of 0.001 was used for all data sets and total number of mapped reads in each sample was normalized to ten million mapped reads. Gene enhancers were assigned to the genomic region as > +2 kb or < -2 kb from a transcription start site (hg19, Refseq). All enhancer peaks among the different conditions in each comparison were merged into one peak set using mergePeaks –size given. Each enhancer was assigned to the nearest transcription start site. Peak-associated genes were defined based on the closest genes to these genomic regions using RefSeq coordinates of genes. Sequencing data were visualized by preparing custom tracks for the UCSC Genome browser. Gene-ontology annotation was determined using the GREAT (http://bejerano.stanford.edu/great/public/html/) tool on each of the six clusters (using a ranked list as input). Pathways were ranked using binomial p-value.

Clustering, correlation and motif analysis

To generate K-mean clusters with RNA-seq, we used Morpheus (https://software.broadinstitute.org/morpheus/) K-means algorithm with the Euclidean distance metric. The clusters were grouped into gene clusters mainly on the basis of pattern of gene expression, also taking into account the quantitative pattern of expression of each cluster. For the correlation analysis, we calculated the Pearson's and Spearman Correlation r values among replicates. For motif analysis, we used HOMER to analyze the enhancers of genes for motifs that are enriched in target gene enhancers under ATAC-seq peaks. Sequences under peaks were compared to random genomic fragments of the same size and normalized G+C content to identify motifs for which the targeted sequences showed enrichment. Occurrences of motifs from

the JASPAR database were identified by running HOMER on the hg19 reference sequence with a detection threshold of $p < 10^{-10}$. Motifs were chosen from Top 10 highly enriched motifs, excluding redundant ones, for display in macrophage and FLS.

RT-qPCR and Statistical analysis

RNA was extracted from $0.5x10^6$ FLS using RNeasy mini kit (Qiagen) and 1µg was reverse transcribed using a First Strand cDNA synthesis kit (Fermentas). qPCR was performed using SYBR Green supermix using the 7500 Fast Real-time PCR system following the manufacturer's protocols, and triplicate reactions were run for each sample. Results are expressed as mean ± SEM and GraphPad Prism Analytical Software Version 5 for Windows was used for statistical analysis including Wilcoxon matched-pairs signed rank test, p-values and correlation coefficient (r) calculation.

Supplementary Figure legends

Supplementary figure S1. FLS RNA-seq analysis and isolation strategy for genes whose expression is sustained in FLS (FSGs) but transient in macrophages. (A) Kinetic analysis of TNF-induced FLS mRNA transcripts visualized on a heatmap (left). RNA-seq analysis of TNF-stimulated FLS (3, 24, 72 h) was performed and differentially expressed 2,116 genes (> 1.5 fold and $p < 0.05$) at each time point were subjected to K-means clustering. Bar graphs (right) represent CPM values and error bars indicate SEM. (B) Ingenuity Pathway Analysis (IPA) for canonical pathways on genes in each cluster defined in figure S1A. (C) RNA-seq correlation analysis of three TNF-stimulated macrophage replicates (3, 6, 24 h) and two TNF-stimulated FLS replicates (1, 3, 24, 72h). r: Spearman rank correlation was estimated. (D) Strategy for

isolating genes whose expression was sustained in FLS sustained and transient in macrophages (FLS sustained genes (FSGs), n=280). (E) Distribution of FSGs in RNA-seq clusters defined in Supplementary figure S1 (A).

Supplementary figure S2. Comparative transcriptomic analysis of the responses to TNF between in vitro generated Mϕ and RA FLS. (A) Venn diagram of TNF-induced genes (> 2 fold and $p < 0.05$) in FLS and macrophages at 3h and 24h of TNF stimulation. Genes significantly up-regulated by TNF only in macrophages (macrophage-specific genes; group 1): 927 genes at 3h of TNF stimulation and 1012 genes at 24h of TNF stimulation. Genes significantly up-regulated by TNF only in RA FLS (FLS-specific genes; group 3): 418 genes at 3 h of TNF stimulation and 588 genes at 24h of TNF stimulation. Genes up-regulated by TNF in both cell types (macrophage and FLS overlapping genes; group 2): 206 genes at 3h of TNF stimulation and 393 genes at 24h of TNF stimulation. Ingenuity Pathway Analysis (IPA) for canonical pathways on macrophage-specific genes (group 1), macrophage and FLS overlapping genes (group 2), and FLS-specific genes (group 3) at 3h and 24h of TNF stimulation. Note the induction of the feedback inhibitory IL-10-STAT3 pathway in macrophages but not FLS.

