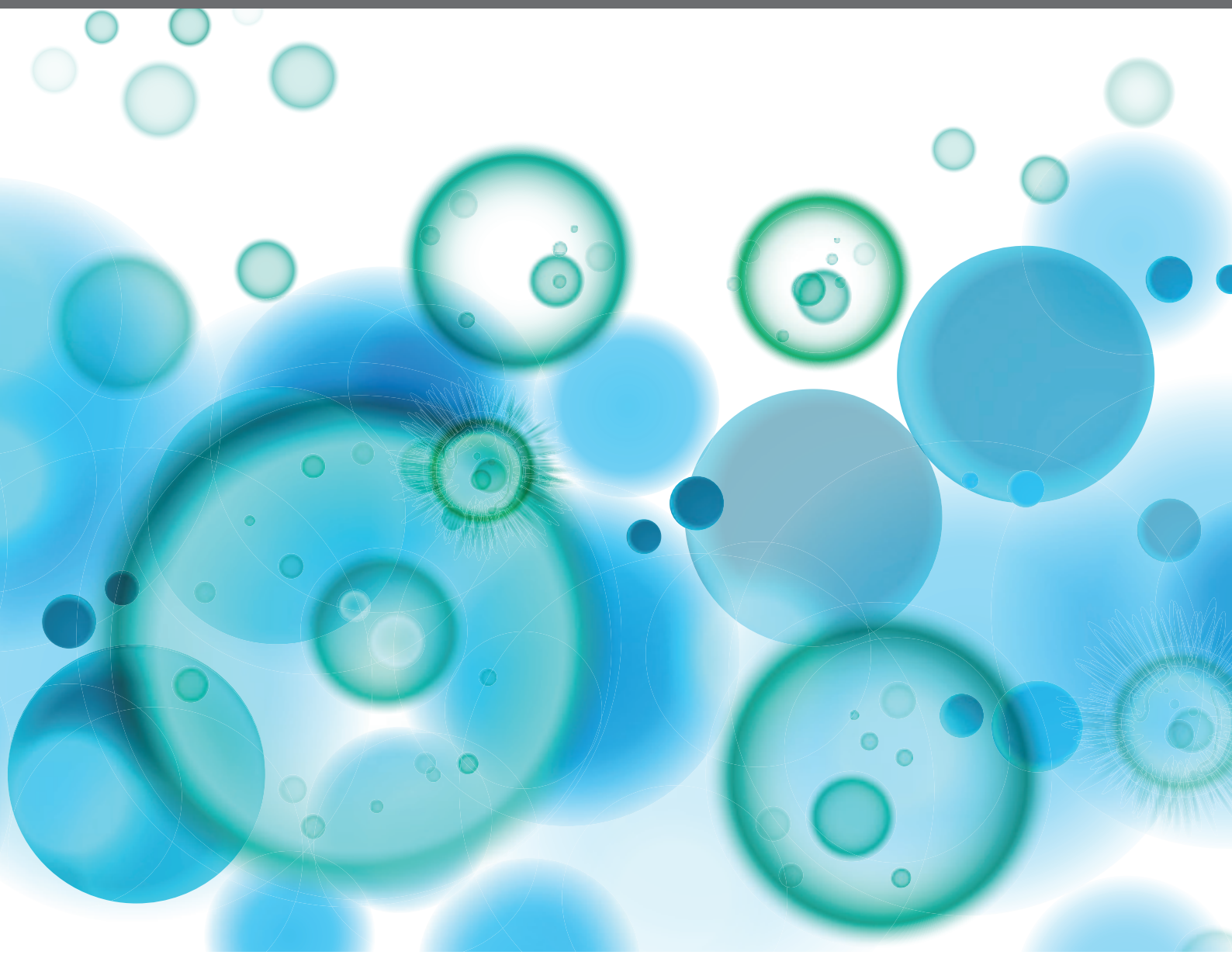
# TRANSCRIPTIONAL AND CHROMATIN REGULATION IN ADAPTIVE AND INNATE IMMUNE CELLS

EDITED BY: Keiko Ozato and Dinah S. Singer
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-723-2
DOI 10.3389/978-2-88963-723-2

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# TRANSCRIPTIONAL AND CHROMATIN REGULATION IN ADAPTIVE AND INNATE IMMUNE CELLS

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Transcription depends on an ordered sequence of events, starting with (i) setting of the enhancer and chromatin environment, (ii) assembly of DNA binding and general transcription factors, (iii) initiation, elongation, processing of mRNA and termination, followed by (iv) creation of epigenetic marks and memory formation. Highlighting the importance of these activities, more than 10% total genes are dedicated to regulating transcriptional mechanisms. This area of research is highly active and new insights are continuously being added to our knowledge.

Cells of the immune system have unique features of gene regulation to support diverse tasks required for innate and adaptive immunity. Innate immunity involves the recognition of external infectious and noxious agents as well as internal cancer cell components, and the elimination of these agents by non-specific mechanisms. Adaptive immunity involves gene rearrangement to achieve highly specific T and B cell responses, imparting the capability of self and non-self discrimination. This requires transcription and epigenetic regulation. Adaptive immunity also employs epigenetic memory, enabling recapitulation of prior transcription. Recent advances in nuclear architecture, chromatin structure, and transcriptional regulation have provided new insights into immune responses. The increased understanding of these molecular mechanisms is now affording opportunities to improve therapeutic strategies for various diseases.

**Citation:** Ozato, K., Singer, D. S., eds. (2020). Transcriptional and Chromatin Regulation in Adaptive and Innate Immune Cells. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-723-2

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frontiers in Immunology

ORIGINAL RESEARCH
published: 10 April 2018
doi: 10.3389/fimmu.2018.00750

# Allergic $T_H2$ Response Governed by B-Cell Lymphoma 6 Function in Naturally Occurring Memory Phenotype CD4$^+$ T Cells

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OPEN ACCESS

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**Specialty section:**
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

**Received:** 11 November 2017
**Accepted:** 26 March 2018
**Published:** 10 April 2018

**Citation:**
Ogasawara T, Kohashi Y, Ikari J, Taniguchi T, Tsuruoka N, Watanabe-Takano H, Fujimura L, Sakamoto A, Hatano M, Hirata H, Fukushima Y, Fukuda T, Kurasawa K, Tatsumi K, Tokuhisa T and Arima M (2018) Allergic $T_H2$ Response Governed by B-Cell Lymphoma 6 Function in Naturally Occurring Memory Phenotype CD4$^+$ T Cells. Front. Immunol. 9:750. doi: 10.3389/fimmu.2018.00750

Transcriptional repressor B-cell lymphoma 6 (Bcl6) appears to regulate $T_H2$ immune responses in allergies, but its precise role is unclear. We previously reported that Bcl6 suppressed IL-4 production in naïve CD4$^+$ T cell-derived memory $T_H2$ cells. To investigate Bcl6 function in allergic responses in naturally occurring memory phenotype CD4$^+$ T (MPT) cells and their derived $T_H2$ ($MPT_H2$) cells, *Bcl6*-manipulated *mice*, highly conserved intron enhancer (hcIE)-deficient *mice*, and reporter mice for conserved noncoding sequence 2 (CNS2) 3′ distal enhancer region were used to elucidate Bcl6 function in MPT cells. The molecular mechanisms of Bcl6-mediated $T_H2$ cytokine gene regulation were elucidated using cellular and molecular approaches. Bcl6 function in MPT cells was determined using adoptive transfer to naïve mice, which were assessed for allergic airway inflammation. Bcl6 suppressed IL-4 production in MPT and $MPT_H2$ cells by suppressing CNS2 enhancer activity. Bcl6 downregulated *Il4* expression in $MPT_H2$ cells, but not MPT cells, by suppressing hcIE activity. The inhibitory functions of Bcl6 in MPT and $MPT_H2$ cells attenuated allergic responses. Bcl6 is a critical regulator of IL-4 production by MPT and $MPT_H2$ cells in $T_H2$ immune responses related to the pathogenesis of allergies.

**Keywords: B-cell lymphoma 6, naturally occurring memory phenotype T cells, allergy, $T_H2$ cells, asthma**

**Abbreviations:** Abs, antibodies; APC, antigen-presenting cell; BAL, Bronchoalveolar lavage; BALF, Bronchoalveolar lavage fluid; Bcl6, B-cell lymphoma 6; BS, binding sequence; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; DC, dendritic cell; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; hcIE, highly conserved intron enhancer; HS, DNase hypersensitive site; KO, knockout; LCR, locus control region; LTR, long terminal repeat; mAbs, monoclonal antibodies; MFI, mean fluorescence intensity; MPT cell, memory phenotype CD4$^+$ T cell; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; NAM-$LT_H2$ cell, $NAT_H2$ cell-derived memory-like $T_H2$ cell; $NAMT_H2$ cell, $NAT_H2$ cell-derived memory cell; $NAT_H2$ cell, naïve CD4$^+$ T cell-derived $T_H2$ cell; OVA, ovalbumin; PCR, polymerase chain reaction; STAT, signal transducer and activator of transcription; TCR, T cell receptor; $T_{FH}$ cell, T follicular helper cell; TG, transgenic; WT, wild-type.

## INTRODUCTION

Allergic asthma is an inflammatory airway disorder mediated by $T_H2$ cells, which produce various effector cytokines (IL-4, IL-5, and IL-13) (1, 2). IL-4 induces signal transducer and activator of transcription (STAT) 6 phosphorylation, causing the protein to translocate to the nucleus, where it induces the expression of *Gata3* (3, 4), a key regulator of $T_H2$ cell differentiation. GATA3 facilitates *Il4*, *Il5*, and *Il13* transcription in $T_H2$ cells (3, 4). In mouse and human allergies, IL-4 initiates $T_H2$ responses and IgE isotype class switching, whereas IL-5 and IL-13 are important for eosinophil infiltration/activation and increased airway hyperreactivity in allergic asthma (1, 2).

The proto-oncogene product B-cell lymphoma 6 (Bcl6) is a sequence-specific transcriptional repressor (5–9). Tissue hypereosinophilia occurs with increased IL-4, IL-5, and IL-13 production in B-cell lymphoma 6 (*Bcl6*)-knockout (KO) mice, suggesting that Bcl6 participates in allergy pathogenesis and that it may be important for reducing $T_H2$ immune responses. However, the T cell-intrinsic function of Bcl6 in $T_H2$ cell responses remains unclear. Bcl6-binding DNA sequences resemble STAT protein-bound motifs (10), indicating that Bcl6 may repress $T_H2$ cytokine expression by competitively inhibiting the binding of STAT factors to GAS sites in target genes (5, 11–13), including $T_H2$ cytokine gene loci (14). We previously identified Bcl6/STAT-binding sequences (BSs) (15) in CNS1 (BS1), IL-4 promoter region (BS2), and DNase hypersensitive site 2 (HS2) (BS3, BS4) and HS3 (BS5, BS6) in intron two and the 3′ region of CNS2 (BS7) in the *Il4* locus; BSIL5 sequences in the *Il5* locus (14); and BSIL13 sequences in the *Il13* locus. We, furthermore, reported that Bcl6 repressed *Il4* and *Il5* expression by binding to genomic DNA in naïve $CD4^+$ T cell-derived memory (NAM) $T_H2$ cells (14, 15), identifying Bcl6 as a critical regulator of $T_H2$ cytokine production in memory $CD4^+$ T cells in addition to its role in the maintenance and survival of the cells (15–17). Conversely, T follicular helper ($T_{FH}$) cell differentiation may result from Bcl6-mediated suppression of the differentiation of other $T_H$ cell lineages *in vivo* (18–20). Thus, the role of Bcl6 in the regulation of $T_H2$ cytokine production in pathophysiological settings remains unclear. We focused on a $CD4^+$ T cell subset, namely, naturally occurring memory phenotype $CD4^+$ T (MPT) cells (21–27). These are derived from $CD4^+$ T cells that naturally exhibit memory cell markers ($CD44^{high}$ $CD25^-$ $CD49b^-$) without antigen stimulation, rather than from memory $CD4^+$ T cells differentiated from naïve $CD4^+$ T cells after antigen stimulation. A small subset of MPT cells and their derived $MPT_H2$ cell populations, but not naïve $CD4^+$ T cell-derived $T_H2$ cells ($NAT_H2$ cells), have an active conserved noncoding sequence 2 (CNS2) 3′ distal enhancer region in the *Il4* locus similar to that in natural killer T cells, producing IL-4 without T cell receptor (TCR)-mediated stimulation (28). CNS2-active MPT cells are candidate cells that initially produce IL-4 to promote $T_H2$ cell differentiation, and thus, they may be involved in allergy pathogenesis, although the mechanisms remain unclear. Because Bcl6 expression is extremely high in CNS2-active MPT cells (29), we hypothesized that Bcl6 regulates allergen-mediated MPT cell activation in $T_H2$ cell-dependent allergies.

## MATERIALS AND METHODS

### Antibodies (Abs) and Reagents

Allophycocyanin-conjugated anti-CD4 monoclonal antibody (mAb, GK1.5), anti-IL-4 mAb (11B11), anti-IFN-γ mAb (R4-6A2), anti-CD62L mAb (MEL-14), anti-CD44 mAb (IM7), PE-conjugated anti-IL-4 mAb (BVD4-1D11), PE-conjugated KJ1-26 (anti-clonotypic mAb for DO11.10 TCR, KJ1-26), anti-CD11c mAb (HL3), unconjugated anti-IL-4 mAb (11B11), anti-IL-12 mAb (C17.8), anti-IFN-mAb (R4-6A2), anti-CD44 mAb (IM7), FITC-conjugated anti-CD49b mAb (DX5), and PerCP-conjugated anti-CD4 mAb (GK1.5) were purchased from BD Bioscience. Anti-STAT5 Abs (C-17), anti-STAT6 Abs (N-20), anti-Bcl6 Abs (N-3), anti-tubulin Abs (H-235), and normal rabbit IgG were purchased from Santa Cruz Biotechnology. FITC-conjugated anti-T1/ST2 (IL-33R) mAb (DJ8) was purchased from MD Bioproducts. Mouse rIL-2, rIL-4, rIL-7, rIL-12, and rIL-33 were purchased from PeproTech. Anti-CD3ε mAbs (145-2C11) were purchased from Cedar Lane. Anti-CD28 mAbs (PV-1) were purchased from Southern Biotechnology. The ovalbumin (OVA) peptide (Loh15: residues 323–339; ISQAVHAAHAEINEAGR) was synthesized by BEX Co. Ltd. (Tokyo, Japan). The Bcl6 inhibitory peptide was synthesized by Scrum Inc. (Tokyo, Japan).

### Animals

*Bcl6*-transgenic (TG) mice with exogenous *Bcl6* under Lck proximal promoter control (17, 30), *Bcl6*-KO mice (31), and highly conserved intron enhancer (hcIE)-KO mice on a BALB/c background (Japan SLC) were described previously (15). CNS2-green fluorescent protein (GFP)-TG mice were gifted by Dr. Masato Kubo (28). Some *Bcl6*-TG, *Bcl6*-KO, and hcIE-KO mice were crossed with OVA-specific TCRαβ (DO11.10) and/or CNS2-GFP-TG mice. All mice were used at 8–12 weeks of age.

### $CD4^+$ T Cell Purification and $T_H$ Cell Induction

Naïve $CD44^{low}$ $CD62L^+$ $CD4^+$ T cells, $CD44^{high}$ $CD62L^-$ $CD4^+$ MPT cells, transferred T cells, dendritic cells (DCs), and T cell-deleted splenocytes were isolated from murine spleens using a cell sorter (FACSVantage, BD Biosciences). Sorted T cells ($2 \times 10^5$ cells/mL) from DO11.10 background mice were stimulated with OVA peptides (Loh15) (1 μg/mL) plus irradiated or $CD11C^+$ DCs ($4 \times 10^4$ cells/mL) or splenocytes ($1 \times 10^6$ cells/mL), depleted of $CD4^+$ and $CD8^+$ T cells, and used as antigen-presenting cells (APCs) in the presence of rIL-2 (25 U/mL) ($T_H0$ condition). In addition to primary TCR-mediated stimulation with OVA, stimulation with soluble anti-CD3 (2 μg/mL) and anti-CD28 mAbs (2 μg/mL) was employed for some experiments. For $T_H1$ or $T_H2$ polarization, cells were cultured in the presence of rIL-12 (100 U/mL)/anti-IL-4 mAb (5 μg/mL) or rIL-4 (1,000 U/mL)/anti-IL-12 mAb (10 μg/mL), as previously described (15). In some experiments, anti-IL-4 mAbs or anti-IFN-γ mAbs were added to the $T_H0$ condition cultures. On days 3 and 5, activated naïve T cells and MPT cells were stimulated with rIL-2 (25 U/mL) and rIL-7 (10 U/mL) following primary stimulation. $NAT_H2$ cells were further cultured with IL-7 for 21 days to yield $NAT_H2$

cell-derived memory-like $T_H2$ (NAM-$LT_H2$) cells, which have a functional phenotype similar to $NAT_H2$ cell-derived memory ($NAMT_H2$) cells *in vivo* (15). Some MPT cells were cultured in the presence of IL-33 (0–100 ng/mL) with or without IL-7 for the appropriate times as shown in each experiment prior to analysis of chromatin immunoprecipitation (ChIP) assays and the effect of TCR stimulation on cytokine production.

## Fluorescence-Activated Cell Sorting (FACS) Analysis

As previously described (15, 17), T cells with or without 8 h of restimulation were treated with monensin (2 μM) for the last 3 h, followed by staining with an appropriate combination of FITC-conjugated anti-KJ1-26, APC-conjugated anti-CD44, and PerCP-conjugated anti-CD4 mAbs. For staining, cells were washed once with FACS buffer (PBS with 3% fetal calf serum and 0.1% sodium azide) and then permeabilized with Perm2 (BD Biosciences) for 10 min at room temperature, followed by two washes in FACS buffer. Finally, cells were stained with an appropriate combination of anti-IFN-γ-APC and anti-IL-4-PE for 30 min at room temperature, washed, and resuspended in FACS buffer for analysis.

## Cytokine Concentrations

IL-4, IL-5, and IL-13 levels in the culture supernatants of cells that were stimulated for 48 h in bronchoalveolar lavage fluid (BALF) were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA). IgE anti-OVA Abs were detected using a mouse anti-OVA IgE Antibody Assay Kit (Chondrex, Redmond, WA, USA).

## mRNA Measurements

cDNA synthesized from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) was used for qRT-polymerase chain reaction (PCR) analysis as described previously (15). Real-time PCR was performed in 25 μL reaction volumes containing iQ SYBR-Green Supermix, 200 nM of each primer, and 0.5 μL of cDNA. The PCR cycle parameters were 3 min at 95°C and 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by melting curve analysis. Relative quantification of cytokine mRNA expression was performed using the comparative Ct method. The relative quantification value of the target in stimulated T cells, normalized to the β-actin gene expression level (endogenous control) and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold), where ΔCt = Ct of the target gene − Ct of the endogenous control gene (β-actin) and ΔΔCt = ΔCt of stimulated samples for target gene − ΔCt of the untreated control as a calibrator for the target gene. All data in stimulated T cells were expressed as arbitrary units relative to the expression level in the corresponding unstimulated T cells. The primers were as follows: β-*actin*: 5′-CCAGCCTTCCTTCTTGGGTAT-3′ (forward), 5′-TGGCATAGAGGTCTTTACGGATGT-3′ (reverse); *Il4*: 5′-TCTCGAATGTACCAGGAGCCATATC-3′ (forward), 5′-AGCACCTTGGAAGCCCTACAGA-3′ (reverse); *Il5*: 5′-CGATGAGGCTTCCTGTCCCTA-3′ (forward), 5′-TTGGAATAGCATTTCCACAGTACCC-3′ (reverse); *Il13*: 5′-CAATTGCAATGCCATCTACAGGAC-3′ (forward), 5′-CGAAACAGTTGCTTTGTGTAGCTGA-3′ (reverse); *Gata3*: 5′-AGAGATTTCAGATCTGGGCAATGG-3′ (forward), 5′-CAGGGACTGATTCACAGAGCATGTA-3′ (reverse); *Bcl6*: 5′-CCGGCTCAATAATCTCGTGAA-3′ (forward), 5′-GGTGCATGTAGAGTGGTGAGTGA-3′ (reverse).

## Chromatin Immunoprecipitation

The ChIP assay was performed as previously described (14, 15). Protein and chromatin in $T_H$ cells were cross-linked by adding formaldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA), after which the cells were lysed in SDS lysis buffer. Subsequently, precleared, sonicated chromatin and protein G agarose (Millipore) were incubated with specific Abs for the protein of interest or control IgG (rabbit). Some of the untreated chromatin was used as an input sample. qPCR was used to quantify the DNA region in the immune-precipitated chromatin and the input DNA. Relative ChIP DNA quantification was performed using the comparative Ct method. The Ct value of ChIP DNA was normalized to that of the input DNA using the following equation: ΔCt (normalized ChIP) = Ct (ChIP) − Ct (input). The normalized Ct values were adjusted to the normalized background Ct value (ΔΔCt [ChIP/IgG] = ΔCt [normalized ChIP] − ΔCt [normalized IgG]). ChIP enrichment above the sample specific background was calculated as $2^{-\Delta\Delta Ct\ (ChIP/IgG)}$ and reported as a fold change. The following primers were used for qPCR: *Il5BS*: 5′-TGGGCCTTACTTCTCCGTGTAACT-3′ (forward), 5′-CTCCAGTGACCCTGATACCTGAAT-3′ (reverse); *Il13BS*: 5′-TTCTACTAGCTCGGGACTCTTCCA-3′ (forward), 5′-ATGGACATGACATGGGAAACCCAG-3′ (reverse); *BS1*: 5′-AGGTCCATGGAAGGGACAGATCA-3′ (forward), 5′-CGGATCCTTTCCTGGAATTGCTGA-3′ (reverse); *BS2*: 5′-TCCAATTGGTCTGATTTCACAGGA-3′ (forward), 5′-ACACCAGATTGTCAGTTATTCTGGGC-3′ (reverse); *BS3*: 5′-ACAGATGTGACAGGCTGATAGTGC-3′ (forward), 5′-GGCCTTTCATTCTCAGTGGTGTGT-3′ (reverse); *BS4*: 5′-CCTGGCTTCTGAGATGCAATGAGT-3′ (forward), 5′-GGGTAAGAGGAAAGCCAGCATGA-3′ (reverse); *BS5*: 5′-TTCAAGGATAAGCAAGTGGCAGGC-3′ (forward), 5′-ATTGGAACTAAGCCAGCCGATGGA-3′ (reverse); *BS6*: 5′-CGCCTCTCCTGTAAGGTACACAAT-3′ (forward), 5′-TTGCCTTGCAACCATGAAGACCTG-3′ (reverse); *BS7*: 5′-CACTCACCAATTTGTCTGGAGGCT-3′ (forward) 5′-ATGGTGATCACAGTCCAAGTCCAG-3′ (reverse).

## Retroviral Vectors With a *d2EGFP* Reporter Gene

A genomic fragment of the *Il4* promoter (p) region (positions −751 to +1 relative to the transcription start site, MGI: 96556), hcIE (222 bp), and CNS2 (337 bp) were amplified by PCR. The fragment of the *Il4* promoter region was subcloned upstream of *d2-enhanced GFP* (*d2EGFP*) in the retrovirus vector pBABE delta Bll(−). Fragments of hcIE (222 bp) or CNS2 (337 bp) were subcloned downstream of *d2EGFP* to generate pBABE delta Bll(−)-*Il4*p-*d2EGFP*-hcIE or pBABE delta Bll(−)-*Il4*p-*d2EGFP*-CNS2, respectively. pBABE delta BII(−) is based on pBABEpuro (3) (gifted by Dr. H. von Melchner, University of Frankfurt Medical School). PCR-based mutagenesis of G3 and BS3 in hcIE and BS7 (1) and (2) in CNS2 was achieved using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene).

