RESEARCH ARTICLE

REVISED Blimp-1 and c-Maf regulate *Il10 and* negatively regulate common and unique proinflammatory gene networks in IL-12 plus IL-27-driven T helper-1 cells [version 2; peer review: 2 approved]

Luke S. Cox[1*], Marisol Alvarez-Martinez[1*], Xuemei Wu[1], Leona Gabryšová[1], Raphaëlle Luisier[2], James Briscoe[3], Nicholas M. Luscombe[2,4], Anne O'Garra[1,5]

[1]Immunoregulation and Infection Laboratory, The Francis Crick Institute, London, England, NW1 1AT, UK
[2]Computational Biology Laboratory, The Francis Crick Institute, London, England, NW1 1AT, UK
[3]Developmental Dynamics Laboratory, The Francis Crick Institute, London, England, NW1 1AT, UK
[4]UCL Genetics Institute, Department of Genetics, Evolution and Environment, University College London, London, England, UK
[5]National Heart and Lung Institute, Imperial College London, London, England, UK

* Equal contributors

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Abstract

Background

CD4+ Th1 cells producing IFN-γ are required to eradicate intracellular pathogens, however if uncontrolled these cells can cause immunopathology. The cytokine IL-10 is produced by multiple immune cells including Th1 cells during infection and regulates the immune response to minimise collateral host damage. In this study we aimed to elucidate the transcriptional network of genes controlling the expression of *Il10* and proinflammatory cytokines, including *Ifng* in Th1 cells differentiated from mouse naive CD4+ T cells.

Methods

We applied computational analysis of gene regulation derived from temporal profiling of gene expression clusters obtained from bulk RNA sequencing (RNA-seq) of flow cytometry sorted naïve CD4+ T cells from mouse spleens differentiated *in vitro* into Th1 effector cells with IL-12 and IL-27 to produce *Ifng* and *Il10,* compared to IL-27 alone which express *Il10* only, or IL-12 alone which express *Ifng* and no *Il10,*

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1. **Leonie Taams**, King's College London, London, UK

2. **Christopher A Hunter**, University of Pennsylvania, Philadelphia, USA

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or medium control driven-CD4+ T cells which do not express effector cytokines. Data were integrated with analysis of active genomic regions from these T cells using an assay for transposase-accessible chromatin with sequencing (ATAC)-seq, integrated with literature derived-Chromatin-immunoprecipitation (ChIP)-seq data and the RNA-seq data, to elucidate the transcriptional network of genes controlling expression of *Il10* and pro-inflammatory effector genes in Th1 cells. The co-dominant role for the transcription factors, *Prdm1* (encoding Blimp-1) and *Maf* (encoding c-Maf) , in cytokine gene regulation in Th1 cells, was confirmed using T cells obtained from mice with T-cell specific deletion of these transcription factors.

Results

We show that the transcription factors Blimp-1 and c-Maf each have unique and common effects on cytokine gene regulation and not only co-operate to induce *Il10* gene expression in IL-12 plus IL-27 differentiated mouse Th1 cells, but additionally directly negatively regulate key proinflammatory cytokines including *Ifng*, thus providing mechanisms for reinforcement of regulated Th1 cell responses.

Conclusions

These data show that Blimp-1 and c-Maf positively and negatively regulate a network of both unique and common anti-inflammatory and pro-inflammatory genes to reinforce a Th1 response in mice that will eradicate pathogens with minimum immunopathology.

Keywords
CD4+ T cells, Th1 cells, IL-10, IFN-γ, Prdm1, Maf

This article is included in the The Francis Crick Institute gateway.

Corresponding author: Anne O'Garra (anne.ogarra@crick.ac.uk)

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REVISED **Amendments from Version 1**

Summary of major differences to the new submission in answer to Reviewers' comments:

Introduction has been modified to clarify the questions being addressed in our study, in context of the published literature. We have now rearranged and expanded our text to address this. The transcription factors that regulate the differentiation and function of Th1 cells producing *Ifng* are well established, including STAT molecules and T-bet, however, whether these transcription factors regulate *Il10* expression as part of the differentiation pathway of Th1 cells has been difficult to decipher (Gabrysova *et al.*, 2014, new reference number to be applied). Whether transcription factors such as Prdm1 or Maf, which positively induce *Il10*expression, do so as part of the differentiation pathway of Th1 cells, or alternatively, simultaneously negatively regulate proinflammatory cytokines in Th1 cells, therefore not contributing to Th1 differentiation but instead reinforcing a regulated Th1 response, is unclear (Gabrysova *et al.*, 2014; Gabrysova *et al.*, 2018, new reference numbers to be applied). At the end of the paragraph where we introduce the publication of Kuchroo *et al.*, 2020, (original reference 13) which showed that Blimp-1 and c-Maf regulation *Il10* expression in IL-27-differentiated "Tr1" cells, we have added another sentence. The role of *Prdm1* and *Maf* in regulating *Il10* and proinflammatory cytokines in IL-10-producing Th1 cells, however, has not been reported. In the Results, we have simplified Figure 2 and Figure 5, and clarified the text, pointing out that no Supplementary Figures can be included. In the Discussion, we have now indicated that the effects of transcription factors on regulation of *Il10* expression are undoubtedly context-specific and may vary according to the T effector cell response. Finally, in the Discussion, we have added a paragraph to reference the reported roles of Blimp-1 and c-Maf in regulation of *Il10* expression in human T cells.

Any further responses from the reviewers can be found at the end of the article

Introduction

CD4+ T helper 1 (Th1) cells are critical in controlling infection by production of the cytokine IFN-γ, which upregulates the expression of MHC-class II molecules on antigen-presenting cells (APC) thus enhancing their capacity to present antigen to activate CD4⁺ T cells, and also activates macrophages to kill intracellular pathogens[1]. However, uncontrolled Th1 responses can cause immunopathology[2]. IL-10 is a regulatory cytokine that has been widely shown to limit immunopathology particularly during infection and intestinal responses to pathobionts[3–6] and mutations in IL-10 or the IL-10R result in inflammatory bowel disease (IBD) in humans[7,8]. Most cells of the immune system can produce IL-10 to limit over-exuberant immune responses and pathology[5,6,9]. Th1 cells have been shown to be the critical source of IL-10 to limit responses to pathogens such as *Toxoplasma gondii*[10] and *Leishmania major*[11] and thus avoid immunopathology. The cytokine IL-27, which has been reported to regulate the immune response by multiple mechanisms[12–14], promotes IL-10 production by effector Th1 CD4⁺ T cells *in vivo* in response to the malaria parasites *Plasmodium chabaudi*[15] and *L. major*[16] infections, providing a critical mechanism for protection from severe immunopathology. The transcription factors that regulate the differentiation and function of Th1 cells producing *Ifng* are well established, including STAT molecules and T-bet, however, whether these transcription factors regulate *Il10* expression as part of the differentiation pathway of Th1 cells has been difficult to decipher[9]. Whether transcription factors such as *Prdm1* or *Maf,* which positively induce *Il10* expression, do so as part of the differentiation pathway of Th1 cells, or alternatively, simultaneously negatively regulate proinflammatory cytokines in Th1 cells, therefore not contributing to Th1 differentiation but instead reinforcing a regulated Th1 response, is unclear[9,17].

Several transcription factors have been shown to regulate IL-10 in T cells[13,17]. Both common and cell-specific transcriptional mechanisms are in place to tightly regulate the expression of *Il10* and proinflammatory gene expression in T cells to ensure a controlled immune response to pathogens and/or other insults[5,6,18–20]. Since transcription factors have multiple gene targets this raises the question as to whether known transcription factors that positively regulate *Il10* may simultaneously negatively regulate proinflammatory cytokine expression in T cells, thus driving a controlled response to control immune responses to pathogens and pathobionts to limit host damage. For example, the transcription factor c-Maf has been shown to induce *Il10* expression directly across multiple T cell subsets both *in vitro* and *in vivo*[5,6,13,18–20] whilst also acting as a negative regulator of *Il2*[17] and Th17 responses[21]. Blimp-1, encoded by the *Prdm1* gene, has also been shown to induce IL-10[13,19,20], although originally described as a global regulator of T cell homeostasis and differentiation *in vivo*[22–26].

Recently Kuchroo *et al.*[13], systematically identified regulators for *Il10* and highlighted *Prdm1* and *Maf* as two central nodes of the *Il10* regulatory circuits that cooperatively promoted IL-10 production in 'Tr1 cells' differentiated *in vitro* with IL-27 and through gain-of-function in Th1, Th2, Th17, and Treg cells[13]. IL-27-driven 'Tr1 cells' lacking both *Prdm1* and *Maf* (DKO) showed an almost complete loss of *Il10* expression. Moreover, expression of several transcription factors shown previously to be important for *Il10* expression, including *Fosl2, Hif1a, Hlx, and Notch1*[27], was found to be abrogated in IL-27-driven 'Tr1 cells' from CD4⁺ T cells deficient in both *Prdm1* and *Maf,* showing the latter to be upstream major controllers of *Il10* gene regulaton[13]. Moreover, DKO 'Tr1 cells' showed a unique reduction in chromatin accessibility in co-inhibitory receptor gene loci such as *Ctla4, Pdcd1* (PD-1), *Tigit, Havcr2* (Tim-3), suggesting that *Prdm1* and *Maf* have complementary but indispensable roles in regulating Tr1 at the transcriptional level and reinforcing the expression of negative immune regulators. The role of *Prdm1* and *Maf* in regulating *Il10* and proinflammatory cytokines in IL-10-producing Th1 cells, however, has as yet not been reported[9,17].

In this study we applied computational analysis of gene regulation derived from temporal profiling of gene expression clusters integrated with analysis of active genomic regions in CD4⁺ Th1 effector cells differentiated with IL-27 plus IL-12, which express *Il10* together with proinflammatory cytokines. Gene expression and active genomic region analysis data

was compared to T cells differentiated in IL-27 alone (named "Tr1 cells"[13]), which express *Il10* but no proinflammatory cytokines, or conversely IL-12 alone, which express proinflammatory cytokines but not *Il10,* all compared to medium control, which do not express cytokines. The aim was to elucidate the transcriptional network of genes controlling expression of *Il10* and pro-inflammatory effector genes in Th1 cells, and identify transcription factors that not only induced *Il10,* and additionally negatively regulated Th1 proinflammatory gene expression, and were therefore not part of the Th1 differentiation pathway. We show that the transcription factors Blimp-1 and c-Maf each have unique and common effects on cytokine gene regulation and not only co-operate to induce *Il10* gene expression in IL-12 plus IL-27 differentiated Th1 cells, but additionally directly negatively regulate key proinflammatory cytokines including *Ifng*, thus providing mechanisms for reinforcing the regulation of Th1 cell responses. Thus, Blimp-1 and c-Maf positively and negatively regulate a network of both unique and common anti-inflammatory and pro-inflammatory genes to reinforce a Th1 response that will allow eradication of pathogens with minimum immunopathology.

Methods

Animals

Mice were bred and maintained under specific pathogen free conditions in accordance with the Home Office UK Animals (Scientific Procedures) Act 1986. Age-matched male or female mice were used for experiments. *Maf*$^{fl/fl}$ mice were provided by M. Sieweke and C. Birchmeier (Max Delbrück Centre for Molecular Medicine, Germany)[28] and backcrossed to C57BL/6J for 10 generations and then crossed to *Cd4*^{Cre} mice to generate *Maf*$^{fl/fl}$ *Cd4*^{Cre} mice as described in 17. *Prdm1*$^{fl/fl}$ mice were purchased from the Jackson Laboratory (Stock Number 008100)[29], and further backcrossed to C57BL/6J for four generations and then crossed to *Cd4*^{Cre} mice to generate *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} mice. *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} and *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ control mice were generated in-house by crossing *Maf*$^{fl/fl}$ *Cd4*^{Cre} with *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} mice. All mouse breeding was performed under strict care and husbandry ensuring no discomfort to the mice. Breeding was carried out in accordance with UK Home Office regulations, under Project License, O'Garra *P5AF488B4,* 30 Apr 18 | Amended: 10 Jan 20 | Expired: 29 Apr 23, and were approved by The Francis Crick Institute Ethical Review Panel before each submission to the UK Home Office. This study adhered to the ARRIVE guidelines[30].

Naïve CD4$^+$ T cell sorting and *in vitro* helper T cell differentiation

For each experiment, spleens were obtained after humane killing of mice by either placing the animal into a secure chamber and filling it gradually with carbon dioxide until the animal was unconscious and until death was confirmed and then followed by cervical dislocation; or by cervical dislocation of the neck, depending on how many mice needed to be humanely killed. Spleens from a total of 5–10 age-matched mice per genotype were homogenized and incubated with unconjugated rat anti-mouse antibodies against B220 (RA3.6B2, DNAX), MHC class-II (M5/114, eBioscience) and CD8 (C291.2.43, DNAX). CD4$^+$ T cells were then negatively enriched using magnetic beads (BioMag, Qiagen). Live naïve CD4$^+$CD62L$^+$CD44^{lo}CD25$^-$ T cells were then sorted to over 95% purity using the following antibodies: CD4 (RM4-5, e450), CD8 (53-6.7, FITC), CD62L (MEL-14, PE-Cy7), CD44 (IM7, PE) and CD25 (PC61.5, APC) (all from eBioscience); and propidium iodide (final concentration 2µg/ml, Sigma) on either a MoFlo XDP or Influx flow cytometer (Beckman Coulter, Inc.). Sorted naïve CD4$^+$ T cells were then plated at 500,000 cells/well in flat-bottom 48-well plates and stimulated with plate-bound anti-CD3 (5µg/ml, 2C11, Harlan) and soluble anti-CD28 (2µg/ml, 37.51, Harlan) for up to 4 days in the presence of no polarizing cytokines for Medium control, IL-12 (rmIL-12p70, 5ng/ml, eBioscience), IL-12+IL-27 (rmIL-12p70, 5ng/ml, eBioscience; rmIL-27, 25ng/ml, R&D); or IL-27 (rmIL-27, 25ng/ml, R&D). All cells were cultured in conditioned RPMI (BE12-702F, Lonza) supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 100U/ml Pen-Strep, 2mM L-glutamine, 1mM Sodium pyruvate, 10mM HEPES (all Lonza) and 0.05mM 2-Mercaptoethanol (Sigma) in a humid incubator at 37°C with 5% carbon dioxide. For each experiment three wells were differentiated per condition to give technical triplicates from the pool of naïve CD4$^+$ T cells per genotype.

Quantitative RT-PCR

RNA was extracted from *in vitro* differentiated T-helper cells using the QIAShredder and RNeasy Mini Kit, or RNeasy Micro kit, both with on-column DNase digestion, according to the manufacturer's instructions (Qiagen). Eluted RNA was then reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) plus RNasin (Promega) according to the manufacturer's instructions, followed by RNaseH (Promega) treatment for 30 min at 37°C. High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to convert RNA into cDNA. Samples were incubated for: 10 min 25°C, 2 hr 37°C, 5 min 85°C in a thermal cycler (Vertiti Thermo Cycler, Applied Biosystems). Residual RNA was digested by RHase H incubation (final concentration 0.03U/µl, Invitrogen) for 30 min at 37°C. Reverse transcribed cDNA was then diluted to 5ng/µl using nuclease-free water (Ambion) and stored at -80°C. TaqMan™ Assay system (Applied Biosystems) was used for RT-qPCR-analysis, reaction mix/primer probes are summarised below. Reactions were carried out in 96-well plates (Applied Biosystems) on either a 7900HT or QuantStudio 3 RT-qPCR machine (Applied Biosystems). For each experiment, RT-qPCR was always performed to confirm the deletion of either *Maf, Prdm1* or both. All genes were analysed relative to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (encoded by the *Hprt*) gene. Delta Ct (ΔCt) was calculated by taking the difference between the Ct value of the gene of interest and the Ct for *Hprt* in a given sample, which was then inputted into the following equation (1.8^- ΔCt)*10^5 to give relative gene expression. For consistency, in the Applied Biosystems qPCR software the Ct threshold was manually set to 0.25 with automatic baseline threshold activated for all experiments and primer probes. cDNA was then analysed for the expression of specific genes on a 7900HT ABI, QS3 or QS5 real-time PCR system, using the TaqMan Universal Master

Mix II – no UNG and the following TaqMan mouse probes (all from Applied Biosystems): *Il10* (mm00439616_m1), *Ifng* (mm01168134_m1), *Tbx21* (mm00450960_m1), *Hprt* (mm03024075_m1). All expression levels were normalised to the internal housekeeping gene *Hprt* and calculated as $1.8^{-(Ct\ Hprt - Ct\ gene)}$ x10^5. Reactions are run on a Thermal Cycler, Veriti model (Applied Biosystems).

Statistical analysis

All figure legends show the number of independent biological experiments performed for each analysis and replicates. For PCR analysis, two-tailed unpaired t-test with 95% confidence interval was used for statistical analysis. All statistical analysis, apart from the sequencing data analysis was carried out with GraphPad Prism 8 (RRID:SCR_002798) software (GraphPad, USA) (*=p≤0.05; **=p≤ 0.01; ***=p≤ 0.001, ****=p≤0.0001). Analyses for sequencing data were performed with R Project for Statistical Computing (RRID:SCR_001905) version 3.6.1 and Bioconductor (RRID:SCR_006442) version 3.9 unless otherwise stated. Error bars and n values used are described in the figure legends.

RNA-seq of *in vitro* differentiated T-helper cells

RNA was extracted using the QIAShredder and RNeasy Mini Kit, or RNeasy Micro kit, both with on-column DNase digestion, according to the manufacturer's instructions (Qiagen). RNA-seq libraries were made with total RNA equally pooled from the technical triplicate wells within an independent biological experiment, using the Illumina Stranded TruSeq Library preparation kit V2 and unique multiplexing indexes, according to the manufacturer's instructions (Illumina). All libraries were then sequenced using the HiSeq 4000 system (Illumina) with paired-end read lengths of 100bp and at least 25 million reads per sample.

ATAC-seq of *in vitro* differentiated T-helper cells

ATAC-seq samples from *in vitro* differentiated T-helper cells were prepared as outlined in 31. For each sample, 50,000 cells were lysed in cold lysis buffer containing 10mM Tris-HCl, pH 7.4, 10mM NaCl, 3mM MgCl2, 0.1% Nonidet™ P40 substitute (all Sigma) and the nuclei incubated for 2 hours at 37°C with 50µl of TDE1/TD transposase reaction mix (Illumina). Tagmented DNA was then purified using the MinElute kit (Qiagen) and amplified under standard ATAC PCR conditions: 72°C for 5 min; 98°C for 30s and thermocycling at 98°C for 10s, 63°C for 30s and 72°C for 1 min for 12 cycles. Each 50µl PCR reaction consisted of: 10µl Tagmented DNA, 10µl water, 25µl NEBNext High-Fidelity 2x PCR Master Mix (NEB), 2.5µl Nextera XT V2 i5 primer and 2.5µl Nextera XT V2 i7 primer (Illumina). NexteraXT V2 primers (Illumina) were used to allow larger scale multiplexing, these sequences were ordered directly from Sigma (0.2 scale, cartridge) and diluted to 100µM with 10mM Tris-EDTA buffer, pH8 (Sigma) and then to 25µM with DEPC-treated water (Ambion) for use in the reaction. Following amplification, ATAC-seq libraries were cleaned up using 90µl of AMPure XP beads (Beckman Coulter) and two 80% Ethanol washes whilst being placed on a magnetic plate stand, before being eluted in 1mM (0.1x) Tris-EDTA buffer, pH8 (Sigma) diluted with DEPC-treated water (Ambion). ATAC-seq libraries were then checked on the TapeStation/BioAnalyser (Agilent) before being sequenced on the HiSeq 4000 system (Illumina), with paired-end read lengths of 50bp and at least 50–80 million uniquely mapped reads per sample.

RNA-seq: pre-processing and quality control (*in vitro* CD4+ T cell datasets)

Paired end RNA-seq reads were quality controlled and adapters were trimmed using skewer (RRID:SCR_001151) software version 0.2.2[32] with the following parameters: "-m pe -q 26 -Q 28 -e -l 30 -L 100", specifying the relevant adapter sequences. Reads were then aligned to mm10 genome and the GENCODE reference transcriptome version M22 using STAR (RRID:SCR_004463) software version 2.7.1[33], excluding multi-mapping reads by setting the parameter "outFilterMultimapNmax" to 1. In order to increase read mapping to novel junctions the parameter "twopassMode" was set to "Basic". Raw gene counts were retrieved using QoRTs software version 1.1.8[34], specifying the "stranded" parameter for the *in vitro* $CD4^+$ T cell datasets due to the nature of the library preparation. Normalized read counts were retrieved using DESeq2 (RRID:SCR_015687) version 1.24.0[35] and rlog transformed in order to visualize gene quantifications.

RNA-seq kinetics analysis of *in vitro* CD4+ T cells

Differentially expressed genes (DEG) at any given time point for IL-12, IL-12+IL-27, and IL-27 compared to medium control were obtained using DESeq2 (fold change >=1.5 and Benjamini-Hochberg adjusted p value<0.05), resulting in a total of 2,300 DEG. Next, the gene expression values of these DEG were subjected to *k*-means clustering using a *k*=9; where the optimal *k* was obtained using the R library "factoextra" (RRID:SCR_016692) (factoextra 2017). The expression values of the 2,300 DEG were standardized per gene (row z score) and plotted in a heatmap. The mean expression and 90% c.i. were obtained for these clusters and plotted.

Transcription factors correlating with *Il10* expression

A gene was considered to be coding for a transcription factor if it was present in at least two of the following references[36–38], or Ingenuity Pathway Analysis (RRID:SCR_008653) (IPA) database (genes annotated as either "transcription regulator" or "ligand-dependent nuclear receptor") (QIAGEN Redwood City, www.qiagen.com/ingenuity).