Supplementary figure S3. ChIP-seq and ATAC-seq analysis of FLS. (A) H3K27ac ChIP-seq and ATAC-seq peaks at each time point of TNF stimulation (3, 24, 72 h) and resting condition (T0). Correlation analysis between two biological replicates for H3K27ac ChIP-seq and ATAC-seq in FLS was done by estimating Pearson correlation coefficient (r) based on the read density of entire peak regions. (B) Venn diagram showing the overlap between H3K27ac ChIP-seq and ATAC-seq peaks in FLS at each time point of TNF stimulation (3, 24, 72 h). (C) Motifs enriched

under ATAC-seq peaks of FLS at resting condition (T0). (D) Heatmap of ATACseq normalized tag density of 9,319 peaks that overlap with H3K27ac peaks in each of the six clusters presented relative to the maximum.

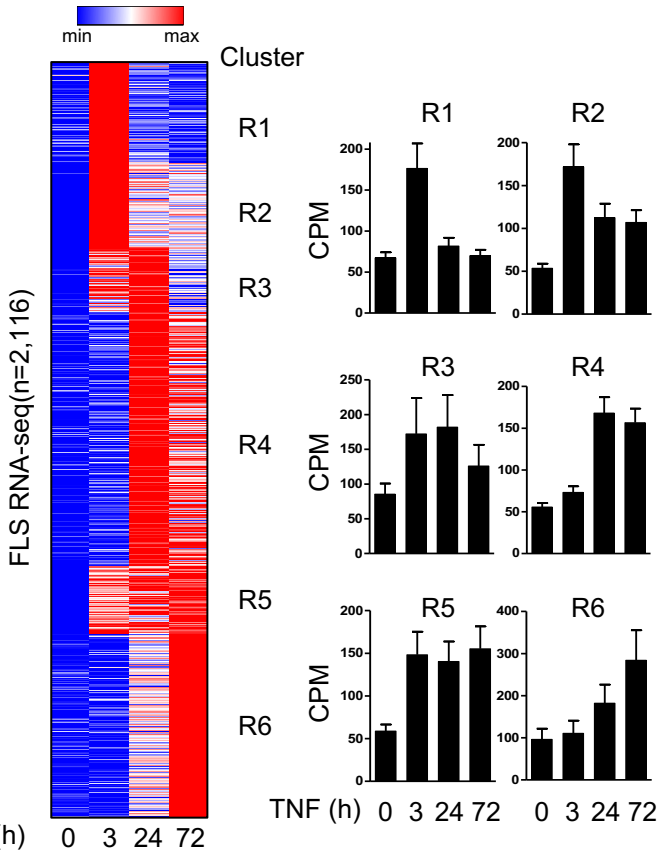
Supplementary figure S4. ChIPseq and ATACseq analysis of enhancers associated with genes that are tolerized in macrophages and expressed in a sustained manner in FLS. (A) Heatmaps of normalized tag densities for H3K27ac ChIP-seq peaks associated with the 80 genes defined in figure 2A. (B) Histogram of H3K27ac-seq normalized tag densities for peaks defined in figure S3A. (C) Histogram of ATAC-seq normalized tag densities at the H3K27ac peaks shown in figure S3A. (D) Venn diagram showing the overlap between H3K27ac ChIP-seq peaks in resting and TNF-stimulated FLS and macrophages at enhancers (> +2 kb or < -2 kb from TSS) associated with the fibroblast sustained genes (FSGs) defined in figure 1A.

Supplementary figure S5. Suppressive I-BET effect on TNF-induced inflammatory gene expressions of FLS. (A) RT-qPCR analysis of mRNA isolated from RA FLS stimulated with TNF (10 ng/ml) in the presence or absence of I-BET (10 μM) for 48 hours. Data are cumulative of three independent replicates. (B) MTT assay to evaluate the impact of I-BET (10 μM, 24 hours) on FLS survival. Data are cumulative of three independent biological replicates from three different RA patients that were used for RNA sequencing (data presented in Figure 5). I-BET had no significant effect on FLS survival over a period of 24 hours (Wilcoxon matched-pairs signed rank test).