Specifically, a fragment of *d2EGFP* cDNA was PCR amplified using an *Xho*I-anchored sense primer (underlined) (5′-CCG CTCGAGTCTAGAGGATCCACCGGTCGC-3′) immediately upstream of the *Xba*I site (+258) and an antisense primer with a *Sal*I-anchored antisense primer (underlined) (5′-ACGC GTCGACTCTAGAGTCGCGGCCGCATC-3′) immediately downstream of the *Xba*I site (+1147) of pd2EGFP. The *Xho*I/*Sal*I fragment of *d2EGFP* was subcloned into a T Easy vector (d2EGFP-T vector). The *Eco*RI-digested *d2EGFP* fragment was blunted and subcloned into a blunted *Not*I/*Nco*I-restricted pMX vector (pMX-d2EGFP). A genomic fragment of the *Il4* promoter region was PCR amplified using the *Eco*RI-anchored sense primer (underlined) (5′-GAATTCCTCCACACTGATGCTGTAGTGC-3′) and *Xho*I-anchored antisense primer (underlined) (5′-CTCGAGG CTAACAATGCTGGC-3′). The subcloned *Il4* promoter fragment was then digested with *Eco*RI and *Xho*I and subcloned into the restricted site of pMX-d2EGFP (pMX-*Il4*p-*d2EGFP*). An *Eco*RI and *Sal*I fragment of pMX-*Il4*p-*d2EGFP* was then subcloned into the *EcoR*I/*Sal*I-restricted pBABE delta Bll(−) to generate pBABE delta Bll(−)-*Il4*p-*d2EGFP*. The vector pBABE delta BII(−) is based on pBABEpuro, with further modifications to completely destroy the endogenous transcriptional regulatory sequences within the retroviral long terminal repeat (LTR). R and U5 are the intact R and U5 regions of MMLV, respectively, en. del. U3 is the SIN U3 found in proviral LTRs after integration of the virus into the host genome, and partial LTR denotes a transcription-competent part of the LTR that is used to drive transcription of the genomic viral RNA in the packaging cells. The hcIE genomic fragments were PCR amplified with the *Xho*I-anchored sense primer (underlined) (5′-CCGCTCGAGCCTTTCTGCCTGCTGCTCTG-3′) and *Sal*I-anchored antisense primer (underlined) (5′-ACGCGTCGAC GAAAAGCAGGCAGTCTGGAG-3′).

Conserved noncoding sequence 2 fragments were obtained by PCR using the *Xho*I-anchored sense primer (underlined) (5′-CCGCTCGAGCTGGAGATTAGAAGTGGAGGCT-3′) and *Sal*I-anchored antisense primer (underlined) (5′-ACGC GTCGACTTTCCTGTCCTCGTCTTTTCCAGT-3′). The hcIE and CNS2 fragments were then inserted in *Sal*I-digested pBABE delta Bll(−)-*Il4*p-*d2EGFP* to generate pBABE delta Bll(−)-*Il4*p-*d2EGFP*-hcIE and pBABE delta Bll(−)-*Il4*p-*d2EGFP*-CNS2, respectively, for reporter gene assays. PCR-based mutagenesis of G3 (5′-CTGATAGTG-3′: +1247 to +1255), BS3 (5′-TTCATGGAA-3′: +1328 to +1336) in hcIE, and BS7 (1) (5′-GTTTTTGAA-3′: +12941 to +12949) and BS7 (2) (5′-TTCCTGGA-3′: +13142 to +13149) in CNS2 in the reporter plasmid were generated using a QuickChange XL Site-Directed Mutagenesis Kit according to the manufacturer's instructions. The underlined nucleotides were substitutes for CTAT for G3 and TT for BS3 and BS7 to generate pBABE delta Bll(−)-*Il4*p-*d2EGFP*-hcIE-MutBS3, pBABE delta Bll(−)-*Il4*p-*d2EGFP*-hcIE-MutG3, pBABE delta Bll(−)-*Il4*p-*d2EGFP*-CNS2-MutB7 (1), and pBABE delta Bll(−)-*Il4p-d2EGFP*-CNS2-MutB7 (2), respectively. Successful PCR and mutation were verified by DNA sequencing.

## Retrovirus Infection

Platinum-E packaging cells (32) were transfected with 1–1.5 μg of DNA of a retrovirus construct mixed with 6 μL of Fugene (Boehringer Mannheim). Virus supernatant was concentrated by centrifugation (8,000 × *g*, 16 h) and added to $T_H2$ cell-inducing cultures on day 2. Intracellular cytokine staining or mean fluorescence intensity (MFI) analysis was performed on day 7 as described previously. Infected cells were subjected to FACS analysis of the intracellular fluorescence of d2EGFP 8 h after restimulation with plate-bound anti-CD3 mAbs.

## Western Blot Analysis

*In vitro*-differentiated $T_H2$ cells were lysed with lysis buffer (1% Nonidet P-40, 5% glycerol, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 μg/mL leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/mL pepstatin A, 10 mM $Na_3VO_4$, and 10 mM NaF). For immunoblotting, anti-Bcl6 or anti-β-tubulin Ab was used. Immunoreactive bands were visualized using a Phototope-HRP Western Blot Detection System (Cell Signaling Technology). For quantitative analysis of Western blots, the intensities of individual bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Antigen-Induced Airway Inflammation

### OVA Challenge and Bronchoalveolar lavage (BAL)

$T_H2$ cells ($1.5 \times 10^7$ or $3 \times 10^7$) were injected intravenously into naïve wild-type (WT) BALB/c mice (day 0), followed by intratracheal challenge with 1% OVA solution (50 μL) twice (days 2 and 3), BAL three times (days 2, 7, and 12), and serum collection. On days 4 and 5, the transferred $T_H2$ cells isolated from whole lungs and BALF were collected from the mice by instilling the lungs with 0.5 mL of PBS six times. Sera on day 14 were analyzed for OVA antigen-specific IgE Abs. In another experiment, a mixture of *Bcl6*-WT, *Bcl6*-TG, or *Bcl6*-KO KJ1-26$^+$ MPT ($2 \times 10^6$ cells) and *Bcl6*-WT KJ1-26$^-$ naïve CD4$^+$ T ($5 \times 10^6$ cells) cells were intravenously transferred into BALB/c *nu*/*nu* mice (day 0). Subsequently, mice were sensitized *via* i.p. injection of 10 μg of OVA plus 1 mg of alum twice (days 1 and 6), followed by intratracheal challenge with OVA twice (days 16 and 17). BAL and pathology examination were performed (day 18), and transferred KJ1-26$^-$ cells were isolated from spleens (day 16). The isolated cells were restimulated with plate-bound anti-CD3 mAbs to analyze cytokine production. The BALF supernatant was stored at −80°C. Each cell pellet was resuspended in PBS for counting and subjected to cytospin. Preparations on slides were stained with Diff-Quick (Sysmex International Reagents, Kobe, Japan) for the differential analysis of cell counts. After BAL, lungs were treated with collagenase II (1 mg/mL) for 30 min at 37°C, and leukocytes were isolated on a Percoll gradient.

### Histologic Examination

After BAL, the left lobes of lungs were extracted, washed with PBS, and fixed in 4% formaldehyde in sodium phosphate buffer for more than 2 days at room temperature. After fixation, lungs were embedded in paraffin and stained with hematoxylin and eosin. Images of each tissue section were captured using a Zeiss Axioscope 2 microscope equipped with a video camera (AxioCam ERc5s, Carl Zeiss, Jena, Germany) and processed using Axiovision V.4 software (Carl Zeiss).

## Statistical Analysis

Statistical significance was determined using *t*-tests (two-tailed) for two groups and Tukey–Kramer or Steel–Dwass multiple comparisons tests for three or more groups. *P* values < 0.05 were considered significant.

# RESULTS

## Bcl6 Represses IL-4 Production by MPT Cells

Splenic CNS2-active MPT cells were detected as a GFP$^+$ subpopulation in reporter gene TG mice (CNS2-GFP-TG) on each *Bcl6* genotype background (28) (**Figure 1A**). Unfortunately, offspring from CNS2-GFP-TG mice on the *Bcl6*-KO background could not be obtained (**Figure 1B**). Although the percentages of GFP$^+$ cells were similar between *Bcl6*-TG and *Bcl6*-WT mice (**Figure 1C**), the IL-4$^+$ MPT cell frequency (**Figure 1D**) and MFI of CNS2-GFP in MPT cells (**Figure 1E**) were inversely correlated with Bcl6 levels. GFP$^+$ MPT cells displayed significant *Il4* expression, which was lower in *Bcl6*-TG cells than in WT cells (**Figure 1F**). *Il4* expression was extremely low in the GFP$^-$ population regardless of Bcl6 levels. The absolute numbers and percentages of IL-4$^+$ MPT cells were also negatively associated with Bcl6 levels (**Figure 1G**), whereas the absolute numbers of GFP$^+$ MPT cells (**Figure 1H**) and MPT cells (**Figure 1I**) among all CD4$^+$ T cells

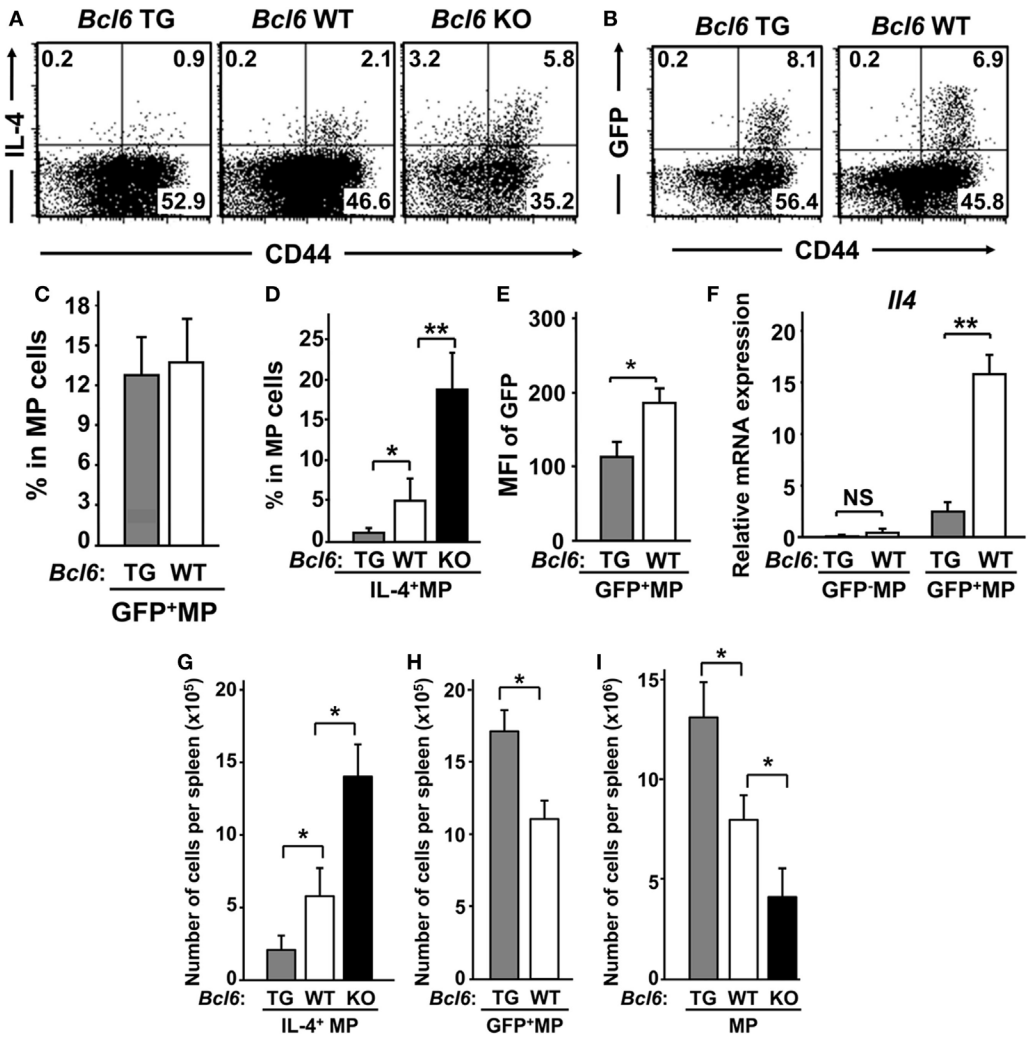


**FIGURE 1** | IL-4$^+$ MPT cells in mice with varying genetic *Bcl6* expression. **(A,B)** FACS analysis of intracellular IL-4$^+$ [**(A)** *Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO] and CNS2-activation-related GFP$^+$ [**(B)** *Bcl6*-TG and *Bcl6*-WT] MPT cells in a CD44$^{high}$ population by gating CD4$^+$ CD49b$^-$ T splenocytes at rest. The presented data are representative of four independent experiments. The numbers in the corners represent the percentages of gated T cells. **(C,D)** Frequency of GFP$^+$ [**(C)** *Bcl6*-TG and *Bcl6*-WT] and IL-4$^+$ [**(D)** *Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO] MPT cells. **(E)** MFI of GFP in MPT cells from *Bcl6*-TG and *Bcl6*-WT mice. **(F)** qRT-PCR analysis of the relative expression of *Il4* in GFP$^-$ and GFP$^+$ MPT cells from *Bcl6*-TG and *Bcl6*-WT spleens. **(G–I)** Absolute cell numbers of populations of IL-4$^+$ [**(G)** *Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO], GFP$^+$ [**(H)** *Bcl6*-TG and *Bcl6*-WT], and total **(I)** MPT cells in one spleen. Data are presented as the mean ± SEM ($n$ = 7–9). $^*P < 0.05$; $^{**}P < 0.01$, comparison between two groups as indicated. Bcl6, B-cell lymphoma 6; CNS, conserved noncoding sequence; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; KO, knockout; MFI, mean fluorescence intensity; MPT cell, memory phenotype CD4$^+$ T cell; NS, not significant; TG, transgenic; WT, wild-type.

were positively correlated with Bcl6 levels. Therefore, Bcl6 may be involved in *Il4* downregulation in MPT cells and MPT cell survival and maintenance. Because it has been reported that the $T_H2$ and $T_H1$ conditions are promotive and inhibitory, respectively, on the maintenance of *Bcl6*-WT CNS2-GFP$^+$ MPT cells (28), we analyzed the effect of Bcl6 on the maintenance of CNS2-GFP$^+$ MPT cells in each culture setting (Figure S1 in Supplementary Material). Regarding the maintenance of GFP$^+$ cells, a promoting effect of the $T_H2$ condition and inhibitory effect of $T_H1$ condition were observed regardless of the *Bcl6* genotype, whereas Bcl6 appears to function as a suppressor for CNS2 activity.

## Bcl6 Represses *Il4* Expression in $T_H2$-Primed MPT Cells

To investigate the function of Bcl6 in the differentiation of MPT cells into $T_H$ cell lineages following TCR stimulation, MPT cells expressing a clonotypic TCR (KJ1-26$^+$) from the spleens of *Bcl6*-TG, *Bcl6*-KO, and *Bcl6*-WT DO11.10 TG mice were cultured under conditions driving them toward the $T_H0$, $T_H1$, or $T_H2$ phenotype, followed by intracellular IL-4 analysis after restimulation with anti-CD3 mAbs (**Figures 2A,B**). Under the $T_H0$ condition, Bcl6 decreased IL-4 production in a concentration-dependent manner, and high Bcl6 expression facilitated IFN-γ induction during

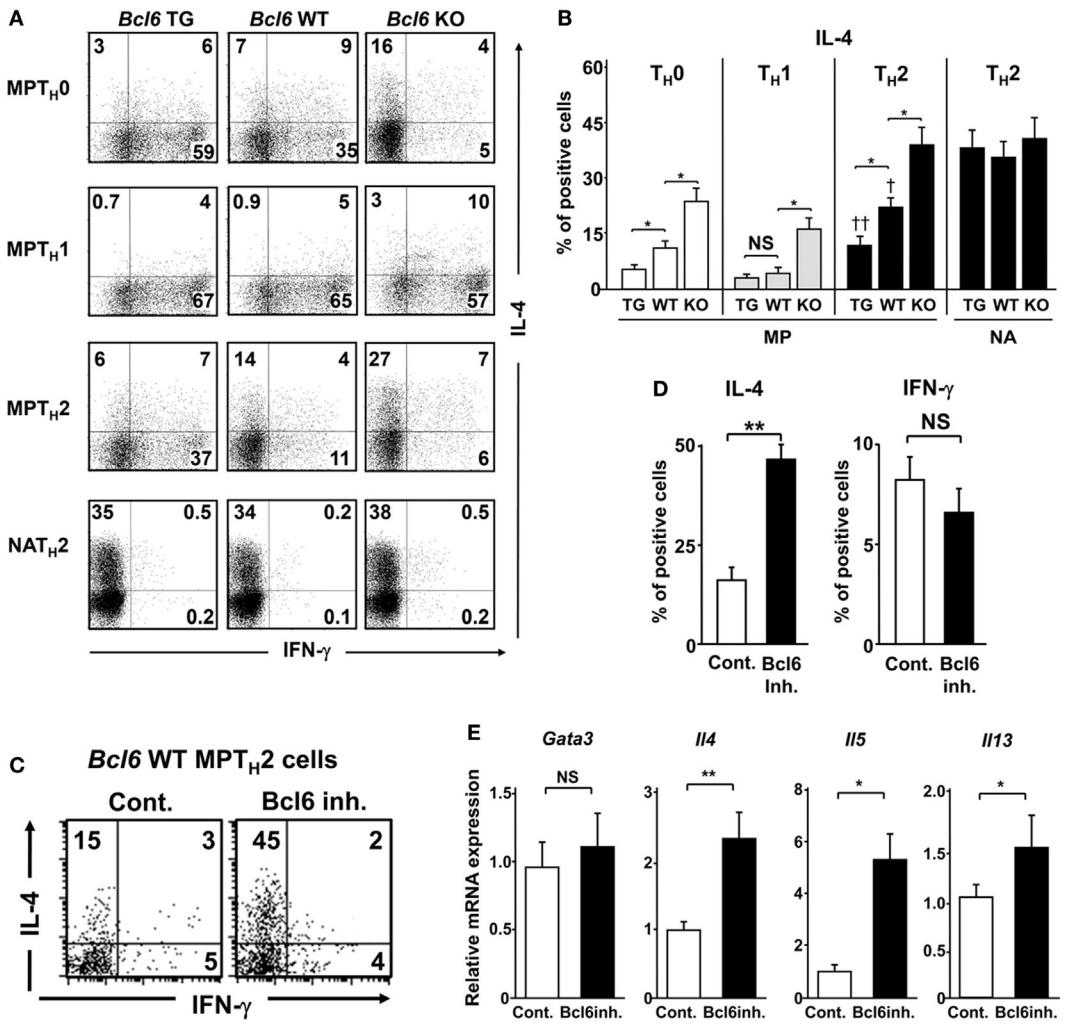


**FIGURE 2** | Regulatory role of Bcl6 in the differentiation of $T_H2$ cells. **(A–E)** KJ1-26$^+$ MPT and NA T cells (CD4$^+$ CD44$^{low}$ CD62L$^+$) among splenocytes from *Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO DO11.10 mice cultured with ovalbumin peptides and antigen-presenting cells *in vitro* for 7 days to produce $T_H0$, $T_H1$, and $T_H2$ cells. Cells were restimulated with anti-CD3 monoclonal antibodies. After 8 h, IL-4- and IFN-γ-producing cells among gated KJ1-26$^+$ CD4$^+$ T cells were analyzed by FACS. **(C–E)** Analysis of cytokine production by *Bcl6*-WT $MPT_H2$ cells treated with a Bcl6 inhibitor (inh.) for 12 h prior to restimulation. **(A,C)** Numbers in the corners represent percentages among gated T cells. **(B,D)** Percentage of IL-4$^+$ (*Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO) cells for each $T_H$ cell type **(B)** and IL-4$^+$ and IFN-γ$^+$ *Bcl6*-WT $MPT_H2$ cells cultured with or without Bcl6 inhibitor **(D)**. **(E)** qRT-PCR analysis of the relative expression of *Gata3*, *Il4*, *Il5*, and *Il13* in restimulated *Bcl6*-WT $MPT_H2$ cells treated with or without a Bcl6 inhibitor. Data are presented as the mean ± SEM ($n$ = 7–8). $^*P < 0.05$, $^{**}P < 0.01$, comparison between two groups is indicated; $^{\dagger}P < 0.05$, compared with *Bcl6*-WT. All results are representative of five independent experiments with similar outcomes, excluding **(C)**, for which four experiments were conducted. Bcl6, B-cell lymphoma 6; Cont., control; FACS, fluorescence-activated cell sorting; KO, knockout; MPT cell, memory phenotype CD4$^+$ T cell; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; NA, naïve; TG, transgenic; WT, wild-type.

$T_H1$ phenotype differentiation. Under the $T_H1$ condition, Bcl6 deficiency in MPT cells preserved IL-4 production, although its level was lower than that under the $T_H0$ condition. Under the $T_H2$ condition, Bcl6 negatively regulated MPT cell-derived $T_H2$ ($MPT_H2$) cell differentiation but not $NAT_H2$ differentiation, as previously reported (15) (**Figures 2A,B**), although Bcl6 could suppress the initial IL-4 production by naïve CD4 T cells under the $T_H0$ condition even when blocking the effects of IFN-γ (Figure S2 in Supplementary Material). Because Bcl6 appears to promote IFN-γ production, which may indirectly affect IL-4 induction, we analyzed a mixed culture of *Bcl6*-WT MPT cells with either *Bcl6*-TG or *Bcl6*-KO cells under the $T_H0$ condition. *Bcl6*-KO MPT cells caused WT cells to skew clearly toward the $T_H2$ phenotype with reduced $T_H1$ skewing, whereas *Bcl6*-TG cells promoted slight $T_H$ skewing (Figure S3 in Supplementary Material), indicating that increased IL-4 production in *Bck6*-KO MPT cells autoaccelerates $T_H2$ cell differentiation by preventing $T_H1$ cell differentiation. Thus, Bcl6 appears to promote IFN-γ production by inhibiting IL-4 production rather than inhibiting IL-4 production *via* the promotion of IFN-γ production.

To confirm the suppressive effects of Bcl6 on $T_H2$ cytokine genes in $MPT_H2$ cells, *Bcl6*-WT $MPT_H2$ cells were treated with a Bcl6 inhibitor (15), followed by restimulation with anti-CD3 mAbs. Bcl6 inhibition augmented IL-4 production but not IFN-γ production (**Figures 2C,D**). $T_H2$ cytokine gene expression was upregulated by the inhibitor without changes in *Gata3* expression (**Figure 2E**), indicating that Bcl6 suppresses *Il4* expression in developing and differentiated $MPT_H2$ cells.