Genes encoding for transcription factors that have expression patterns correlating with *Il10* gene expression were analysed (absolute Pearson's correlation >0.7) from Days 1 to 4 in all conditions. A linear regression model was fitted for the top 9 transcription factors positively correlating with *Il10* expression.

Genome-wide differential footprint detection with BaGFoot software

To identify potential transcription factors underlying the gene expression changes occurring between Day 2 and Day 3, we applied the BaGFoot software on our ATAC-seq data[39], using

all ATAC-seq peaks identified in each treatment condition at Day 2 and Day 3. BaGFoot predicts these changes by searching for TF-binding motif matches in regions with altered ATAC-seq insertion patterns between the two days. We used 318 motifs of class A and B quality in the HOCOMOCO (RRID:SCR_005409) database v11[40]. BaGFoot does not consider replicates for the analysis, thus we performed two pair-wise comparisons (using all biological replicates) for each condition and calculated the average changes in accessibility and footprint-depth. Results are displayed as bagplots, using a fence of factor 2. We identified TFs with potentially altered binding between Day 2 and Day 3 by identifying the outliers of the multivariate distribution, as assessed by the Mahalanobis distance of each TF to the multivariate distribution[39]. The statistical significance of these distances was tested using a Chi-square distribution followed by a Benjamini-Hochberg correction for multiple-testing, as recommended by BaGFoot, and shown as tables.

Differential gene expression analysis of *in vitro* CD4$^+$ T cells: *Cd4*^{Cre}-mediated deletion of transcription factors versus floxed controls

The DEG of CD4$^+$ T cells with *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf*, or both *Prdm1* and *Maf* against their corresponding floxed controls were obtained for all conditions (Medium, IL-12, IL-12+IL-27, and IL-27) using DeSeq2 with the default thresholds (Benjamini-Hochberg adjusted p value<0.1), this resulted in 208, 561, and 802 for cells with *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf*, and both *Prdm1* and *Maf*, respective.

Singular Value Decomposition analysis and biological interpretation

Singular Value Decomposition (SVD) analysis was performed as in 17 on each set of samples that shared a genetic background: a) *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} was analysed with *Prdm1*$^{fl/fl}$, b) *Maf*$^{fl/fl}$ *Cd4*^{Cre} with *Maf*$^{fl/fl}$, and c) *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} with *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$. Prior to the SVD analysis, the rlog-normalized gene counts were "centered" by subtracting the mean expression per gene. The average values of the right-singular vectors, which relate the association of each sample to a component, were plotted as bar-plots.

For each right-singular vector three linear models were fitted: 1) a full linear model containing the variables for the differentiation condition and the genotype, 2) a reduced model containing only the differentiation condition, and 3) a reduced model containing only the genotype. In order to identify the association of each component with the condition and/or genotype the Akaike Information Criterion (AIC) score was calculated and an analysis of variance (ANOVA) with a Chi-squared-test was performed between the full model (1) versus the reduced ones (2 and 3). The component capturing the *Cd4*^{Cre}-mediated transcription factors deletion was chosen using the following criteria:

a) The component where the AIC of the reduced genotype model is lower than AIC of the full mode (line-plot)

b) The component with the smallest p value in the ANOVA (heatmap). Only statistically significant values (BH adjusted p value <0.05) were plotted for visualisation.

c) The component in which the average values of the right-singular vectors diverge in sign for *Cd4*^{Cre}-mediated transcription factors deletion *vs.* floxed controls shown as histograms.

The left-singular vectors, which relate the contribution of a gene to a component, were segregated between positive and negative values, and each set was subjected to *k*-means clustering (*k*=2). The genes belonging to the most positive and negative clusters were selected for further examination and they are referred in the text as the "SVD components associated with *Cd4*^{Cre}-mediated deletion".

For each genetic background the standardised expression values (row z score) of genes belonging to the component capturing the *Cd4*^{Cre}-mediated transcription factors deletion are shown in the heatmaps.

To further dissect the genes mostly affected by the *Cd4*^{Cre}-mediated transcription factors deletion a matrix containing the fold-changes of the three genetic backgrounds (*Prdm1*, *Maf*, and *Prdm1xMaf*) was created and subjected to *K* means clustering (k=7).

Data annotation

Gene Ontology (GO)[41,42] (RRID:SCR_002811) enrichment was assessed using the R package "topGO" (RRID:SCR_014798)[43]. The top 100 GO terms of "biological processes" were further synthesized using REViGO (RRID:SCR_005825)[44] with allowed similarity=0.4. The top 10 GO terms were shown for annotation.

ATAC-seq: pre-processing and quality control of *in vitro* CD4+ T cells

Paired end ATAC-seq reads were quality controlled and adapters were trimmed using Skewer software version 0.2.2[32] with the following parameters: "-m pe -q 26 -Q 30 -e -l 30 -L 50", specifying "CTGTCTCTTATACAC" as reference adapter sequence to remove. Quality controlled reads were then aligned to mm10 genome using BWA-MEM (RRID:SCR_010910)[45] with (Picard toolkit 2018 (RRID:SCR_006525)) and SAMtools (RRID:SCR_002105) 1.3.1[46] was used to discard discordant alignments and/or with low mapping qualities (mapQ<30). In order to account for transposase insertion, reads were shifted +4bp in the forward and -5bp in the reverse strand; moreover, read-pairs that spanned >99bp were excluded from further analyses as they would span nucleosomes[31].

Identification of open chromatin sites

MACS2 (version 2.1.1) (RRID:SCR_013291) was used to identify ATAC-seq peaks using the following parameters: "parameters --keep-dup all --nomodel --shift -100 --extsize 200; q-value < 0.01", in order to identify enrichment of Tn5 cutting sites[47].

Differentially accessible site detection *in vitro* CD4+ T cells

In order to identify open chromatin sites that differed in accessibility between *Cd4*^{Cre}-mediated transcription factor deletion and floxed controls, DiffBind (RRID:SCR_012918) software

version 2.0.2[48] was used with the following parameters: "dba.count:minOverlap=0, score= DBA_SCORE_RPKM, bRemoveDuplicates=FALSE, bUseSummarizeOverlaps= TRUE; dba.analyze: method=DBA_DESEQ2, bFullLibrarySize=T" for each condition and genetic background. An ATAC-seq peak was considered to represent remodelled chromatin if the absolute fold-change>1.5 and FDR<0.05.

Identification of c-Maf and Blimp-1 putative binding sites

c-Maf ChIP-seq raw fastq files were obtained from GSE40918[21] and Blimp-1 ChIP-seq raw fastq files were obtained from GSE79339[49] and GSE66069[50]. Trimmomatic version 0.36 was used for quality control and trim adapter sequences using the following parameters: "HEADCROP:2 TRAILING:25 MINLEN:26"[51]. Trimmed reads were aligned to mouse genome mm10 with Bowtie (RRID:SCR_005476) 1.1.2[52] with the parameters: "y -m2 --best --strata -S". MACS2 2.1.1 was used with default parameters to identify ChIP-seq peaks, and peaks with a q-value<0.01 were defined as statistically significant binding sites. For each transcription factor, a final peak set was generated from the union of the statistically significant binding sites identified in each biological replicate (c-Maf) or GEO dataset (Blimp-1). This resulted in 45,727 ChIP-seq binding sites for c-Maf and 16,893 binding sites for Blimp-1. CRUNCH suite[53] was used to infer the c-Maf motif, as the source dataset provided biological replicates and a suitable input control. On the other hand, the motif of Blimp-1 was taken from HOCOMOCO database v11[40] as two distinct ChIP-seq datasets were used, neither with biological replicates, thus not suitable for analysis with CRUNCH. These identified motifs were used as input for FIMO software[54] the sequences underlying the ATAC-seq peaks were scanned for motif-matches in order to identify further putative binding sites of c-Maf and Blimp-1.

Visualisation of genome browser tracks

"bamCoverage" from DeepTools (RRID:SCR_016366) 2.4.2 was used to normalize ATAC-seq data to RPKMs and the R package "ggbio" (RRID:SCR_003313) was used to visualize the genome browser tracks[55]. The CNS sites marked have the following coordinates (Table 1).

Integration of ATAC-seq, ChIP-seq, motifs, and RNA-seq from *in vitro* CD4+ T cells. ChIP-seq-identified binding sites of c-Maf and Blimp-1 were filtered using the ATAC-seq peaks in order to obtain those binding sites that were biologically relevant to our *in vitro* $CD4^+$ T cells datasets. At this stage, each ATAC-seq had assigned an overlapping ChIP-seq peak, a motif, or none. A gene was assigned to an ATAC-seq peak based on distance proximity, if the peak was within +/- 3kb of the gene body coordinates, using the R package "ChIPSeeker" (RRID:SCR_021322)[56]. Thus, a gene was a direct target of c-Maf and/or Blimp-1 if said gene was annotated with an ATAC-seq peak containing a putative binding site from either transcription

Table 1. Coordinates correspond to mm10 genome.

Gene	Chromosome	start	end	CNS	Citation
Il10	1	130999687	130999976	-20	19
Il10	1	131010529	131010907	-9	19
Il10	1	131015400	131015593	-4.5	19
Il10	1	131019438	131019696	-0.5	19
Il10	1	131025957	131026170	6.45	57
Prdm1	10	44444389	44444891	14	19
Prdm1	10	44459445	44459716	-1	19
Prdm1	10	44459873	44460126	-1.5	19
Prdm1	10	44460425	44460609	2	19
Ifng	10	118435228	118435850	-6	58
Ifng	10	118419035	118419610	-22	58
Ifng	10	118406839	118407520	-34	58
Ifng	10	118458481	118459017	18	58
Ifng	10	118460275	118460853	20	58
Ifng	10	118470550	118471067	29	58
Maf	8	115707132	115707487	-0.5	59
Maf	8	115707694	115708065	-1	59

factor (identified by motif or ChIP-seq). To quantitatively rank abundance of c-Maf or Blimp-1 binding sites in each gene, we applied the same approach as before in 17, additionally the likelihood of c-Maf or Blimp-1 to regulate a gene was calculated using the Binding and Expression Target Analysis (BETA) (RRID:SCR_005396) software, with the following parameters: "plus -g mm10 --da 0.5 --df 1 -c 1".

Direct targets of c-Maf and/or Blimp-1 are depicted in the gene regulatory networks. For all networks, *Maf* and *Prdm1* nodes were added, in order to visualize target genes of Blimp-1 and c-Maf. These gene regulatory networks show the integration of all these "omic" datasets and were generated using the "igraph" R package[60]. Each node represents a gene, and the size of a node represents the left-singular vectors obtained from the SVD analysis, thus relating the effect on expression the *Cd4*^{Cre}-mediated deletion of *Prdm1* and/or *Maf* had on a gene. The edges depict the relationship of a target gene with c-Maf and Blimp-1, the thickness of the edge shows the likelihood of a gene being a target of either c-Maf or Blimp-1 as assessed by the BETA software. The colouring of the edge shows if the target gene has a c-Maf (green), Blimp-1 (pink), or c-Maf and Blimp-1 (blue) binding site assigned. For visualization purposes, the size of the maximum left-singular vector was fixed to be equal to the 2nd highest; additionally, the labels of gene names were only added for the 50 most affected genes according to the SVD analysis. Scores used to generate the networks are available in Supplementary Table 7 as *Underlying data*[30].

Results

Expression of *Prdm1* and *Maf* correlates with *Il10* expression in Th1 cells

We previously showed that T cell-specific deletion of *Maf* resulted in the maximal reduction of IL-10 production by Th1 cells *in vivo* as compared to other T cell subsets[17]. However, since this effect was incomplete, we set out to identify additional transcription factors that positively regulate *Il10* in Th1 cells. To achieve this, we first analysed changes in temporal gene expression *in vitro* in naïve CD4$^+$ T cells stimulated with anti-CD3 and anti-CD28 as described in the Methods, and differentiated these over time with IL-27 plus IL-12 into CD4$^+$ Th1 effector cells which express *Il10* together with proinflammatory cytokines such as *Ifng,* and compared to T cells differentiated in IL-27 alone (named "Tr1 cells"[13]), which express *Il10* but no proinflammatory cytokines, or conversely IL-12 alone, which express proinflammatory cytokines including *Ifng* but not *Il10,* all compared to medium control, which do not express cytokines. The combination of IL-12 and IL-27 has been shown *in vivo* to be required for maximal levels of IFN-γ and IL-10 production by CD4$^+$ Th1 cells[12,15,16]. Cells were cultured under these different conditions for 4 days and sampled at each time point for RNA-based next-generation sequencing (RNA-seq). Cells clustered distinctly for the most part according to time point of differentiation (Figure 1a, Figure 1b), with the principal component 1 separating days 1 and 2 from days 3 and 4 (Figure 1b; Supplementary Table 1 in *Underlying data*[30]).

Co-regulated clusters of gene expression were revealed using k-means clustering (Figure 1c; Supplementary Table 2 in *Underlying data*[30]). Clusters 3, 2 and 6 each expressed effector T cell cytokines genes, including *Il10, Ifng and Il21*, respectively (Figure 1c). Expression of *Il10* (Cluster 3) was seen in IL-12+IL-27 or IL-27 driven T cells, peaking at days 3 and 4 of culture, although other genes within this Cluster 3 were also increased similarly in Th1 cells driven by IL-12 alone (Figure 1c and Figure 1d). By contrast, expression of *Ifng* within Cluster 2, was maximal in IL-12+IL-27 driven Th1 cells from days 2–4 of culture. *Ifng* showed delayed induction in IL-12 alone driven Th1 cells, and a small increase in IL-27 alone driven T cells by day 2, which then decreased with time to the levels seen in medium control cultures (Figure 1c and d). Expression of *Ifng* clustered with expression of the transcription factor *Jun* (Figure 1c). Collectively the expression of the genes in Cluster 6 was induced by IL-12+IL-27 and IL-27 alone and to a lesser extent in IL-12 driven Th1 cells and was maximal from days 1–4 of culture (Figure 1c and d). Expression of *Il21* was also observed within this Cluster 6 and was maximally induced in IL-12 + IL-27 and IL-27 alone driven T cells by day 1 to day 4 of culture, however IL-12 driven Th1 cells only started to express *Il21* by days 3 and 4 of culture as compared to medium controls (Figure 1c and d). Our findings suggest a role for IL-21 in autocrine expansion of T effectors rather than as a regulator of *Il10* as has been previously suggested[61,62], since it is produced maximally by Th1 cells, which do not produce IL-10.

Il10 expression clustered with the transcription factors *Nfatc2, Hif1a* and *Nfil3* (Cluster 3), while *Il21* expression clustered with the transcription factors *Prdm1, Maf* and *Batf* (Cluster 6), all of which were highly expressed upon culture with IL-12+IL-27 or IL-27 alone (Figure 1c). Regardless, the transcription factors showing the highest positive correlation with *Il10* expression were first *Prdm1*, then *Id2, Asb2, Hlx, Nfatc2* and *Maf* (Figure 1e), in keeping with the literature[13]. Of transcription factors previously reported to regulate *Il10* expression, only expression of *Prdm1* and *Maf,* was significantly increased under IL-12+IL-27 and IL-27 alone conditions only, reaching maximal levels when *Il10* expression was observed at days 3 and 4 and showing the strongest correlation with *Il10* expression, while their expression was not observed under IL-12 or medium alone conditions (Figure 2a). Importantly expression of *Batf*, previously suggested to regulate *Il10* in Th2 cells[63], which was not revealed as correlating with *Il10* expression in the analysis in Figure 1e, did not correlate with *Il10* expression under IL-12+IL-27 and IL-27 alone conditions, but rather was maximally expressed on days 1 and 2, rapidly diminishing by days 3 and 4, while it's expression increased with time in IL-12 alone differentiated Th1 cells which did not express Il10 (Figure 2b), therefore suggesting a broader function than the regulation of *Il10* (Figure 2b). Other transcription factors that have been associated with *Il10* gene expression including *Hif1a* and *Nfil3*[13,17], which although expressed under IL-12+IL-27 and IL-27 alone conditions, showed less of a correlation with *Il10* expression, and were found to increase with time in Th1 cells differentiated with IL-12 only

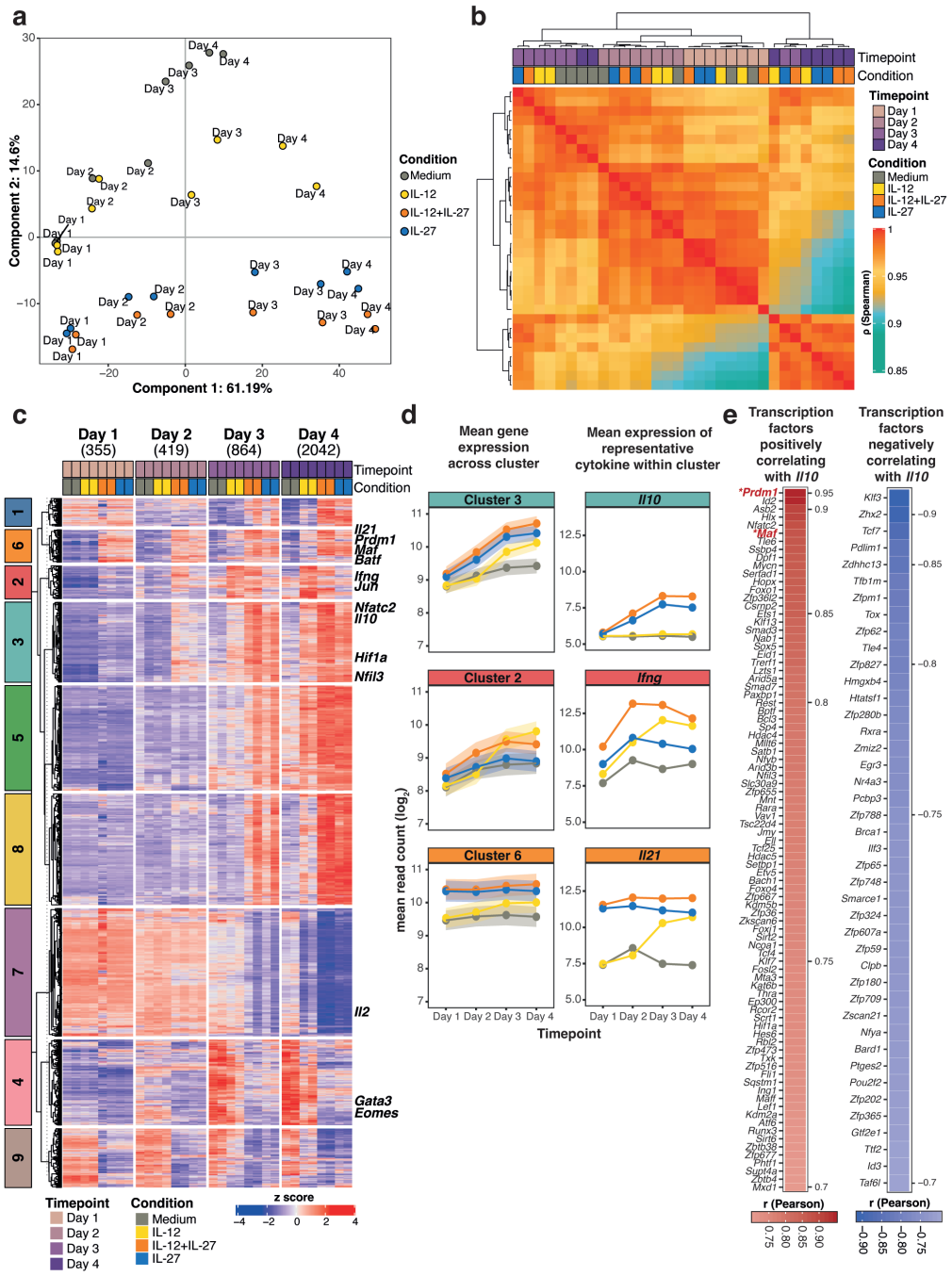


Figure 1. Temporal gene regulation by IL-27 in CD4⁺ T cells is accompanied by clusters of candidate transcription factors that correlate with *Il10* expression, including Blimp-1 and c-Maf. RNA-seq analysis of flow cytometry sorted naïve CD4⁺ T cells activated *in vitro* with anti-CD3 and anti-CD28 antibodies and differentiated in the presence of Medium (no cytokines), IL-12, IL-12+IL-27, or IL-27 from Day 1 to Day 4. **a**, Principal component analysis (PCA) showing the two most dominant variables, time point (PC1: Day 1-2 versus Day 3-4) and differentiation condition (PC2: presence versus absence of IL-27 during differentiation). **b**, Unsupervised hierarchical clustering of a pair-wise Spearman correlation of samples encompassing CD4⁺ T cells differentiated *in vitro* in the presence of Medium, IL-12, IL-12+IL-27, or IL-27 from Day 1 to Day 4. **c**, Heatmap visualization of differentially expressed genes per condition compared to Medium (fold change >=1.5 and BH adjusted p value<0.05), partitioned into 9 clusters using *k*-means clustering. The values above the heatmap between parentheses show the number of differentially expressed genes at each time point. **d**, Gene expression profiles depicting the mean gene expression ±90% confidence intervals (c.i.) across all genes for clusters 3, 2 and 6 accompanied by mean gene expression for representative cytokines *Il10*, *Ifng* and *Il21* respectively. **e**, All transcription factors annotated in the mouse genome positively correlating (Pearson's r>0.7) and negatively correlating (Pearson's r<0.7) with *Il10* expression in *in vitro* CD4⁺ T cells differentiated in the presence of Medium, IL-12, IL-12+IL-27, or IL-27 from Day 1 to Day 4. Data from n=2 biological replicates.