Supplementary Data References

1. Lee A, Qiao Y, Grigoriev G, et al. Tumor necrosisfactor alpha induces sustained signaling and a prolonged and unremitting inflammatory response in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2013;65(4):928-38. doi: 10.1002/art.37853
2. Sohn C, Lee A, Qiao Y, et al. Prolonged tumor necrosis factor alpha primes fibroblast-like synoviocytes in a gene-specific manner by altering chromatin. *Arthritis Rheumatol* 2015;67(1):86-95. doi: 10.1002/art.38871
3. Park SH, Kang K, Giannopoulou E, et al. Type I interferons and the cytokine TNF cooperatively reprogram the macrophage epigenome to promote inflammatory activation. *Nat Immunol* 2017;18(10):1104-16. doi: 10.1038/ni.3818
4. Buenrostro JD, Wu B, Chang HY, et al. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol* 2015;109:21 29 1-9. doi: 10.1002/0471142727.mb2129s109

A



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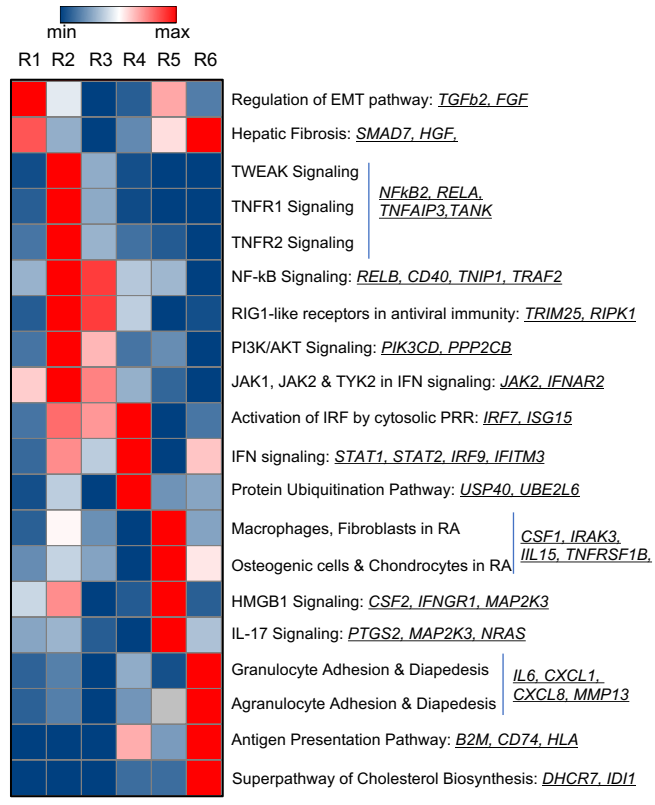
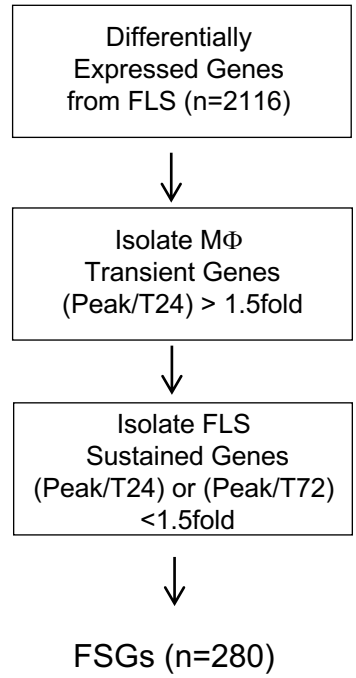


C

RNAseq Correlation

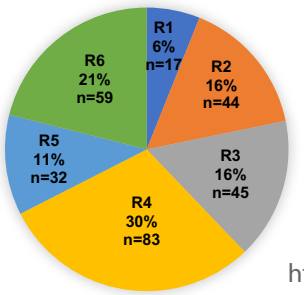
	FLS (n=2)	MΦ (n=3)
	r (spearman rank correlation)	
0h	0.98	0.89 - 0.97
1h	0.98	–
3h	0.97	0.89 - 0.97
6h	–	0.90 - 0.96
24h	0.97	0.90-0.97
72h	0.97	–

D



E

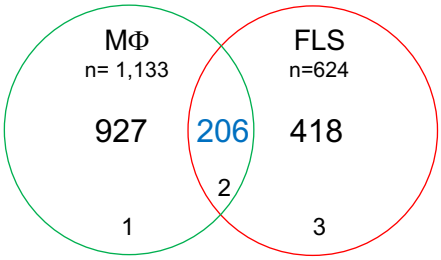
FSGs Distribution in RNA-seq Cluster (n=280)