## Bcl6 Negatively Regulates the Histone Modification of $T_H2$ Cytokine Loci in $MPT_H2$ Cells

Because unprimed MPT cells express higher Bcl6 levels than naïve $CD4^+$ T cells (29), *Bcl6* expression levels in the MPT and $MPT_H2$ cells of CNS2-GFP-TG mice with *Bcl6*-WT background were analyzed at rest (**Figure 3A**). *Bcl6* expression in $GFP^+$ MPT and $GFP^-$ $MPT_H2$ cells was increased by sevenfold and threefold, respectively, compared with that in $GFP^+$ $MPT_H2$ cells. $NAT_H2$ cells had markedly lower *Bcl6* expression than $GFP^+$ $MPT_H2$ cells. *Bcl6* expression in $GFP^+$ MPT cells was slightly increased compared with that in $GFP^-$ MPT cells. Consistent with the mRNA levels, Bcl6 protein expression was lower in $GFP^+$ $MPT_H2$ cells than in $GFP^-$ $MPT_H2$ cells (**Figure 3B**). Bcl6 protein levels in MPT cells from *Bcl6*-WT mice were higher than those in $MPT_H2$ cells, whereas the protein levels in $GFP^+$ MPT cells were slightly higher than those in $GFP^-$ MPT cells. To address Bcl6 function, $T_H2$ cytokine production by $MPT_H2$ cells from *Bcl6*-WT-CNS2-GFP-TG mice was analyzed. $T_H2$ cytokine protein (**Figure 3C**) and transcript levels (**Figure 3D**) were significantly greater in the $GFP^+$ population than in the $GFP^-$ population following stimulation, implying that Bcl6 function may be inhibited depending on its quantity and/or quality and that this inhibition may be involved in $T_H2$ cytokine production in $MPT_H2$ cells. Conversely, IFN-γ protein (**Figure 3C**) and transcript levels (**Figure 3D**) were undetectable and minimal, respectively, in both the $GFP^+$ and $GFP^-$ populations. Because Bcl6 binds to BSs (except BSIL13) (**Figure 3E**, top) and thereby reduces $T_H2$ cytokine production in $NAMT_H2$ cells (15), Bcl6 binding to each site in $MPT_H2$ cells was analyzed by ChIP (**Figure 3E**, bottom). In *Bcl6*-WT and *Bcl6*-TG $MPT_H2$ cells, Bcl6 binding was observed at all BS sites excluding BSIL13, BS1, and BS2. $GFP^+$ cells had significantly less Bcl6 binding than $GFP^-$ cells among *Bcl6*-WT and *Bcl6*-TG $MPT_H2$ cells, whereas Bcl6 binding was augmented in *Bcl6*-TG $MPT_H2$ cells. Thus, Bcl6 repressor functions may be regulated qualitatively (e.g., its binding ability) and quantitatively by its binding to $T_H2$ cytokine gene foci. To investigate the effects of Bcl6 of STATs on histone modification in these foci, ChIP was performed for STAT5 and STAT6 binding to BSs and for histone H3 acetylation in $MPT_H2$ cells (**Figure 3F**). STAT6 binding was marginal, whereas STAT5 binding was significantly decreased depending on Bcl6 levels, as indicated by attenuated histone acetylation.

## Bcl6 Represses *Il4* Expression by Binding to CNS2 in $MPT_H2$ Cells

B-cell lymphoma 6, but not STAT proteins, binds to BS7 (**Figures 3D,E**) in the major *Il4* regulatory region. Although no significant Bcl6-mediated interaction was observed between BS7 in CNS2 regarding *Il4* regulation in $NAMT_H2$ cells (15), CNS2 enhancer activity may be suppressed by Bcl6 through BS7 binding. FACS analysis indicated that GFP MFI levels related to CNS2 activation in MPT cells, including at two mutated sites, namely, BS7 (1) and (2) (**Figure 4A**), were inversely correlated with Bcl6 levels (**Figure 1E**). Therefore, the role of Bcl6 in enhancing activity in $MPT_H2$ cells from *Bcl6*-WT or *Bcl6*-KO mice was investigated using a retrovirus reporter gene transfer vector (**Figure 4B**) designed to assess *Il4* promoter (p) activity by measuring the MFI for d2EGFP, a reporter protein, following stimulation with anti-CD3 and anti-CD28 mAbs (**Figures 4C,D**). Additionally, a CNS2 sequence containing WT or mutated BS7, that is, Mu-BS7 (1)-CNS2 and Mu-BS7 (2)-CNS2, were inserted downstream of *d2EGFP* (**Figure 4B**). The MFI for d2EGFP with CNS2-WT elements in *Bcl6*-KO cells was higher than that in *Bcl6*-WT cells. The MFI was augmented by mutations in both BS7 (1) and (2) in *Bcl6*-WT cells, whereas that of *Bcl6*-KO cells was not significantly changed (**Figures 4C,D**). Thus, Bcl6 mediated CNS2 suppression in $MPT_H2$ cells and presumably in unprimed MPT cells.

## Bcl6 Represses *Il4* Expression by Binding to hcIE in $MPT_H2$ Cells

Another *Il4* regulatory region, HS2 (1.2 kbp) located in intron 2, is a critical regulatory region for GATA3 binding-mediated *Il4* expression in $NAT_H2$ cells in HS2-KO mice (33) (**Figure 5A**, top). A 222 bp DNA sequence of the hcIE region (Mouse Genome Informatics accession no. 5897323) (15) including BS3 and the GATA site (G3) in HS2 (**Figure 5A**, top) was studied. *Gata3* expression was low in unprimed MPT cells from *Bcl6*-WT and *Bcl6*-TG mice regardless of CNS2 activation, whereas MPT cells under the $T_H2$ condition exhibited similar *Gata3* gene induction in *Bcl6*-WT and *Bcl6*-TG cells. Gene expression was augmented, particularly in $GFP^+$ cells, and attenuated in a Bcl6-dependent manner in $MPT_H0$ cells. However, further *Gata3* expression

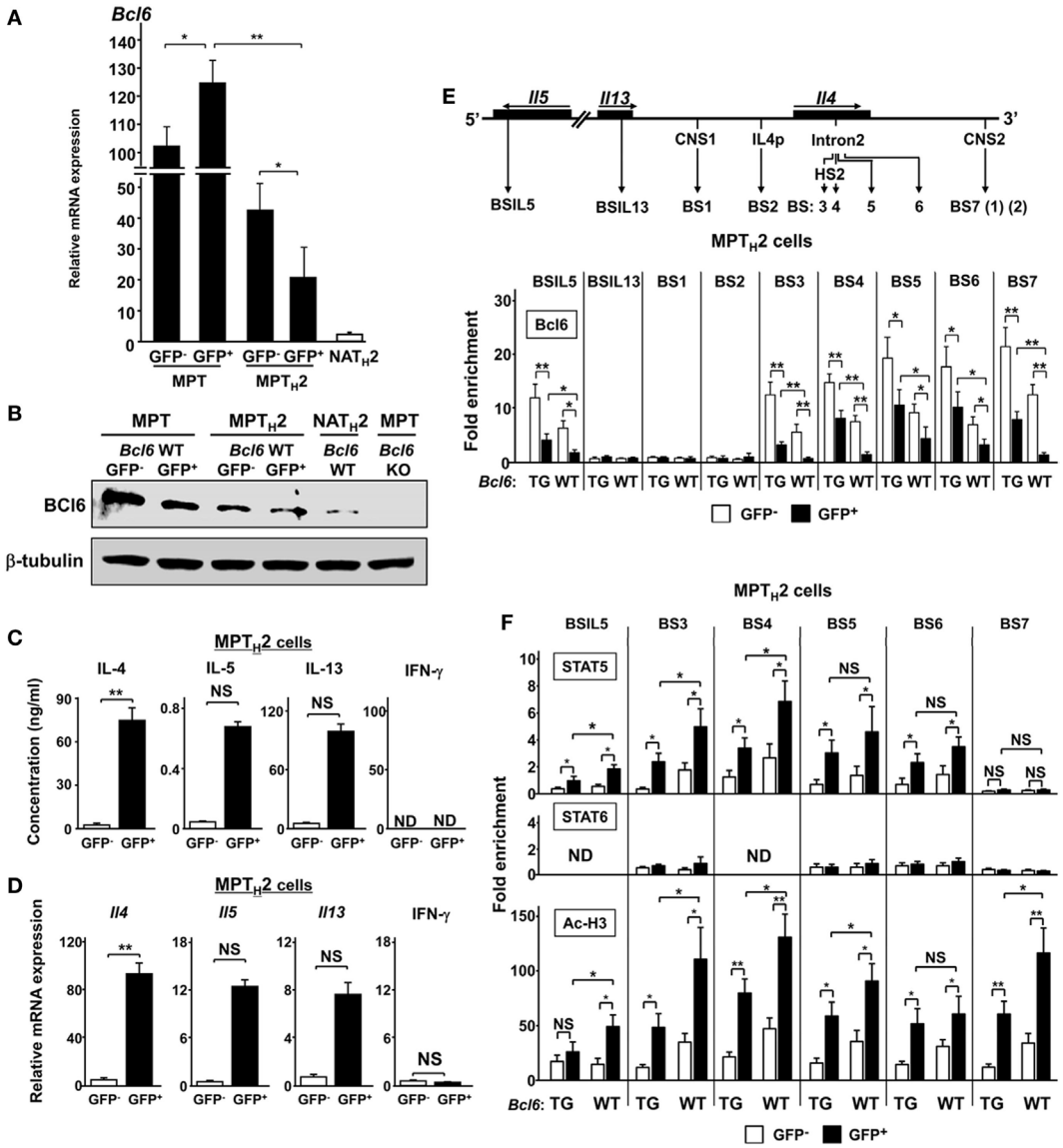


**FIGURE 3** | Role of Bcl6 and signal transducer and activator of transcription (STAT) binding to the *Il4* locus MPT cells. **(A)** *Bcl6* mRNA levels in GFP$^+$ and GFP$^-$ MPT cells, GFP$^+$ and GFP$^-$ MPT$_H$2 cells, and NA T$_H$2 cells, as measured by qRT-PCR. **(B)** Western blot analysis of Bcl6 protein in GFP$^+$ and GFP$^-$ MPT cells MPT (*Bcl6*-WT) and MPT cells (*Bcl6*-KO) in the spleen and GFP$^+$ and GFP$^-$ MPT$_H$2 cells (*Bcl6*-WT). Data are representative of three independent experiments. **(C,D)** KJ1-26$^+$ cells among MPT cells from the spleens of *Bcl6*-WT-CNS2-GFP-TG DO11.10 mice were cultured with ovalbumin peptides and antigen-presenting cells *in vitro* for 7 days under T$_H$2 conditions. Cells were restimulated with anti-CD3 and anti-CD28 monoclonal antibodies. After 48 h, IL-4, IL-5, IL-13, and IFN-γ levels in culture supernatants were measured by ELISA **(C)**. After 8 h, the mRNA levels of *Il4*, *Il5*, *Il13*, and *Ifn*-γ were measured by qRT-PCR **(D)**. [**(E)**, top] Diagram of T$_H$2 cytokine gene loci, with regulatory regions indicated by arrows [CNS, gene promoter regions (p), and Bcl6/STAT (BSs): IL5BS in *Il5*; IL13BS in *Il13* intron 1; BS1 and BS7 (1) (2) in CNS1 and CNS2, respectively; BS2 in *Il4*p; and BS3, BS4, and BS5 in *Il4* intron 2]. **(E,F)** Bcl6 levels [**(E)** bottom], STAT5 and STAT6 binding, and Ac-H3 **(F)** at each BS were analyzed by chromatin immunoprecipitation assay for CNS2-active (GFP$^+$) (closed bar) and CNS2-inactive (GFP$^-$) (open bar) MPT$_H$2 cells. All results are representative of three **(A,C,D)** or four **(E,F)** independent experiments with similar outcomes. Data are presented as the mean ± SEM ($n$ = 7–9). $^*P < 0.05$, $^{**}P < 0.01$, comparison between two groups is indicated. Ac-H3, acetylated histone H3; CNS, conserved noncoding sequence; BS, binding sequence; Bcl6, B-cell lymphoma 6; GFP, green fluorescent protein; KO, knockout; ND, not detected; MPT cell, memory phenotype CD4$^+$ T cell; MPT$_H$2 cell, MPT cell-derived T$_H$2 cell; NA, naïve; TG, transgenic; WT, wild-type.

in MPT$_H$2 cells was not significantly affected by Bcl6 levels (**Figure 5A**, bottom). We investigated the enhancer activity using a reporter construct, uncovering that Bcl6 inhibited hcIE function in MPT$_H$2 cells (Figures S4A–C in Supplementary Material). Similarly, ChIP demonstrated that GATA3 binding to G3 was increased in CNS2-active GFP$^+$ MPT$_H$2 cells compared with that in unprimed GFP$^+$ MPT cells and was significantly attenuated in *Bcl6*-TG background cells (**Figure 5B**). Thus, Bcl6 repressed *Il4* expression by downregulating GATA3-mediated hcIE activity in MPT$_H$2 but not MPT cells.

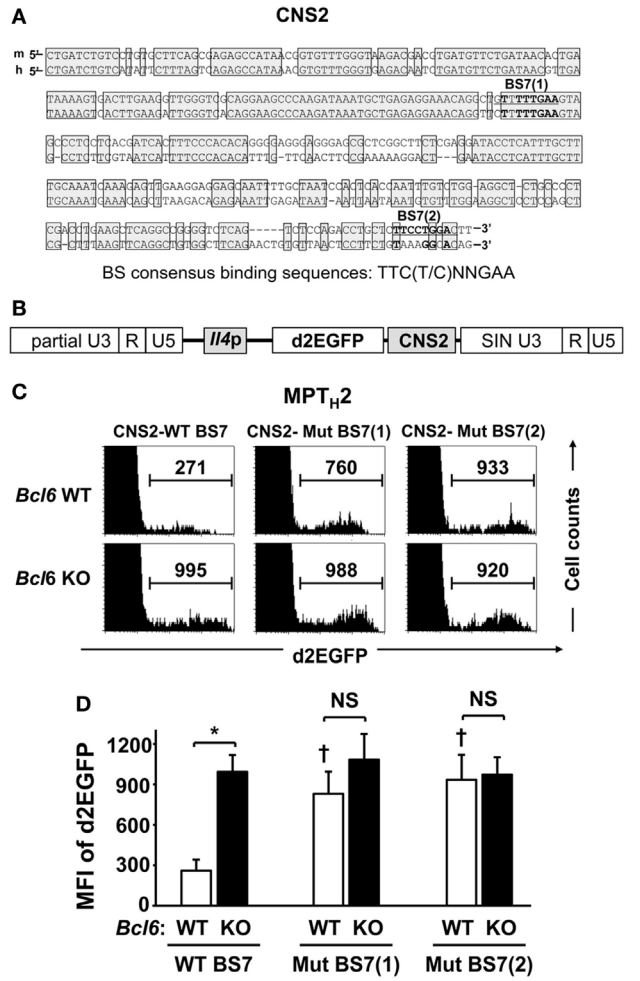


**FIGURE 4** | Role of Bcl6 in the CNS2 enhancer activity of $MPT_H2$ cells. **(A)** A conserved sequence (positions +12805 to +13151 relative to the transcription start site; Mouse Genome Informatics accession no. 96556) in the CNS2 region of mice is shown with human CNS2, including BS7 (1) and (2). Conserved sequences between mice and humans are indicated by shaded boxes. **(B,C)** Splenic *Bcl6*-KO and *Bcl6*-WT MPT cells were cultured under $T_H2$ conditions, and a retrovirus containing the *d2EGFP* reporter gene, with CNS2-WT BS7 **(B)**, CNS2-Mut BS7 (1), or CNS2-Mut BS7 (2), was introduced into $T_H2$ cells on day 2 of culture. After 7 days of culture, cells were restimulated with anti-CD3 monoclonal antibodies for 8 h and subjected to FACS analysis of the intracellular MFI of d2EGFP. **(C)** Histograms of FACS analysis are representative of eight to nine independent experiments. Numbers in each column represent the MFI of d2EGFP. **(D)** Mean values of the MFI of d2EGFP are indicated. Data are presented as the mean ± SEM ($n$ = 8–9). *$P$ < 0.05, comparison between two groups is indicated; †$P$ < 0.05, compared with CNS2-WT. Bcl6, B-cell lymphoma 6; CNS, conserved noncoding sequence; d2EGFP, d2-enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; MPT, memory phenotype $CD4^+$ T; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; Mut, mutant; KO, knockout; WT, wild-type.

To further examine the role of hcIE in $T_H2$ cytokine production, we generated hcIE-KO mice and observed markedly diminished IL-4 production in hcIE-KO $NAT_H2$ and $NAMT_H2$ cells (15). Intracellular cytokine analysis revealed a similar frequency of IL-$4^+$ populations in unprimed MPT cells in WT and hcIE-KO background mice, whereas IL-$4^+$ $MPT_H2$ cell development was impaired without changes in *Gata3* expression following hcIE deletion (**Figures 5C,D**). Bcl6 binding was augmented at BS4, BS5, and BS6 in intron 2 but not at CNS2 (BS7) in hcIE-KO $MPT_H2$ cells compared with that in hcIE-WT background cells (**Figure 5E**), indicating that hcIE activity dampens Bcl6-mediated suppressor activity for intron 2 except at the CNS2 region.

## Bcl6 Suppresses Initial IL-4 Production in MPT Cells and $T_H2$ Cell Differentiation

Because IL-4 production by MPT cells plays an important role in $NAT_H2$ cell differentiation (28), to address the effects of Bcl6 on MPT cell function, *Bcl6*-WT-naïve KJ1-$26^+$ $CD4^+$ T cells were cocultured with KJ1-$26^-$ MPT cells from *Bcl6*-TG, *Bcl6*-KO, or *Bcl6*-WT mice in the presence of soluble anti-CD3 and CD28 mAbs and irradiated $CD11c^+$ DCs as the $T_H0$ condition. Differentiation of IL-4-producing KJ1-$26^+$ $NAT_H2$ cells varied inversely with Bcl6 levels in KJ1-$26^-$ MPT cells, whereas IFN-γ-producing $NAT_H1$ cells differentiated in the opposite direction (**Figures 6A,B**). Because MP cell-derived IFN-γ may affect $NAT_H2$ cell differentiation, we analyzed the $T_H2$ skewing of naïve $CD4^+$ T cells cocultured with MPT cells by excluding the effect of endogenous IFN-γ. Although $T_H2$ skewing became prominent in the coculture in the presence of anti-IFN-γ Abs regardless of the Bcl6 genotype, the skewing was still suppressed in the presence of *Bcl6*-TG MPT cells. Therefore, Bcl6 plays an important role in suppressing MPT cell function to skew naïve $CD4^+$ T cells toward the $T_H2$ phenotype (**Figures 6A,B**). Furthermore, regardless of the *Bcl6* genotype, intrinsic IL-4 in MPT cells was involved in preserving the $T_H2$ cell phenotype (Figures S5A,B in the Supplementary Material).

As CNS2-active MPT cells are essential for inducing $T_H2$ responses following immunization in an allergic murine model (28), we examined Bcl6 function in the MPT cell-induced response during the development of allergic immunity in BALB/c *nu/nu* mice undergoing adoptive transfer of *Bcl6*-WT-naïve $CD4^+$ T cells (KJ1-$26^+$) and MPT cells (KJ1-$26^-$) from each respective *Bcl6* genotype. Following OVA challenge in the mice, the numbers of all inflammatory cells, neutrophils, eosinophils (left), and KJ1-$26^+$ T cells (right) in whole lung tissues were significantly increased, being inversely correlated with Bcl6 levels in the transferred MPT cells (**Figures 7A,B**). In BALF from the recipients, the $T_H2$ cytokine concentrations of IL-4, IL-5, and IL-13, but not IFN-γ, were decreased after the last OVA challenge, with this effect being dependent on Bcl6 levels in the transferred MPT cells (**Figure 7C**). In KJ1-$26^+$ T cells (naïve-derived $T_H$ cells) from the spleens of recipients after the last OVA challenge, $T_H2$ cytokine mRNA expression (*Il4*, *Il5*, and *Il13*) was decreased depending on Bcl6 levels in the transferred MPT cells (**Figure 7D**). OVA-specific IgE levels in the sera were increased, in accordance with increased cytokine production after the last challenge (**Figure 7E**). This finding indicates that Bcl6 suppressed the development of allergic inflammation by reducing MPT cell function to facilitate $NAT_H2$ cell differentiation.

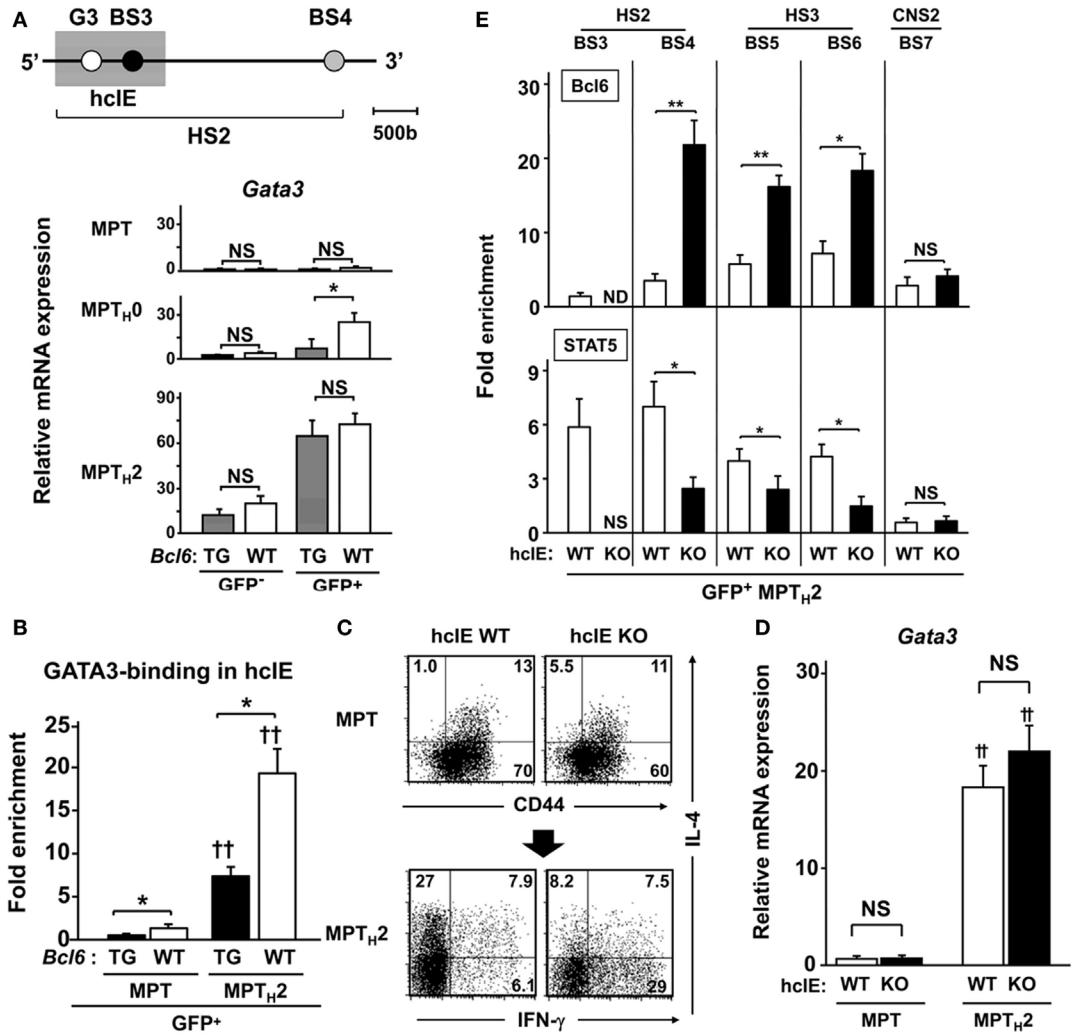


**FIGURE 5** | Role of Bcl6 in hcIE activity in $MPT_H2$ cells. **(A–D)** KJ1-26$^+$ cells among splenic MPT cells were cultured with ovalbumin peptides and antigen-presenting cells *in vitro* under $T_H0$ or $T_H2$ conditions. [**(A)** top] Diagram of the HS2 region in *Il4* intron 2, indicating regulatory regions. The shaded square indicates the hcIE region including the GATA3-binding site (G3) and BS3 within HS2. **(A)** *Gata3* mRNA levels in GFP$^+$ and GFP$^-$ MPT, $MPT_H0$, and $MPT_H2$ cells derived from *Bcl6*-TG and *Bcl6*-WT mice on a CNS2-GFP-TG background. **(B)** GATA3 binding to G3 analyzed by ChIP assays for GFP$^+$ MPT and $MPT_H2$ cells on a CNS2-GFP-TG background. **(C,D)** Analysis of splenic MPT cells or $MPT_H2$ cells derived from hcIE-KO or hcIE-WT mice. **(A)** FACS analysis of intracellular cytokine populations of MPT cells by gating CD4$^+$ CD49b$^-$ T cells in the resting phase and $MPT_H2$ cells restimulated with anti-CD3 monoclonal antibodies. The numbers in the corners represent the percentages among the gated T cells. **(D)** *Gata3* mRNA levels were measured by qRT-PCR for MPT and $MPT_H2$ cells derived from hcIE-KO and hcIE-WT mice. **(E)** Bcl6 levels and STAT5 binding to each BS were analyzed by ChIP assay for GFP$^+$ $MPT_H2$ cells from hcIE-KO or hcIE-WT mice on a CNS2-GFP-TG background. All results are representative of three **(A,B)** or five **(C–E)** independent experiments with similar outcomes. Data are means ± SEMs ($n$ = 9–10). *$P$ < 0.05, comparison between two groups as indicated; †$P$ < 0.05, ††$P$ < 0.01, compared with the MPT cells. Bcl6, B-cell lymphoma 6; BS, binding sequence; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; FACS, fluorescence-activated cell sorting; hcIE, highly conserved intron enhancer; HS, DNase hypersensitive site; KO, knockout; MPT cell, memory phenotype CD4$^+$ T cell; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; ND, not detected; NS, not significant; TG, transgenic; WT, wild-type.