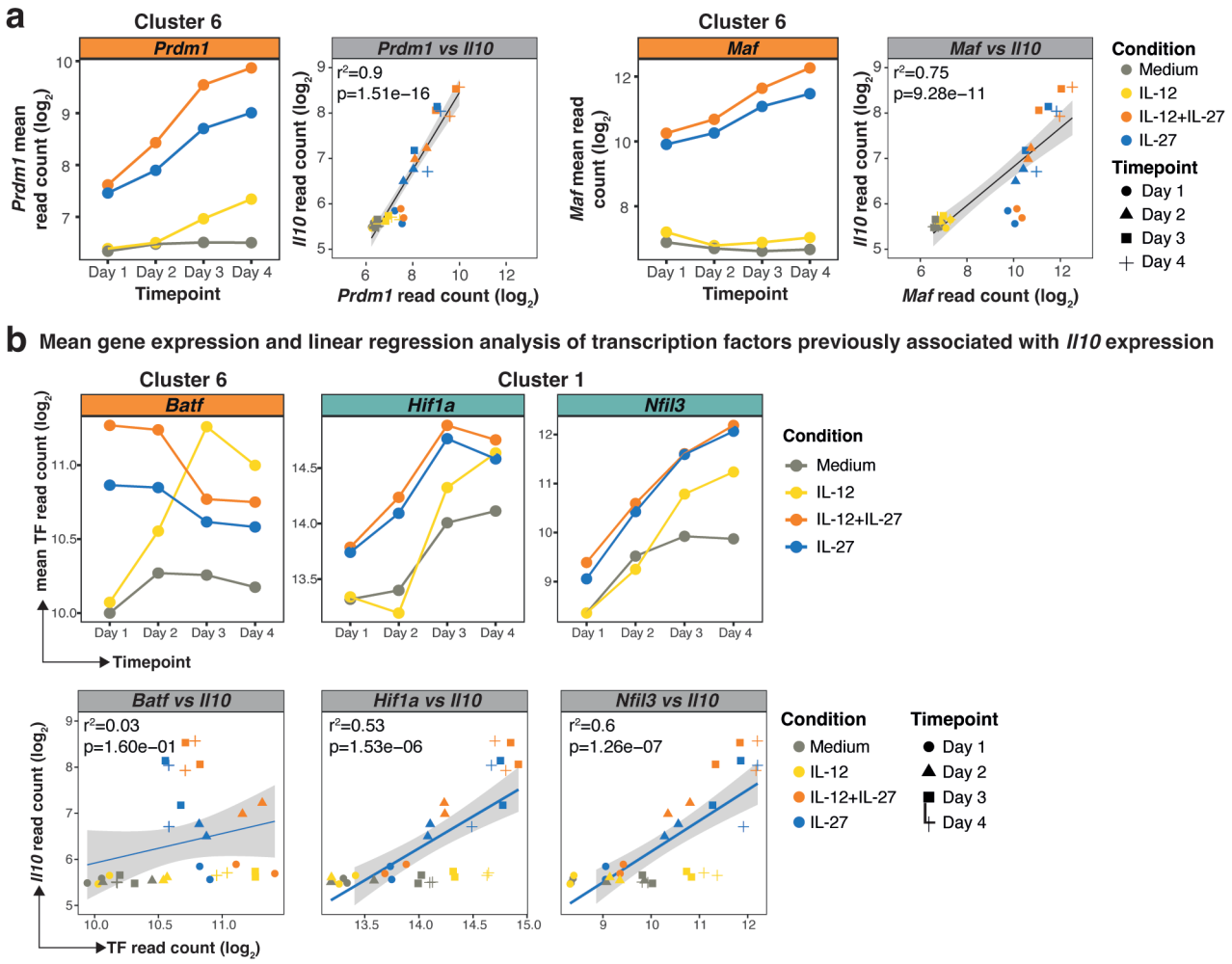
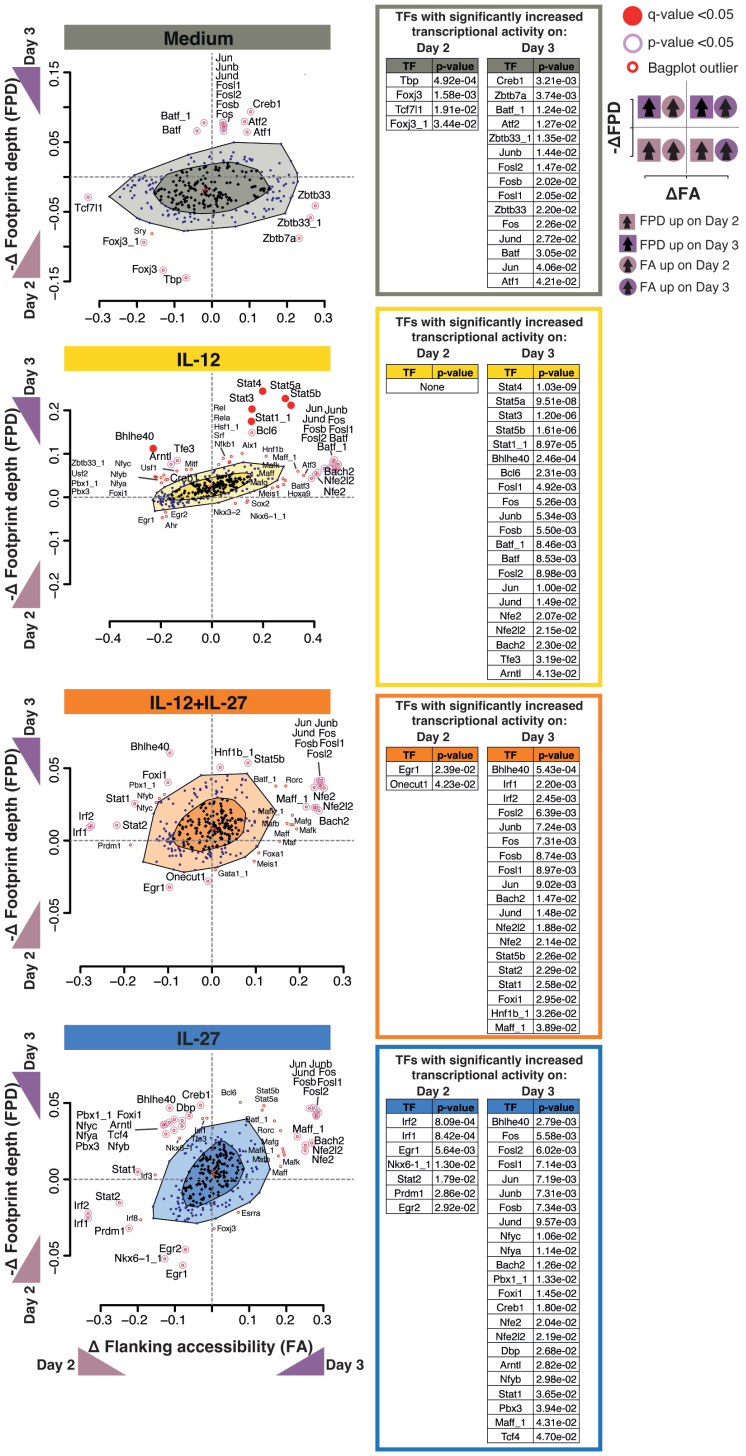


Figure 2. Blimp-1 and c-Maf strongly positively correlate with IL-27 induced *Il10* expression in differentiating naïve CD4⁺ T cells. RNA-seq analysis of flow cytometry sorted naïve CD4⁺ T cells activated *in vitro* with anti-CD3 and anti-CD28 antibodies and differentiated in the presence of Medium (no cytokines), IL-12, IL-12+IL-27, or IL-27 from Day 1 to Day 4. **a**, Mean gene expression of *Prdm1* and *Maf,* both transcription factors positively correlating with *Il10* expression, accompanied by linear regression of *Prdm1* and *Maf* against *Il10* expression across all conditions and timepoints. **b**, Mean gene expression profiles (top panel) of transcription factors previously associated with *Il10* expression. Linear regression (bottom panel) of these transcription factors against *Il10* expression across all conditions and timepoints.

which do not express *Il10*, therefore again suggesting a broader role for these transcription factors in Th1 differentiation (Figure 2b).

To further investigate global changes in transcriptional activity in CD4⁺ naïve T cells cultured as above, we used the assay for transposase-accessible chromatin plus sequencing (ATAC-seq) to reveal functionally active genomic regions at days 2 and 3, timepoints, which marked key transcriptional changes during the differentiation of CD4⁺ naïve T cells into Th1 cells (cultured as in Figure 1a). The 'bivariate genomic footprinting' (BaGFoot) software[39] was applied to the ATAC-seq data, to detect global changes in transcription factor binding activity (genome-wide) occurring between day 2 and day 3 under the different conditions. Differences in binding activity are assessed by BaGFoot software by quantifying the differences in Tn5 transposition within a transcription factor motif, by measuring the 'footprint depth' (Figure 3, y axis) and 'flanking accessibility' (Figure 3, x axis) and comparing these metrics between timepoints. A transcription factor bound to chromatin has a high footprint depth and a high flanking accessibility.

Only IL-27 and IL-12+IL-27 cultured T cells showed increased transcriptional activity for *Prdm1* (higher activity at day 2 vs day 3) and *Maf* (higher activity at day 3 vs day 2), reinforcing a role for these transcription factors in regulating transcriptional programs in IL-12+IL-27 and IL-27 cultured T cells. On the other hand, the AP-1 family members, *e.g.* Jun and Fos, showed increased transcriptional activity at day 3 across all cytokine differentiation conditions including medium control (Figure 3) suggesting a broader role in T helper cell activation/differentiation. *Batf* transcriptional activity was only evident



Medium			
Day 2		Day 3	
TF	p-value	TF	p-value
Tbp	4.92e-04	Creb1	3.21e-03
Foxj3	1.58e-03	Zbtb7a	3.74e-03
Tcf7l1	1.91e-02	Batf_1	1.24e-02
Foxj3_1	3.44e-02	Atf2	1.27e-02
		Zbtb33_1	1.35e-02
		Junb	1.44e-02
		Fosl2	1.47e-02
		Fosb	2.02e-02
		Fosl1	2.05e-02
		Zbtb33	2.20e-02
		Fos	2.26e-02
		Jund	2.72e-02
		Batf	3.05e-02
		Jun	4.06e-02
		Atf1	4.21e-02

IL-12			
Day 2		Day 3	
TF	p-value	TF	p-value
None		Stat4	1.03e-09
		Stat5a	9.51e-08
		Stat3	1.20e-06
		Stat5b	1.61e-06
		Stat1_1	8.97e-05
		Bhlhe40	2.46e-04
		Bcl6	2.31e-03
		Fosl1	4.92e-03
		Fos	5.26e-03
		Junb	5.34e-03
		Fosb	5.50e-03
		Batf_1	8.46e-03
		Batf	8.53e-03
		Fosl2	8.98e-03
		Jun	1.00e-02
		Jund	1.49e-02
		Nfe2	2.07e-02
		Nfe2l2	2.15e-02
		Bach2	2.30e-02
		Tfe3	3.19e-02
		Arntl	4.13e-02

IL-12+IL-27			
Day 2		Day 3	
TF	p-value	TF	p-value
Egr1	2.39e-02	Bhlhe40	5.43e-04
Onecut1	4.23e-02	Irf1	2.20e-03
		Irf2	2.45e-03
		Fosl2	6.39e-03
		Junb	7.24e-03
		Fos	7.31e-03
		Fosb	8.74e-03
		Fosl1	8.97e-03
		Jun	9.02e-03
		Bach2	1.47e-02
		Jund	1.48e-02
		Nfe2l2	1.88e-02
		Nfe2	2.14e-02
		Stat5b	2.26e-02
		Stat2	2.29e-02
		Stat1	2.58e-02
		Foxi1	2.95e-02
		Hnf1b_1	3.26e-02
		Maff_1	3.89e-02

IL-27			
Day 2		Day 3	
TF	p-value	TF	p-value
Irf2	8.09e-04	Bhlhe40	2.79e-03
Irf1	8.42e-04	Fos	5.58e-03
Egr1	5.64e-03	Fosl2	6.02e-03
Nkx6-1_1	1.30e-02	Fosl1	7.14e-03
Stat2	1.79e-02	Jun	7.19e-03
Prdm1	2.86e-02	Junb	7.31e-03
Egr2	2.92e-02	Fosb	7.34e-03
		Jund	9.57e-03
		Nfyc	1.06e-02
		Nfya	1.14e-02
		Bach2	1.26e-02
		Pbx1_1	1.33e-02
		Foxi1	1.45e-02
		Creb1	1.80e-02
		Nfe2	2.04e-02
		Nfe2l2	2.19e-02
		Dbp	2.68e-02
		Arntl	2.82e-02
		Nfyb	2.98e-02
		Stat1	3.65e-02
		Pbx3	3.94e-02
		Maff_1	4.31e-02
		Tcf4	4.70e-02

Figure 3. Blimp-1 and c-Maf have increased differential binding between Day 2 and Day 3 of culture only under IL-12+IL+27 and IL-27 cytokine driving conditions. BaGFoot analysis of transcription factors with putative genome-wide changes in chromatin binding between Day 2 and Day 3, as assessed by Tn5 insertion patterns (obtained with ATAC-seq) in *in vitro* activated and differentiated CD4⁺ T cells in the presence of Medium (no cytokines), IL-12, IL-12+IL-27, or IL-27. Presented as the change in change in the 'flanking accessibility' of motifs (ΔFA) plotted against the 'footprint depth' (–ΔFPD); wedges along axes indicate direction and degree of change in transcription factor binding between Day 2 and Day3. Dark shading in the "bagplots" indicates a region with no change in transcription factor binding patterns, and light shading indicates a region in which most non-significant minor changes in binding occur. Transcription factors with significant change in binding are found as outliers outside the "bagplot". A table of P values is provided for the outliers of the "bagplot" and indicates the statistical confidence assigned to the differential binding between Day 2 and Day 3 of a transcription factor in each CD4⁺ T cell differentiation condition.

in cells cultured in medium alone or IL-12; and Stat 3, 4 and 5 transcriptional activity was only detected under IL-12 conditions, which were not accompanied by *Il10* gene expression, again implicating these transcription factors in broader roles in Th1 cell activation/differentiation. Detection of STAT activity and a transcriptome more similar to T cells cultured in medium or in IL-12 alone at days 1 and 2 (Figure 1a), suggests that Th1 cells cultured with IL-12, which do not express *Il10* were temporally and qualitatively different with respect to global transcriptional activity, to Th1 cells cultured with IL-12+IL-27 and IL-27 alone, where both conditions lead to *Il10* expression, although proinflammatory cytokine expression was only observed in IL-12+IL-27 driven Th1 cells.

Reduction or abrogation of *Il10* expression in IL-12+IL-27 and IL-27-driven CD4+ T cells upon deletion of *Prdm1, Maf* or both *Prdm1* and *Maf,* is accompanied by increased *Ifng* expression

Since expression of *Il10* in IL-12+IL-27 and IL-27-driven CD4$^+$ T cells appeared to correlate strongly with expression of *Prdm1* as well as *Maf* over time, while *Ifng* expression appeared to be reduced at peak times under these conditions, we wished to determine the requirement of *Prdm1* and/or *Maf* in the regulation of both cytokines. To address this, naïve CD4$^+$ T cells from *Prdm1*$^{fl/fl}$ *Cd4*^{Cre}, *Maf*$^{fl/fl}$ *Cd4*^{Cre}, *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} and respective floxed control mice, were differentiated into Th1 cells, with IL-12 or IL-12+IL-27, and IL-27 alone or medium controls (as in Figure 1) and expression of *Il10, Ifng* and *Tbx21* was first assessed by RT-PCR (Figure 4). Th1 cells differentiated with IL-12+IL-27 and IL-27-driven T cells showed significant levels of *Il10* expression, which was diminished in the absence of *Prdm1* or *Maf* (Figure 4a–c). Effects on T cells differentiated with IL-27 alone are in keeping with findings of Zhang *et al.*, in 'Tr1 cells'[13]. In addition to the effects that we observed on *Il10* expression in the IL-12+IL-27-driven Th1 cells, the absence of *Prdm1, Maf* or both transcription factors, resulted in an increase in *Ifng* expression, while the expression of the Th1/IFN-specific transcription factor *Tbx21*[64] was not significantly affected (Figure 4a–c).

Deciphering the transcriptional programs regulated by Blimp-1 and c-Maf in CD4$^+$ T cells

To determine the role of Blimp-1 and c-Maf on the regulation of cytokine gene networks in an unbiased fashion, naïve CD4$^+$ T cells from *Prdm1*$^{fl/fl}$ *Cd4*^{Cre}, *Maf*$^{fl/fl}$ *Cd4*^{Cre}, *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} and respective floxed control mice, were differentiated with IL-12 or IL-12+IL-27, and IL-27 or medium controls as described for Figure 1 and Figure 4. Cells were sampled at day 3 and processed for RNA-seq, which was then subjected to bioinformatics analyses (Figure 5; Supplementary Table 4 in *Underlying data*[30]). Hierarchical clustering of a Pearson's correlation analyses in each genotype revealed that the greatest variations in gene expression were cytokine driven, while differences resulting from transcription factor deletion were difficult to discern (Figure 5a–c). Variance captured by singular-value-decomposition (SVD) components (Figure 5d-f) allowed clustering of differential gene expression according to the cytokine-driven conditions and additionally the effects of transcription factor deletion, supported by biological pathway analysis (Figure 5 and Figure 6; Supplementary Table 5 in *Underlying data*[30]). The variance explained by the SVD components capturing the *Cd4*^{Cre}-mediated transcription factors deletion were: 1.77% for the *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} (Component 7); 1.62% for the *Maf*$^{fl/fl}$ *Cd4*^{Cre} (Component 6); and 3.45% for the *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} (Component 5) (Figure 5d–f). It is of note, that while deletion of *Prdm1* and *Maf* in CD4$^+$ T cells demonstrated the effects of Blimp-1 and c-Maf in regulating *Il10* and negatively regulating common and unique proinflammatory gene networks in IL-12+IL-27-driven Th1 cells and to a lesser extent in IL-27 driven "Tr1" cells, these effects were less pronounced than gene expression changes resulting from TCR-stimulation and culture in the cytokines, IL-12, IL-12+IL-27, or IL-27 (Figure 5d–f, Component 1, which constitute 58-69% of the response) and (Figure 5d–f, Component 2 or Component 3, which constitute 14–16% or 4–8% of the response, respectively).

Heatmap values of fold-changes for genes within the combined SVD components associated with *Cd4*^{Cre}-mediated transcription factor deletion in cells differentiated with IL-12+IL-27 or IL-27 were subjected to *k*-means clustering to identify clusters of genes that were most affected by the different transcription factors in an unbiased fashion (Figure 5g and j) Supplementary Table 6 in *Underlying data*[30]). The average fold-changes for each cluster showed *k*-means Cluster 2 to be the most decreased, and Cluster 1 the most increased in *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} and *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre}, but less so in *Maf*$^{fl/fl}$ *Cd4*^{Cre} T cells, as compared to floxed controls, when differentiated with IL-12+IL-27 (Figure 5g-i). Cluster 2 contained the genes most decreased in expression including *Il10, Lars2 and Id2* (Figure 5h). Conversely, Cluster 1, contained a large number of proinflammatory effector genes that were the most increased in expression including, *Il3, Penk, Eomes, Il23r, Cd200, Ifng, Il2* and *Csf2* (Figure 5i). When differentiated with IL-27 alone the average fold-changes in the T cells for each cluster showed *k*-means Cluster 5 to be the most decreased, and Cluster 1 the most increased, in all transcription factor deficient T cells including *Prdm1*$^{fl/fl}$ *Cd4*^{Cre}, *Maf*$^{fl/fl}$ *Cd4*^{Cre} and *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre}, as compared to floxed controls (Figure 5j-l). Cluster 5, containing the genes most decreased in expression included *Il10, Timp1, Pdpn, Rorc* and *Hlx* (Figure 5k). Conversely, Cluster 1, containing proinflammatory effector genes that were the most increased in expression included, *Eomes, Ifng, Il2, Csf2 and Penk* (Figure 5l), suggesting that in addition to upregulating *Il10,* Blimp-1 *(Prdm1)* and c-Maf may directly repress proinflammatory genes to reinforce a suppressive response.