Supplementary Figure S1

A

TNF 3h

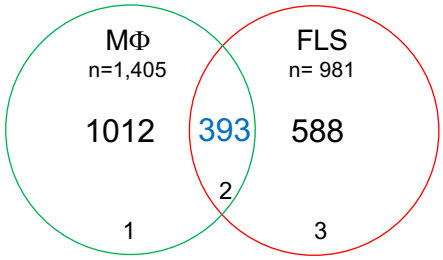


MΦ Canonical Pathway	-log(p-value)
STAT3 Pathway	5.77
Th1 and Th2 Activation Pathway	5.24
Role of Osteoblasts, Osteoclasts and Chondrocytes in RA	5.18
Role of Macrophages, Fibroblasts and Endothelial Cells in RA	5.01
IL-6 Signaling	4.97
IL-10 Signaling	4.39

MΦ & FLS Canonical Pathway	-log(p-value)
TNFR2 Signaling	18.6
Induction of Apoptosis by HIV1	17
Death Receptor Signaling	16.9
TWEAK Signaling	15.7
Role of PKR in Interferon Induction and Antiviral Response	14.7
4-1BB Signaling in T Lymphocytes	14.4

FLS Canonical Pathway	-log(p-value)
Role of IL-17F in Allergic Inflammatory Airway Diseases	9.53
Role of Pattern Recognition Receptors for Bacteria and Viruses	6.69
Interferon Signaling	5.54
Role of IL-17A in Arthritis	5.38
Hepatic Fibrosis / Hepatic Stellate Cell Activation	5.17
Regulation of the Epithelial-Mesenchymal Transition Pathway	4.89

TNF 24h



MΦ Canonical Pathway	-log(p-value)
Superpathway of Cholesterol Biosynthesis	8.51
Cholesterol Biosynthesis I	6.76
Cholesterol Biosynthesis II	6.76
Cholesterol Biosynthesis III	6.76
Molecular Mechanisms of Cancer	6.63
LXR/RXR Activation	5.39

MΦ & FLS Canonical Pathway	-log(p-value)
Agranulocyte Adhesion and Diapedesis	15.1
Granulocyte Adhesion and Diapedesis	14.8
Neuroinflammation Signaling Pathway	14.1
Activation of IRF by Cytosolic Pattern Recognition Receptors	14
TREM1 Signaling	12.7
Role of Macrophages, Fibroblasts and Endothelial Cells in RA	12.1

FLS Canonical Pathway	-log(p-value)
Interferon Signaling	7.86
Antigen Presentation Pathway	6.47
Cell Cycle Control of Chromosomal Replication	4.98
Protein Ubiquitination Pathway	4.18
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.09
Crosstalk between Dendritic cells and NK cells	4.08