## Bcl6 Attenuates the Synergistic Effect of $MPT_H2$ Cells and NAM-$LT_H2$ Cells on Allergic Responses

IL-4 levels were affected by Bcl6 in $NAMT_H2$ cells, as previously reported (15). We focused on the functional difference in the spatiotemporal dynamics between $MPT_H2$ and $NAMT_H2$ cells. In the current study, NAM-$LT_H2$ cells were analyzed as memory cells derived from naïve CD4$^+$ T cells. In the resting phase, $MPT_H2$ cells constitutively express *Il4*, the expression of which is reduced in a Bcl6-dependent manner. Following 1 h of restimulation, *Il4* expression in $MPT_H2$ cells was increased to similar levels in each Bcl6 genotype, and the expression occurred earlier than that in *Bcl6*-WT-NAM-$LT_H2$ cells. *Il4* expression levels were decreased in most $MPT_H2$ cells, but not *Bcl6*-KO cells, in a Bcl6-dependent manner at 8 h after restimulation (**Figure 8A**). In NAM-$LT_H2$ cells, *Il4* expression levels were low in the resting phase and increased after restimulation. The expression levels in

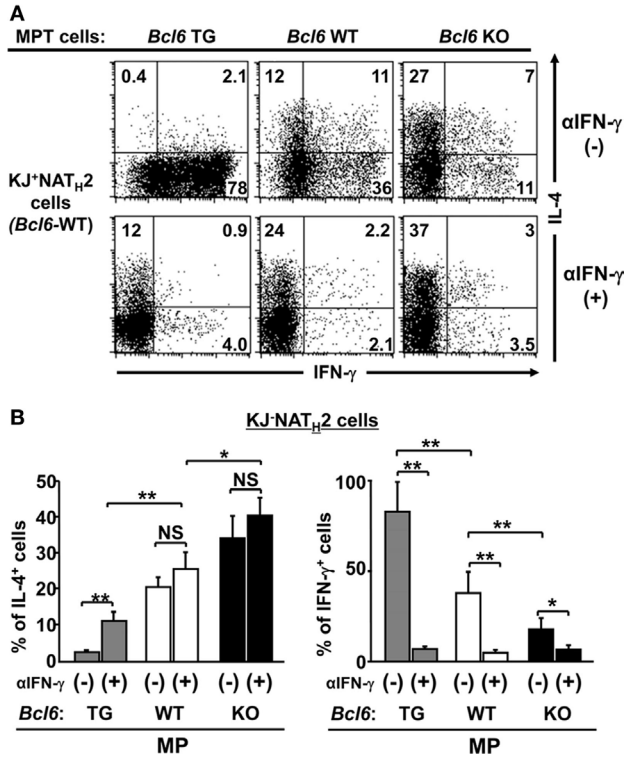


**FIGURE 6** | Role of Bcl6 in initial IL-4 production by MPT cells to induce $NAT_H2$ cells *in vitro*. **(A,B)** *Bcl6*-WT KJ1-26$^+$ naïve CD4$^+$ T cells were cocultured with KJ1-26$^-$ MPT cells (*Bcl6*-TG, *Bcl6*-WT, or *Bcl6*-KO) in the presence of soluble anti-CD3 and CD28 mAbs and irradiated CD11c$^+$ DCs as the $T_H0$ condition with or without anti-IFN-γ Abs. **(A)** FACS analysis of intracellular cytokines in each effector T cell type derived from KJ1-26$^+$ naïve CD4$^+$ T cells are presented as a representative figure among three independent experiments after restimulation with anti-CD3 mAbs. Numbers in the corners denote the percentages of gated KJ1-26$^+$ CD4$^+$ T cells. **(B)** Frequency of the populations of IL-4$^+$ and IFN-γ$^+$ KJ1-26$^+$ T cells after reactivation. All results are representative of three independent experiments with similar outcomes. Data are presented as the mean ± SEM ($n$ = 8–9). $^*P < 0.05$; $^{**}P < 0.01$. Ab, antibody; Bcl6, B-cell lymphoma 6; DC, dendritic cell; KO, knockout; mAb, monoclonal antibody; MPT cell, memory phenotype CD4$^+$ T cell; $NAT_H2$ cell; naïve CD4$^+$ T cell-derived $T_H2$ cell; TG, transgenic; WT, wild-type.

*Bcl6*-WT-NAM-$LT_H2$ cells were high, similar to those in *Bcl6*-KO $MPT_H2$ cells at 8 h after restimulation (**Figure 8A**). The protein levels of IL-4 and IL-5, but not of IL-13, were consistent with the *Il4* expression pattern in each $T_H2$ cell type (Figure S6 in Supplementary Material).

After adoptive transfer of each cell type ($MPT_H2$ cells or NAM-$LT_H2$ cells) with a DO11.10 genetic background into WT BALB/c *nu/nu* mice, cell migration into lung tissues following OVA antigen challenge was determined and presented as percentages (**Figure 8B**) and absolute cell numbers (**Figure 8C**). Among *Bcl6*-WT cells, $MPT_H2$ cells had greater migratory capability compared with NAM-$LT_H2$ cells at 24 h. The migration of $MPT_H2$ cells decreased sequentially, whereas that of NAM-$LT_H2$ cells increased at 48 h. The migration of *Bcl6*-KO $MPT_H2$ cells was further augmented compared with that of *Bcl6*-WT cells. Next, we assessed the role of Bcl6 in interactions between $MPT_H2$ and *Bcl6*-WT-NAM-$LT_H2$ cells during allergic responses. WT BALB/c mice were adoptively transferred with combinations of each type of KJ1-26$^+$ $T_H2$ cells and sequentially challenged with OVA (**Figures 8D,E**). When *Bcl6*-WT-NAM-$LT_H2$ or *Bcl6*-WT $MPT_H2$ cells were transferred, $T_H2$ cytokine levels (IL-4, IL-5, and IL-13) in the BALF were similar among recipients, whereas *Bcl6*-KO $MPT_H2$ cells induced a fourfold to sevenfold increase in $T_H2$ cytokine levels. Combined transfer of *Bcl6*-WT-NAM-$LT_H2$ and *Bcl6*-WT $MPT_H2$ cells resulted in synergistic cytokine production, which was further augmented when *Bcl6*-KO $MPT_H2$ cells were transferred instead of *Bcl6*-WT $MPT_H2$ cells (**Figure 8D**). The numbers of inflammatory cells, including eosinophils and lymphocytes, in the BALF (**Figure 8E**) were increased, in accordance with the increased production of cytokines, indicating that Bcl6 plays a critical role in regulating the functions of $MPT_H2$ cells, which precede $NAMT_H2$ cells in the development of local allergic pathology.

## IL-33 Reinforces IL-4 Production by MPT Cells

Because we previously reported the effects of IL-33 on Bcl6-mediated histone modification in memory $T_H2$ cells to augment IL-4 production (15), we focused in this study on the effect of IL-33 on MPT cells. FACS analysis demonstrated no significant difference in the cell-surface expression of ST2, an IL-33R subunit on MPT cells, between *Bcl6*-TG and *Bcl6*-WT mice (**Figures 9A,B**). ST2 was preferentially expressed on GFP$^+$ MPT cells rather than GFP$^-$ cells. When MPT cells were cultured in the presence of IL-7 for 6 days followed by IL-33 administration (**Figure 9C**, top), the frequency (**Figure 9C**) and absolute number (**Figure 9D**) of IL-4$^+$ MPT cells increased in a concentration-dependent manner at 8 h following the last IL-33 dose. The effect of IL-33 on IL-4$^+$ MPT cells was significantly reduced in *Bcl6*-TG cells compared with that in WT cells (**Figures 9C,D**). Consistent with the priming effect of IL-33, we observed elevated levels of histone acetylation at BS sites in the *Il4* locus with increased STAT5 histone association and decreased Bcl6 histone association. These effects of IL-33 on histone modification were attenuated in *Bcl6*-TG cells (**Figure 9E**).

## DISCUSSION

The function of Bcl6 to regulated $T_H2$ cytokine production is unclear. We found that Bcl6 negatively regulated IL-4 gene expression in MPT cells and their derived $MPT_H2$ cells. Bcl6 inhibition significantly augmented IL-4 production by WT $MPT_H2$ cells. Furthermore, IL-4 expression was reduced in T cell-specific *Bcl6*-TG MPT and *Bcl6*-TG $MPT_H2$ cells, indicating a suppressive function of T cell-intrinsic Bcl6. CNS2 contains multiple putative binding sites for RBP-J, a critical modulator of notch signaling (34). CNS2 is regulated by notch signals to control initial IL-4 expression in MPT cells (28). We demonstrated that Bcl6 binds to CNS2, leading to suppression of its enhancer activity in $MPT_H2$ cells. Bcl6 antagonizes notch-dependent transcription (35, 36). However, *Rbpj* deletion does not alter epigenetic markers on the

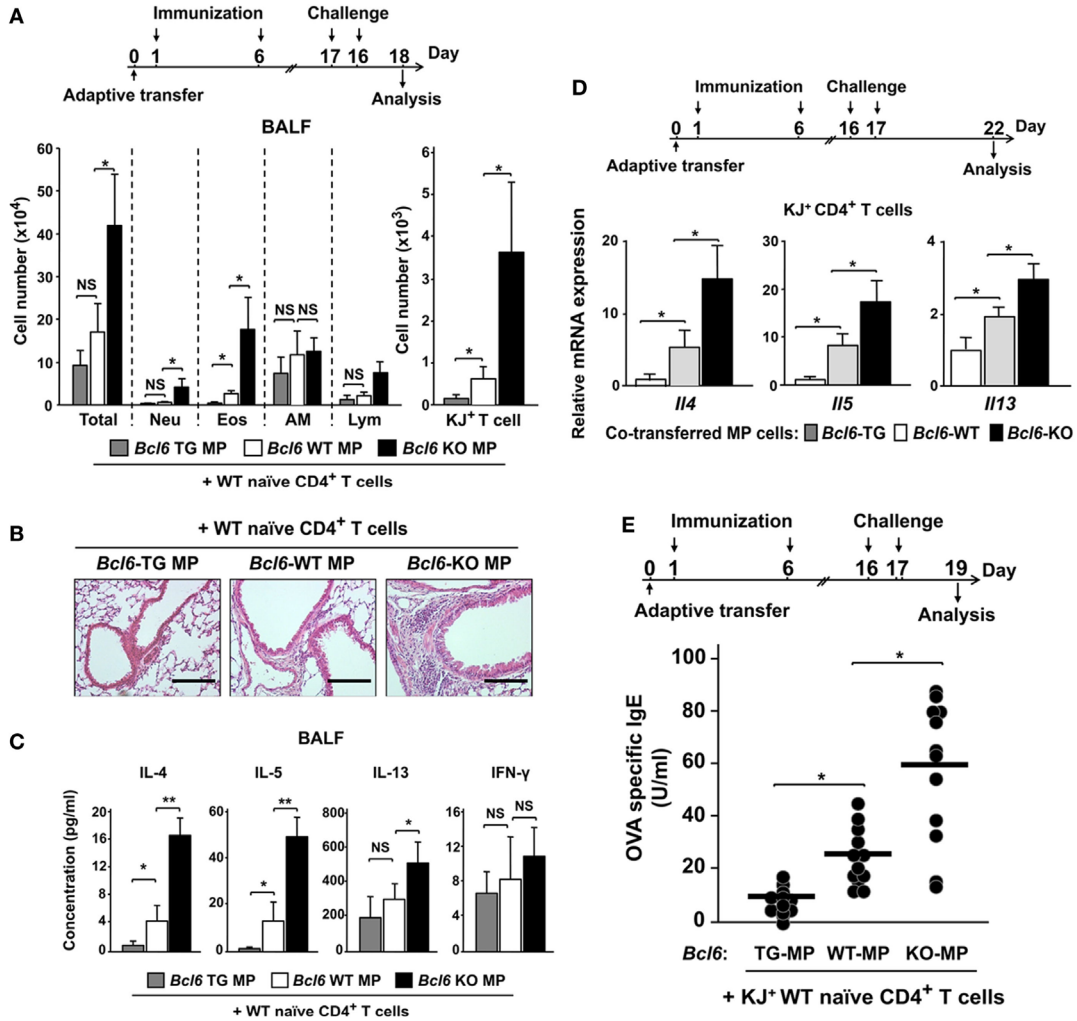


**FIGURE 7** | Role of Bcl6-mediated MPT cell functions in $NAT_H2$ differentiation in an allergic murine model. [**(A)** top] Mixture of purified KJ1-26$^-$ MPT cells (*Bcl6*-WT or *Bcl6*-KO) and KJ1-26$^+$ WT naïve CD4$^+$ T cells were transferred into BALB/c *nu/nu* mice intravenously (day 0). These mice were immunized with alum-conjugated OVA and then intratracheally challenged with OVA. [**(A)** bottom] Absolute cell numbers of Neu, Eos, AM, and Lym in BALF, **(B)** hematoxylin and eosin-stained, formalin-fixed lung sections (magnification: 200×), and **(C)** $T_H2$ cytokine levels in the BALF of recipient mice 48 h after the last OVA challenge. **(D)** Relative *Il4*, *Il5*, and *Il13* expression mRNA in splenic KJ1-26$^+$ T cells restimulated with anti-CD3 monoclonal antibodies 5 days after the last challenge. **(E)** OVA-specific IgE antibody titers in sera from each recipient of *Bcl6*-WT $NAT_H2$ cells, plus $MPT_H2$ cells transferred from *Bcl6*-TG, *Bcl6*-WT, or *Bcl6*-KO mice 2 days after the last challenge. All results are representative of four independent experiments with similar outcomes. Data are presented as the mean ± SEM ($n$ = 5–7). $^*P < 0.05$, $^{**}P < 0.01$, comparison between two groups is indicated. AM, alveolar macrophages; BALF, bronchoalveolar lavage fluid; Bcl6, B-cell lymphoma 6; Eos, eosinophils; KJ$^+$, KJ1-26-positive; KO, knockout; Lym, lymphocytes; MPT cell, memory phenotype CD4$^+$ T cell; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; $NAT_H2$ cell; naïve CD4$^+$ T cell-derived $T_H2$ cell; Neu, neutrophils; NS, not significant; OVA, ovalbumin; TG, transgenic; WT, wild-type.

CNS2 site in $T_{FH}$ cells (29). Thus, to elucidate the positive regulatory mechanism of the activation of CNS2, a target of Bcl6 in MPT cells, further analysis is required.

GATA3 binding in the HS2 enhancer region is critical for $NAT_H2$ (15, 35) and $NAMT_H2$ cells (15). However, extremely low GATA3 expression might not be associated with IL-4 production in MPT cells. We demonstrated that GATA3-mediated hcIE activation is not essential for IL-4 production by MPT cells (**Figures 5B–D**). However, $MPT_H2$ cell differentiation requires hcIE enhancer activity, which induces permissive histone modification of the *Il4* locus by cooperating with STAT5 and GATA3 (37). Bcl6 directly bound to and interfered with hcIE function in $MPT_H2$ cells. Accordingly, we suggest that diverse Bcl6 functions regulate IL-4 production in $MPT_H2$ and MPT cells. The locus control region (LCR) at the Rad50 gene is also extremely important for $T_H2$ cytokine expression. This region is considered to be involved in coordinating $T_H2$ cytokine genes including IL-4. We previously reported the GATA3-binding site and Bcl6/STAT-binding sites in conserved regions ($T_H2$LCR) in the Rad50 gene in another study (15). We also reported that Bcl6 binding in the LCR is augmented by disruption of hcIE in *Il4*, indicating that Bcl6-mediated $T_H2$LCR organizes $T_H2$ cytokine gene including IL-4. Therefore, $T_H2$LCR may be implicated in *Il4* regulation in CNS2-active MPT cells. To elucidate the role

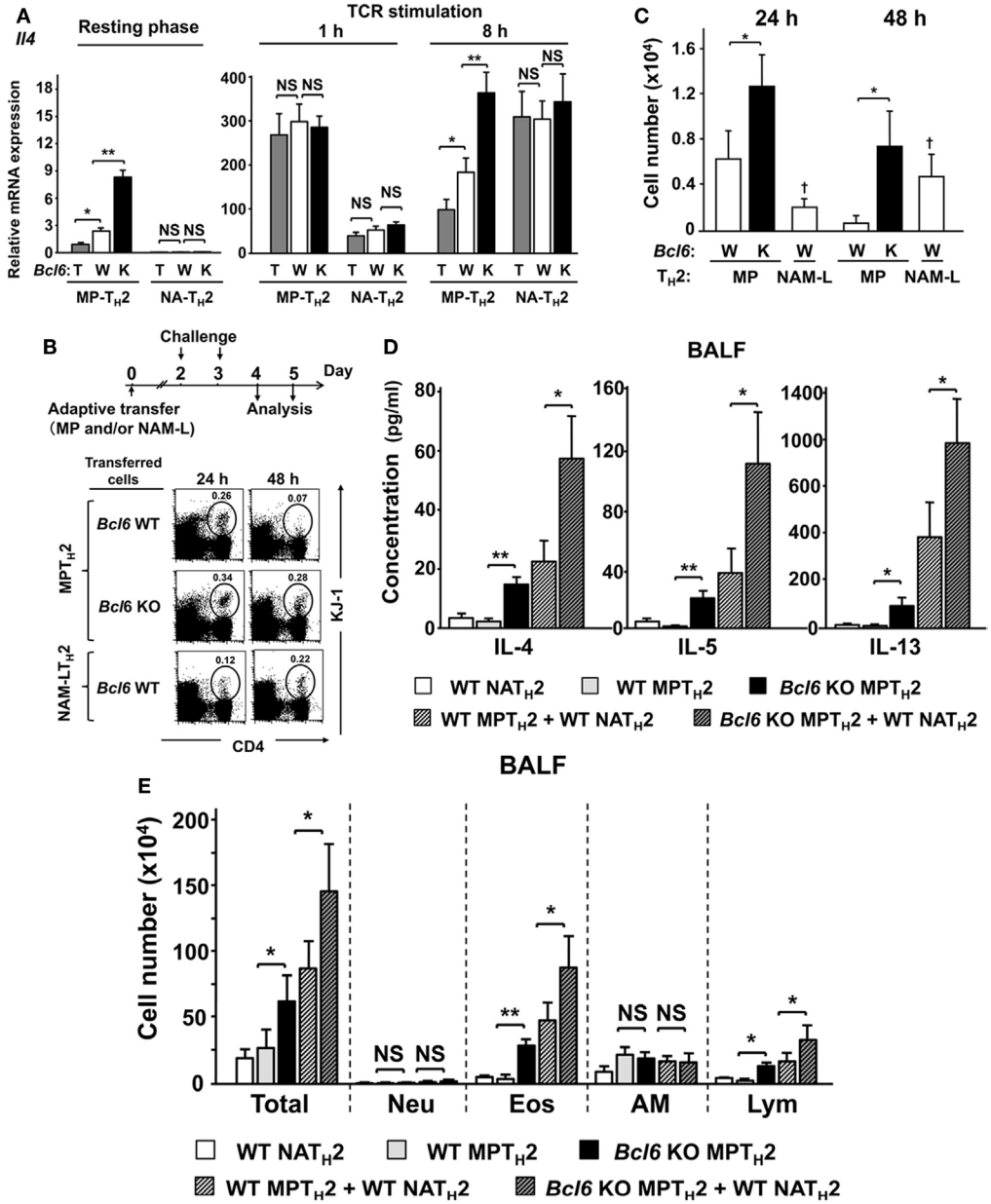


**FIGURE 8** | Role of Bcl6 in interactions between $MPT_H2$ and naïve $NAT_H2$ cells in allergy pathogenesis. **(A–E)** KJ1-26$^+$ $MPT_H2$ cells and $NAT_H2$ cells were differentiated from the spleens of *Bcl6*-TG (T), *Bcl6*-WT (W), and *Bcl6*-KO (K) mice in the presence of OVA peptides and antigen-presenting cells in $T_H2$ conditions. **(A)** *Il4* mRNA levels in each $T_H2$ cell type were measured by qRT-PCR at rest and at 1 and 8 h after restimulation with anti-CD3 monoclonal antibodies. **(B–E)** *Bcl6*-WT BALB/c *nu/nu* mice were administered KJ1-26$^+$ $MPT_H2$ cells ($3 \times 10^7$), KJ1-26$^+$ $NAT_H2$ cells ($3 \times 10^7$), or combinations of $MPT_H2$ ($1.5 \times 10^7$) and $NAT_H2$ cells ($1.5 \times 10^7$) *via* adoptive transfer (day 0). **(B)** Representative FACS data for donor cells in circles with their percentages among total CD4$^+$ T cells in whole lungs from recipients at 24 and 48 h after the last intratracheal OVA challenge. **(C)** Absolute numbers of KJ1-26$^+$ cells in the lungs, **(D)** $T_H2$ cytokine levels, and **(E)** cell types in the bronchoalveolar lavage fluid 48 h after the last challenge. All results are representative of four independent experiments with similar outcomes. Data are presented as the mean ± SEM ($n$ = 8–10). $^*P < 0.05$, $^{**}P < 0.01$, comparison between two groups is indicated **(A,B,D,E)**; $^\dagger P < 0.05$, compared with $MPT_H2$ cells. AM, alveolar macrophages; Bcl6, B-cell lymphoma 6; Eos, eosinophils; FACS, fluorescence-activated cell sorting; KO, knockout; Lym, lymphocytes; MPT cell, memory phenotype CD4$^+$ T cell; $MPT_H2$ cell, MPT cell-derived $T_H2$ cell; $NAT_H2$ cell; naïve CD4$^+$ T cell-derived $T_H2$ cell; Neu, neutrophils; NS, not significant; OVA, ovalbumin; TCR, T cell receptor; TG, transgenic; WT, wild-type.

of $T_H2$LCR, further studies using region-deficient mice are required.