GO enrichment analysis was also applied to each SVD component associated with *Cd4*^{Cre}-mediated deletion of either *Prdm1*, *Maf,* or both *Prdm1* and *Maf* (Figure 6a–f). Contributing genes were partitioned into "positively associated" (black outline) or "negatively associated" (orange outline) with the SVD component and their expression values visualized in a heatmap (Figure 6a, 6c and 6e) and annotated using the biological processes within the GO database (Figure 6b, 6d and 6f). An increase in expression of *Ifng* and Th1-associated and T cell activation-associated pathways was observed across all

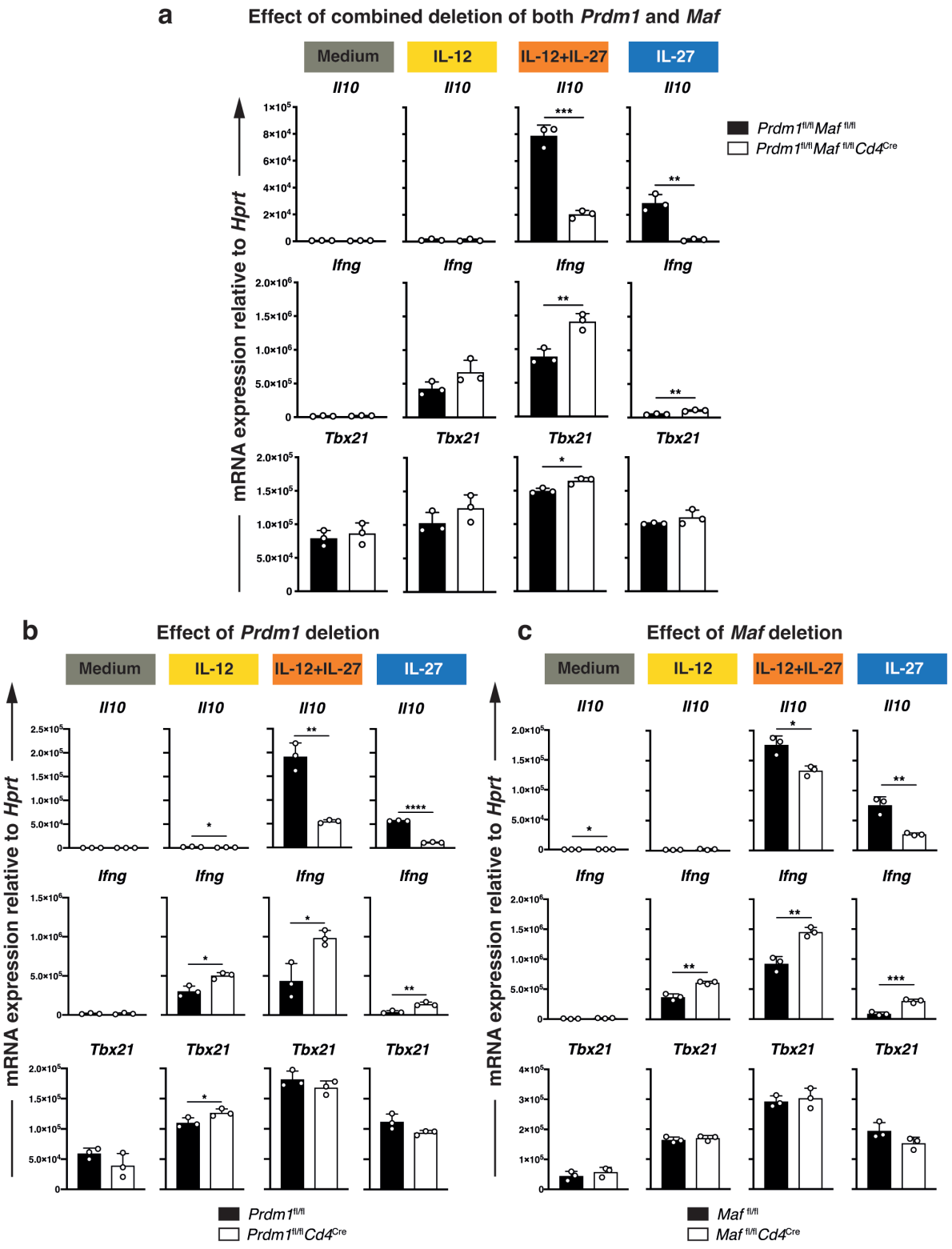


Figure 4. Blimp-1 and c-Maf promote the expression *Il10*, whilst repressing *Ifng* in CD4⁺ T cells without affecting differentiation capacity in presence of IL-12, IL-12+IL-27, or IL-27. Naïve CD4⁺ T cells activated *in vitro* with anti-CD3 and anti-CD28 antibodies and differentiated in the presence of Medium (no cytokines), IL-12, IL-12+IL-27, or IL-27 to Day 3. **a**–**c**, Real-time quantitative PCR analysis of *Il10, Ifng* and *Tbx21* expression upon *Cd4*^{Cre}-mediated deletion of **a**, both *Prdm1* and *Maf*, and either **b**, *Prdm1* or **c**, *Maf* alone. Graphs show mean of technical triplicates ±SD. Data representative of n=3 independent biological experiments.

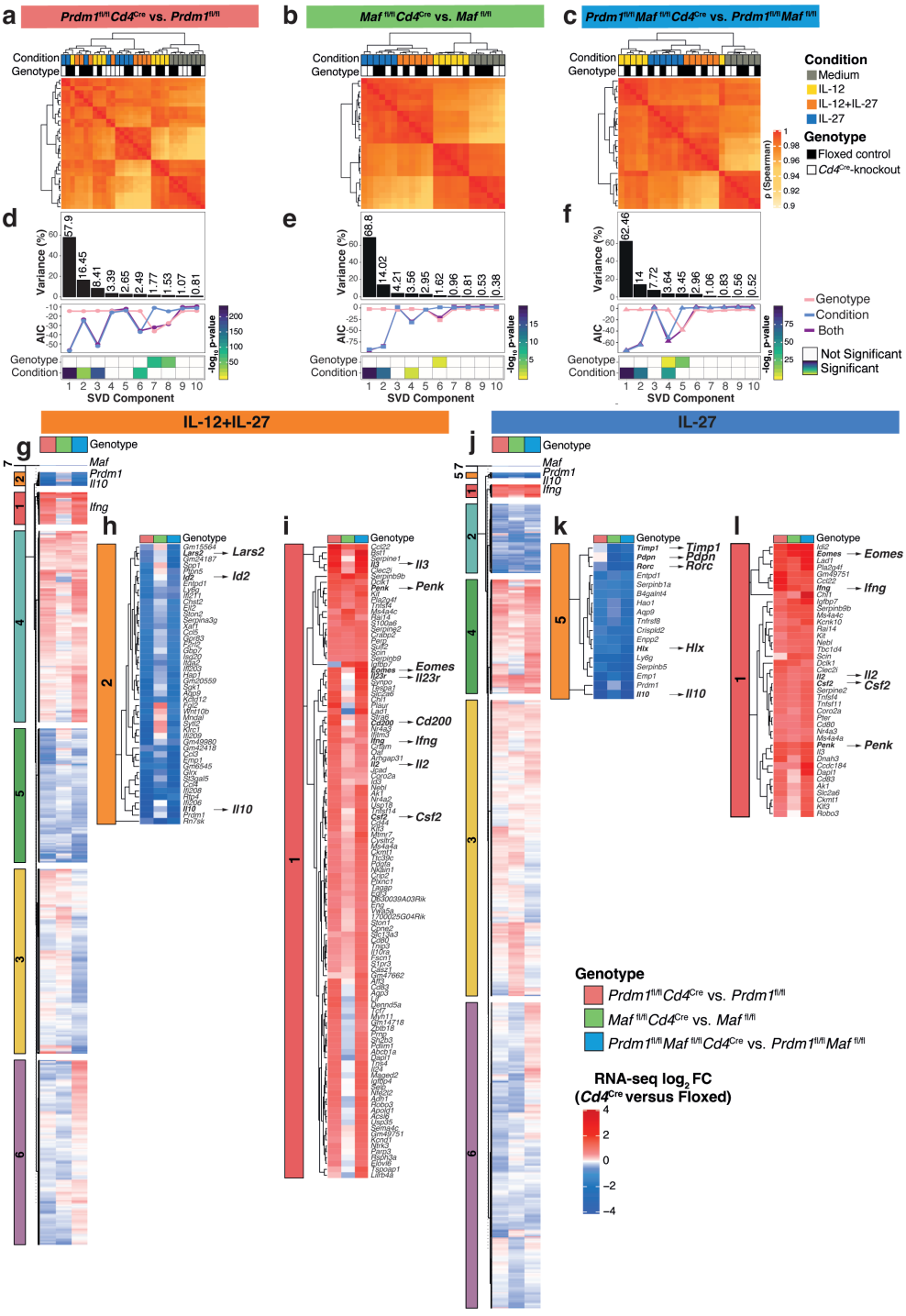


Figure 5. Deciphering the transcriptional programs regulated by Blimp-1 and c-Maf in CD4⁺ T cells. RNA-seq analysis of CD4+ T cells differentiated *in vitro* with Medium, IL-12, IL-12+IL-27, or IL-27 on Day 3 with *Cd4*^{Cre}-mediated transcription factor deletion. **a**–**c**, Clustering of Spearman correlation showing the effect of deletion of **a**, *Prdm1*, **b**, *Maf*, or **c**, both *Prdm1* and *Maf* on gene expression across all conditions. **d**–**f**, Singular value decomposition (SVD) analysis identifying changes in gene expression upon deletion of **d**, *Prdm1*, **e**, *Maf*, or **f**, both *Prdm1* and *Maf*. Histograms depict percentage of variance explained for the first ten SVD-components. To statistically determine the variables associated with each component, the Akaike information criterion (AIC) score (line-plot) and ANOVA p values (X^2 test; heatmap) were calculated. **g** and **j** Heatmap of fold-changes for genes within the SVD components associated with *Cd4*^{Cre}-mediated transcription factor deletion in cells differentiated with **g**, IL-12+IL-27 and j, IL-27 alone. Fold-change values were subjected to *k*-means clustering to unbiasedly identify genes most affected. **h**-**i** and **k**-**l**. Heatmap of genes most affected by *Cd4*^{Cre}-mediated transcription factor deletion in **h**-**i** IL-12+IL-27 and **k**-**l**, IL-27 alone differentiated cells (extracted from heatmaps in **g** and **j**). Data from n=3–4 biological replicates.

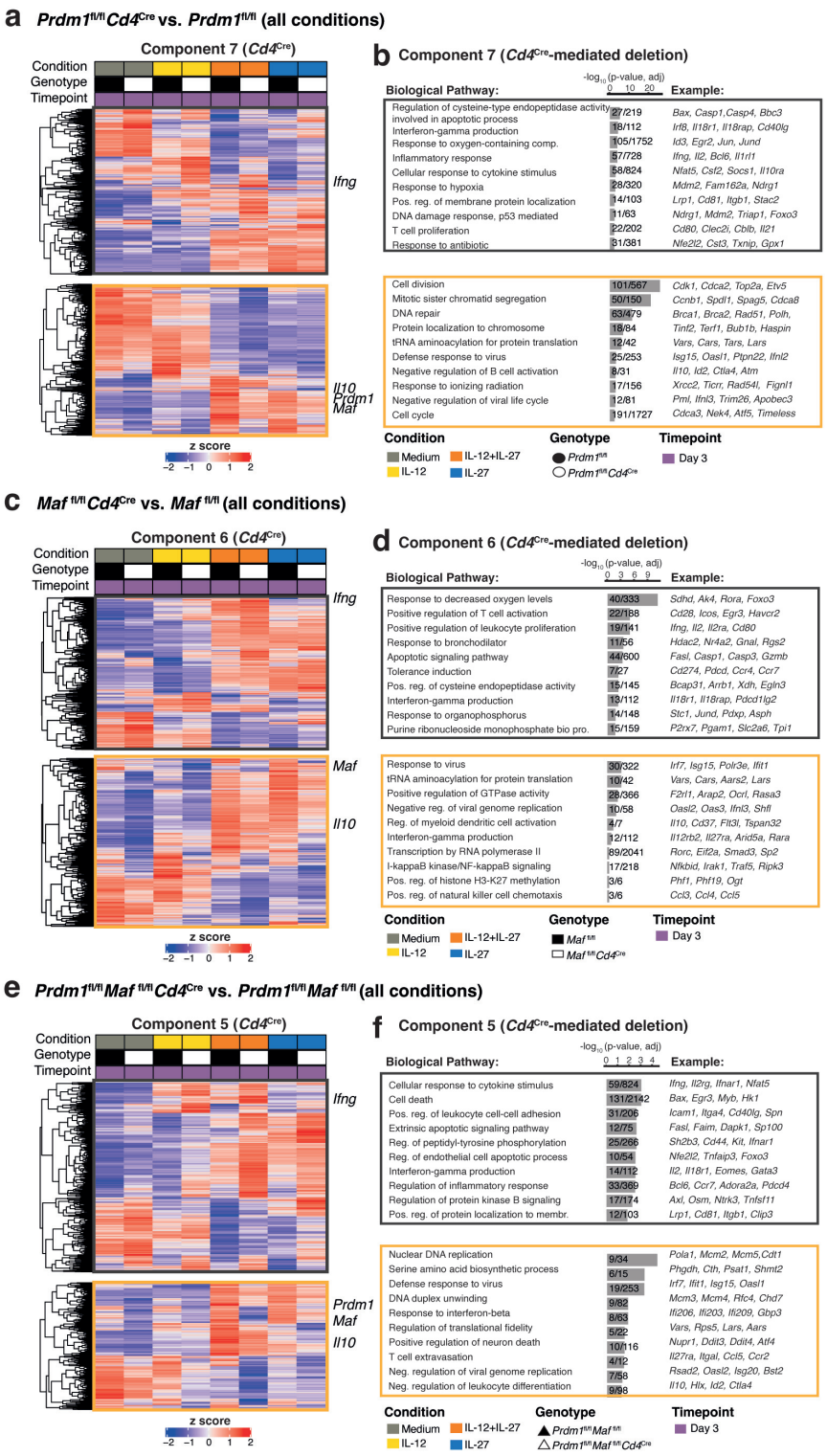


Figure 6. Deciphering the transcriptional programs regulated by Blimp-1 and c-Maf in *in vitro* differentiated CD4+ T cells from dominant IL-27 and IL-12 driven transcriptional changes. a, **c**, **e**, For each SVD component associated with *Cd4*^{Cre}-mediated deletion of either **a**, *Prdm1*, **c**, *Maf,* or **e**, both *Prdm1* and *Maf* contributing genes were partitioned into "positively associated" (black outline) or "negatively associated" (orange outline) with the SVD component and their expression values visualized in a heatmap. **b**, **d**, **f**, Gene ontology biological pathways enriched within the SVD component associated with *Cd4*^{Cre}-mediated deletion of either **b**, *Prdm1*, **d**, *Maf,* or **f**, both *Prdm1* and *Maf.* Data from n=3–4 biological replicates.

cytokine-driven conditions even in the absence of detectable *Il10* expression, such as in Th1 cells driven by IL-12 alone, and was the most pronounced in *Prdm1*$^{fl/fl}$ *Cd4*^{Cre} and *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} but less so in *Maf*$^{fl/fl}$ *Cd4*^{Cre} T cells, as compared to floxed controls (Figure 6a–f; black outline). Conversely, accompanying the decrease in *Il10* expression in IL-12+IL-27 and/or IL-27-driven cultures, decreased expression of *Lars2, Id2, Hlx, Timp1, Pdpn* and *Rorc*, was observed in *Prdm1*$^{fl/fl}$ *Cd4*^{Cre}, *Maf*$^{fl/fl}$ *Cd4*^{Cre} and *Prdm1*$^{fl/fl}$ *Maf*$^{fl/fl}$ *Cd4*^{Cre} T cells, as compared to floxed controls, but *Lars2* and *Id2* were reduced to a lesser degree in *Maf*$^{fl/fl}$ *Cd4*^{Cre} T cells in IL-12+IL-27-driven cultures (Figure 5g–h); Supplementary Tables 5 and 6[30]), suggesting that Blimp-1 and c-Maf regulate common and distinct genes/pathways to enforce a regulated immune response. These data support the alternative *k*-means clustering analysis approach (Figure 5g–l) described above.

Prdm1 and *Maf* have complementary roles in regulating *Il10* and *Ifng* expression in IL-12, IL-12+IL-27 and IL-27-driven T cells

To identify the molecular mechanisms whereby Blimp-1 and c-Maf affected gene regulation in *in vitro* differentiated CD4$^+$ T cells from the different CD4-specific transcription factor deleted mice, we used the assay for transposase-accessible chromatin plus sequencing (ATAC-seq) to reveal changes in functionally active genomic regions across each cytokine-driven condition. Consistent with the RNA-seq profile (Figure 5a–c), ATAC-seq revealed that the cytokines added during culture was the most dominant variable shaping the open chromatin landscape of *in vitro* differentiated CD4$^+$ T cells (Figure 7a–c). Specifically, the principal component 1 (explaining 42–56% of the variance) segregated *in vitro* differentiated CD4$^+$ T cells in the presence of IL-12 or medium from those differentiated in the presence of IL-12+IL-27 or IL-27. Moreover, major chromatin remodelling occurring between Day 2 and Day 3 was observed in all *in vitro* differentiated CD4$^+$ T cells in the presence of cytokines. However, no major changes in accessibility were observed upon *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf,* or both *Prdm1* and *Maf* (Figure 7a–f). Together these results highlight a role for IL-27 (including IL-27 plus IL-12) in influencing the open chromatin landscape of *in vitro* differentiated CD4$^+$ T cells and suggests that c-Maf and Blimp-1 do not drive chromatin remodelling in order to perform their gene regulation functions, as has been

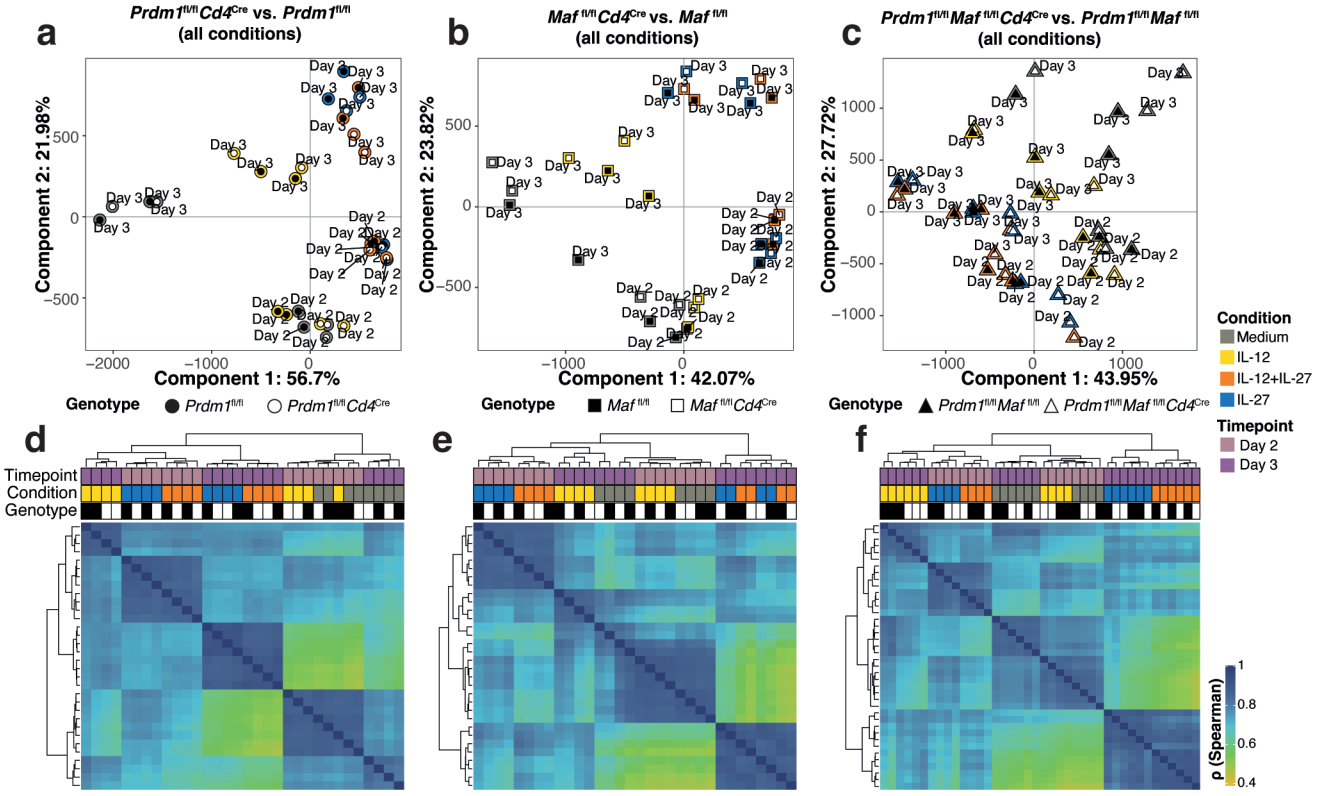


Figure 7. ATAC-seq reveals chromatin remodelling occurring between Day 2 and Day 3 in *in vitro* differentiated CD4$^+$ T cells in the presence of IL-27, but no major changes in accessibility upon *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf,* or both *Prdm1* and *Maf*. ATAC-seq analysis of CD4$^+$ T cells differentiated *in vitro* in the presence of Medium, IL-12, IL-12+IL-27, or IL-27 on Day 2 and Day 3 with *Cd4*^{Cre}-mediated deletion of either *Prdm1*, *Maf*, or both *Prdm1* and *Maf,* and corresponding floxed controls. **a**–**c**, PCA plots showing PC1, explaining cell differentiation in the presence of IL-27, versus PC2, explaining transition from Day 2 to Day 3. **d**–**f**, Unsupervised hierarchical clustering of a pair-wise Spearman correlation of read coverages underlying ATAC-seq peaks called in *in vitro* differentiated CD4+ T cells in the presence of Medium, IL-12, IL-12+IL-27, or IL-27 from Day 2 to Day 3.

shown for c-Maf[21]. It would appear from our findings that cytokines are the main drivers of changes in chromatin remodelling while changes in chromatin remodelling are not observed in the absence of Prdm1, Maf or both transcription factors (Figure 7a-f). This indicates that Blimp-1 and c-Maf do not themselves induce chromatin remodelling in these differentiating Th1 cells, as previously reported for c-Maf in Th17 cells *in vitro*[21] and as we have reported for c-Maf in *ex-vivo* T cells from infection models in other contexts[17].