Supplementary Figure S2

A

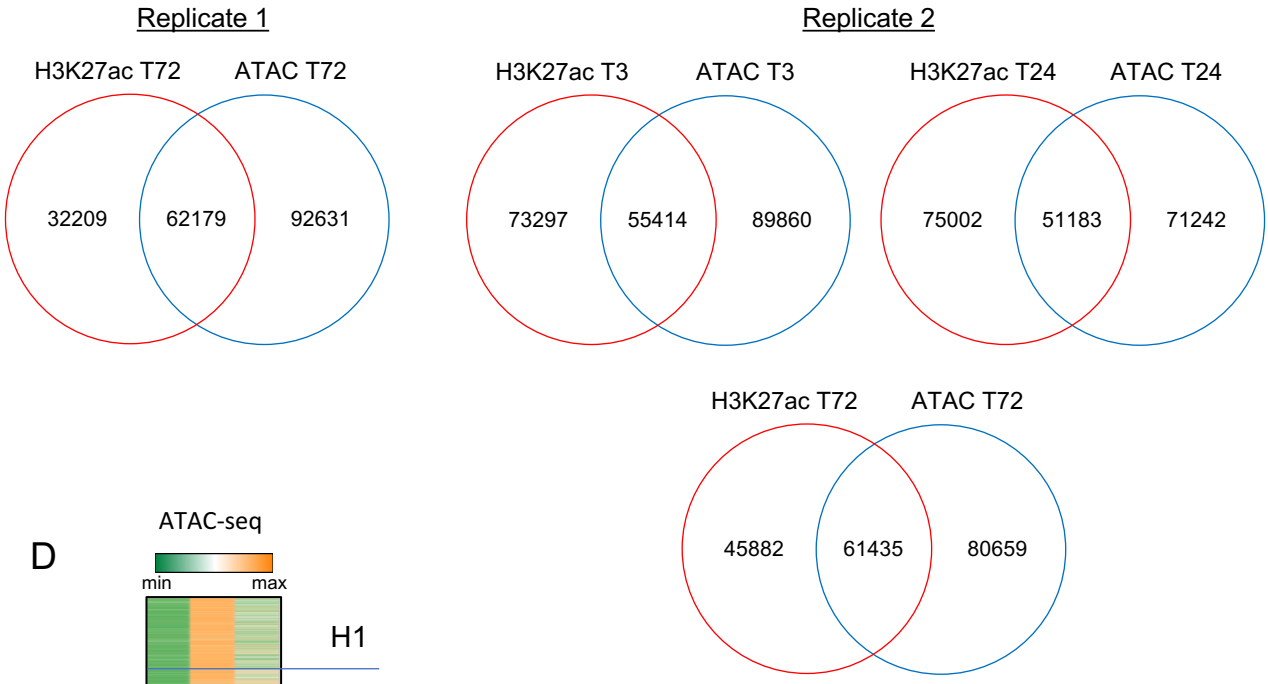
FLS H3K27ac

	Replicate 1	Replicate 2	r
T0	94261 peaks	132235 peaks	0.91
T3	–	133699 peaks	–
T24	–	130600 peaks	–
T72	96844 peaks	111555 peaks	0.94

FLS ATAC-seq

	Replicate 1	Replicate 2	r
T0	158771 peaks	130823 peaks	0.99
T3	–	160536 peaks	–
T72	169224 peaks	160251 peaks	0.99

B



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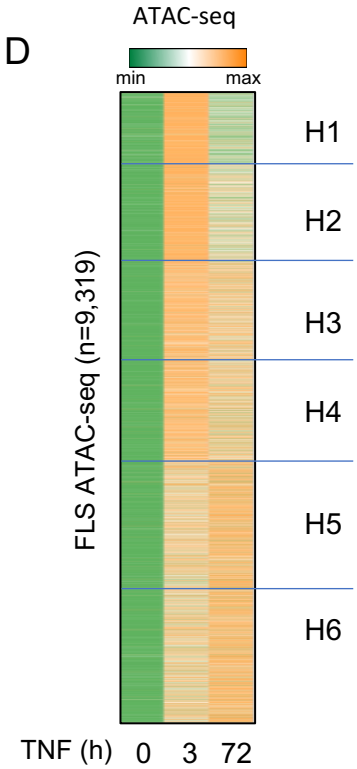
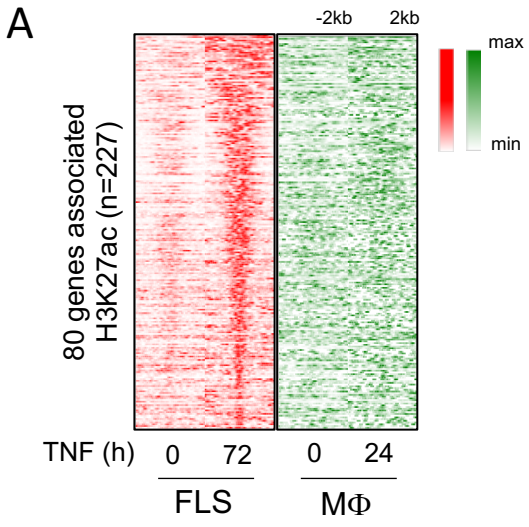


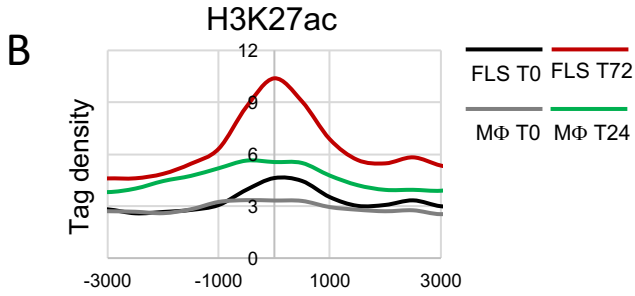
C

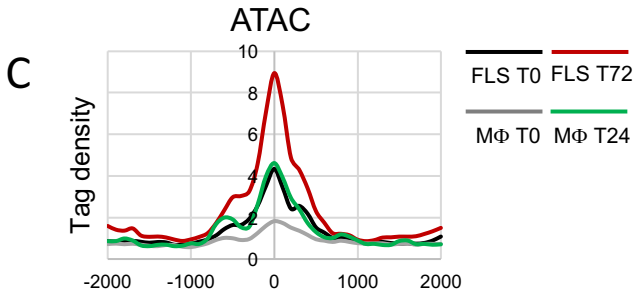
Motif Analysis (Resting FLS)