B-cell lymphoma 6 has various regulatory functions associated with cell viability and cytokine production, although the detailed molecular mechanisms have not been clarified. We observed that CNS2-active MPT cells contained high Bcl6 levels that declined following augmented IL-4 production under $T_H2$ priming conditions. Intriguingly, in *Bcl6*-WT $MPT_H2$ cells,

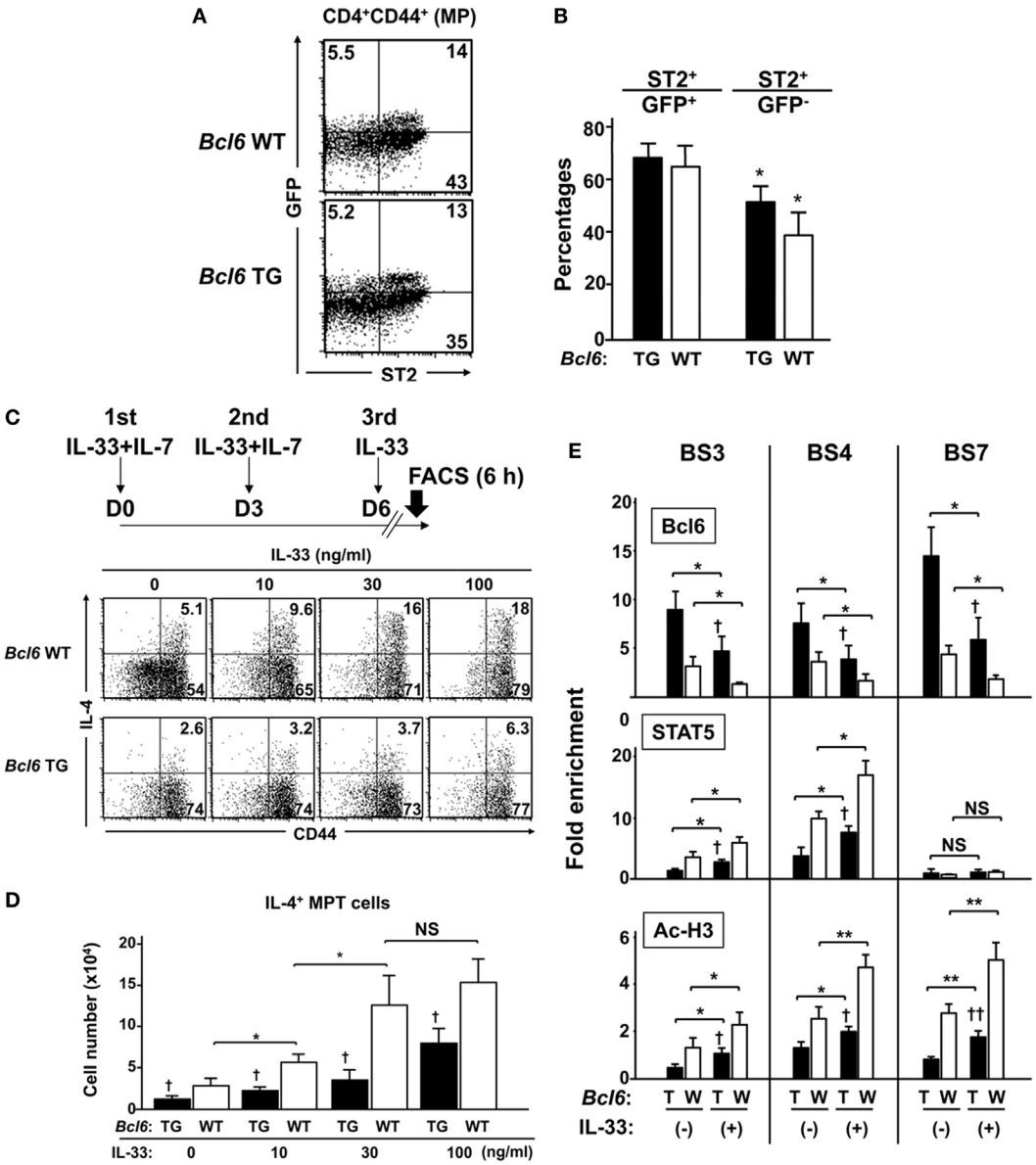


**FIGURE 9** | IL-33 reinforces IL-4 production by MPT cells through functional competition against the suppressor activity of Bcl6. **(A,B)** FACS analysis of splenic CNS2-GFP-TG MPT cells from *Bcl6*-TG and *Bcl6*-WT mice at rest. **(A)** Data show the expression of GFP and ST2 gated cells among all CD4$^+$ CD44$^+$ cells (representative of six independent experiments). **(B)** Percentages of ST2$^+$ cells among GFP$^+$ and GFP$^-$ MPT cells. **(C,D)** IL-33 was added to the culture of MPT cells from *Bcl6*-TG and *Bcl6*-WT mice three times in the presence of IL-7. [**(C)** top] Six hours after the last IL-33 dose, MPT cells were analyzed for intracellular IL-4 levels. Numbers indicate the percentage of IL-4$^+$ cells among all MPT cells. [**(C)** bottom] FACS analysis data are representative of four independent experiments. **(D)** Absolute numbers of IL-4$^+$ MPT cells 8 h after the last IL-33 dose. **(E)** ChIP analysis of Bcl6 and STAT5 binding and Ac-H3 at each BS in CNS2-GFP$^+$ MPT cells from *Bcl6*-TG (T) and *Bcl6*-WT mice(W). Cells were primed with or without IL-33 three times in the presence of IL-7. Analysis was performed 8 h after the last IL-33 dose. All results are representative of three **(A–D)** or four **(E)** independent experiments with similar outcomes. Data are presented as the mean ± SEM ($n$ = 6–7). $^*P < 0.05$, $^{**}P < 0.01$, comparison between two groups is indicated; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with WT. Ac-H3, acetylated histone H3; Bcl6, B-cell lymphoma 6; BS, binding sequence; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; MPT cell, memory phenotype CD4$^+$ T cell; TG, transgenic; WT, wild-type.

the CNS2-active population exhibited markedly lower *Bcl6* levels and higher *Il4* levels than the CNS2-inactive population. Greater Bcl6 mRNA levels in CNS2-active MPT cells than in the CNS2-inactive population in WT mice have been reported (29), whereas we observed slight differences in expression between these two populations. However, Bcl6 protein levels in CNS2-active *Bcl6*-WT MPT cells were inversely decreased relative to those in the CNS2-inactive MPT cells. Therefore, when pleiotropic Bcl6 effects are required in the same cellular environment, its function may be quantitatively controlled at transcriptional, translational, or post-transcriptional levels.

We previously demonstrated that $T_H2$ cytokine genes are negatively regulated by Bcl6 through chromatin remodeling and that interactions between Bcl6 and STAT5 are physiologically implicated in histone modulation and consequently cytokine production in $NAMT_H2$ cells rather than $NAT_H2$ cell differentiation (15). In a previous report, we advocated that STAT5 and GATA3 cooperate in permissive histone modification of the *Il4* locus by binding to hcIE and that STAT5- and GATA3-mediated epigenetic activity of hcIE may be controlled by directly and/or indirectly preventing the Bcl6-mediated silencing. In addition, Bcl6 binding to BS4, BS5, and BS6 in the *Il4* locus was augmented upon hcIE disruption in differentiating $T_H2$ cells. Therefore, even in the presence of high levels of Bcl6, *Bcl6*-TG naïve $CD4^+$ T cells could differentiate into $T_H2$ cells under the $T_H2$ full commitment condition. Conversely, when naïve *Bcl6*-TG, *Bcl6*-WT, and *Bcl6*-KO $CD4^+$ T cells are stimulated under the $T_H0$ condition, IL-4 production by restimulated $CD4^+$ T cells was reduced in a Bcl6 level-dependent manner. Therefore, we propose that the repressor activity of Bcl6 in the *Il4* locus including hcIE and CNS2 can be determined in functional balance with transcriptional activators, such as GATA3, STATs, and RBP-J, in both $MPT_H2$ and $NAT_H2$ cells. Accordingly, both quantitative and qualitative Bcl6 functional modifications, such as reduced binding activity (15), may be implicated in the gene regulation of *Il4*. Notably, we observed that Bcl6 binding to the *Il4* locus is higher in CNS2-$GFP^-$ *Bcl6*-TG $MPT_H2$ cells than in $GFP^+$ B*cl6*-TG cells. Because enhancers can generally regulate transcription by interacting with enhancers or promoters *via* chromatin looping mechanisms, we propose that CNS2 may also stimulate *Il4* transcription *via* physical interactions with hcIE, which may influence and organize Bcl6/STAT binding in hcIE. Therefore, Bcl6 binding to the *Il4* locus may exceed STAT5 binding *via* Bcl6-mediated inhibition of CNS2 activity.

In earlier reports, we and other groups uncovered that Bcl6 has no significant intrinsic function in the differentiation of naïve $CD4^+$ T cells into $T_H1/T_H2$ cells in full commitment experiments *in vitro*. In later studies focusing on $T_{FH}$ cells, Bcl6 suppressed effector T cells, including $T_H1$, $T_H2$, and $T_H17$ cells, resulting in the induction of $T_{FH}$ cell differentiation. The current study indicated that Bcl6 promotes IFN-γ production *via* by inhibiting IL-4 production in activated naïve $CD4^+$ T cells and MPT cells in some experimental settings, rather than inhibiting IL-4 production by promoting IFN-γ production.

Contrarily, we previously reported that Bcl6 plays an important anti-apoptotic role in effector-derived memory precursor $CD4^+$ T cells, suggesting that Bcl6 is involved in long-term memory T cell survival (17, 30, 38). We observed that the numbers of splenic MPT cells and, intriguingly, CNS2-active $GFP^+$ MPT cells were positively associated with intrinsic Bcl6 levels, whereas the MFI of GFP was reduced in *Bcl6*-TG cells. Recently, CNS2-active $GFP^+$ $CD4^+$ T cells in secondary lymphoid tissues were found to have a high *Bcl6* expression phenotype, similar to $T_{FH}$ cells (29). Bcl6 is a master regulatory factor for $T_{FH}$ cell differentiation. However, a substantial *Bcl6*-KO MPT cell population exists, and we suggested that CNS2-active MPT cells are not necessary as part of the $T_{FH}$ cell lineage. Although the molecular mechanism is unclear, Bcl6 may be implicated in, but not essential for, the development and/or maintenance of MPT and $MPT_H2$ cells.

$NAMT_H2$ cells have an important role in chronic allergic responses (15), although the relationship between $NAMT_H2$ and $MPT_H2$ cells is unclear. We observed that $T_H2$ cytokine production peaked and declined earlier in *Bcl6*-WT-$MPT_H2$ cells than in WT-NAM-$LT_H2$ cells. Moreover, the migratory function of $MPT_H2$ cells was superior to that of NAM-$LT_H2$ cells, albeit due to an unknown mechanism. Because CNS2 and *Il4* are constitutively activated in $MPT_H2$ cells but not in $NAMT_H2$ cells (15), $MPT_H2$ cells might influence $NAMT_H2$ cell function in chronic allergy. Accordingly, $MPT_H2$ cells organize $T_H2$ immune responses directly and/or indirectly by regulating $NAMT_H2$ cell function, resulting in allergy enhancement.

IL-4 production by CNS2-active MPT cells induced $T_H2$ responses by inducing the differentiation of $NAT_H2$ cells from naïve $CD4^+$ T cells and their self-differentiation into $MPT_H2$ cells following immunization (28). We confirmed initial IL-4 production from MPT cells in this study. Because CNS2-active MPT cells do not belong to the $T_{FH}$ cell lineage derived from naïve $CD4^+$ T cells (29) but they rather develop from selected thymocytes among those expressing other MHC class II markers (39), $IL\text{-}4^+$ MPT cells might develop independently of naïve $CD4^+$ T cells during thymic differentiation. In that case, sequentially differentiated $MPT_H2$ cells as well as MPT cells contribute to the early pathology of some allergies.

When considering the nature of Bcl6 in MPT and $MPT_H2$ cells in pathologic conditions, we should determine whether *Bcl6* expression can be modified without artificial gene manipulation at both protein and RNA levels. Recently, we reported that a $T_H2$-promoting factor, namely, IL-33-mediated breakdown of Bcl6 in $NAMT_H2$ cells, is likely involved in allergies (15) given the effect of IL-33 on both MPT and $NAMT_H2$ cells. Therefore, the IL-33/Bcl6 axis might participate in allergy pathology *via* the regulation of *Il4* in MPT cells to promote disease development in $MPT_H2$ and $NAMT_H2$ cells, contributing to the maintenance and exacerbation of disease pathology.

In summary, the current study provides evidence for a novel role of Bcl6 in the functional regulation of MPT and $MPT_H2$ cells, implying interplay between Bcl6 and transcriptional activators to promote the production of relevant $T_H2$ cytokines, particularly IL-4. Thus, $T_H2$ cell-promoting factors that suppress Bcl6 function may represent crucial therapeutic targets for $T_H2$ cell-mediated diseases.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Chiba University Resolution on Use of Animals in Research. The protocol was approved by the Institutional Animal Care and Use Committee at Chiba University School of Medicine. The mice were maintained under specific pathogen-free conditions in the animal center of Chiba University Graduate School of Medicine.

## AUTHOR CONTRIBUTIONS

MA and TO jointly designed the experiments and directed the study and wrote the manuscript. MA, TO, YK, JI, TT, NT,

HW-T, LF, AS, HH, and MH performed the experiments. MA, TO, MH, YF, and KK analyzed the data and generated the figures. YF, KT, TT, and TF provided reagents and/or support for the analysis.

## ACKNOWLEDGMENTS

The authors thank S. Satake for technical support and S. Nakamura for secretarial services. This work was supported in part by Grants-in-Aid for Scientific Research (C) (JP17K10005: MA; JP24591460: MA) from the Japan Society for the Promotion of Science.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00750/full#supplementary-material.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 August 2018
doi: 10.3389/fimmu.2018.01828

# Blimp-1-Mediated Pathway Promotes Type I IFN Production in Plasmacytoid Dendritic Cells by Targeting to Interleukin-1 Receptor-Associated Kinase M

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**Specialty section:**
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

**Received:** 22 March 2018
**Accepted:** 24 July 2018
**Published:** 07 August 2018

**Citation:**
Ko Y-A, Chan Y-H, Liu C-H, Liang J-J, Chuang T-H, Hsueh Y-P, Lin Y-L and Lin K-I (2018) Blimp-1-Mediated Pathway Promotes Type I IFN Production in Plasmacytoid Dendritic Cells by Targeting to Interleukin-1 Receptor-Associated Kinase M. Front. Immunol. 9:1828. doi: 10.3389/fimmu.2018.01828

Plasmacytoid dendritic cells (pDCs) are a specialized subset of DCs capable of rapidly producing copious amounts of type I IFN (IFN-I) in response to viral infections. The mechanism regulating rapid production of IFN-I after pDCs are exposed to viral nucleic acids remains elusive. Here, we show that the transcription factor Blimp-1 is promptly induced in pDCs after exposure to TLR7 and TLR9 ligands *via* a unique Ras-related C3 botulinum toxin substrate (Rac)-mediated pathway. Deletion of the *Prdm1* gene encoding Blimp-1 impaired production of IFN-I, but not other cytokines, upon viral infection or treatment with CpG DNA in pDCs. Accordingly, mice lacking Blimp-1 in DCs failed to produce IFN-I after CpG stimulation and did not mount proper antiviral responses following flavivirus infection. The development of pDCs in bone marrow as well as the induction of several activation markers, such as CD86, CD69, and MHCII, by CpG stimulation was generally not affected by the absence of Blimp-1. Mechanistically, we found that Blimp-1 controls the activation of IKKα and IRF7 by directly suppressing *interleukin-1 receptor-associated kinase 3* (*Irak3*), a negative regulator of TLR signaling, in pDCs. Together, we identify a Blimp-1-dependent pathway that rapidly facilitates IFN-I production by relieving interleukin-1 receptor-associated kinase M, encoded by *Irak3*, in pDCs.

**Keywords: plasmacytoid dendritic cell, type I interferon, Blimp-1, interleukin-1 receptor-associated kinase M, antiviral response**

## INTRODUCTION

Plasmacytoid dendritic cells (pDCs) are a distinctive subset of DCs with low abundance and a short lifespan (1). They produce copious amounts of type I IFN (IFN-I) by utilizing highly expressed TLR7 and TLR9 to sense pathogen-derived single-stranded RNA and unmethylated DNA, respectively (2–4). Besides IFN-I, pDCs also secret proinflammatory cytokines to combat early phase infection, including IL-6, IL-12, and TNF-α. These responses are accompanied by

the upregulation of MHCII and co-stimulatory molecules that allow bridging activation of adaptive immunity (5). Aberrant pDC-derived IFN-I production is associated with the activation and expansion of auto-reactive T and B cells in autoimmune diseases (6). However, despite the importance of pDCs in the antiviral response and autoimmunity, the underlying regulatory pathways that contribute to the rapid large-scale production of IFN-I remain elusive.

Blimp-1, a transcription factor, is critical for regulating differentiation of mature B cells into plasma cells (7). It also plays important roles in several other immune cell lineages. For example, Blimp-1 negatively regulates the homeostasis of $CD8^-$ conventional DCs (cDCs) and is essential for cDC maturation in response to stimulation (8). In particular, Blimp-1 participates in the regulation of the tolerogenic function of DCs. DC-specific deletion of *Prdm1*, the gene encoding Blimp-1, results in a lupus-like syndrome in female mice that is characterized by elevated serum autoantibodies, enhanced germinal center formation, and increased follicular T helper cells (9). However, whether Blimp-1 plays a functional role in pDCs remains unknown. Given that TLR ligands can induce Blimp-1 in several immune cell lineages (10), we here would like to investigate whether Blimp-1 is involved in the regulation of IFN-I production in pDCs.

# MATERIALS AND METHODS

## Mice

*Prdm1*$^{f/f}$ mice (11) were crossed with CD11c-Cre or R26CreER mice, both purchased from The Jackson Laboratory, to generate *Prdm1*$^{f/f}$CD11c-Cre$^{+/-}$ (CKO-11c), *Prdm1*$^{f/f}$ER-Cre$^{+/-}$ (CKO-ER), and their littermate control *Prdm1*$^{f/f}$CD11c-Cre$^{-/-}$ (Ctrl-11c) or *Prdm1*$^{f/f}$ER-Cre$^{-/-}$ (Ctrl-ER) mice. To avoid the autoimmune phenotypes of female CKO-11c mice (9), only male CKO-11c and male littermate control mice were used in all experiments. *Tlr7* knockout (KO) (12) and Blimp-1-yellow fluorescent protein (YFP) reporter mice (13) were purchased from The Jackson Laboratory, and *Tlr9* KO (obtained from Dr. Shizuo Akira) (14) mice were paired with wild-type C57BL/6 mice (purchased from the National Laboratory Animal Center, Taipei, Taiwan). All mice were housed and bred in the specific pathogen free conditions in the animal facility of Institute of Cellular and Organismic biology at Academia Sinica. Animal experimental protocols were approved by IACUC of Academia Sinica.

## Reagents

Type-A CpG oligonucleotides (ODN2216), type-C CpG oligonucleotides (ODN2395), Imiquimod (R837), and poly(I:C) were purchased from InvivoGene. The lipopolysaccharide (*E. coli.* O26:B6) was obtained from Sigma-Aldrich Co. For virus infection, influenza A virus (H1N1/WSN, from Dr. Jia-Tsrong Jan), herpes simplex virus-1 (KOS strain, from Dr. Chia-Chi Ku), respiratory syncytial virus (A2 strain, from Dr. Joe Yen-Hung Chow), and Japanese encephalitis virus (JEV RP-9 strain, from Dr. Yi-Ling Lin) were used. In some experiments, the FLpDCs were pretreated with Rac inhibitor, EHop-016 (Calbiochem) for 1 h, followed by CpG-A or R837 stimulation.

## *In Vivo* Challenge and Plaque Assay

A neurovirulent JEV strain, RP-9, was used for the induction of encephalitis in CKO-11c and Ctrl-11c mice following the procedures described previously (15). Briefly, mice were anesthetized and intracerebrally injected with 10 μl of PBS to damage the brain–blood barrier followed by intraperitoneal inoculation with $5 \times 10^4$ PFU of RP-9 virus. Sera were collected at indicated time points after infection and the mice were observed daily for 14 days to record lethality. Anti-PDCA-1 antibody (BX444; BioXcell) and rat IgG1 isotype control (HRPN; BioXcell) antibody were used to test the significance of pDCs in JEV infection *in vivo* and were injected three times (250 μg/injection) at 24-h intervals before infection.

For *in vivo* CpG-A challenge, 5 μg ODN2216 was mixed with 30 μl DOTAP, the liposomal transfection reagent, and incubated at room temperature for 15 min. Mice were intravenously injected with CpG-A plus DOTAP, or DOTAP alone. After 6 h, IFN-α and cytokines in sera were determined as previously described (16, 17).

To quantify JEV virus amounts, whole brain homogenates were harvested from Ctrl-11c and CKO-11c mice 6 days after JEV infection. BHK-21 cells were used for plaque assays as described previously (15). Briefly, brain homogenates were serially diluted and added into 80% confluent BHK-21 cells. After 2 h, the supernatant was removed and the infected BHK-21 cells were overlaid with 1% agarose-RPMI solution (SealPlaque, FMC BioProducts), followed by incubation at 37°C. Four days later, cells were fixed and stained with 0.5% crystal violet, and then the plaque numbers were counted.

## Cell Preparation, Stimulation, and Transfection

Splenic $CD11c^+$ DCs were enriched by using positive selection with mouse CD11c microbeads (Miltenyl Biotec), the $CD11c^{int}$ $B220^+$Siglec-$H^+$ pDCs, or $CD11c^{high}B220^-$Siglec-$H^-$ cDCs were sorted by cell sorter and cultured in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. FLpDCs were generated as previously described (18). Bone marrow (BM) cells were harvested from the femurs and tibiae of mice. Red blood cells were lyzed and single cell suspensions were cultured in RPMI 1640 supplemented with 50 ng Flt3 ligand (PeproTech) at a density of $1 \times 10^6$ cells/ml for 9 days. To delete *Prdm1* allele *in vitro*, BM cultures from CKO-ER and Ctrl-ER mice were supplied with 500 nM 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich). Nine days later, $CD11c^+$ cells that were at least 90% confluent were used to enrich $CD11c^+B220^+Bst2^+$Siglec-$H^+$ pDCs after B220 microbeads isolation (Miltenyl Biotec). The purified pDCs were stimulated with 1 μM CpG-A (InvivoGen), CpG-C (InvivoGen), or 2 μg/ml R837 (InvivoGen). cDCs were treated with 50 ng/ml poly(I:C) (InvivoGen) or 10 ng/ml LPS (Sigma-Aldrich) at a density of $1 \times 10^6$ cells/ml for the indicated time points. For virus infection, pDCs ($1 \times 10^6$ cells/ml) were infected with influenza H1N1

(WSN strain) at a titer of $1 \times 10^4$ TCID50/ml. Herpes simplex virus 1 (HSV-1) (KOS strain) and respiratory syncytial virus (RSV A2 strain) were applied at an MOI of 1 and JEV (RP9 strain) was used at an MOI of 10 for 24 h.

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation with Ficoll-Paque at 400 × *g* for 30 min without brake at 22°C. The mononuclear cells were carefully isolated from the interphase and the BDCA2$^+$ pDCs were further purified by plasmacytoid dendritic cell isolation kit II (Miltenyl Biotec). In some experiments, the purified pDCs were stimulated with 1 μM CpG-A or influenza H1N1 (WSN strain) at a titer of $10^4$ TCID50/ml for 24 h. Blood samples were from Taipei Blood Center. The consent procedures of collection of samples from healthy donors were approved by the Academia Sinica Research Ethics Committee.

To knock down interleukin-1 receptor-associated kinase M (IRAK-M) expression, the FLpDCs generated from Ctrl-ER or CKO-ER mice were isolated and transfected with small-interfering RNA (siRNA) against *interleukin-1 receptor-associated kinase 3* (*Irak3*) or the control siRNA by TurboFect (Thermo Scientific). The transfection procedure was performed as previously described (19). Briefly, 1.5 μg siRNA were diluted in 50 μl serum-free RPMI1640 containing 1 μl TurboFect for 15 min at room temperature. After incubation, the mixtures were added to FLpDCs in a final volume of 550 μl. The target sense sequences were synthesized by TOOLS Biotechnology Co. The Irak3 siRNA sequences are #1:5′-GGGAAGACUUUCCGUUAAATT-3′, #2:5′-GGCUG GAUGUUCGUCAUAUTT-3′, and #3:5′-GCAGAGUUCUACC AUAAAUTT-3′, and the FAM tagged control sequences are 5′-UUCUCCGAACGUGUCACGUTT-3′.