Analysis of public c-Maf and Blimp-1 ChIP-seq datasets against our ATAC-seq data from T cells differentiated with IL-27+IL-12, IL-27 or IL-12, as compared to medium control, confirmed binding of Blimp-1 and c-Maf at accessible chromatin regions within the *Il10* locus (Figure 8a). These findings further support the hypothesis that Blimp-1 and c-Maf may be critical regulators of IL-10 in multiple settings including Th1 cells differentiated with IL-12+IL-27 in addition to in "Tr1" cells differentiated with IL-27 as reported[13]. We additionally show here that both transcription factors also bind accessible chromatin within the *Ifng* locus (Figure 8b). Thus, binding of both Blimp-1 and c-Maf to the *Il10* and *Ifng* loci indicate that Blimp-1 and c-Maf are direct positive regulators of *Il10,* and direct negative regulators of *Ifng* (Figure 8a and b). Blimp-1 and c-Maf showed binding to the *Il10* and *Ifng* locus at distinct sites, which suggests complementary action to induce *Il10* while reducing *Ifng* expression (Figure 8a and b). Distinct c-Maf and Blimp-1 *Ifng* binding sites were also observed by ATAC-seq analysis and these increased in T cells differentiated with IL-12 and IL-12+IL-27 and to a lesser extent in IL-27-driven T cells (Figure 8b). These accessible binding sites were not affected by T -cell specific depletion of Prdm-1 and Maf, or both transcription factors, as compared to controls.

Gene regulatory networks derived from multiomic data integration highlight shared and unique targets of Blimp-1 and c-Maf

We further integrated our RNA-seq data and ATAC-seq data with data obtained by analysis of public Blimp-1 and c-Maf ChIP-seq datasets[21,49] and public motif data to identify genes that were targets of Blimp-1 and c-Maf (as depicted in Figure 9 schematic). These results were confirmed by BETA software, which integrates ChIP-seq analyses and gene-expression data to identify target genes (Figure 9). Gene regulatory networks derived from the multiomic data integration highlighted unique and shared targets between Blimp-1 and c-Maf affected by *Cd4*^{Cre}-mediated deletion of *Prdm1, Maf* or both *Prdm1* and *Maf* (Figure 10 and Figure 11). Specific clusters from the RNA expression data (Figure 5g and 5j) were chosen for our depiction as gene regulatory networks in Figure 10 and Figure 11, because these clusters contained direct targets of Blimp-1 and/or c-Maf that were the most affected at the RNA expression level upon Cd4Cre-mediated deletion of Prdm1, Maf, or both Prdm1 and Maf eg. for IL-12+IL-27 found in Clusters 2,1,7 in Figure 5g; and for IL-27 alone found in Clusters 5, 7, 1 in Figure 5j.

Direct targets of Blimp-1 and/or c-Maf that were the most affected upon *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf*, or both *Prdm1* and *Maf* with IL-12+IL-27 were found in Clusters 2,1,7 from Figure 5g and for IL-27 alone in Clusters 5, 7, 1 from Figure 5j, and are depicted as gene regulatory networks in Figure 10a, 10c and 10e and Figure 10b, 10d and 10f respectively. In the networks shown in Figure 10a, 10c and 10e (IL-12+IL-27) and in Figure 10b, 10d and 10e (IL-27 alone), the nodes correspond to genes affected upon *Cd4*^{Cre}-mediated deletion of the transcription factors, with the node size reflecting the contribution of a gene to the SVD component associated with the deletion of the transcription factors; the node colouring represents the fold change of the *Cd4*^{Cre}-mediated knockouts compared to the floxed control gene expression (red up, blue down-regulated). The edge colour depicts if Blimp-1 (pink), c-Maf (green), or both Blimp-1 and c-Maf (blue) have binding sites assigned to the target genes; whilst the thickness of the edge shows the likelihood of c-Maf and/or Blimp-1 regulating a gene according to the BETA software. Data incorporates the RNA-seq and ATAC-seq biological replicates as outlined in the Figure 9 schematic.

In IL-12+IL-27-differentiated Th1 cells, shared targets included *Il10, Id2, Ccl3, Ccl4, Ccl5,* which were reduced upon *Cd4*^{Cre}-mediated deletion of *Prdm1*, both *Prdm1* and *Maf* and to a lesser extent *Maf,* as compared to floxed controls (Figure 10a, 10c and 10e; Supplementary Table 7[30]). Both *Prdm1* and *Maf* were shown to be direct targets of each other and were reduced in the absence of the reciprocal transcription factor (Figure 10a, 10c and 10e; Supplementary Table 7[30]). Blimp-1 and/or c-Maf targets of genes which were upregulated in Th1 cells differentiated with IL-12+IL-27 upon *Cd4*^{Cre}-mediated deletion of both *Prdm1* and *Maf,* but to a lesser extent upon *Prdm1*, and to a far lesser degree upon *Maf* deletion, as compared to floxed controls, included *Ifng, Il2, Il3, Tcf7, Tnfsf4, Cd80, Cd83, Eomes, Serpine1/2, Penk, Cd200, Il23r* (Figure 10a, 10c and 10e; Supplementary Table 7[30]). Under these differentiation conditions unique direct targets of c-Maf included *Il3* and *Stra6 (*encoding the Vitamin A Receptor) and of Blimp-1 included *Penk,* and these genes were the most upregulated in the Th1 cells upon *Cd4*^{Cre}-mediated deletion of either *Prdm1* or both *Prdm1* and *Maf,* and to a lesser extent upon *Maf* deletion, as compared to floxed controls (Figure 10a, 10c and 10e; Supplementary Table 7[30]). In T cells differentiated with IL-27 alone, shared targets again included *Il10,* but also *Timp1, Pdpn, Rorc,* and these were reduced upon *Cd4*^{Cre}-mediated deletion of both *Prdm1* and *Maf* and either *Prdm1* or *Maf* alone, as compared to floxed controls (Figure 10b, 10d and 10f). Shared targets of Blimp-1 and c-Maf which were upregulated in IL-27 differentiated T cells upon *Cd4*^{Cre}-mediated deletion of either *Prdm1* or both *Prdm1* and *Maf,* and to a much lesser extent in *Maf,* as compared to floxed controls included *Il2, Ifng, Tnfsf4* and *Eomes* (Figure 10b, 10d and 10f; Supplementary Table 7[30]). Unique targets of Blimp-1 were revealed in IL-27 differentiated T cells and included *Hlx,* which was reduced and *Penk* which was increased upon *Cd4*^{Cre}-mediated deletion of *Prdm1,* as compared to floxed controls (Figure 10b, 10d and 10f; Supplementary Table 7[30]). Unique targets of c-Maf included *Il3* which was increased upon *Cd4*^{Cre}-mediated deletion of both *Prdm1* and *Maf* and either *Prdm1* or *Maf* alone (Figure 10b, 10d and 10f; Supplementary

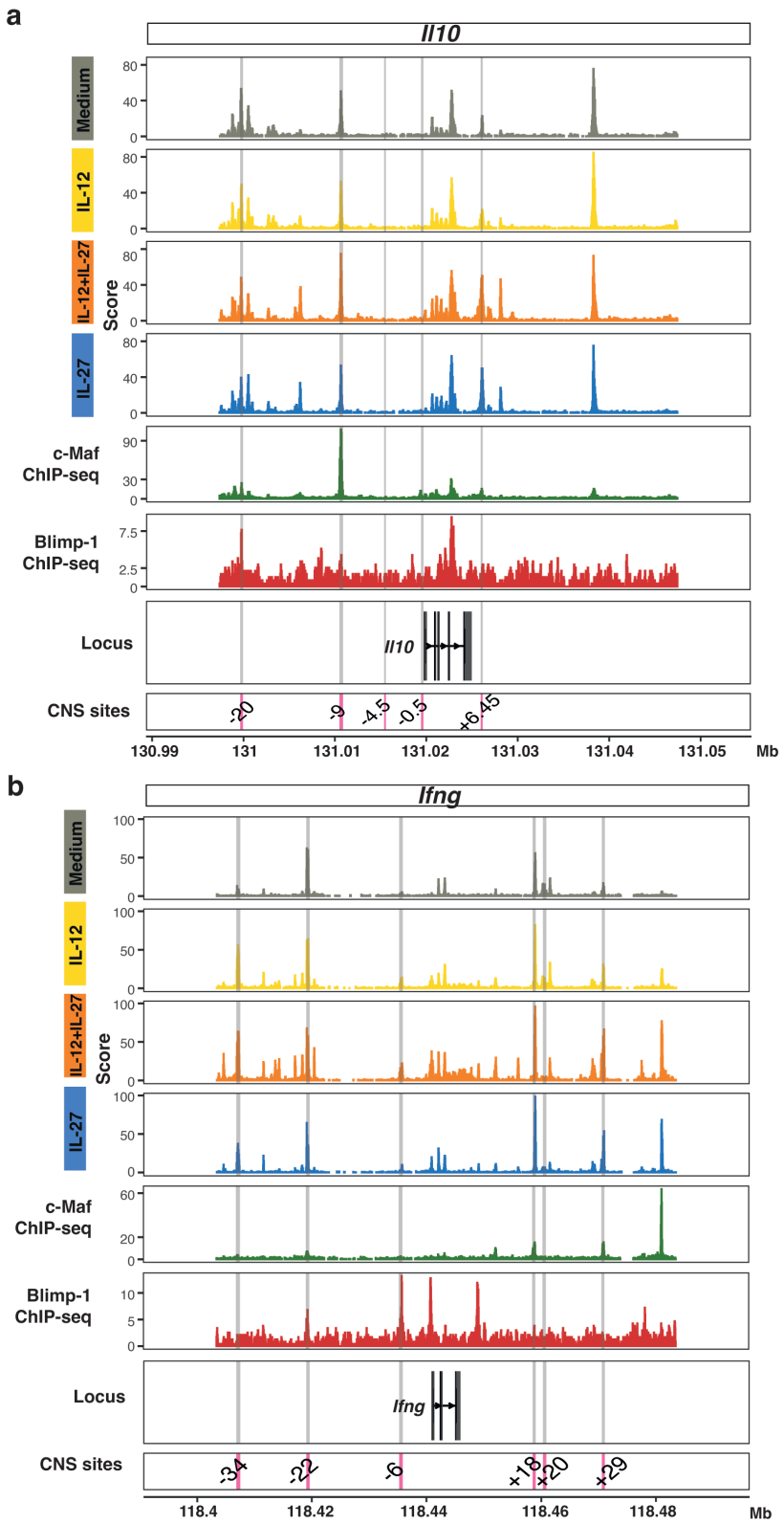


Figure 8. Integration of multiomic datasets identifies *Il10* and *Ifng* as targets for reciprocal regulation by Blimp-1 and c-Maf. Genome browser tracks of ATAC-seq data for **a**, *Il10* and **b**, *Ifng* from Day 3 $CD4^+$ T cells differentiated in the presence of Medium, IL-12, IL-12+IL-27, and IL-27. ChIP-seq tracks for c-Maf (green, GSE40918) and Blimp-1 (red, GSE79339) are shown. CNS reported in the literature for *Il10* and *Ifng* are highlighted in grey shading and labelled in the bottom track. Data from n=2–3 biological replicates.

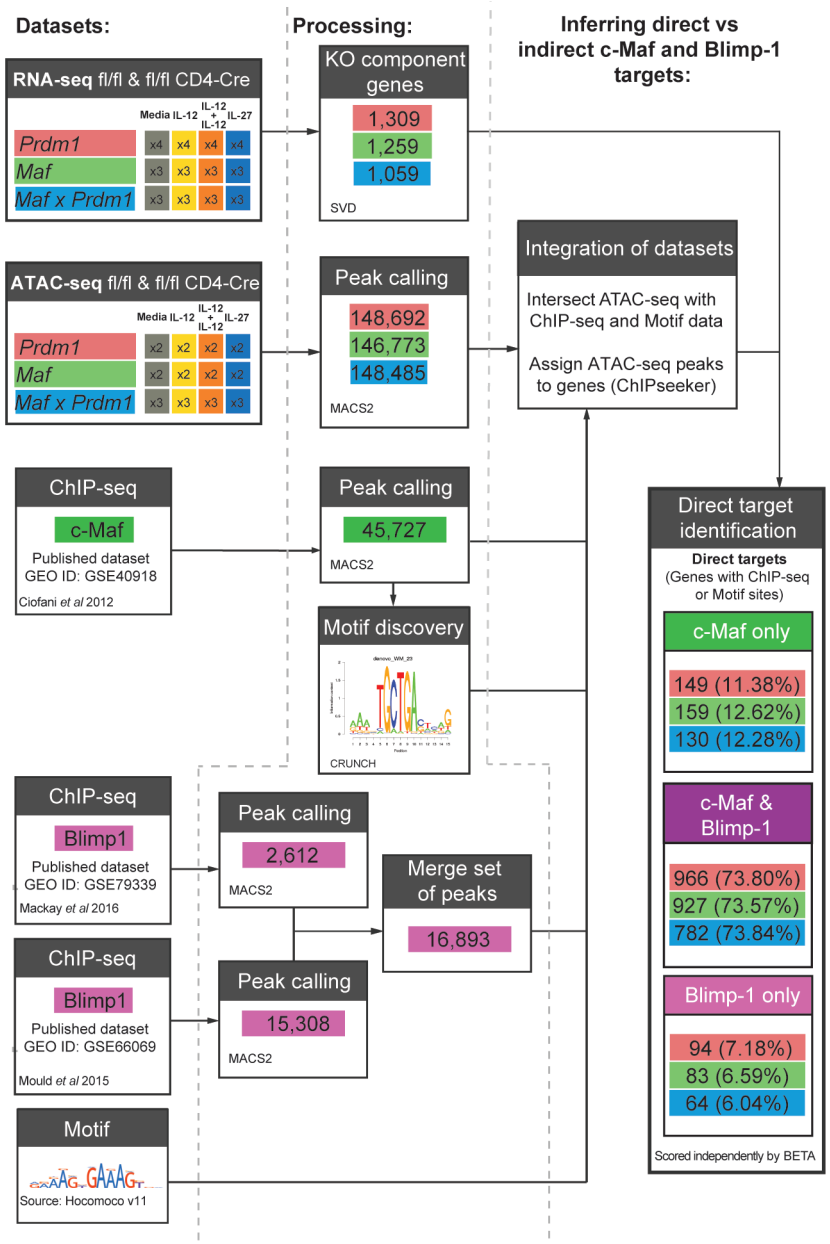


Figure 9. Schematic of framework applied to integrate multiomic datasets. Framework schematic for the identification of putative direct target genes of Blimp-1 and c-Maf in CD4+ T cells differentiated *in vitro* in the presence of Medium, IL-12, IL-12+IL-27, or IL-27 on Day 3, with replicates indicated. For each condition, Blimp-1 (GSE79339 and GSE66069) and c-Maf (GSE40918) ChIP-seq peaks and their corresponding motifs were filtered with and associated to the ATAC-seq peaks to identify the biologically relevant binding sites of Blimp-1 and c-Maf. Each ATAC-seq peak was associated to a gene based on distance proximity, thus, allowing the association of changes in the transcriptome with the binding of Blimp-1 and/or c-Maf (see Methods). Data from n=2–4 biological replicates.

Table 7[30]). Under IL-27 differentiation conditions both *Prdm1* and *Maf* were also shown to be direct targets of each other and were reduced in the absence of the reciprocal transcription factor (Figure 10b, 10d and 10f; Supplementary Table 7[30]).

Additionally, further network analysis of the IL-12+IL-27 CD4 T differentiated cell data (Figure 11a, 11c and 11e; Supplementary Table 7[30]) was applied to "Cluster 5" (from Figure 5g) with addition of the *Prdm1* and *Maf* genes and to IL-27 CD4 T differentiated cell data (Figure 11b, 11d and 11f; Supplementary Table 7[30]) to "Cluster 6" of Figure 5j again with addition of the *Prdm1* and *Maf* genes. Although weakly affected target genes were observed in these clusters that were affected by $Cd4^{Cre}$-mediated deletion of *Prdm1*, both *Prdm1* and *Maf* as compared to floxed controls, these clusters included *Tigit, Lag3* in keeping with previous reports[13,65].

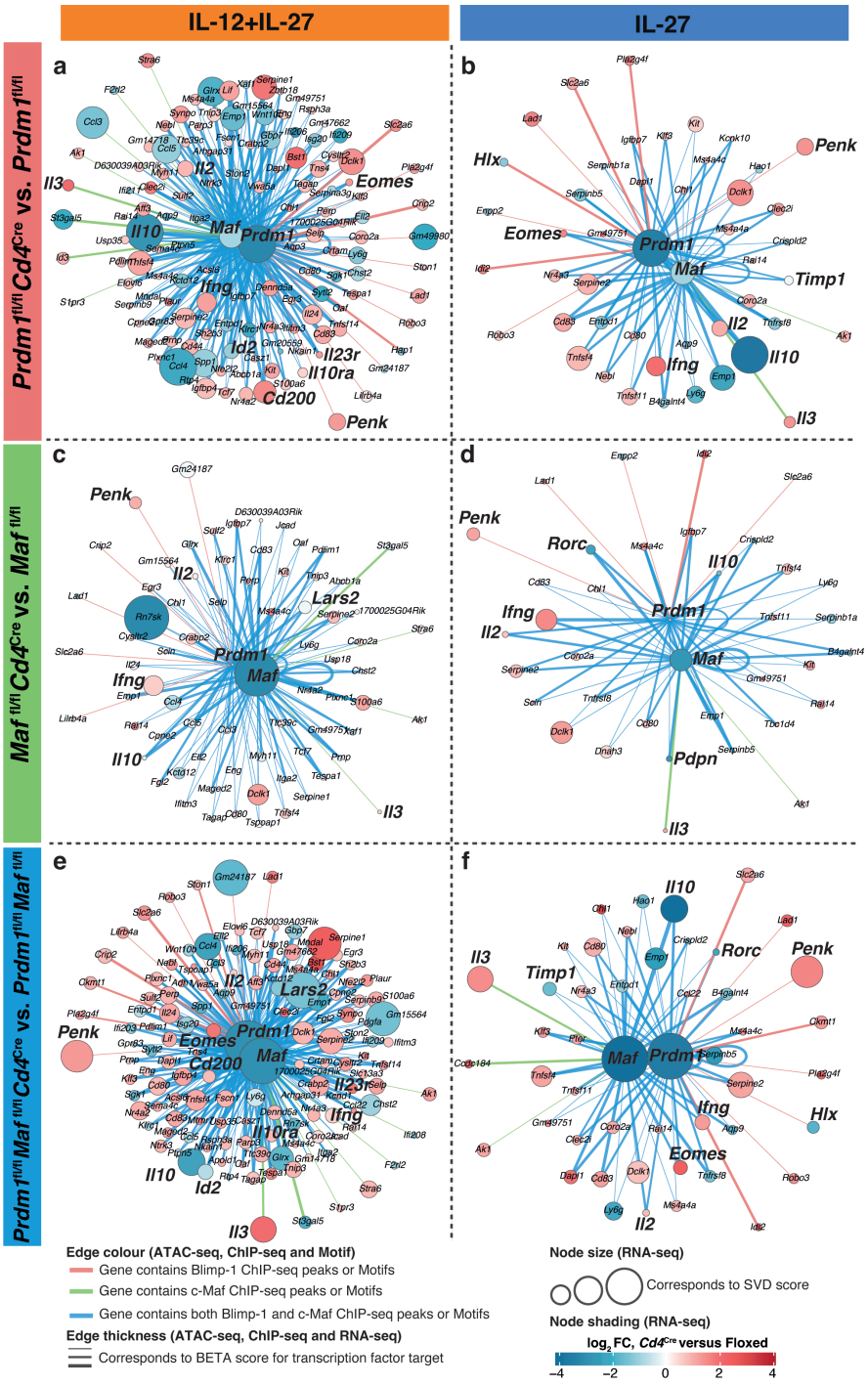


Figure 10. Gene regulatory networks derived from multiomic data integration highlight a majority of shared targets between Blimp-1 and c-Maf affected by *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf*, or both *Prdm1* and *Maf*. RNA-seq, ATAC-seq, ChIP-seq, and motifs were integrated (see Methods and Figure 9) to derive gene regulatory networks of the direct targets of Blimp-1 and/or c-Maf. Direct targets of Blimp-1 and/or c-Maf that were most affected upon *Cd4*^{Cre}-mediated deletion of **a**, *Prdm1*, **b**, *Maf*, or **c**, both *Prdm1* and *Maf* with IL-12+IL-27 (Clusters 2,1,7 from Figure 5j) and IL-27 alone (Clusters 5, 7, 1 from Figure 5m) differentiated CD4+ T cells *in vitro*. In the networks, the nodes correspond to genes affected upon *Cd4*^{Cre}-mediated deletion of the transcription factors. The node size reflects the contribution of a gene to the SVD component associated with the deletion of the transcription factors. The node colouring represents the fold change of the *Cd4*^{Cre}-mediated knockouts compared to the floxed control gene expression. The edge colour depicts if Blimp-1 (pink), c-Maf (green), or both Blimp-1 and c-Maf (blue) have binding sites assigned to the target genes; whilst the thickness of the edge shows the likelihood of c-Maf and/or Blimp-1 regulating a gene according to the BETA software. Data incorporates the RNA-seq and ATAC-seq biological replicates outlined in Figure 9 schematic. Data from n=2–4 biological replicates.