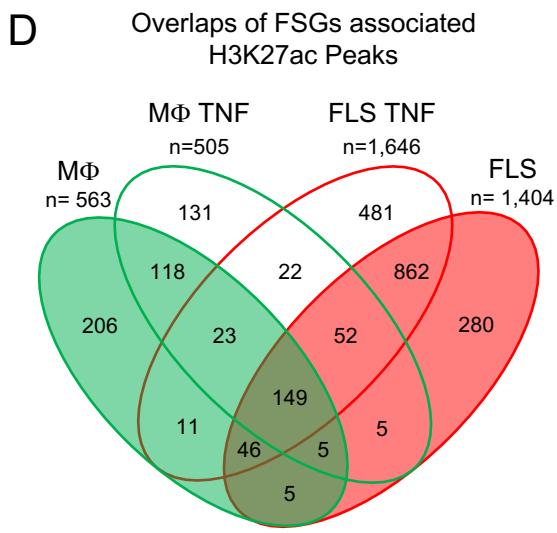
Motif	TF	P-value
	AP-1	1 x 10^{-5726}
	CTCF	1 x 10^{-1583}
	Sp1	1 x 10^{-928}
	MED-1	1 x 10^{-781}
	EBF1	1 x 10^{-562}
	CEBP	1 x 10^{-461}

Supplementary Figure S3

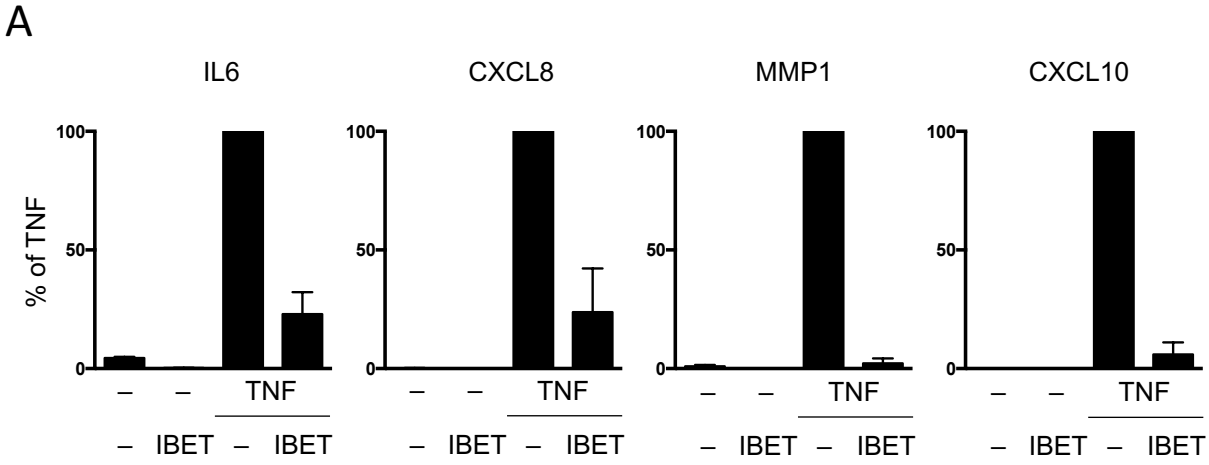


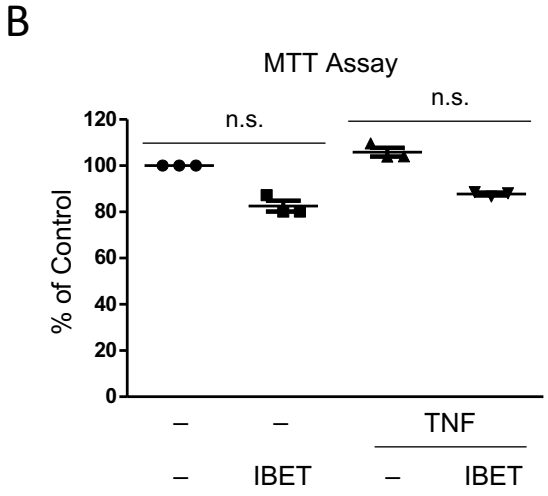






Supplementary Figure S4





Supplementary Figure S5