## RNA Isolation and RT-Quantitative PCR (RT-qPCR)

Total RNAs were extracted by Isol-RNA Lysis Reagent (5 PRIME), and subjected to reverse transcription by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Gene specific primer sets were used to perform the qPCR analysis by using Applied Biosystems StepOne™ Real-Time PCR System. Taqman probe sets including mouse *Prdm1* (Mm 01187285_m1) and human *PRDM1* (Hs 00153357_m1) were purchased from Applied Biosystems. The specific primer sequences for SYBR green detection are listed below: *Ifna4*, 5′-GCAATGACCTCCATCAGCAGCT-3′, and 5′-GTGGAAGTATGTCCTCACAGCC-3′; *Ifna5*, 5′-G GATGTGACCTTCCTCAGACTC-3′, and 5′-CACCTTCTCCT GTGGGAATCCA-3′; *Ifnb1*, 5′-GCCTTTGCCATCCAAGAGA TGC-3′, and 5′-ACACTGTCTGCTGGTGGAGTTC-3′; *Il6*, 5′-A CAAGTCGGAGGCTTAATTACACAT-3′,and5′-AATCAGAAT TGCCATTGCACAA-3′; *Il12p40*, 5′-TTGAACTGGCGTTGGA AGCACG-3′, and 5′-CCACCTGTGAGTTCTTCAAAGGC-3′; *Tnfa*, 5′-GACCCTCACACTCAGATCATCTTCT-3′, and 5′-CC TCCACTTGGTGGTTTGCT-3′; *Irak3*, 5′-CTGCAAAGTGGT GCTGGATGAC-3′, and 5′-GCTTTGCAGAGAAGTTCCGAG G-3′; *Tcf4*, 5′-CCTCCAATCCTTCAACTCCTGTG-3′, and 5′-T CCAAACGGTCTTCGATTCGGC-3′; *Ikzf1*, 5′-CCACCACGA GATGGCAGAAGAC-3′, and 5′-GGCATGTCTGACAGGCAC TTGT-3′; *Irf8*, 5′-CAATCAGGAGGTGGATGCTTCC-3′, and 5′-GTTCAGAGCACAGCGTAACCTC-3′; *Tlr7*, 5′-GTGATGC TGTGTGGTTTGTCTGG-3′, and 5′-CCTTTGTGTGCTCCTG GACCTA-3′; *Tlr9*, 5′-GCTGTCAATGGCTCTCAGTTCC-3′, and 5′-CCTGCAACTGTGGTAGCTCACT-3′; *Actin*, 5′-CAT TGCTGACAGGATGCAGAAGG-3′, and 5′-TGCTGGAAGG TGGACAGTGAGG-3′.

## Nuclear and Cytoplasmic Proteins Extraction and Immunoblotting

Cell cytoplasmic and nuclear extracts were obtained by using NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's protocols (ThermoFisher). Immunoblotting was performed as previously described (8). The blots were probed with anti-IRF7 antibody (EPR4718; abcam), anti-Lamin-B (M-20; Santa Cruz Biotechnology), anti-IKKα (Cell Signaling), anti-AKT (Cell Signaling), anti-Osteopontin (Abcam), anti-p65 (C-20; Santa Cruz Biotechnology), anti-P50 (Santa Cruz Biotechnology), anti-STAT1 (Cell Signaling), anti-IRAK-M (ProSci), and anti-Blimp-1 (Abcam). The activation of IRF7, IKKα/β, AKT, and STAT1 were detected by phospho-specific antibodies against pIRF7 (Ser471/472; D6M2I; Cell Signaling), pIKKα/β (Ser176/180; 16A6; Cell Signaling), pAKT (Ser473; D9E; Cell Signaling), and pSTAT1 (Tyr701; 58D6; Cell Signaling). Representative blots from at least two independent experiments were shown.

Rac1 activation was detected by Rac1 activation assay kit (Abcam). Briefly, the total cell lysates were harvested from stimulated FLpDCs and incubated with PAK1 PBD beads at 4°C for 1 h. Rac1-GTP precipitate and the total lysate controls were analyzed by western blot analysis. Rac1 was detected by a specific mouse monoclonal antibody.

## ELISA

The supernatant from stimulated pDC culture or the serum collected from the CpG-A injected or JEV infected mice was harvested and subjected to ELISA analysis to determine the levels of IFN-α (PBL Assay Science), IL-6, and TNF-α (eBioscience) following the manufacturer's protocols. Finally, 2 N $H_2SO_4$ was added to stop the reaction and absorbance at 450 nm was measured using a microplate reader (SpectraMax M2).

## Flow Cytometry Analysis and Antibodies

Single cell suspensions were prepared for surface staining of the cells with fluorochrome-conjugated antibodies against Flt3 (A2F10), Bst2 (ebio927), and Siglec-H (ebio440c) were purchased from eBioscience, B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD3 (145-2c11), and CD86 (GL1) were purchased from BD, CD69 (H1.2F3), CD19 (6D5), CD49b (Dx5), MHCII (M5/114.15.2), and Ly-6c (HK1.4) were purchased from BioLegend, and Ly49Q (2E6) were purchased from Medical & Biological Laboratories Co. After incubating on ice for 15 min, the cells were washed twice and analyzed by BD FACS canto II flow cytometer. In some experiments, mouse splenic pDCs and cDCs were sorted by BD FACS Aria II system.

## Chromatin Immunoprecipitation (ChIP) Assay

To detect Blimp-1 binding to the endogenous target sites, a ChIP assay was performed according to previously described procedures (20). Basically, $5 \times 10^7$ Flt3L cultured pDCs from C57BL/6 mice were stimulated with 1 μM CpG-A for 4 h and fixed with 1% formaldehyde at 37°C for 15 min and quenched with 125 mM glycine. The sheared chromatins were incubated with goat anti-Blimp-1 antibody (Abcam) or goat IgG isotype antibody (Abcam) at 4°C overnight. The antibody-chromatin immunocomplexes were pulled down by the protein-G magnetic beads and eluted at 65°C for 30 min. Immuneprecipitated DNA was isolated and analyzed by real-time qPCR. The primer sequences used in qPCR are listed below: site 1, 5′-AGGAATCTTGGTGACAATTTGGC-3′, and 5′-GACGGTAAAAGCTAGGGTGCTCT-3′; site 2, 5′-CCAAAATGATGGACTGTGGCC-3′, and 5′-CCCTGATGAAAGCAGATTCGG-3′; site 3, 5′-GCAAAGTGGCCCGATTGAGAGTA-3′, and 5′-CGGCCTTCAAAACAAAATGTTCTG-3′; site 4, 5′-TGTTGTTCTTCCTATGGGGTTGC-3′, and 5′-AACCATTGGACTGAGCACAGGGT-3′; site 5, 5′-TCTGAGTTTGACGCCCCAGTACA-3′, and 5′-TGCGCAAGTGCACATGTACATGA-3′; and *Gapdh*, 5′-GGGTTCCTATAAATACGGACTGC-3′, and 5′-CTGGCACTGCACAAGAAGA-3′.

## Statistical Analysis

Statistical significance was determined by using the two-tailed unpaired Student's *t*-test. Data represent mean ± SEM. The differences in mouse survival between two groups were analyzed by log-rank (Mantel-Cox) test. Results from independent biological replicates were used in statistical analysis. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

# RESULTS

## Blimp-1 Is Induced After TLR7/9 Stimulation in pDCs

We first examined the expression of Blimp-1 in pDCs after stimulation. Human PBMCs were isolated from healthy donors and the BDCA2$^+$ pDCs were purified. Compared with the pDCs treated with medium alone, Blimp-1 expression in pDCs was upregulated after treatment with CpG-A or influenza virus (H1N1) infection (**Figures 1A,B**), which induced high IFN-I production (**Figure 1C**). We then examined if Blimp-1 is expressed in mouse pDCs, characterized as CD11c$^{int}$B220$^+$Siglec-H$^+$Bst2$^+$ (21–23) (Figure S1 in Supplementary Material). The Blimp-1-YFP reporter mice that express YFP under the control of Blimp-1 regulatory element (13) were used to track the expression of Blimp-1. Similar to human pDCs, a rapid induction of Blimp-1 in mouse splenic pDCs was detected 3 h after intravenous injection of DOTAP/CpG-A, as compared with the DOTAP injected group (**Figure 1D**). This rapid induction of Blimp-1 was also observed after exposure of Flt3-ligand-cultured bone marrow (BM)-derived pDCs (FLpDCs) to the CpG-A as compared with the medium treated FLpDCs (**Figure 1E**). However, to our surprise, a lack of TLR9 did not affect Blimp-1 expression (**Figure 1F**). Given that TLR7 and TLR9 are endosomal receptors, and that TLR ligands transiently stimulate endocytosis in DCs (24), we suspected the induction of Blimp-1 in FLpDCs may occur upstream of TLR activation. Rac-1, a small G protein, is activated by stimulation with TLR9 ligand; however, this occurs independently of TLR9 activation (25). Indeed, the induction of Blimp-1 in FLpDCs was significantly reduced following the treatment with EHop-016, a Rac inhibitor that docks at the guanine nucleotide exchange factor (GEF) binding pocket of Rac to inhibit Rac activation (26) (**Figure 1G**). In addition, Blimp-1 can be induced by R837, the TLR7 ligand, in FLpDCs, but the induction of Blimp-1 was diminished when TLR7 is deficient (**Figure 1H**). This finding was correlated with the defective Rac-1 activation after R837 treatment in TLR7 KO FLpDCs (**Figure 1I**). Moreover, inhibition of Rac activity also decreased Blimp-1 induction after R837 treatment in FLpDCs (**Figure 1J**). These combined data suggest that Blimp-1 induction in pDCs is mediated by Rac activation soon after exposure to TLR7 and TLR9 ligands.

## Blimp-1 Is Essential for IFN-I Production in pDCs

To determine the functions of Blimp-1 in pDCs, we generated mice carrying a conditionally deleted *Prdm1* allele. *LoxP*-flanked *Prdm1* (*Prdm1$^{f/f}$*) mice were crossed with mice expressing Cre recombinase under the control of the integrin alpha X (Itgax/CD11c) promoter, CD11c-cre, to obtain mice with a DC-specific *Prdm1* deletion, hereafter referred to as CKO-11c mice. Blimp-1 deletion efficiency was ascertained at both genomic DNA and mRNA levels in splenic pDCs and cDCs (Figures S2A,B in Supplementary Material), as well as in BM CD11c$^+$ cells (Figures S2C,D in Supplementary Material). Because of the gender-specific autoimmune phenotype in female CKO-11c mice (9), only male mice were used in this study. First, we examined whether Blimp-1 regulated pDC development. The absolute counts of splenic pDCs from CKO-11c mice were similar to the littermate control, Ctrl-11c, mice (**Figure 2A**). According to the mouse model of sequential pDC development (23), reduced Blimp-1 in BM DC lineages did not appear to alter pDC development because the expression of various markers representing pDC developmental stages was comparable between BM pDCs in CKO-11c and Ctrl-11c mice (**Figure 2B**). Previous studies demonstrated that for pDCs to develop from progenitors in BM, several critical factors are required including Flt3, and the transcription factors E2-2 (encoded by *Tcf4*), Ikaros (encoded by *Ikzf3*), and IRF8 (23, 27). Cell surface Flt3 and the transcription factor mRNA levels were consistently equivalent in Ctrl-11c and CKO-11c pDCs (**Figures 2B,C**). Therefore, Blimp-1 may not be important for the development of pDCs.

Because the ability to produce large quantities of IFN-I is the hallmark of pDCs (28), we next examined whether Blimp-1 is involved in IFN-I production by pDCs. Blimp-1 was originally identified as binding to the positive regulatory domain I (PRDI) element of the *IFN-β* promoter and negatively regulating IFN-β expression (29). To our surprise, a significant reduction in IFN-α was detected in the sera of CpG-A/DOTAP treated CKO-11c

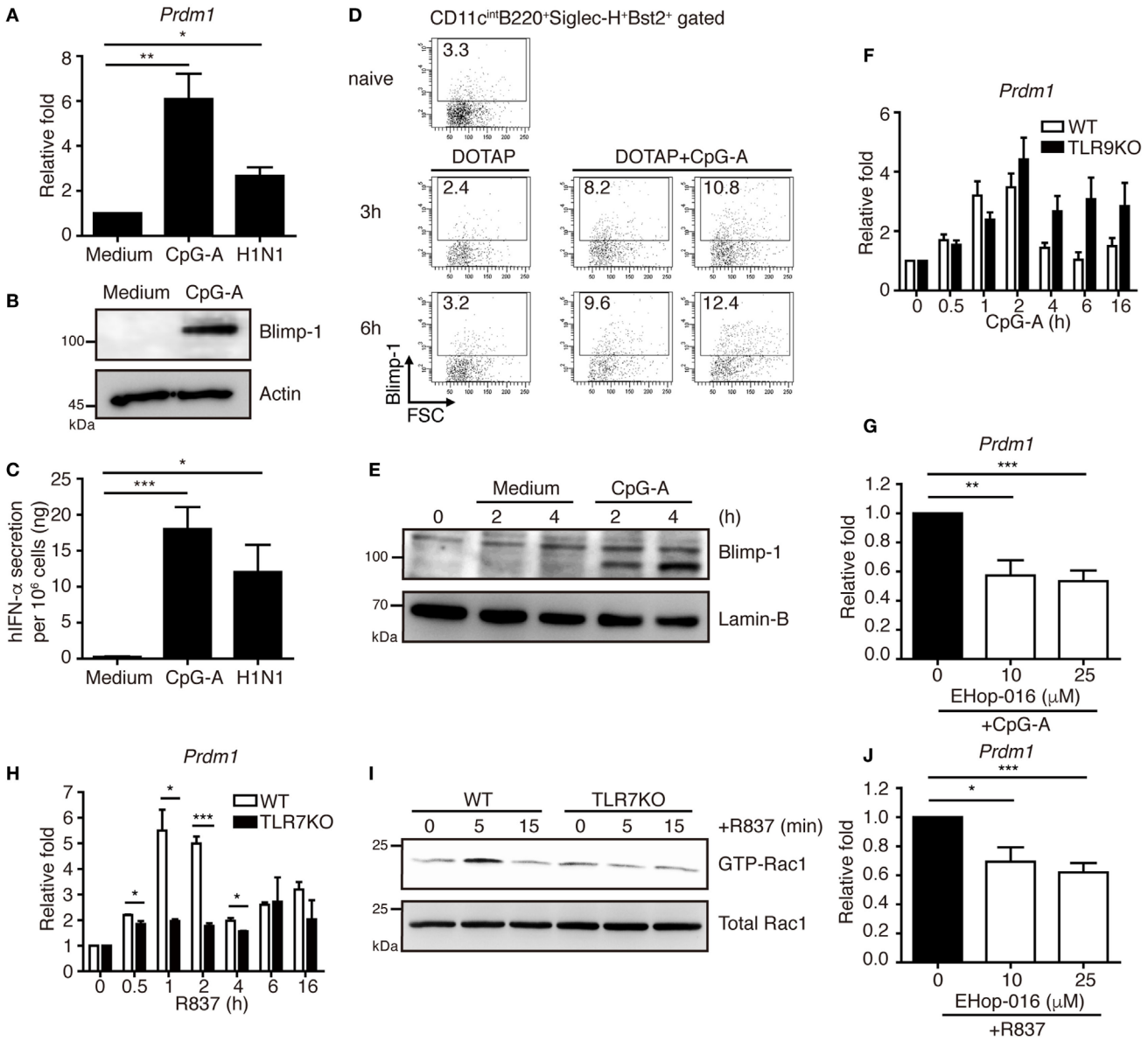


**FIGURE 1** | Blimp-1 was induced *via* Rac activation after TLR7/9 ligand treatment in plasmacytoid dendritic cells (pDCs). **(A)** RT-quantitative PCR (RT-qPCR) showing Blimp-1 mRNA in human pDCs 24 h after treatment with medium alone, 1 μM CpG-A and H1N1 at a titer of $10^4$ TCID50/ml. **(B)** Blimp-1 protein levels were determined by immunoblotting in human pDCs 24 h after treatment with medium alone or 1 μM CpG-A. **(C)** ELISA showing the levels of IFN-α produced by human pDCs as described in **(A)**. **(D)** Blimp-1-yellow fluorescent protein reporter mice were intravenously injected with DOTAP alone or DOTAP + CpG-A. The frequency of Blimp-1$^+$ pDCs in splenic CD11c$^{int}$B220$^+$Siglec-H$^+$Bst2$^+$ gate was examined at indicated time after infection. The frequency of Blimp-1$^+$ pDCs from untreated group (naïve) was shown for comparison. **(E)** Nuclear Blimp-1 protein levels were detected by immunoblotting in mouse FLpDCs stimulated with medium alone or 1 μM CpG-A at indicated time points. Freshly isolated FLpDCs at 0 h, before addition of medium alone or CpG-A, were also used as the control. **(F)** RT-qPCR showing the Blimp-1 mRNA levels in *Tlr9* knockout (KO) FLpDCs treated with 1 μM CpG-A. **(G)** RT-qPCR showing Blimp-1 mRNA levels in FLpDCs after 1 h pre-treatment with EHop-016 and further treatment with 1 μM CpG-A for 1 h. **(H)** RT-qPCR showing the Blimp-1 mRNA levels in *Tlr7* KO FLpDCs treated with 2 μg/ml R837 for 1 h. **(I)** Rac1 activation determined by PAK1 PBD agarose beads pulled down and immunoblotting with antibody against Rac1 in FLpDCs from WT and TLR7 KO mice after stimulation with 2 μg/ml R837. **(J)** RT-qPCR showing Blimp-1 mRNA expression in FLpDCs after 1 h pre-treatment with EHop-016 and further treatment with 2 μg/ml R837 for 1 h. Data represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n$ = 3–6 in **(A)**, 4–7 in **(C)**, 3 in **(F)**, 5–6 in **(G)**, 3 in **(H)**, and 4–5 in **(J)**]. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

mice compared with that of Ctrl-11c mice, while DOTAP injection did not induce IFN-α in both Ctrl-11c and CKO-11c mice (**Figure 3A**). By contrast, comparable amounts of proinflammatory cytokines IL-6 and TNF-α were detected in CpG-A treated Ctrl-11c and CKO-11c mice (**Figures 3B,C**). These results suggest a role for Blimp-1 in the control of IFN-I production. To verify whether intrinsic Blimp-1 expression in pDCs contributes to IFN-I production, splenic pDCs were isolated from Ctrl-11c

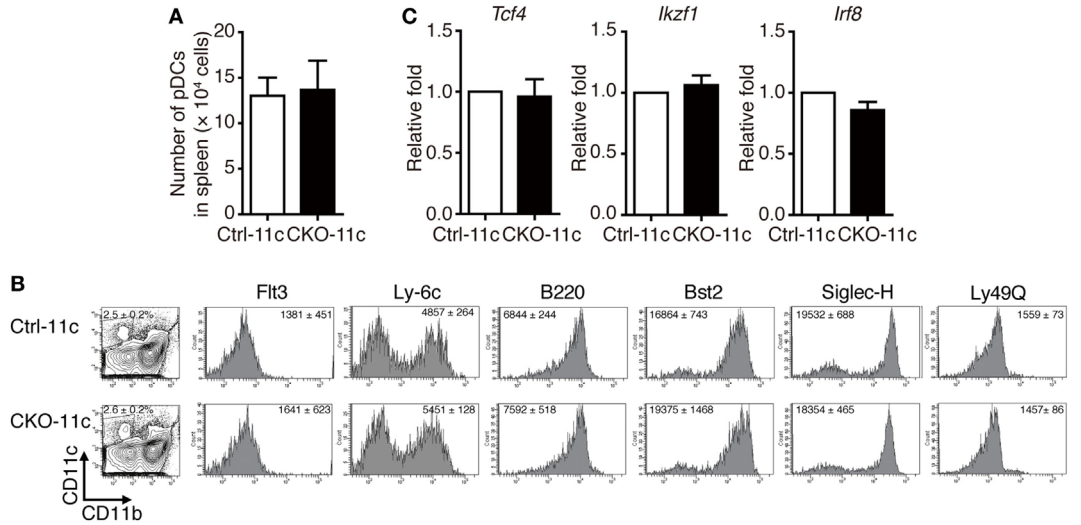


**FIGURE 2** | Comparable plasmacytoid dendritic cell (pDC) development in CKO-11c mice and Ctrl-11c mice. **(A)** pDC numbers in the spleen of Ctrl-11c and CKO-11c mice were enumerated. **(B)** Flow cytometric analysis showing the expression of various pDC maturation markers in CD11b−CD11c+ bone marrow cells isolated from Ctrl-11c or CKO-11c mice. The frequency of the $CD11b^{-}CD11c^{+}$ population and the mean fluorescence intensity of the staining in each histogram are indicated. **(C)** RT-quantitative PCR showing the mRNA expression levels of E2-2 (*Tcf4*), Ikaros (*Ikzf1*), and IRF8 in splenic pDCs isolated from Ctrl-11c or CKO-11c mice. Results represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 3$ in **(A–C)**].

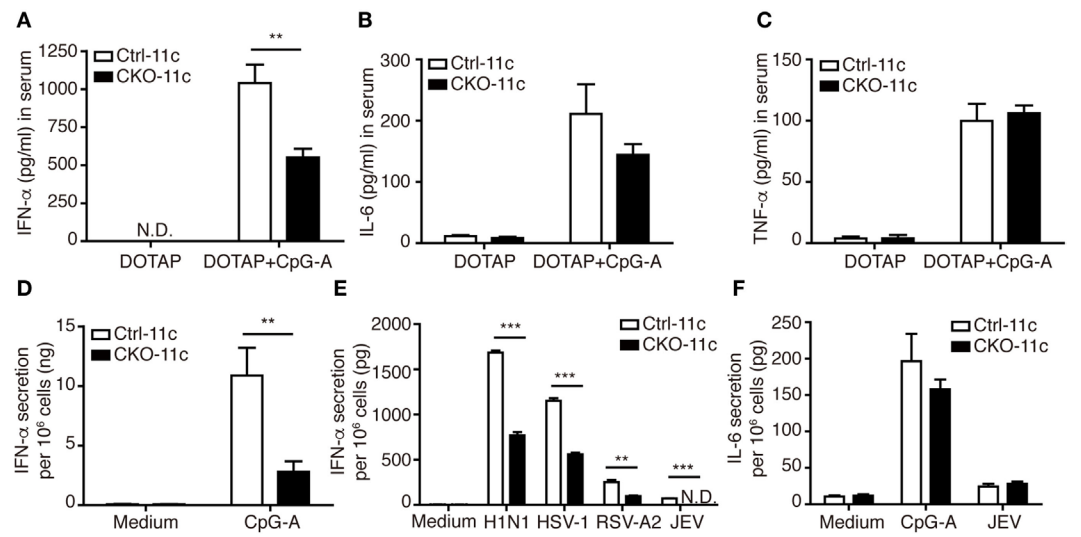


**FIGURE 3** | Type I IFN (IFN-I) production was impaired in CKO-11c mice and in plasmacytoid dendritic cells (pDCs) from CKO-11c mice. **(A)** ELISA showing the levels of IFN-α production in the serum of Ctrl-11c and CKO-11c mice 6 h after intravenous injection with 5 μg CpG-A + DOTAP or DOTAP alone. **(B,C)** ELISA determining the levels of IL-6 **(B)** and TNF-α **(C)** in serum from Ctrl-11c and CKO-11c mice from panel **(A)**. **(D,E)** ELISA determining the levels of IFN-α production at 24 h in medium alone treated, 1 μM CpG-A-stimulated or virus-infected splenic pDCs isolated from Ctrl-11c and CKO-11c mice. **(F)** ELISA measurement of the levels of IL-6 produced by Ctrl-11c and CKO-11c splenic pDCs at 24 h after treatment with medium alone, 1 μM CpG-A and Japanese encephalitis virus (JEV) at MOI of 10. Data represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 3$ in DOTAP, 5–7 in CpG-A + DOTAP in **(A)**, 3–4 in **(B,C)**, 3 in medium, 6 in CpG-A treated group in **(D)**, 3 in **(E)**, and 3–4 in **(F)**]. $^{**}p < 0.01$; $^{***}p < 0.001$. N.D. = not detectable.

and CKO-11c mice and stimulated with CpG-A and viruses including several single-stranded RNA viruses; influenza H1N1 virus, RSV-A2, and JEV, as well as a double-stranded DNA virus, HSV-1. Remarkably, IFN-α production by all stimuli was reduced in pDCs lacking Blimp-1 (**Figures 3D,E**). However, IL-6 production by pDCs was not affected in the absence of Blimp-1 (**Figure 3F**). Similar numbers of viable cells were found in control and Blimp-1-deficient splenic pDCs after treatment (Figures S2E in Supplementary Material). These data indicated that Blimp-1 plays a crucial role in antiviral responses in pDCs.