In summary we show that c-Maf and Blimp-1 are direct targets of each other, which regulate each other to directly induce *Il10* gene expression in Th1 cells differentiated in IL-12+IL-27 (Figure 12). We show that *Il10* is a direct target of both transcription factors in Th1 cells as previously reported for 'Tr1 cells' differentiated with IL-27 alone[13]. We additionally show that both

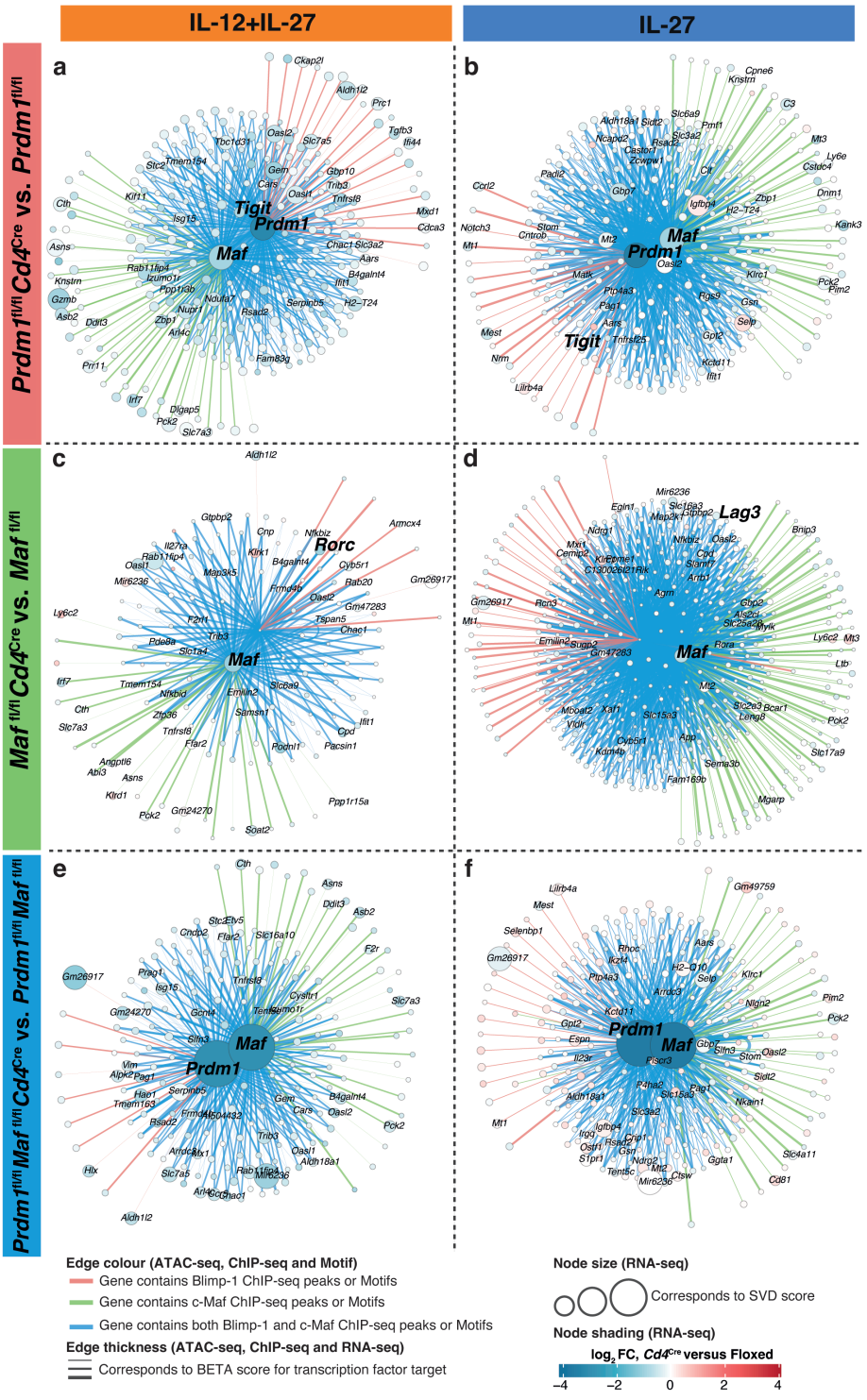


Figure 11. Gene regulatory networks derived from multiomic data integration point to known and new shared targets between Blimp-1 and c-Maf. Gene regulatory networks of the direct targets of Blimp-1 and/or c-Maf were derived, as in Figure 10, for clusters that contained genes that had been previously been reported to be regulated by c-Maf and Blimp-1 and were affected upon *Cd4*^{Cre}-mediated deletion of **a**, *Prdm1*, **b**, *Maf*, or **c**, both *Prdm1* and *Maf* with IL-12+IL-27 (Clusters 5 from Figure 5g) and IL-27 alone (Clusters 6 from Figure 5j) differentiated CD4+ T cells *in vitro*.

c-Maf and Blimp-1 can directly bind to the proinflammatory cytokine loci *Ifng, Il2* and *Id2* in IL-12+IL-27 differentiated Th1 cells and negatively regulate their expression, thus enforcing a controlled Th1 effector response. Moreover, c-Maf binds and positively regulates *Stra6* and binds and negatively regulates *Il3;* while Blimp-1 binds and positively regulates *Id2* and binds and negatively regulates *Cd200* and *Eomes* (Figure 12) in Th1 cells differentiated with IL-12 plus IL-27. Importantly, these genes are most strongly affected by *Cd4*^{Cre}-mediated deletion of both *Prdm1* and *Maf.*

Our study shows that the transcription factors Blimp-1 and c-Maf are co-dominant positive regulators of *Il10* in IL-12+IL-27-driven Th1 cells as was recently reported for IL-27-driven "Tr1" cells[13]. We additionally show that both Blimp-1 and c-Maf also negatively co-regulate common and unique proinflammatory gene networks in both IL-12 plus IL-27 differentiated Th1 cells and IL-27-differentiated "Tr1"cells. These data demonstrate that together Blimp-1 and c-Maf control a network of genes, specifically inducing *Il10* expression, while negatively regulating proinflammatory molecules, to ensure a tightly regulated IL-12-driven Th1 effector responses to limit host damage.

Discussion

Using computational inference of gene regulation derived from temporal gene cluster profiling and analysis of active genomic regions in Th1 cells differentiated with IL-12 and IL-27 or IL-12 alone, which produce proinflammatory cytokines but differ with respect to *Il10* expression, we show that Blimp-1 and c-Maf are co-dominant transcriptional regulators of *Il10* gene expression. We confirm these findings using T-cell specific deletion of these transcription factors and show that both transcription factors additionally negatively regulate a network of proinflammatory effector genes in Th1 cells through their indirect and direct action on shared and distinct effector target genes, thus reinforcing a controlled Th1 cell response.

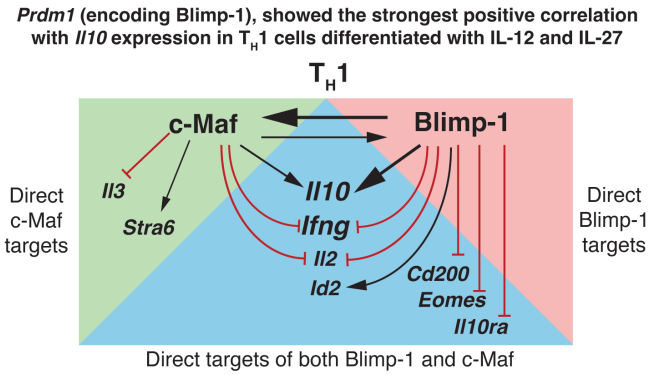


Figure 12. Comprehensive transcriptomic analysis reveals that Blimp-1 and c-Maf regulate *Il10*, cross-regulate each other, but also negatively regulate common and unique proinflammatory gene networks. Summary schematic of some of the findings herein presented in regards of the genes regulated by c-Maf and Blimp-1. The vast majority of direct targets detected were shared between c-Maf and Blimp-1, however they were affected to different extents upon the *Cd4*^{Cre}-mediated deletion of *Prdm1*, *Maf*, or both *Prdm1 and Maf*.

Temporal profiling of gene expression has been reported to facilitate the development of regulatory transcriptional networks dictating the differentiation of naïve CD4$^+$ T cells in Th17 cells[21,66], and Th2 cells[67], leading to the discovery of key regulators of activation and differentiation and reinforcing the general principles for T helper cell differentiation. We herein applied clustering algorithms and temporal profiling to gene expression data together with analysis of active genomic regions to reveal the transcriptional networks regulating *Il10* expression against that of the Th1-specific proinflammatory cytokine *Ifng*. Data from these analyses were compared across Th1 cells differentiated with IL-12 plus IL-27, which expressed high levels of both *Ifng* and *Il10*, and Th1 cells differentiated with IL-12 alone, which expressed high levels of *Ifng* but no *Il10*, alongside T cells differentiated with IL-27 alone, often referred to as 'Tr1 cells'[13], which expressed *Il10* but little to no *Ifng* and control CD4$^+$ T cells cultured in medium alone where *Il10* or effector cytokine expression is below the level of detection. This comparative analysis of *Il10* expression against *Ifng* and other effector cytokines allowed us to identify transcription factors predicted to be positive regulators of *Il10*. Additionally, this computational analysis allowed us not only to identify putative co-dominant transcription factors regulating *Il10* but additionally to determine any effects on *Ifng* and effector cytokine gene expression and transcription factors associated with Th1 cell differentiation. Th1 cells producing IFN-γ and IL-10 have been shown to be required for control of Th1 cell responses *in vivo* during chronic infection with intracellular pathogens to inhibit collateral host damage[10,11,15,16], whereas Th1 cells producing only IFN-γ have been associated with acute infection[68,69]. CD4$^+$ T cells producing IL-10 only in the absence of proinflammatory cytokines have been described[70,71] and often referred to as 'Tr1 cells'[13,72], which *in vivo* have been reported in the intestine[70,73,74] although it is unclear whether they are effector Th cells which have diminished their proinflammatory cytokine expression whilst maintaining IL-10 production in the intestine[73] or under certain metabolic conditions[75]. Therefore, a detailed knowledge of transcriptional regulation of *Il10* expression and effector cytokines such as *Ifng* and accompanying Th1 effector cytokines may provide therapeutic avenues to target inflammatory disease.

Analysis of transcription factors revealed that *Prdm1* showed the most significant positive correlation with *Il10* expression followed by *Id2, Asb2, Hlx, Nfatc2* and *Maf,* some of which we and others have previously reported as regulators of *Il10*[9,13,19,20,69]. Other proposed transcription factors reported to regulate *Il10* expression in T cells, such as *Hif1a* and *Nfil3*[9,13,20,69] showed only a slight correlation with *Il10* expression in Th1 cells differentiated with IL-12 plus IL-27 and were also expressed in Th1 cells differentiated with IL-12 alone, which did not express *Il10,* suggesting that they are not directly involved in *Il10* gene expression, but may play a broader role in the

differentiation of Th1 cells. Likewise, the transcription factor *Batf,* which has been previously reported to regulate *Il10* expression in differentiating Th2 cells[63] was actually down-regulated in both IL-12 plus IL-27 and IL-27 alone differentiation conditions, whilst increasing under IL-12 alone differentiation conditions, and showed very poor correlation with *Il10* expression. Thus, as discussed earlier for IL-21, it is possible that a number of these transcription factors are required for the differentiation and possibly proliferation of T helper cells rather than for direct positive regulation of *Il10* gene expression. Alternatively, the capacity of these transcription factors to regulate *Il10* may be cell and/or context dependent, for example regulating *Il10* in some effector T cells and not others, as previously discussed[9,17].

Analysis of transcriptional activity from ATAC-seq data, using BaGFoot software[39] revealed differential transcriptional activity for the transcription factors *Prdm1* and *Maf* between day 2 and day 3 in IL-12 plus IL-27 differentiated Th1 cells and IL-27 differentiated T cells, which both express *Il10.* These transcription factors were not found to be significantly active between day 2 and day 3 in IL-12 differentiated Th1 cells or medium control cultured CD4$^+$ T cells, which do not express *Il10,* suggesting that these transcription factors may be co-dominant regulators of *Il10.* By contrast, the transcriptional activity of the AP-1 family member, *Batf,* was only evident in cells cultured in medium alone or IL-12, which do not express *Il10*. Other AP-1 family members *Jun* and *Fos* showed increased transcriptional activity across all conditions including in IL-12-driven Th1 cells producing *Ifng* and no *Il10,* as well as in *Il10* expressing cells. This suggests that Batf and other AP-1 family members may be pioneer factors involved in Th cell differentiation, as previously suggested[76], rather than major regulators of *Il10*[63] and endorses the role of Jun and Fos as enhancers of *Il10* gene regulation[6,57]. Increased transcriptional activity of *Stat 3, 4* and *5* was most pronounced under IL-12 conditions in Th1 cells expressing *Ifng* but not *Il10.* Transcriptional activity of *Bhlhe40*, a known negative regulator of IL-10[17] and which we have previously shown to be negatively regulated by c-Maf, was found to be increased in cells cultured in IL-12, IL-12+IL-27 and IL-27, supporting its role as a regulator of *Il10*[17]. Our findings suggesting that *Prdm1* and *Maf* are co-dominant regulators of *Il10* in IL-12 plus IL-27 differentiated Th1 effector cells are in keeping with the report from Kuchroo *et al.*[13], who recently computed a transcriptional network induced by IL-27 in CD4$^+$ T cells, termed 'Tr1 cells', expressing *Il10,* but little to no *Ifng.* Hence *Prdm1* and *Maf* not only promote *Il10* expression in a T cell regulatory setting such as 'Tr1 cells' as reported, but additionally appear to be co-dominant regulators of *Il10* in an effector Th1 setting accompanying high levels of *Ifng.* Indeed, specific deletion of *Prdm1, Maf* and the combination of both these transcription factors in IL-12 plus IL-27 differentiated Th1 effector cells expressing *Ifng,* confirmed their co-dominant role in regulating *Il10* gene expression in these pro-inflammatory cells. Thus, *Prdm1* and *Maf* are not only central hubs in regulating the expression of *Il10* in IL-27 differentiated 'Tr1 cells' where they were confirmed to control a regulatory circuit of multiple other transcriptional modulators using Prdm1/Maf DKO 'Tr1 cells'[13], but as we now show, also regulate *Il10* expression in a proinflammatory Th1 effector setting.

Commitment of T helper cells to specific subsets requires induction of master transcription factors that induce specific transcriptional programs that direct a specific T cell subset towards terminal differentiation while restricting the fates of other T cell subsets[77]. Thus, we questioned whether *Prdm1* and *Maf* may be part of the network for Th1 cell differentiation with *Il10* expression accompanying terminal differentiation of these cells to provide feedback regulation, or alternatively contribute to a regulated Th1 response by antagonising the expression of *Ifng* and other proinflammatory molecules. The absence of *Prdm1* and *Maf* in IL-12 plus IL-27 differentiated Th1 cells actually resulted in an increase in *Ifng* expression, showing that while *Prdm1* and *Maf* synergistically promote *Il10* expression, they negatively regulate the expression of the effector cell programme, reflected by increased expression of *Ifng,* thus controlling Th1 effector responses. It is likely that this co-dominant transcriptional regulation by *Prdm1* and *Maf* is in place to ensure a controlled Th1 response against chronic infection with intracellular pathogens to minimise accompanying pathology. The negative regulation of *Ifng* that we observed was most pronounced in Th1 cells differentiated in IL-12 plus IL-27. This was in contrast to the discussion from Kuchroo *et al.*[13], in IL-27-only driven IL-10 producing 'Tr1 cells', which expressed minimal to no *Ifng,* that although *Prdm1* and *Maf* synergistically promoted IL-10 production, they did not inhibit production of T helper cell signature cytokines. This may reflect the differential effects on distinct T cell subsets. However, our findings using in-depth clustering of RNA-seq data demonstrated that T-cell specific deletion of *Prdm1*, *Maf*, or both transcription factors, led to an increase in several proinflammatory genes in both IL-27 differentiated T cells as well as IL-12 plus IL-27 differentiated Th1 cells, although to a much larger extent (expression and number of genes) in the Th1 cells. Although we found that the absence of *Prdm1, Maf* or both transcription factors resulted in an increase in *Ifng* expression, the expression of the Th1/IFN-specific transcription factor *Tbx21*[64] was not significantly affected, suggesting that Blimp-1 and c-Maf may potentially have direct effects on the *Ifng* gene itself. This was supported by combining ATAC-seq and ChiP-seq data, which clearly revealed both unique and overlapping Blimp-1 and c-Maf sites not only in the *Il10* locus, but additionally in the *Ifng* locus. Moreover, Th1 cells differentiated in IL-12 (together with IL-27 or not) resulted in increased chromatin accessibility in the *Ifng* locus possibly enhancing the regulatory action of *Prdm1* and *Maf.*

Relevant to our study, in recent years Blimp-1 and c-Maf have been associated with transcriptional signatures from human disease such as colitis and rheumatoid arthritis[78,79]. The conserved nature of these transcription factors between mouse and humans suggests similar transcriptional mechanisms for cytokine gene regulation operate in mouse and humans. In support of this, SNPs in Blimp-1 have been associated with elevated IFN-g expression in colitis patients[79] and Blimp-1 has been shown to bind conserved CNS sites in human and mouse in the *Ifng/IFNG* and *Tbx21/TBX21* loci in T cells and NK

cells[21,80]. Likewise, c-Maf has been shown to be regulated by the cholesterol pathway and regulate *IL10* expression by human Th1 cells[74] in keeping with regulation of *Il10* expression in mouse Th1 cells as we report herein. Very recently both Blimp-1 and c-Maf have been reported to co-regulate $CD4^+$ T cell derived IL-10 in Crohn's patients[81] and identified in Th1/Tr1 cells from malaria patients[82,83]. However, the exact dynamic role of both Blimp-1 and c-Maf play in the regulation of the expression of cytokines, and other important inflammatory genes needs further research.

Additionally, gene regulatory networks derived from multiomic data integration highlighted a large number of shared and some unique targets of Blimp-1 and c-Maf which showed positive and negative regulatory effects on gene expression. Accompanying these dominant effects upon T cell-specific deletion of *Prdm1* and *Maf* on *Il10* expression in IL-12 plus IL-27 driven Th1 cells, was a decrease in other genes including *Id2, Lars2* and *Tigit,* whilst in IL-27 driven T cells the decrease in *Il10* was accompanied by a decrease in expression of genes including *Timp1, Hlx, Tigit*, *Rorc,* in keeping with the reports from Kuchroo on *Il10*-only producing 'Tr1 cells'[13], while deletion of both *Prdm1* and *Maf* in IL-12 plus IL-27 differentiated Th1 cells led to an increased number as well as level of proinflammatory gene expression including, *Ifng, Il23r, Eomes, Il2,Il3, Penk* and *Cd200* and others, this was mostly less marked in the IL-27 alone differentiated cells, although many were found to be shared targets of both *Prdm1* and *Maf.* A limitation of this study is firstly that the regulation of *Il10* versus proinflammatory cytokines by Blimp-1 and c-Maf was firstly only demonstrated at a transcriptional level and consequent effects on protein production were not the focus of the study. Secondly, the effects of T cell-specific deletion of *Prdm1* and *Maf* on *Il10* and proinflammatory cytokine expression was only investigated *in vitro*. Our continuing studies will further address the role of Blimp-1 and c-Maf in regulation of cytokine responses *in vivo,* and the physiological consequences of T cell-specific deletion of these transcription factors in response to pathogens and/or pathobionts.

In summary, we have shown that *Prdm1* and *Maf* are co-dominant transcription factors that induce *Il10* gene expression, together with a cluster of genes including other transcription factors and co-inhibitory receptor genes, indicating their role in establishing an immunoregulatory gene programme in T cells. In addition, our findings show that both *Prdm1* and *Maf* also negatively regulate a number of proinflammatory genes including *Ifng, Il23r, Eomes, Il2, Il3, Penk* and *Cd200* and others, most strongly in Th1 cells, demonstrating their major role in controlling Th1 responses to allow eradication of pathogens with minimum pathology.

Data availability

Underlying data

The materials, data and any associated protocols that support the findings of this study are available from the corresponding author upon request. The RNA-seq and ATAC-seq datasets have been deposited in the NCBI Gene Expression Omnibus (GEO) database with the primary accession number GSE197789. Publicly available datasets used in this study include GSE40918, GSE79339, and GSE66069.

GEO: Blimp-1 and c-Maf regulate Il10 and negatively regulate common and unique proinflammatory gene networks in IL-12 plus IL-27-driven T helper-1 cells [Mus musculus (house mouse)]. Accession number GSE197789; https://identifiers.org/geo:GSE197789[84]

GEO: A validated regulatory network for Th17 cell specification [Mus musculus (house mouse)]. Accession number GSE40918; https://identifiers.org/geo:GSE40918[21]

GEO: Hobit and Blimp1 instruct a universal transcriptional program of tissue-residency in lymphocytes [Mus musculus (house mouse)]. Accession number GSE79339; https://identifiers.org/geo:GSE79339[49]

GEO: Analysis of Blimp-1 and Irf-1 genomic binding in wild type and Prdm1/Blimp-1 mutant embryonic gut [Mus musculus (house mouse)]. Accession number GSE66069; https://identifiers.org/geo:GSE66069[50]

Figshare: Blimp-1 and c-Maf regulate *Il10* and negatively regulate common and unique proinflammatory gene networks in IL-12 plus IL-27-driven T helper-1 cells. https://doi.org/10.6084/m9.figshare.23592393[30]

This project contains the following underlying data:

- SupplementaryTable1_KineticsRawGeneCounts.xlsx
- SupplementaryTable2_KineticsDifferentialGeneExpression_KmeansCluster.xlsx
- SupplementaryTable3_TF_Cytokines_GeneLists.xlsx
- SupplementaryTable4_CD4creDeletion_FloxCtrls_RawNormGeneCounts
- SupplementaryTable5_SVDresults.xlsx
- SupplementaryTable6_SVD_CD4CreDeletionComponent_KmeansClusterFoldChanges.xlsx
- SupplementaryTable7_DirectTargets_Blimp1-cMaf_NetworkValues.xlsx

Reporting guidelines

Repository: ARRIVE checklist for 'Blimp-1 and c-Maf regulate *Il10 and* negatively regulate common and unique proinflammatory gene networks in IL-12 plus IL-27-driven T helper-1 cells'. https://doi.org/10.6084/m9.figshare.23592393[30]

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https://identifiers.org/geo:GSE197789

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 08 December 2023

https://doi.org/10.21956/wellcomeopenres.22787.r70937

✔ **Leonie Taams**
King's College London, London, England, UK

I would like to thank the authors for their detailed and comprehensive replies and edits; I am happy to state that I am satisfied with these revisions.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunoregulation (wet-lab based) with limited expertise in bioinformatic analysis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 08 December 2023

https://doi.org/10.21956/wellcomeopenres.22787.r70938

✔ **Christopher A Hunter**
Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA

This is good to go. In particular the explanation for why all the data sets are included (as part of a resources paper) makes this clearer to this reviewer.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 19 October 2023

https://doi.org/10.21956/wellcomeopenres.21798.r67170

?