To exclude the possibility that this result might be caused by impaired pDC development that was not readily detected in our analysis, we crossed *Prdm1*$^{f/f}$ mice with mice carrying the inducible estrogen receptor/cre (ER-cre) in ubiquitous tissues (30). The resulting inducible *Prdm1* KO mice, termed CKO-ER mice, had almost 70% inducible deletion of *Prdm1* in FLpDCs after induction with 4-hydroxytamoxifen (4-OHT) (Figure S3A in Supplementary Material). Blimp-1 mRNA and protein levels were also significantly decreased in the 4-OHT treated FLpDCs from CKO-ER mice (**Figures 4A,B**). Of note, Blimp-1 protein expression was detected early at 15 min after CpG-A stimulation in 4-OHT treated FLpDCs from littermate controls, Ctrl-ER mice (**Figure 4B**). We verified that the deletion of *Prdm1 in vitro* during FL-mediated BM culture did not affect pDC development (Figures S3B,C in Supplementary Material). TLR7 and TLR9 expression was comparable between 4-OHT-treated FLpDCs derived from CKO-ER and Ctrl-ER mice (Figure S3D in Supplementary Material). We also ensured that 4-OHT had no obvious effects on Blimp-1 induction (Figure S3E in Supplementary Material). It is noted that we consistently showed defective IFN-I induction after CpG stimulation in 4-OHT-treated FLpDCs derived from CKO-ER mice compared with those from Ctrl-ER mice (**Figures 4C,D**); and there was no change in IL-6, IL-12p40, or TNF-α (**Figures 4C,E**). In addition to CpG-A, Blimp-1 mRNA levels were increased in FLpDCs after CpG-C treatment (Figure S3F in Supplementary Material). Consistently, IFN-α production was reduced in Blimp-1-deficient FLpDCs, while the production of IL-6 and TNF-α was comparable between control and Blimp-1-deficient FLpDCs after CpG-C treatment (Figure S3G in Supplementary Material).

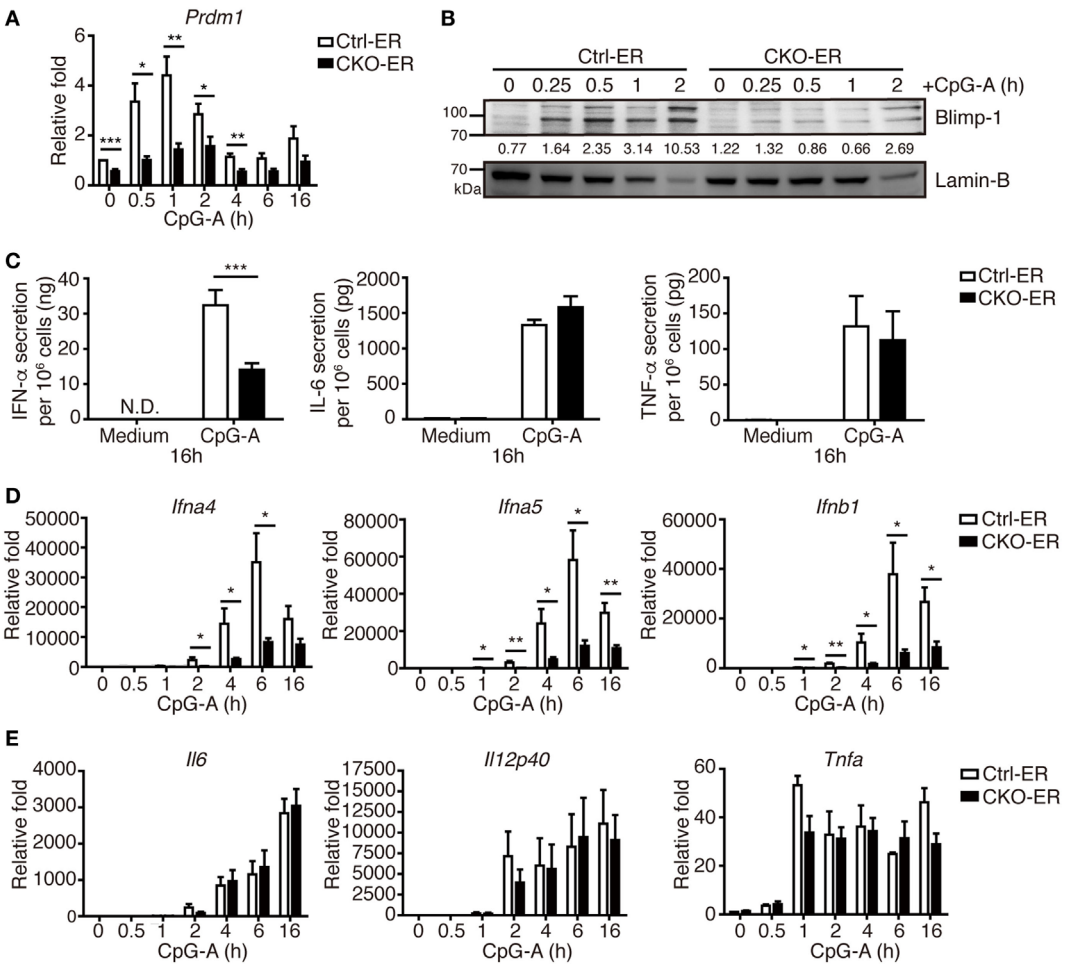


**FIGURE 4** | Inducible deletion of *Prdm1* confirmed the important role of Blimp-1 in type I IFN (IFN-I) production in plasmacytoid dendritic cells (pDCs). **(A,B)** RT-quantitative PCR (RT-qPCR) **(A)** and immunoblotting **(B)** showing Blimp-1 mRNA and protein levels in FLpDCs from Ctrl-ER and CKO-ER mice treated with 500 nM 4-OHT and then stimulated with 1 μM CpG-A at indicated time points. The quantitation of Blimp-1 in **(B)** was presented by the ratios of Blimp-1 band intensity vs. Lamin-B band intensity at each time point. **(C)** 4-OHT treated FLpDCs cultured from Ctrl-ER and CKO-ER mice were stimulated with 1 μM CpG-A or medium alone for 16 h, followed by ELISA to measure the levels of IFN-α, IL-6, and TNF-α production. **(D,E)** RT-qPCR showing mRNA levels of IFN-I **(D)** and proinflammatory cytokines **(E)** in 4-OHT treated FLpDCs from Ctrl-ER and CKO-ER mice after stimulation with 1 μM CpG-A at indicated time points. Data represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 6$ in **(A)**, 3 in medium, 9–14 in CpG-A treatment in **(C)**, 7 in **(D)**, and 3–4 in **(E)**]. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

The reduced production of IFN-α in FLpDCs lacking Blimp-1 was not caused by enhanced cell death because the frequency of Annexin V-positive cells was similar in Blimp-1-deficient and control FLpDCs at 6 and 16 h after treatment (**Figure 5A**). Furthermore, Blimp-1-deficient FLpDCs appear to be activated in a similar manner to control FLpDCs, as evidenced by the comparable induction of CD86, CD69, and MHCII expression following CpG-A treatment (**Figure 5B**). Combined, these results show that Blimp-1 expression in pDCs selectively controls IFN-I production.

## Impaired Antiviral Responses in Blimp-1 Deficient-Mice

Type I IFN induction is essential for fighting viral infection, replication, and pathogenesis. Because pDCs with reduced Blimp-1 expression had defective IFN-I production after viral infection, we examined the importance of Blimp-1 in antiviral responses *in vivo*. The infection of JEV, a flavivirus, is highly sensitive to IFN-I production, but it is unaffected in mice lacking components of adaptive immunity (15, 31). Furthermore, our data showed that IFN-α production was reduced in Blimp-1-deficient pDC culture after JEV infection. Toward this end, we first examined the importance of pDCs in the clearance of JEV infection in mice. According to the reported procedures (32), which depleted the mouse pDCs *in vivo* but avoided the inadvertent depletion of other immune cells activated after virus infection, we injected mice with three shots of anti-PDCA-1 antibody or isotype control antibody at 24-h intervals only before JEV infection (Figure S4A in Supplementary Material). Administration of anti-PDCA-1 antibody with this strategy caused nearly complete depletion of pDCs in mouse spleen (Figures S4B–D in Supplementary Material), which was linked with increased mortality and reduced IFN-α production in sera after JEV infection (Figures S4E,F in Supplementary Material). The frequency of other immune cell types, including cDCs, myeloid cells, B cells, T cells, NK cells, sand NKT cells, at before or 3 days after JEV infection was not affected by anti-PDCA-1 antibody treatment (Figures S4G,H in Supplementary Material). To address further the roles of Blimp-1 in antiviral responses, Ctrl-11c and CKO-11c mice were intraperitoneally injected with JEV, followed by intracerebral damage of the brain–blood barrier. We found that CKO-11c mice were much more susceptible to JEV infection and have higher mortality rate (**Figure 6A**). This increased susceptibility was correlated with diminished serum IFN-α levels quickly after JEV infection (**Figure 6B**) and elevated viral titers in the brain (**Figures 6C,D**). However, the IL-6 levels were comparable in the sera of Ctrl-11c and CKO-11c mice (**Figure 6E**). Therefore, Blimp-1-mediated pathway is critical for the antiviral response against JEV infection.

## Blimp-1 Regulates IRF7 Activation

Given that pDCs with reduced Blimp-1 expression had impaired IFN-I production, we determined whether the TLR-mediated signaling pathway is affected by reduced Blimp-1. Endosomal TLR7 and TLR9 are abundantly expressed in pDCs, with MyD88 serving as a mediator to provoke downstream kinase cascades

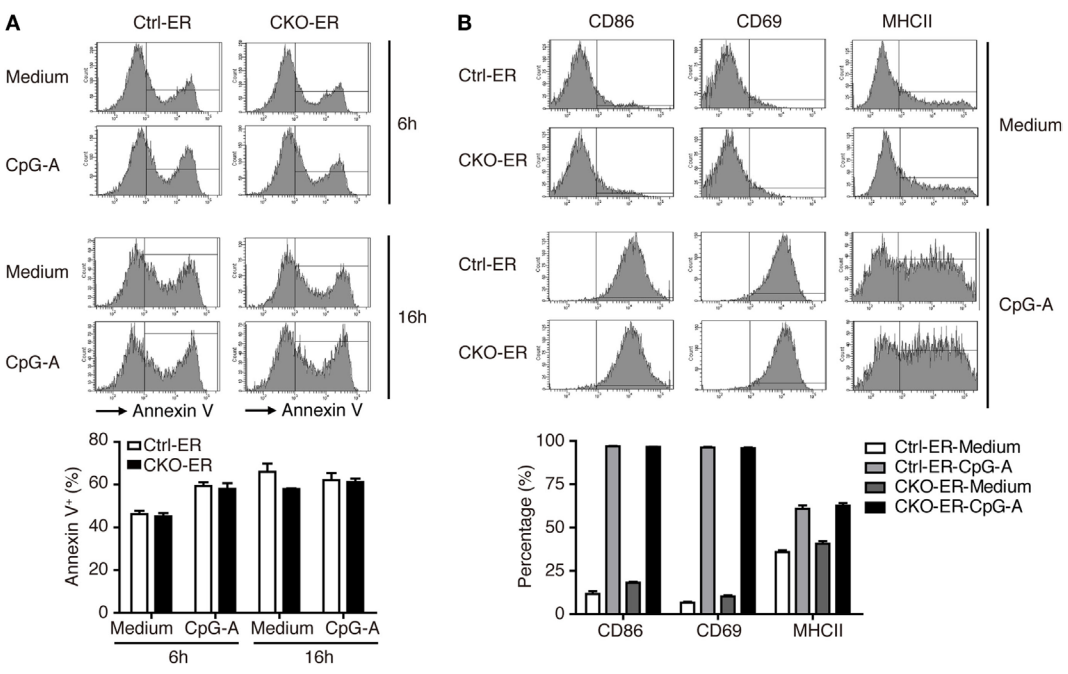


**FIGURE 5** | Activation and apoptosis were not affected in Blimp-1-deficient FLpDCs. **(A)** Flow cytometric analysis showing the frequency of Annexin V positive cells in 4-OHT treated Ctrl-ER or CKO-ER FLpDCs stimulated with 1 μM CpG-A or medium alone for 6 and 16 h. **(B)** Flow cytometric analysis of CD86, CD69, and MHCII expression on 4-OHT treated Ctrl-ER or CKO-ER FLpDCs with or without 16 h of 1 μM CpG-A stimulation. The positive frequency of each marker is indicated. Results represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 3$ in **(A,B)**].

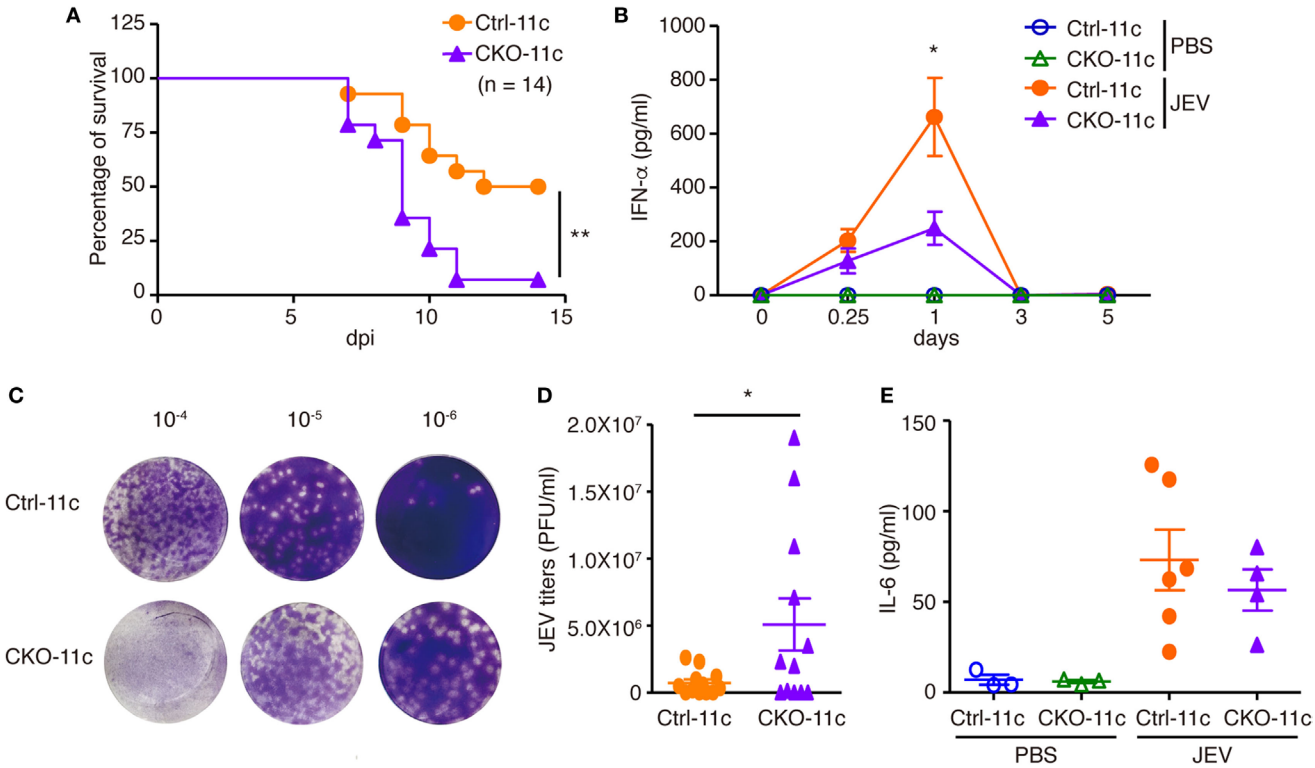


**FIGURE 6** | Impaired antiviral responses in mice lacking Blimp-1 in DCs. **(A)** Survival rates of Ctrl-11c or CKO-11c mice infected with Japanese encephalitis virus (JEV) ($5 \times 10^4$ pfu) by intraperitoneal (i.p.) injection followed by intracerebral (i.c.) needle injury. dpi: days post-infection. **(B)** ELISA measurement of IFN-α at different days post-PBS injection or post-JEV infection in Ctrl-11c and CKO-11c mice serum. **(C,D)** Plaque assay showing JEV titers in Ctrl-11c and CKO-11c mouse brains at 6 days post-infection **(C)**. Quantitation of results from panel **(C)** is **(D)**. **(E)** ELISA showing serum IL-6 levels 24 h after JEV infection or PBS injection in Ctrl-11c and CKO-11c mice. Data in **(A)** was analyzed by log-rank (Mantel-Cox) test ($n = 14$). Data in **(B,D,E)** represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 10$–13 in 0.25 and 1 dpi and 3–5 in 0, 3, and 5 dpi and PBS injected group in **(B)**, 12 in **(D)**, and 3–6 in **(E)**]. $^*p < 0.05$; $^{**}p < 0.01$.

and IRF7 activation. The phosphorylation and translocation of IRF7 are essential for IFN-I production in pDCs. *Irf7* KO pDCs exhibit dramatically reduced IFN-I production, but do not have reductions in other proinflammatory cytokines (33). We found that nuclear levels of IRF7 following CpG-A stimulation were greatly reduced in 4-OHT-treated FLpDCs derived from CKO-ER mice (**Figure 7A**). This reduction may be caused by defective phosphorylation of IRF7 at Ser437/438 (**Figure 7B**), because phosphorylation is required for IRF7 activation (34). Moreover, IKKα, osteopontin (OPN), and PI3K selectively regulate IFN-I production in pDCs by promoting the phosphorylation of IRF7 (35–37). IKKα activation was decreased following CpG-A treatment in Blimp-1-deficient pDCs (**Figure 7C**); however, OPN and the activation of PI3K downstream factor, AKT, were comparable irrespective of the presence of Blimp-1. Unlike IRF7, canonical NF-κB, p65, and p50 were activated normally by CpG-A stimulation in 4-OHT treated FLpDCs derived from CKO-ER mice (**Figure 7D**). This result is consistent with our notion that cytokine production is not generally affected in stimulated pDCs in the absence of Blimp-1. The IFN-I produced in the early phase response to TLR ligands amplified a positive feedback loop that signals through interferon α/β receptor (IFNAR) to activate the JAK–STAT pathway (38). To determine whether impaired IFN-I production in Blimp-1-deficient pDCs was caused by perturbed IFNAR signaling, we examined the activation of STAT1 after CpG-A stimulation in 4-OHT treated CKO-ER and Ctrl-ER FLpDCs. STAT1 phosphorylation at Tyr701 was reduced in Blimp-1-deficient FLpDCs (**Figure 7E**), signifying a reduction in STAT1 activation. However, this effect was attributed to perturbed production of IFN-I by Blimp-1-deficient pDCs, because comparable levels of phosphorylated STAT1 were detected in the control and Blimp-1-deficient FLpDCs after culture supplementation with mouse IFN-α (**Figure 7F**).

## Blimp-1 Inhibits IRAK-M Expression in pDCs

Blimp-1 was first identified as suppressing IFN-β expression after virus infection in a human bone osteosarcoma cell line, MG63 (29). Unexpectedly, our results demonstrated that Blimp-1 positively regulates IFN-I production following virus infection in pDCs, suggesting a cell type-specific effect. To confirm this, similar levels of IFN-β production were detected in the splenic cDCs isolated from CKO-11c and Ctrl-11c mice following stimulation with the TLR3 ligand poly(I:C) or the

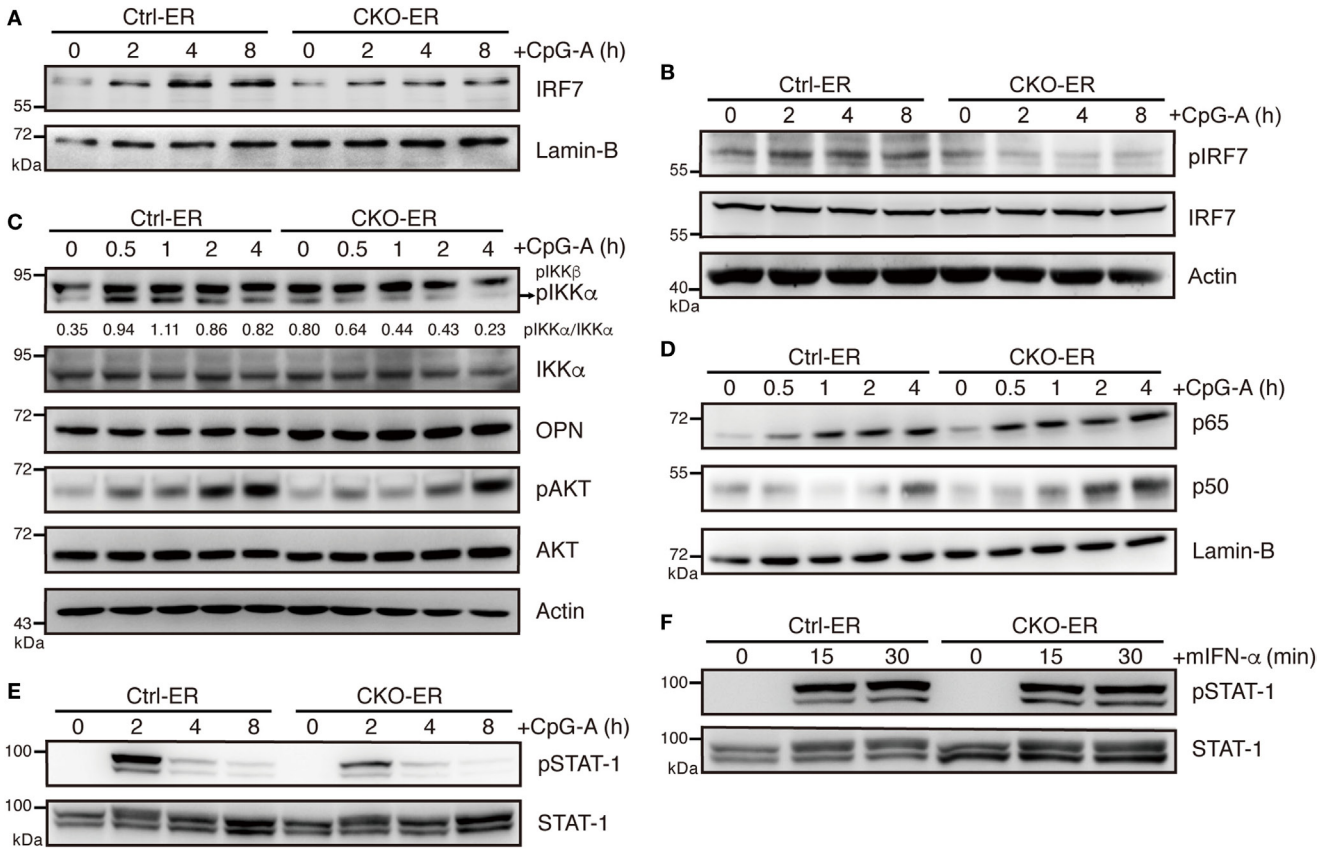


**FIGURE 7** | Impaired IKKα and IRF7 activation in plasmacytoid dendritic cells (pDCs) with reduced Blimp-1 expression. **(A)** Immunoblot analysis of nuclear extracts showing the levels of IRF7 in 4-OHT treated Ctrl-ER and CKO-ER FLpDCs following stimulation with 1 μM CpG-A at indicated time points. **(B)** Immunoblot analysis of total cell lysates showing the levels of IRF7 phosphorylation at Ser471/472 in 1 μM CpG-A stimulated Ctrl-ER and CKO-ER FLpDCs. **(C)** Immunoblot analysis showing the levels of phospho-IKKα at Ser176/180, OPN and phospho-AKT at Ser473, in 4-OHT treated and CpG-A stimulated Ctrl-ER and CKO-ER FLpDCs. The quantification of pIKKα was presented by the ratios of pIKKα band intensity vs. IKKα band intensity at each time point. **(D)** Immunoblot analysis using nuclear extracts of 4-OHT treated Ctrl-ER and CKO-ER FLpDCs showing the levels of p50 and p65 translocation after 1 μM CpG-A treatment at indicated time points. **(E)** Immunoblot analysis showing the levels of phosphorylated STAT1 at Tyr701 at the indicated time points in Ctrl-ER and CKO-ER FLpDCs treated with 4-OHT and then stimulated with 1 μM CpG-A at the indicated time points. **(F)** Immunoblot analysis showing levels of phosphorylated STAT1 at Tyr701 in FLpDCs in the presence of 500 U/ml mIFN-α.