Christopher A Hunter
Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA

The overarching premise of this study is that transcriptionally distinct subsets of T lymphocytes are required for control and eradication of intracellular pathogens, however the transcriptional networks within specialized T cell subsets that regulate key cytokines required for control, including IFN-g and IL-10 remains unclear. Here they take a multi-modal approach to investigate the transcriptional networks underlying cytokine production within CD4 T cells treated in Th1 polarizing conditions in vitro. Utilizing various combinations of the cytokines IL-12 and IL-27. They generate and present original RNA and ATAC-seq datasets to elucidate the co-dominant role of *Prdm-1* (Blimp-1) and *Maf* (c-Maf) in regulating expression of IL-10 in Th1 cells, and this is reinforced and affirmed using mice with T-cell specific deletion of these transcription factors. This is similar (in some ways) to previous studies that used public and in-house transcriptional datasets to identify Prdm-1 and Maf as co-regulators of IL-10 production by Tr1 cells differentiated in vitro with IL-27.

Our understanding of the molecular mechanisms that underly the integration of cytokine signals within a cell and drive functional cell states is continues to develop and this study provides a piece of the puzzle through their comprehensive analysis of the integration of signals for the IL-12 family members at the transcriptional and epigenetic level. The undertaking to generate and analysis high-dimensional datasets is no easy task and the breadth of the undertaking really makes this a nice resource data set. This iteration does distinguish itself from the previous report on Prdm-1 and Maf regulating IL-10 expression in Tr1 cells and its genuinely important to know what is robust and reproducible so there is clear value to this report. The authors do try to get the most out of their novel datasets, but in places the manuscript is dense and it might help to remove redundant figures and focus the narrative to drive home their points that Prdm-1 and Maf are regulators of IL-10 expression in a variety of conditions. However, as currently written the difference in transcriptional regulation between Tr1 and Th1 cells is unclear and the data sets need to be more fully synthesized. If I was reviewing this for a colleague – my suggestion would be to look again at the original sentence at the end of the first paragraph of the Introduction. Maybe consider re-working the sentiment that the transcriptional mechanisms that regulate cytokine production by Th1 cells including their production of IL-10 are as yet unclear. It

undersells what is = a rich literature on this topic that for example led to the identification of T-bet and how STATs promote Th1 responses and IL-10 production. Maybe this can be used to better rfame how the current study moves the field.

The comments below are provided to help with clarity and are not meant to be prescriptive and new experiments or reanalysis is not requested.

Figure 1 – no comments. Favorite figure.

Figure 2 – These transcriptional data have already been presented within Figure 1, the only difference is the visualization used to display the data. The authors have already presented these genes and determined them to be DEGs in Figure 1C, and we are not sure how/if presenting the read counts provides any new insight. The authors already show a pearson's correlation in Figure 1e and use the second half of Figure 2 to show correlations derived from a linear regression. It is unclear whether these graphs provide a novel lens to interpret these data. Perhaps Figure 1 is sufficient for the claims made in Figure 2?

Figure 3 – the authors take advantage of a software package BaGFoot to analysis ATAC-seq data but there is not sufficient description of the package to allow a reader to interpret the graphs. The authors highlight that these depictions show increased transcriptional activity for Prdm1 and Maf, but does this provide additional insight beyond what is shown by figure 1. Perhaps just needs to be better integrated.

Figure 4 – Good as is. We like the figure and the written description.

Figure 5 – The authors move onto T-cell specific depletion of Prdm-1 and Maf and transcriptional analysis of these cells via RNA-seq. Is the variance (G-I) redundant with the graphs (D-F)?

Figure 6 – all of these genes are already shown in Figure 5 and seems redundant.

Figure 7 – The interpretation of the PCA analysis for this ATAC-seq data is similar to what has been previously established, although it is important to see the consistency. The authors go on to say that there is no chromatin remodeling, but there are differences in the visualizations of the heatmap. For example. it appears that the absence of Maf alone induces a similar chromatin remodeling to the absence of both Prdm1 and Maf. Are we interpreting this wrong?

Figure 8 – Maybe there is a mis-understanding (on our part). How does the IL-10 and IFN-g locus look in the ATAC-seq data generated from T -cell specific depletion of Prdm-1 and Maf as shown in Figure 7. These data would be more interesting to see rather than the WT cells (described in the figure legend?).

Fig 9, 10 and 11. The authors have done a lot of analysis but it would be genuinely helpful if they could provide clarity in the discussion as to why these genes are interesting. For example, the focus on Cluster 5 is unclear.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cytokines and T cells

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Nov 2023

Anne O'Garra

Christopher A Hunter Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA ***We thank this reviewer for his comments and suggestions which have helped to clarify apsects of our manuscript, however we would like to explain that our reason for providing all of these complementary analyses is in view of the fact this manuscript is submitted as a Resource paper and that Wellcome Open Research does not allow Supplementary Figures, or data not shown. Inclusion of all these complementary analyses will highlight these data which will be accessible for further analyses to specialist readers with further interest as the RNA-seq and ATAC-seq data will all be deposited in the GEO for public access. We have clarified our manuscript upon the suggestions of this reviewer and added or replaced text as we explain below.***

The overarching premise of this study is that transcriptionally distinct subsets of T lymphocytes are required for control and eradication of intracellular pathogens, however the transcriptional networks within specialized T cell subsets that regulate key cytokines required for control, including IFN-g and IL-10 remains unclear. Here they take a multi-modal approach to investigate the transcriptional networks underlying cytokine production within CD4 T cells treated in Th1 polarizing conditions in vitro. Utilizing various combinations of the cytokines IL-12 and IL-27. They generate and present original RNA and ATAC-seq datasets to elucidate the co-dominant role of *Prdm-1* (Blimp-1) and *Maf* (c-Maf) in regulating expression of IL-10 in Th1 cells, and this is reinforced and affirmed using mice

with T-cell specific deletion of these transcription factors. This is similar (in some ways) to previous studies that used public and in-house transcriptional datasets to identify Prdm-1 and Maf as co-regulators of IL-10 production by Tr1 cells differentiated in vitro with IL-27[AO1] . Our understanding of the molecular mechanisms that underly the integration of cytokine signals within a cell and drive functional cell states is continues to develop and this study provides a piece of the puzzle through their comprehensive analysis of the integration of signals for the IL-12 family members at the transcriptional and epigenetic level. The undertaking to generate and analysis high-dimensional datasets is no easy task and the breadth of the undertaking really makes this a nice resource data set. This iteration does distinguish itself from the previous report on Prdm-1 and Maf regulating IL-10 expression in Tr1 cells and its genuinely important to know what is robust and reproducible so there is clear value to this report. The authors do try to get the most out of their novel datasets, but in places the manuscript is dense and it might help to remove redundant figures and focus the narrative to drive home their points that Prdm-1 and Maf are regulators of IL-10 expression in a variety of conditions. However, as currently written the difference in transcriptional regulation between Tr1 and Th1 cells is unclear and the data sets need to be more fully synthesized. If I was reviewing this for a colleague – my suggestion would be to look again at the original sentence at the end of the first paragraph of the Introduction. "However, the transcriptional mechanisms that regulate cytokine production by Th1 cells including their production of IL-10 are as yet unclear." ***Thank you to the Reviewer for pointing this out. With respect to this original sentence at the end of the first paragraph of the Introduction copied above, we have expanded, rearranged and clarified what the underlying questions in the field are also quoting a relevant published review and paper as indicated below and with changes to the text of the manuscript.***

Maybe consider re-working the sentiment that the transcriptional mechanisms that regulate cytokine production by Th1 cells including their production of IL-10 are as yet unclear. It undersells what is a rich literature on this topic that for example led to the identification of T-bet and how STATs promote Th1 responses and IL-10 production. Maybe this can be used to better frame how the current study moves the field. ***We agree that this was not clear and have now clarified the questions and current literature by adding the following paragraph in the Introduction as follows: "The transcription factors that regulate the differentiation and function of Th1 cells producing Ifng are well established, including STAT molecules and T-bet, however, whether these transcription factors regulate Il10 expression as part of the differentiation pathway of Th1 cells has been difficult to decipher (Currently Reference 9: Gabrysova et al., 2014, Current Topics Micro&Imm). Whether transcription factors such as Prdm1 or Maf, which positively induce Il10 expression, do so as part of the differentiation pathway of Th1 cells, or alternatively, simultaneously negatively regulate proinflammatory cytokines in Th1 cells, therefore not contributing to Th1 differentiation but instead reinforcing a regulated Th1 response, is unclear (Gabrysova, ref 9, 2014; Gabrysova, Ref. 16, 2018).".***

The comments below are provided to help with clarity and are not meant to be prescriptive and new experiments or reanalysis is not requested. ***Thank you for the useful feedback which we have attended to.***

Figure 1 – no comments. Favorite figure. ***We are pleased that the reviewer appreciates this***

figure.

Figure 2 – These transcriptional data have already been presented within Figure 1, the only difference is the visualization used to display the data. The authors have already presented these genes and determined them to be DEGs in Figure 1C, and we are not sure how/if presenting the read counts provides any new insight. The authors already show a pearson's correlation in Figure 1e and use the second half of Figure 2 to show correlations derived from a linear regression. It is unclear whether these graphs provide a novel lens to interpret these data. Perhaps Figure 1 is sufficient for the claims made in Figure 2? ***We thank the Reviewer for pointing out that our data were seemingly repetitive, and we have now clarified the text to describe the added value of Figure 2a and b, over-and-above what was provided in Figure 1. We have however removed Figure 2c to clarify our message. This clarification below has been added to the manuscript text: "Of transcription factors previously reported to regulate Il10 expression, only expression of Prdm1 and Maf, was significantly increased under IL-12+IL-27 and IL-27 alone conditions only, reaching maximal levels when Il10 expression was observed at days 3 and 4 and showing the strongest correlation with Il10 expression, while their expression was not observed under IL-12 or medium alone conditions (Figure 2a). Importantly expression of Batf, previously suggested to regulate Il10 in Th2 cells (Tussiwand, Nature, original reference 75), which was not revealed as correlating with Il10 expression in the analysis in Figure 1e, did not correlate with Il10 expression under IL-12+IL-27 and IL-27 alone conditions, but rather was maximally expressed on days 1 and 2, rapidly diminishing by days 3 and 4, while it's expression increased with time in IL-12 alone differentiated Th1 cells which did not express Il10 (Figure 2b), therefore suggesting a broader function than the regulation of Il10 (Figure 2b). Other transcription factors that have been associated with Il10 gene expression including Hif1a and Nfil3 [13, 16], which although expressed under IL-12+IL-27 and IL-27 alone conditions, showed less of a correlation with Il10 expression, and were found to increase with time in Th1 cells differentiated with IL-12 only which do not express Il10, therefore again suggesting a broader role for these transcription factors in Th1 differentiation (Figure 2b[AO2])". We trust this simplification of Figure 2 and this clarification of the text will satisfy the Reviewer, particularly given the nature of this manuscript submission as a Resource, and secondly since we cannot add Supplementary figures or data not shown.***

Figure 3 – the authors take advantage of a software package BaGFoot to analysis ATAC-seq data but there is not sufficient description of the package to allow a reader to interpret the graphs. The authors highlight that these depictions show increased transcriptional activity for Prdm1 and Maf, but does this provide additional insight beyond what is shown by figure 1. Perhaps just needs to be better integrated. ***We apologise for the lack of clarity of the BaGFoot analysis of the ATAC-seq data and have expanded the description of the package accordingly as well as clarifying the text regarding the results. We again believe that these data add support at the level of transcriptional activity, over-and-above that which is provided in Figure 1. Moreover, since the manuscript was submitted as a Resource, and these data cannot be provided as a Supplementary Figure, we believe they are important to show and described at the level we have now done, in order not to detract but rather support the main story of the manuscript. These data will then be accessible for further analyses to specialist readers with further interest as the ATAC-seq data will be deposited in the GEO for public access. We have replaced the text as shown below to clarify as the***

Reviewer suggested. "To further investigate global changes in transcriptional activity in CD4$^+$ naïve T cells cultured as above, we used the assay for transposase-accessible chromatin plus sequencing (ATAC-seq) to reveal functionally active genomic regions at days 2 and 3, timepoints, which marked key transcriptional changes during the differentiation of CD4$^+$ naïve T cells into Th1 cells (cultured as in Figure 1a). The 'bivariate genomic footprinting' (BaGFoot) software[39] was applied to the ATAC-seq data, to detect global changes in transcription factor binding activity (genome-wide) occurring between day 2 and day 3 under the different conditions. Differences in binding activity are assessed by BaGFoot software by quantifying the differences in Tn5 transposition within a transcription factor motif, by measuring the 'footprint depth' (Figure 3, y axis) and 'flanking accessibility' (Figure 3, x axis) and comparing these metrics between timepoints. A transcription factor bound to chromatin has a high footprint depth and a high flanking accessibility. Only IL-27 and IL-12+IL-27 cultured T cells showed increased transcriptional activity for Prdm1 (higher activity at day 2 vs day 3) and Maf (higher activity at day 3 vs day 2), reinforcing a role for these transcription factors in regulating transcriptional programs in IL-12+IL-27 and IL-27 cultured T cells. On the other hand, the AP-1 family members, e.g. Jun and Fos, showed increased transcriptional activity at day 3 across all cytokine differentiation conditions including medium control (Figure 3) suggesting a broader role in T helper cell activation/differentiation. Batf transcriptional activity was only evident in cells cultured in medium alone or IL-12; and Stat 3, 4 and 5 transcriptional activity was only detected under IL-12 conditions, which were not accompanied by Il10 gene expression, again implicating these transcription factors in broader roles in Th1 cell activation/differentiation. Detection of STAT activity and a transcriptome more similar to T cells cultured in medium or in IL-12 alone at days 1 and 2 (Figure 1a), suggests that Th1 cells cultured with IL-12, which do not express Il10 were temporally and qualitatively different with respect to global transcriptional activity, to Th1 cells cultured with IL-12+IL-27 and IL-27 alone, where both conditions lead to Il10 expression, although proinflammatory cytokine expression was only observed in IL-12+IL-27 driven Th1 cells."

Figure 4 – Good as is. We like the figure and the written description. ***Thank you.***

Figure 5 – The authors move onto T-cell specific depletion of Prdm-1 and Maf and transcriptional analysis of these cells via RNA-seq. Is the variance (G-I) redundant with the graphs (D-F)? ***We have modified Figure 5 and removed Figure 5g-i as suggested by the reviewer and corrected Figure 5 in the manuscript text accordingly. We thank the Reviewer for pointing this out as it clarifies the figure by removing redundancy.***

Figure 6 – all of these genes are already shown in Figure 5 and seems redundant. ***We respectfully believe that these data are important to keep in the manuscript since they provide supporting data with respect to pathways involved which were discussed at various sections of the text. We argue that this is important to include, in view of the fact that Wellcome Open Research does not allow Supplementary Figures, or data not shown, and will be of value to researchers studying this specific area of transcriptional regulation of cytokines, especially since the manuscript is submitted as a Resource.***

Figure 7 – The interpretation of the PCA analysis for this ATAC-seq data is similar to what has been previously established, although it is important to see the consistency. The

authors go on to say that there is no chromatin remodeling, but there are differences in the visualizations of the heatmap. For example. it appears that the absence of Maf alone induces a similar chromatin remodeling to the absence of both Prdm1 and Maf. Are we interpreting this wrong? ***We are assuming that the Reviewer's comment "****is similar to what has been previously established****" refers to Figure 1 PCA which was RNA-seq of wild type mouse T cells. We emphasise that the PCA of ATAC-seq data in Figure 7 is distinct from the PCA plot of the RNA-seq data in Figure 1 which shows cytokine conditions only, but no knockouts, whereas this Figure 7 is ATAC-seq data PCA of T cells at kinetic points of cytokine-driven conditions. The only other figure in the manuscript showing ATAC-seq data is Figure 3, which again has only cytokine conditions and no knockouts. Figures 5 and 6 are RNA-seq data showing Spearman, Singular Value Decomposition (SVD) and then clustering of only differentially expressed genes by RNA-seq not showing a PCA plot. The ATAC-seq PCA data and Spearman in Figure 7 is of ATAC-seq data, and shows T cells driven under different cytokine conditions from the different T cell-specific transcription deleted mice – Figure 7 a, b and c, PCA, and then, Figure 7b,c and d, Spearman. We firstly shortened the sentence to clarify our message and secondly added an extra line in bold below to our text referring to data in Figure 7 to clarify these findings. A major point of our findings is: "It would appear from our findings that cytokines are the main drivers of changes in chromatin remodelling while changes in chromatin remodelling are not observed in the absence of Prdm1, Maf or both transcription factors, as we have previously reported in a different context for c-Maf [20]". We have now added these lines to the text of our manuscript to summarise and clarify the take-home message of our findings. We trust that this will now clarify these issues. "**Moreover, major chromatin remodelling occurring between Day 2 and Day 3 was observed in all *in vitro* differentiated CD4 $^{+}$ T cells in the presence of cytokines. **However, no major** changes in accessibility were observed upon *Cd4* ^{Cre}-mediated deletion of *Prdm1*, *Maf,* or both *Prdm1* and *Maf* (Figure 7a–f). Together these results highlight a role for IL-27 (including IL-27 plus IL-12) in influencing the open chromatin landscape of *in vitro* differentiated CD4 $^{+}$ T cells and suggests that c-Maf and Blimp-1 do not drive chromatin remodelling in order to perform their gene regulation functions, as has been shown for c-Maf [20] . ***It would appear from our findings that cytokines are the main drivers of changes in chromatin remodelling while changes in chromatin remodelling are not observed in the absence of Prdm1, Maf or both transcription factors (Figure 7a-f). This indicates that Blimp-1 and c-Maf do not themselves induce chromatin remodelling in these differentiating Th1 cells, as previously reported for c-Maf in Th17 cells in vitro[20] and as we have reported for c-Maf in ex-vivo T cells from infection models in other contexts[16]".***

Figure 8 – Maybe there is a mis-understanding (on our part). How does the IL-10 and IFN-g locus look in the ATAC-seq data generated from T -cell specific depletion of Prdm-1 and Maf as shown in Figure 7. These data would be more interesting to see rather than the WT cells (described in the figure legend?). ***The data on the IL-10 and IFN-g loci are from the WT T cells differentiated under the different cytokine conditions only, as those in T cells bearing T -cell specific depletion of Prdm-1 and Maf, or both, showed no difference in terms of chromatin accessibility as compared to controls – this is why we did not show them. We have added the line below to the text of the manuscript to clarify this point. "These accessible binding sites were not affected by T -cell specific depletion of Prdm-1 and Maf, or both transcription factors, as compared to controls."***

Fig 9, 10 and 11. The authors have done a lot of analysis but it would be genuinely helpful if they could provide clarity in the discussion as to why these genes are interesting. For example, the focus on Cluster 5 is unclear. ***Figure 9 provides the schematic of the integration of the multiomic data. The reason that specific clusters were chosen, (from Figure 5g and 5j) for depiction as gene regulatory networks in Figures 10 and 11, is because these clusters contained direct targets of Blimp-1 and/or c-Maf and were the most affected at the RNA expression level upon Cd4Cre-mediated deletion of Prdm1, Maf, or both Prdm1 and Maf eg. for IL-12+IL-27 found in Clusters 2,1,7 in Figure 5g; and for IL-27 alone found in Clusters 5, 7, 1 in Figure 5j, We now provide an explanation at the beginning of the data of why these clusters were selected: "Specific clusters from the RNA expression data (Figure 5g and 5j) were chosen for our depiction as gene regulatory networks in Figures 10 and 11, because these clusters contained direct targets of Blimp-1 and/or c-Maf that were the most affected at the RNA expression level upon Cd4Cre-mediated deletion of Prdm1, Maf, or both Prdm1 and Maf eg. for IL-12+IL-27 found in Clusters 2,1,7 in Figure 5g; and for IL-27 alone found in Clusters 5, 7, 1 in Figure 5j."***

We thank the reviewer for the helpful comments for clarifying our manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Report 05 October 2023

https://doi.org/10.21956/wellcomeopenres.21798.r67171

?

Leonie Taams
King's College London, London, England, UK

This manuscript compellingly demonstrates that Maf and Blimp1 are transcriptional regulators of both Il10 and Ifng gene expression during mouse naïve CD4+ T cell differentiation in the presence of IL-12 and IL-27. Whilst both Maf and Blimp1 had been associated previously with positive regulation of Il10 expression, the results presented here demonstrate that they in parallel negatively regulate Ifng, as well as other Th1-related effector molecules including Il2 and Id2.

The data presented in the paper consist of RNAseq, ATACseq and qPCR data of naïve CD4+ T cells differentiated in the absence or presence of IL-12, IL-27 or IL-12 plus IL-27, using cells from either wt or from CD4+ T cell-conditional KO mice for Maf, Prdm1 or the combination.

The authors employ an impressive range of sophisticated and complex computational modelling tools to analyse the datasets. This reviewer is not qualified to comment on these tools or the analysis approach; input from a qualified bioinformatician expert would be valuable in this

respect. I will therefore focus my comments on the overall concept and interpretation of the results of the study.

Abstract:
It would be useful to indicate clearly in the abstract that the data relate to mouse CD4+ T cells only.

Introduction:
It would help to more clearly spell out the differences in experimental design of the starting material (i.e. the cell culture conditions) between the approach taken in this manuscript and ref 13, as readers who are less familiar may not immediately appreciate how the two studies differentiate.

Results page 9, relating to figure 1d: The authors comments *"[…] expression of Il10 in IL-12+IL-27 and IL-27-driven CD4+ T cells appeared to correlate strongly with expression of Prdm1 as well as Maf over time, while Ifng expression appeared to be repressed at peak times under these conditions…".* I think that based on the data in Figure 1d, 'repressed' is too strong a word to use; 'reduced' seems to be more fitting.

Figure 4: the authors state "*Th1 cells differentiated with IL-12+IL-27 and IL-27-driven T cells showed significant levels of Il10 expression, which was diminished in the absence of Prdm1 or Maf, and to the greatest extent in the absence of both transcription factors (Figure 4a–c).*" I would suggest to remove the final part "and to the greatest extent in the absence of both transcription factors" as this is not that obvious from the actual figure: when comparing Fig 4a and 4b the data for IL10 are virtually similar for the double KO and the Prdm1 single KO cells.

Figures 5 & 6: these figures are the result from highly complex bioinformatical analysis approaches and I found both the narrative and the depiction difficult to understand and interpret. Whilst the complexity and sophistication of the approach are to be applauded and with the appreciation that for some readers these figures will contain valuable information and data, this reviewer wonders to what extent these sections help versus hinder the clarity of the manuscript. In other words, are the analysis and its associated narrative and depictions essential to go from Figure 4 to Figure 7? Could the information perhaps be shortened and placed as Suppl data instead?

Figure 11: the added value is not entirely clear to this reviewer, and given that the narrative on this figure is rather short, I wonder if this could be removed.

Discussion:
It would be very informative if the authors could add a specific section in the discussion highlighting to what extent c-Maf and Blimp-1 are known to regulate *IL10* and *IFNG* expression in human CD4+ T cells, under similar conditions or otherwise, and/or what the gaps in knowledge are in this context with respect to human CD4+ T cells.

There are occasions where the authors a bit too strongly exclude a role for certain transcription factors that previously were found to regulate IL10 expression. An example can be found here (discussion page 22): "*Likewise, the transcription factor Batf, which has been previously reported to regulate Il10 expression in differentiating Th2 cells was actually downregulated in both IL-12 plus IL-27*

and IL-27 alone differentiation conditions, whilst increasing under IL-12 alone differentiation conditions, and showed very poor correlation with Il10 expression. Thus, as discussed earlier for IL-21, it is likely that a number of these transcription factors are required for the differentiation and possibly proliferation of T helper cells rather than for direct positive regulation of Il10 gene expression."

It is also possible that these other transcriptional regulators have the capacity to regulate Il10, but that this is cell and/or context dependent, i.e. depending on the naïve/effector state of the cell, their polarisation or differentiation. In other words, it would be helpful to acknowledge that the regulation of Il10 and Ifng by Blimp-1 and c-Maf as described in the manuscript is tested specifically and solely in the context of the two cytokines and their combination in mouse naïve CD4+ T cells.

Finally, a short section on the limitations of this study would be helpful, i.e. acknowledge that this work is all done at the transcriptional level, and that it has not been formally tested that Blimp-1/c-Maf overexpression alone or in combination leads to direct upregulation of IL-10 and downregulation of IFNg at the protein level.

Minor:
Page 3: the following sentence is very long and not very clear: "Although IL-27 regulates the effector function of IL-12 driven Th1 cells, producing high levels of IFN-γ, by inducing the production of IL-10 to limit and control Th1-mediated immunopathology in vivo12,14,15,27, the mechanisms of transcriptional regulation of Il10 and proinflammatory cytokines in Th1 cells are unclear."

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunoregulation (wet-lab based) with limited expertise in bioinformatic analysis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Nov 2023

Anne O'Garra

Leonie Taams King's College London, London, England, UK

This manuscript compellingly demonstrates that Maf and Blimp1 are transcriptional regulators of both Il10 and Ifng gene expression during mouse naïve CD4+ T cell differentiation in the presence of IL-12 and IL-27. Whilst both Maf and Blimp1 had been associated previously with positive regulation of Il10 expression, the results presented here demonstrate that they in parallel negatively regulate Ifng, as well as other Th1-related effector molecules including Il2 and Id2. The data presented in the paper consist of RNAseq, ATACseq and qPCR data of naïve CD4+ T cells differentiated in the absence or presence of IL-12, IL-27 or IL-12 plus IL-27, using cells from either wt or from CD4+ T cell-conditional KO mice for Maf, Prdm1 or the combination. The authors employ an impressive range of sophisticated and complex computational modelling tools to analyse the datasets. This reviewer is not qualified to comment on these tools or the analysis approach; input from a qualified bioinformatician expert would be valuable in this respect. I will therefore focus my comments on the overall concept and interpretation of the results of the study.

Abstract: It would be useful to indicate clearly in the abstract that the data relate to mouse CD4+ T cells only. **Thank you this has been corrected.**

Introduction: It would help to more clearly spell out the differences in experimental design of the starting material (i.e. the cell culture conditions) between the approach taken in this manuscript and ref 13, as readers who are less familiar may not immediately appreciate how the two studies differentiate. ***Thank you to the Reviewer, this is a good point and we have introduced text at appropriate stages of the Introduction and Results to indicate what is different and thus what are the advances of our study over the published Ref 13 study. Firstly, we have rearranged the Introduction, to spell out the question at the end of the first paragraph regarding the regulation of Il10 expression in Th1 cells and re-writing the lines to spell out our question more clearly; this text had been originally included at the end of the paragraph describing the findings of Ref. 13, but is better placed here at the beginning of our Introduction: "The transcription factors that regulate the differentiation and function of Th1 cells producing Ifng are well established, including STAT molecules and T-bet, however, whether these transcription factors regulate Il10 expression as part of the differentiation pathway of Th1 cells has been difficult to decipher (Gabrysova et al., 2014, Current Topics Micro&Imm; Currently Reference 9:). Whether transcription factors such as Prdm1 or Maf, which positively induce Il10 expression, do so as part of the differentiation pathway of Th1 cells, or alternatively, simultaneously negatively regulate proinflammatory cytokines in Th1 cells, therefore not contributing to Th1 differentiation but instead reinforcing a regulated Th1 response, is unclear (Gabrysova, ref 9, 2014; Gabrysova, Ref. 16, 2018)".*** **Then at the end of the 3rd paragraph describing the Ref 13 study we have now**

added this line: "The role of *Prdm1* and *Maf* in regulating *Il10* and proinflammatory cytokines in IL-10-producing Th1 cells has as yet not been reported." And in the last paragraph of the Introduction, we have added lines to the text (shown in *bold italics below*), within the original final paragraph of the Introduction, to clarify our approach and therefore our novel findings: "In this study we applied computational analysis of gene regulation derived from temporal profiling of gene expression clusters integrated with analysis of active genomic regions in CD4 $^{+}$ Th1 effector cells differentiated with IL-27 ***plus IL-12, which express Il10 together with proinflammatory cytokines. Gene expression and active genomic region analysis data was compared to T cells differentiated in IL-27 alone (named "Tr1 cells" [13]), which express Il10 but no proinflammatory cytokines, or conversely IL-12 alone, which express proinflammatory cytokines but not Il10, all compared to medium control, which do not express cytokines. The aim was to elucidate the transcriptional network of genes controlling expression of Il10 and pro-inflammatory effector genes in Th1 cells, and identify transcription factors that not only induced Il10, and additionally negatively regulated Th1 proinflammatory gene expression, and were therefore not part of the Th1 differentiation pathway*.** We show that the transcription factors Blimp-1 and c-Maf each have unique and common effects on cytokine gene regulation and not only co-operate to induce *Il10* gene expression in IL-12 plus IL-27 differentiated Th1 cells, but additionally directly negatively regulate key proinflammatory cytokines including *Ifng*, thus providing mechanisms for reinforcing the regulation of Th1 cell responses. Thus, Blimp-1 and c-Maf positively and negatively regulate a network of both unique and common anti-inflammatory and pro-inflammatory genes to reinforce a Th1 response that will allow eradication of pathogens with minimum immunopathology. " ***We have also now clarified our culture conditions at the beginning of our Results section, which together with our rationale now provided at the end of the Introduction we believe now clarifies our approach and its difference with that of the Ref 13 study – leading then to novel findings: "To achieve this, we first analysed changes in temporal gene expression in vitro in naïve CD4 $^{+}$ T cells stimulated with anti-CD3 and anti-CD28 as described in the Methods, and differentiated these over time with IL-27 plus IL-12 into CD4 $^{+}$ Th1 effector cells which express Il10 together with proinflammatory cytokines such as Ifng, and compared to T cells differentiated in IL-27 alone (named "Tr1 cells" [13]), which express Il10 but no proinflammatory cytokines, or conversely IL-12 alone, which express proinflammatory cytokines including Ifng but not Il10, all compared to medium control, which do not express cytokines."***

Results page 9, relating to figure 1d: The authors comments *"[…] expression of Il10 in IL-12+IL-27 and IL-27-driven CD4+ T cells appeared to correlate strongly with expression of Prdm1 as well as Maf over time, while Ifng expression appeared to be repressed at peak times under these conditions…".* I think that based on the data in Figure 1d, 'repressed' is too strong a word to use; 'reduced' seems to be more fitting. ***Thank you – we have changed the word "repressed" to "reduced".***

Figure 4: the authors state "*Th1 cells differentiated with IL-12+IL-27 and IL-27-driven T cells showed significant levels of Il10 expression, which was diminished in the absence of Prdm1 or Maf, and to the greatest extent in the absence of both transcription factors (Figure 4a–c).*" I would suggest to remove the final part "and to the greatest extent in the absence of both transcription factors" as this is not that obvious from the actual figure: when comparing Fig

4a and 4b the data for IL10 are virtually similar for the double KO and the Prdm1 single KO cells. ***We thank the Reviewer, we have removed "and to the greatest extent in the absence of both transcription factors" from the "final part "of this sentence***.

Figures 5 & 6: these figures are the result from highly complex bioinformatical analysis approaches and I found both the narrative and the depiction difficult to understand and interpret. Whilst the complexity and sophistication of the approach are to be applauded and with the appreciation that for some readers these figures will contain valuable information and data, this reviewer wonders to what extent these sections help versus hinder the clarity of the manuscript. In other words, are the analysis and its associated narrative and depictions essential to go from Figure 4 to Figure 7? Could the information perhaps be shortened and placed as Suppl data instead? ***Unfortunately, Wellcome Open Research does not allow for Supplementary Figures and hence the inclusion here in Main figures. We believe that these data are important to keep in the manuscript since they provide supporting data with respect to firstly the genes reduced in the different transcription factor deleted T cells (Figure 5). Also the clusters in Figure 5 are of fundamental importance to Figures 10 and 11. In fact we have added extra text on the rationale for why specific clusters were chosen, (from Figure 5g and 5j) for depiction as gene regulatory networks in Figures 10 and 11, which is because these clusters contained direct targets of Blimp-1 and/or c-Maf and were the most affected at the RNA expression level upon Cd4Cre-mediated deletion of Prdm1, Maf, or both Prdm1 and Maf eg. for IL-12+IL-27 found in Clusters 2,1,7 in Figure 5g; and for IL-27 alone found in Clusters 5, 7, 1 in Figure 5j, We now provide an explanation at the beginning of the data regarding Figures 10 and 11 as to why these clusters were selected which shows the importance of keeping Figure 5, although we have however simplified Figure 5 by removing Fig 5g-i. "Specific clusters from the RNA expression data (Figure 5g and 5j) were chosen for our depiction as gene regulatory networks in Figures 10 and 11, because these clusters contained direct targets of Blimp-1 and/or c-Maf that were the most affected at the RNA expression level upon Cd4Cre-mediated deletion of Prdm1, Maf, or both Prdm1 and Maf eg. for IL-12+IL-27 found in Clusters 2,1,7 in Figure 5g; and for IL-27 alone found in Clusters 5, 7, 1 in Figure 5j." Secondly, we believe the pathways involved (Figure 6), which were discussed at various sections of the text, should be kept especially since Wellcome Open Research does not allow Supplementary Figures, or data not shown, and we believe these Figures will be of value to researchers studying this specific area of transcriptional regulation of cytokines, especially since the manuscript is submitted as a Resource, and will point them to data which they could further analyse which will be deposited in the GEO for public use.***

Figure 11: the added value is not entirely clear to this reviewer, and given that the narrative on this figure is rather short, I wonder if this could be removed. **The reason for including Figure 11 is to compare with Ref 13, Zhang et al., 2020, which we did describe and discuss in the text – since those in Figure 10 were distinct from those described in Ref. 13, again this was discussed. Again we believe it is important to keep this figure and text since *Wellcome Open Research does not allow Supplementary Figures, or data not shown, and especially since the manuscript is submitted as a Resource, which could be further analysed as all will be deposited in the GEO for public use, but showing the figures is essential to guide the reader to the GEO data, as well as for the content in itself.***

Discussion: It would be very informative if the authors could add a specific section in the discussion highlighting to what extent c-Maf and Blimp-1 are known to regulate *IL10* and *IFNG* expression in human CD4+ T cells, under similar conditions or otherwise, and/or what the gaps in knowledge are in this context with respect to human CD4+ T cells. **We have added the paragraph below to the Discussion, with the listed references – to be included - as marked in the text - by Editorial office.** ***Relevant to our study, in recent years Blimp-1 and c-Maf have been associated with transcriptional signatures from human disease such as colitis and rheumatoid arthritis (Mijnheer, 2021; Ellinghaus, 2013). The conserved nature of these transcription factors between mouse and humans suggests similar transcriptional mechanisms for cytokine gene regulation operate in mouse and humans. In support of this, SNPs in Blimp-1 have been associated with elevated IFN-g expression in colitis patients (Ellinghaus, 2013) and Blimp-1 has been shown to bind conserved CNS sites in human and mouse in the Ifng/IFNG and Tbx21/TBX21 loci in T cells and NK cells (Cimmino, 2008; Smith, 2010). Likewise, c-Maf has been shown to be regulated by the cholesterol pathway and regulate IL10 expression by human Th1 cells (Perucha, 2019) in keeping with regulation of Il10 expression in mouse Th1 cells as we report herein. Very recently both Blimp-1 and c-Maf have been reported to co-regulate CD4^{+} T cell derived IL-10 in Crohn's patients (Ahlers, 2022) and identified in Th1/Tr1 cells from malaria patients (Nifeffer, 2023; Edwards, 2023). However, the exact dynamic both Blimp-1 and c-Maf play in the regulation of the expression of cytokines, and other important inflammatory genes needs further research, particularly given that there is a lack of ChIP-seq datasets from individual Th-cell subsets for both of these transcription factors in both mouse and human.***

- Mijnheer, G. *et al.* Conserved human effector Treg cell transcriptomic and epigenetic signature in arthritic joint inflammation. *Nat Commun* **12**, 2710, doi:10.1038/s41467-021-22975-7 (2021).
- Ellinghaus, D. *et al.* Association between variants of PRDM1 and NDP52 and Crohn's disease, based on exome sequencing and functional studies. *Gastroenterology* **145**, 339-347, doi:10.1053/j.gastro.2013.04.040 (2013).
- Cimmino, L. *et al.* Blimp-1 Attenuates Th1 Differentiation by Repression of ifng, tbx21, and bcl6 gene expression. *The Journal of Immunology* **181**, 2338-2347, doi:10.4049/jimmunol.181.4.2338 (2008). **Was referenced in first submission, needs re-referencing.**
- 21 Cimmino L, Martins GA, Liao J, et al.: Blimp-1 Attenuates Th1 Differentiation by Repression of *ifng, tbx21*, and *bcl6* gene expression. *J Immunol.* 2008;181(4):2338–2347. 18684923 10.4049/jimmunol.181.4.2338
- Smith, M. A. *et al.* PRDM1/Blimp-1 controls effector cytokine production in human NK cells. *J Immunol* **185**, 6058-6067, doi:10.4049/jimmunol.1001682 (2010).
- Perucha, E. *et al.* The cholesterol biosynthesis pathway regulates IL-10 expression in human Th1 cells. *Nat Commun* **10**, 498, doi:10.1038/s41467-019-08332-9 (2019).**Was referenced in first submission – needs re-referencing.**
- 74 Perucha E, Melchiotti R, Bibby JA, et al.: The cholesterol biosynthesis pathway regulates IL-10 expression in human Th1 cells. *Nat Commun.* 2019;10(1): 498. 30700717 10.1038/s41467-019-08332-9 6353904
- Ahlers, J. *et al.* A Notch/STAT3-driven Blimp-1/c-Maf-dependent molecular switch induces IL-10 expression in human CD4(+) T cells and is defective in Crohn s disease patients. *Mucosal Immunol* **15**, 480-490, doi:10.1038/s41385-022-00487-x (2022).

- Edwards, C. L. *et al.* IL-10-producing Th1 cells possess a distinct molecular signature in malaria. *J Clin Invest* **133**, doi:10.1172/JCI153733 (2023).
- Nideffer, J. & Jagannathan, P. Type I regulatory T cells in malaria: of mice and men. *J Clin Invest* **133**, doi:10.1172/JCI166019 (2023).

There are occasions where the authors a bit too strongly exclude a role for certain transcription factors that previously were found to regulate IL10 expression. An example can be found here (discussion page 22): "*Likewise, the transcription factor Batf, which has been previously reported to regulate Il10 expression in differentiating Th2 cells was actually downregulated in both IL-12 plus IL-27 and IL-27 alone differentiation conditions, whilst increasing under IL-12 alone differentiation conditions, and showed very poor correlation with Il10 expression. Thus, as discussed earlier for IL-21, it is likely that a number of these transcription factors are required for the differentiation and possibly proliferation of T helper cells rather than for direct positive regulation of Il10 gene expression."* It is also possible that these other transcriptional regulators have the capacity to regulate Il10, but that this is cell and/or context dependent, i.e. depending on the naïve/effector state of the cell, their polarisation or differentiation. In other words, it would be helpful to acknowledge that the regulation of Il10 and Ifng by Blimp-1 and c-Maf as described in the manuscript is tested specifically and solely in the context of the two cytokines and their combination in mouse naïve CD4+ T cells. ***We thank the reviewer for pointing this out. We have amended the text in the Discussion accordingly: "Alternatively, the capacity of these transcription factors to regulate Il10 may be cell and/or context dependent, for example regulating Il10 in some effector T cells and not others, as previously discussed (Gabrysova, ref 9, 2014; Gabrysova, Ref. 16, 2018).[AO1] "***

Finally, a short section on the limitations of this study would be helpful, i.e. acknowledge that this work is all done at the transcriptional level, and that it has not been formally tested that Blimp-1/c- Maf overexpression alone or in combination leads to direct upregulation of IL-10 and downregulation of IFNg at the protein level. ***We thank the Reviewer for this suggestion. We have added the following text to our Discussion to address this issue. "A limitation of this study is firstly that the regulation of Il10 versus proinflammatory cytokines by Blimp-1 and c-Maf was firstly only demonstrated at a transcriptional level and consequent effects on protein production were not the focus of the study. Secondly, the effects of T cell-specific deletion of Prdm1 and Maf on Il10 and proinflammatory cytokine expression was only investigated in vitro. Our continuing studies will further address the role of Blimp-1 and c-Maf in regulation of cytokine responses in vivo, and the physiological consequences of T cell-specific deletion of these transcription factors in response to pathogens and/or pathobionts".***

Minor: Page 3: the following sentence is very long and not very clear: "Although IL-27 regulates the effector function of IL-12 driven Th1 cells, producing high levels of IFN-γ, by inducing the production of IL-10 to limit and control Th1-mediated immunopathology in vivo12,14,15,27, the mechanisms of transcriptional regulation of Il10 and proinflammatory cytokines in Th1 cells are unclear." ***We thank the Reviewer for pointing this out. We have shortened this sentence into two sentences as suggested but also added more text to answer issues raised by the other Reviewer regarding the latter half of this sentence and the question being addressed in this study.*** **Is the work clearly and accurately presented and does it cite the current literature?** Partly ***We have added more text to clarify the***

questions being addressed in this study in the Introdcution. We have added a paragraph to the Discussion, with reference to the literature in human, with the listed references – to be added by Editorial office. The added text to the Discussion to address this issue is as follows "Relevant to our study, in recent years Blimp-1 and c-Maf have been associated with transcriptional signatures from human disease such as colitis and rheumatoid arthritis (Mijnheer, 2021; Ellinghaus, 2013). The conserved nature of these transcription factors between mouse and humans suggests similar transcriptional mechanisms for cytokine gene regulation operate in mouse and humans. In support of this, SNPs in Blimp-1 have been associated with elevated IFN-g expression in colitis patients (Ellinghaus, 2013) and Blimp-1 has been shown to bind conserved CNS sites in human and mouse in the Ifng/IFNG and Tbx21/TBX21 loci in T cells and NK cells (Cimmino, 2008; Smith, 2010). Likewise, c-Maf has been shown to be regulated by the cholesterol pathway and regulate IL10 expression by human Th1 cells (Perucha, 2019) in keeping with regulation of Il10 expression in mouse Th1 cells as we report herein. Very recently both Blimp-1 and c-Maf have been reported to co-regulate $CD4^+$ T cell derived IL-10 in Crohn's patients (Ahlers, 2022) and identified in Th1/Tr1 cells from malaria patients (Nifeffer, 2023; Edwards, 2023). However, the exact dynamic role of both Blimp-1 and c-Maf play in the regulation of the expression of cytokines, and other important inflammatory genes needs further research."

Competing Interests: None