TLR4 ligand LPS (Figures S5A,B in Supplementary Material). IRAK-M is induced in macrophages after LPS stimulation and acts as a negative regulator of TLR signaling by preventing the activation of IRAK-4/IRAK-1 (39). We tested if inhibition of IRAK-M may be required to activate the TLR-mediated signaling cascade in pDCs efficiently and whether Blimp-1 is involved in this regulation. We found that Blimp-1-deficient FLpDCs had increased IRAK-M mRNA and protein expression following CpG-A stimulation, in contrast with the downregulation of IRAK-M in stimulated Ctrl-ER FLpDCs (**Figures 8A,B**). According to previously identified Blimp-1 consensus binding sequences (40, 41), five putative Blimp-1 binding sites were identified within 5 kb upstream and downstream of the *Irak3* transcriptional start site (TSS, **Figure 8C**). Chromatin isolated from CpG-A stimulated FLpDCs was used to perform a ChIP assay to verify its binding by Blimp-1. A significant binding was present at site 3 located 1,909 bp upstream of the *Irak3* TSS (**Figure 8D**).

To determine whether increased IRAK-M expression contributes to the defective IFN-I production in Blimp-1-deficient pDCs, we used siRNAs to knockdown IRAK-M expression. 4-OHT treated CKO-ER and Ctrl-ER FLpDCs were transfected with siRNA-pools containing three different siRNAs specific to *Irak3* and stimulated with CpG-A (Figure S6A in Supplementary Material). IFN-α production was elevated after the knockdown of *Irak3* in CpG-A stimulated Ctrl-ER FLpDCs (**Figure 8E**). Notably, the knockdown of *Irak3* effectively restored the production of IFN-α in stimulated CKO-ER FLpDCs (**Figure 8E**), in contrast to the reduced production of IFN-α observed when these cells are transfected with control siRNA (siCtrl). The effect of siIrak3-pools on IFN-α production was specific because the knockdown efficiency of each individual siIrak3 was correlated with their effect on the restoration of IFN-α production in CKO-ER FLpDCs (Figures S6B,C in Supplementary Material). IRAK-M inhibited the production of proinflammatory cytokines, including IL-12p40, TNF-α, and IL-6, in stimulated macrophages

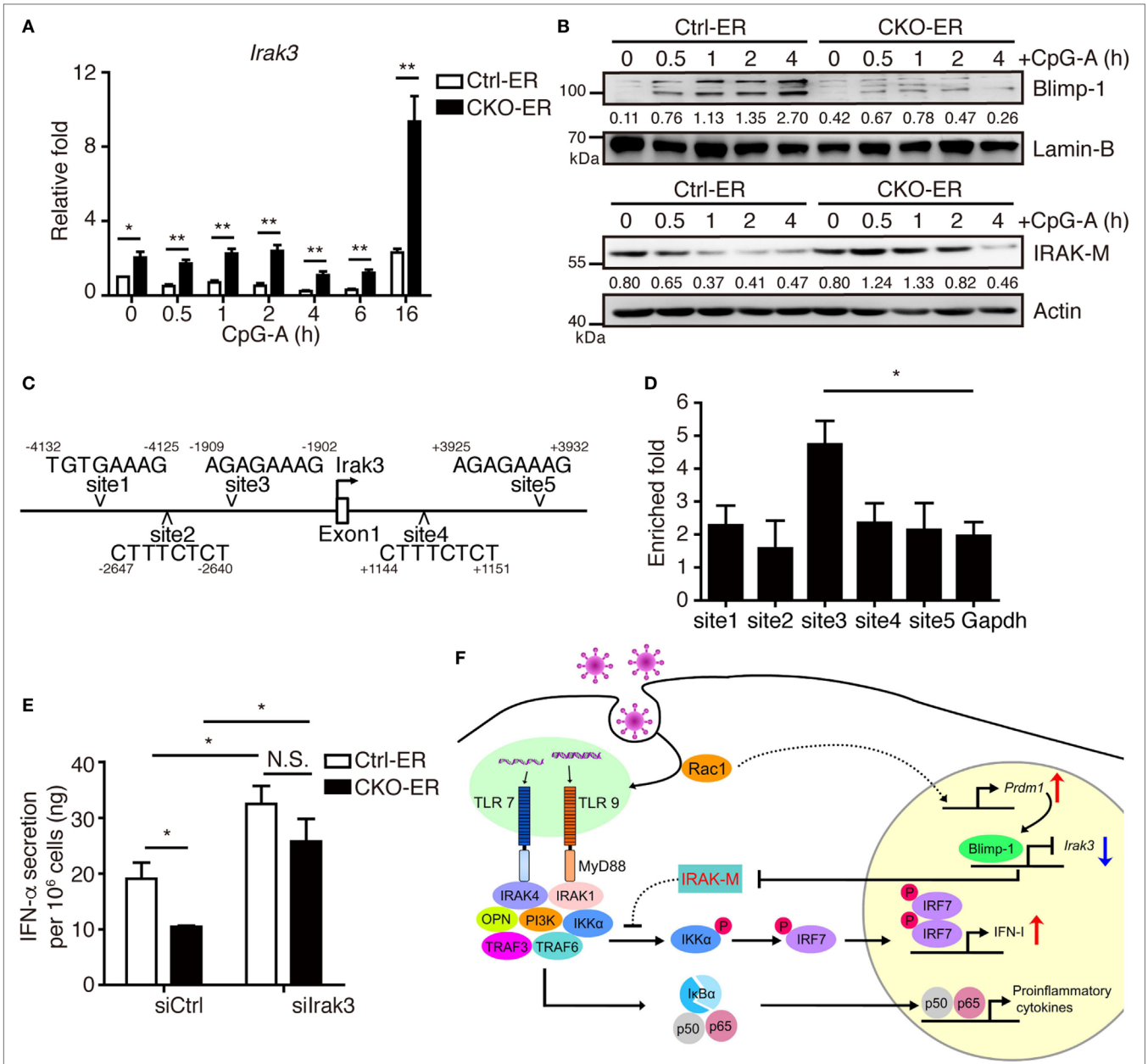


**FIGURE 8** | Increased IRAK-M expression in pDCs lacking Blimp-1 contributes to impaired IFN-I production. **(A,B)** RT-qPCR showing *Irak3* mRNA levels **(A)**, IRAK-M and Blimp-1 protein levels **(B)** in Ctrl-ER and CKO-ER FLpDCs treated with 4-OHT and then stimulated with 1 μM CpG-A. The quantitation of Blimp-1 in **(B)** was presented by the ratios of Blimp-1 band intensity vs. Lamin-B band intensity at each time point. The quantitation of IRAK-M in **(B)** was presented by the ratios of IRAK-M band intensity vs. actin band intensity at each time point. **(C)** Five putative Blimp-1 consensus binding sites were identified within 5 kb upstream and downstream of the *Irak3* transcriptional start site (TSS, indicated by an arrow). **(D)** ChIP assay using chromatin isolated from FLpDCs following 4 h stimulation with 1 μM CpG-A showing the levels of binding of Blimp-1 at various putative sites. *Gapdh* was used as the negative control locus. **(E)** IFN-α production by Blimp-1-deficient and control FLpDCs transfected with control siRNA (siCtrl) or siRNA-pools with three different siRNAs against *Irak3* (siIrak3) and stimulated with 1 μM CpG-A for 16 h. **(F)** Model of the action of Blimp-1 in the regulation of induction of IFN-I signaling in pDCs. Abbreviations: Rac, Ras-related C3 botulinum toxin substrate; IRAK-M, interleukin-1 receptor-associated kinase M; OPN, osteopontin; pDCs, plasmacytoid dendritic cells; IFN-I, type I IFN; siRNA, small-interfering RNA; *Irak3*, *interleukin-1 receptor-associated kinase 3*; ChIP, chromatin immunoprecipitation; RT-qPCR, RT-quantitative PCR. Data represent the mean ± SEM and were analyzed by two-tailed unpaired Student's *t*-test [$n = 4$ in **(A)** and 3 in **(D,E)**]. $^{*}p < 0.05$; $^{**}p < 0.01$. N.S. = no significant difference.

(39). However, unexpectedly, our results suggest that IRAK-M depletion in pDCs did not influence the production of IL-6 and TNF-α (Figures S6D,E in Supplementary Material). Collectively, our results show that Blimp-1-dependent suppression of *Irak3* may accelerate IFN-I production, but not affect cytokine production, in pDCs.

## DISCUSSION

Virus infection and stimulation by various pattern recognition receptors stimulation may trigger the expression of Blimp-1 (10, 29). Blimp-1 was originally reported to be a transcription repressor that binds to PRDI element of *IFN-β* gene promoter and inhibits sustained IFN-β expression after Sendai virus infection in human bone osteosarcoma cell lines (29). In macrophages, Blimp-1 was also reported to directly suppress the expression of murine chemokine (C-C motif) ligand 8 (*CCL8*) that modulates host defense against bacterial pathogens (42). Here, we showed that Blimp-1 was induced in pDCs, the professional IFN-I producing cells that limit viral infection, after TLR7 and TLR9 stimulation. However, to our surprise, we found that Blimp-1 did not inhibit IFN-β production; instead, Blimp-1 promotes IFN-I production and antiviral defense in pDCs. The pathway involved in the induction of Blimp-1 is unique in pDCs, which may not crucially depend on the conjugation of TLRs and ligands as suggested by our contrasting results from the kinetics of induction of Blimp-1 in TLR7- and TLR9-deficient pDCs. We show here that Rac is important for the induction of Blimp-1 in pDCs.

Rac1 is a small G protein that belongs to the Rho GTPase family, which controls many cellular events such as actin reorganization (43). Rac1 is activated by DOCK2 and acts upstream of TLR7 and TLR9 to produce IFN-I in pDCs. Rac is required for the non-specific endocytosis, macropinocytosis, in splenic DCs (44), and the endocytic capacity of DCs is enhanced after stimulation with TLR ligands (24). The activation of Rac1 has also been implicated in virus infection. Studies showed that Rac1 is involved in the suppression of H1N1 virus replication (45), and that activation of Rac1 after HSV-1 infection downregulates virus infectivity (46). Furthermore, Rac activation promotes caveolin-mediated JEV internalization (47). We suspect that Blimp-1 activation after virus infection in pDCs is also Rac-dependent. Using a potent Rac inhibitor, EHop-016, which inhibits Rac activity by targeting to the GEF binding pocket of Rac (26), we demonstrated that Blimp-1 induction in response to the stimulation with TLR7 and TLR9 ligands depends on the activation of Rac. Although, our and others' data (25) indicate that Rac1 is activated after stimulation with TLR7 and TLR9 ligands in pDCs, we cannot rule out the possibility that other Rac family members may also involve as EHop-016 inhibits all Rac family members. Although we found that the induction of Blimp-1 in pDCs is mediated through Rac-1, we here do not know the exact mechanisms causing the defective Rac-1 activation in *Tlr7* KO pDCs. Studies have just begun to reveal that the regulation of these two endosomal TLRs, TLR7 and TLR9, may be quite different. For example, TLR9, but not TLR7, needs UNC93B1, a multipass transmembrane protein, to traffic from plasma membrane to the endosome (48). TLR9 requires UNC93B1-mediated recruitment of AP-2 to ship into endolysosomes, while TLR7 utilizes alternative trafficking pathways. In terms of their functions, in lupus-prone mice, TLR7 and TLR9 have opposing roles in inflammation: TLR9 is required for inflammatory regulation but TLR7 promotes lymphocytes activation and serum IgG production (49). Therefore, it is possible that TLR7, but not TLR9, employs a feedback upregulation for Rac-1 activation.

We here find that the development of pDCs is not affected by Blimp-1 because the absolute pDC numbers in the spleen, the mRNA levels of various key transcription factors, and the expression of pDC markers were not altered by the deletion of *Prdm1*. Furthermore, the activation of pDCs following stimulation with TLR9 ligands was not influenced by the absence of Blimp-1. This is in contrast to our previous findings showing the role of Blimp-1 in cDCs where Blimp-1 deficiency led to the impaired up-activation of MHCII and other activation markers after TNF-α and stimulation with various TLR ligands in BM-derived DCs (8), showing the cell type-specific action of Blimp-1. We suspect that in pDCs, Blimp-1 may participate in the regulation of TLR downstream signaling independent to the activation of pDCs. Endosomal TLR7 and TLR9 are highly expressed in pDCs compared with other splenic DC subsets (50). Upon stimulation, TLR7 and TLR9 undergo conformational changes and recruit downstream factors to form the cytoplasmic transductional translational processor that transduces signals through phosphorylation and unbiquitination (51), finally activating IRF7 for robust IFN-I production. The expression of IRF7 was also controlled by NFATC3 in pDCs (52). In Blimp-1-deficient pDCs, impaired IRF7 phosphorylation and nuclear translocation was found; however, NF-κB activation and proinflammatory cytokine production were not affected. IKKα, osteopontin, and PI3K are necessary for IFN-I production, but not the secretion of other proinflammatory cytokines, by promoting the activation of IRF7 in pDCs (35–37). Our findings that Blimp-1 affects IKKα, IRF7 activation, and IFN-I production support these previous reports. IFN-I produced in response to TLR ligands in the early phase amplifies a positive feedback loop that signals through activation of JAK–STAT pathway *via* IFNAR (38). Our finding that impaired STAT-1 activation in Blimp-1-deficient pDCs was restored by supplemental IFN-α excludes the idea that Blimp-1 acts downstream of IFNAR signaling in IFN responses. Our data demonstrated the importance and the action of Blimp-1 in the sequential pathways of IFN-I production in pDCs. Furthermore, the function of Blimp-1 in the regulation of IFN-I in pDCs is cell type specific.

Interleukin-1 receptor-associated kinase M is a negative regulator of TLR signaling (39), but its expression kinetics appears to differ among cell types. Low expression of IRAK-M was reported in macrophages in the steady state, and both mRNA and protein levels were increased at 6–24 h after LPS stimulation (39). Furthermore, in *Irak3* KO macrophages, increased IL-12p40, TNF-α, and IL-6 production was observed after stimulation with various pathogen-associated molecular patterns (39). However, in human pDCs stimulated with R837, the high levels of IRAK-M declined rapidly. Moreover, knockdown of IRAK-M in human pDCs increased IFN-I production after TLR7 stimulation (19). We found that IRAK-M mRNA and protein levels were increased in TLR9-stimulated Blimp-1-deficient FLpDCs. According to our ChIP data, Blimp-1 directly bound to the promoter region of *Irak3* at 1,909 bp upstream of the TSS, suggesting that Blimp-1 may directly suppress the expression of IRAK-M. Therefore, the Blimp-1-mediated suppression of *Irak3* might be important for the regulation of IFN-I production in pDCs because impaired IFN-I production

was restored after knockdown of *Irak3* in Blimp-1 deficient FLpDCs in response to TLR9 simulation.

Our findings regarding the role of Blimp-1 in the regulation of IFN-I production in pDCs may have clinical relevance, such as in viral infection. IFN-I is one of the most important mediators against viral infection (53). Mosquito-borne JEV belongs to the Flaviviridae family, which causes up to 70,000 viral encephalitis cases annually (54). Previous studies have demonstrated the necessity of IFN-I in JEV infection both *in vitro* and *in vivo* (15, 31). We here also showed that anti-PDCA-1 administration significantly accelerates the death of JEV infected mice. Administration of anti-PDCA-1 antibody may affect other non-pDC cell types, particularly after viral infection (55). To avoid the inadvertent effects on deleting other immune cells, we stopped the anti-PDCA-1 antibody administration after JEV infection. With this approach, we found that the frequency and cell numbers of other cell lineages, including cDCs, myeloid cells, B cells, T cells, NK cells, and NKT cells, remained unchanged between anti-PDCA-1 antibody and control antibody treated groups before and 3 days after JEV infection. Therefore, our results indicated the importance of pDC-induced IFN-I production for defense against JEV infection. More importantly, decreased IFN-α production in serum and elevated virus replication in the brain were observed in CKO-11c mice after JEV infection. Furthermore, similar to the effect of Blimp-1 on the production of proinflammatory cytokines after stimulation with TLR ligands in pDCs, there were no differences in the cytokine production after JEV infection in CKO-11c mice. A negative role of TRIM29 in DNA virus infection in DCs through inhibiting the expression of stimulator of interferon genes, a key molecule in cytosolic DNA-sensing pathway, has been reported (56). Our results showed that Blimp-1 is important for the production of IFN-I in pDCs after CpG treatment and HSV-1 infection. It will be interesting to determine the role of Blimp-1 in DNA virus infection *in vivo*.

In conclusion, we demonstrated a Rac-mediated pathway is involved in the induction of Blimp-1 following the exposure of pDCs to TLR ligands. Blimp-1 suppresses *Irak3*, which efficiently relieves the negative regulation of TLR signaling and allows increased IFN-I production (**Figure 8F**). The Rac/Blimp-1/IRAK-M/IFN-I pathway identified in this study may be a new target pathway to selectively modulate the levels of IFN-I, but not cytokines, for the control of antiviral responses.

## ETHICS STATEMENT

Animal experimental protocols were approved by IACUC of Academia Sinica. The consent procedures of collection of samples from healthy donors were approved by the Academia Sinica Research Ethics Committee.

## AUTHOR CONTRIBUTIONS

K-IL conceived and designed the study. Y-AK, Y-HC and J-JL performed the experiments. Y-AK, Y-HC, J-JL and K-IL analyzed the data. C-HL, T-HC, Y-PH and Y-LL provided crucial animals and reagents. Y-AK. and K-IL wrote the manuscript.

## FUNDING

This work was supported by grants from Academia Sinica (AS-105-TP-B-08-01, 2316-1070800, 107DCA0100005), Taiwan, and Ministry of Science and Technology (104-2320-B-001-016-MY3 and 106-0210-01-15-02).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01828/full#supplementary-material.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 22 November 2018
doi: 10.3389/fimmu.2018.02738

# Chromatin Accessibility and Interactions in the Transcriptional Regulation of T Cells

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During T cell differentiation and activation, specific stimuli, and a network of transcription factors (TFs) are involved in orchestrating chromatin accessibility, establishing enhancer-promoter interactions, and regulating gene expression. Over the past few years, there have been new insights into how chromatin interactions coordinate differentiation during T cell development and how regulatory elements are programmed to allow T cells to differentially respond to distinct stimuli. In this review, we discuss recent advances related to the roles of TFs in establishing the regulatory chromatin landscapes that orchestrate T cell development and differentiation. In particular, we focus on the role of TFs (e.g., TCF-1, BCL11B, PU.1, STAT3, STAT5, AP-1, and IRF4) in mediating chromatin accessibility and interactions and in regulating gene expression in T cells, including gene expression that is dependent on IL-2 and IL-21. Furthermore, we discuss the state of knowledge on enhancer-promoter interactions and how autoimmune disease risk variants can be linked to molecular functions of putative target genes.

**Keywords: transcription factors, chromatin accessibility, T cells, STAT5, ChIA-PET, chromatin interactions**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *10 September 2018*
***Accepted:*** *06 November 2018*
***Published:*** *22 November 2018*

***Citation:***
*Li P and Leonard WJ (2018) Chromatin Accessibility and Interactions in the Transcriptional Regulation of T Cells. Front. Immunol. 9:2738. doi: 10.3389/fimmu.2018.02738*

## INTRODUCTION

Transcriptomic profiles determine the phenotype and function of cells, and this process is tightly controlled by various transcription factors (TFs), epigenetics, and chromatin interactions to define transcriptional patterns in response to cellular signals. More specifically, control of gene expression depends not only on the binding of sequence-specific TFs to target DNA sequences, but also on chromatin accessibility, which is controlled by the proper packaging of DNA/nucleosomes (chromatin) within the nucleus, leading to the arrangement of the genome into distinct spatial structures. Differences in chromatin composition can determine gene expression profiles in cells by providing relative accessibility (open or closed) of key regions to TFs that bind to DNA. Within the immune system, upon cellular stimulation by extracellular signals (e.g., via the T cell receptor (TCR) or cytokines), chromatin composition is modified through the concerted actions of signal-specific TFs and chromatin modifiers via a dynamic process. Naturally occurring genetic mutations in binding sites for TFs that alter the chromatin landscape can potentially disrupt or establish chromatin interactions, thereby resulting in altered gene expression profiles, and predisposing to cancer, autoimmune disease, allergy, immunodeficiency, or other immune disorders. In this review, we focus on the cooperative actions of TFs that play critical roles in shaping the chromatin landscape and accessibility in early and mature T cell development, and how these dynamic changes can alter gene expression profiles.

## TRANSCRIPTION FACTORS THAT ESTABLISH CHROMATIN LANDSCAPE DURING EARLY T CELL DEVELOPMENT

Lineage specific transcription factors (LSTFs) or master regulators are expressed at critical times during lymphoid development or differentiation, and they contribute to cell type determination. During the development of T cells in the thymus, master regulators such as TCF-1, BCL11B, GATA3, PU.1, and RUNX family TFs are critical for T-lineage commitment (**Figure 1**) (1–3). To properly regulate gene expression, TFs must recognize and bind to their sequence-specific DNA binding sites (motifs). Access to regulatory regions in the genome is tightly controlled by chromatin structure. Genome-wide analysis using Hi-C technology, a method coupling 3C (Chromosome Conformation Capture) methodology with high-throughput DNA sequencing (4), has revealed that the genome can be divided into spatially separated regions or "compartments," which are composed of smaller Topologically Associating Domains (TADs) that can be brought into close proximity to each other by chromatin looping (4–6).

During early T cell development, hematopoietic stem cells develop into T cell progenitor cells, termed $CD4^{-}CD8^{-}$ or double-negative (DN) thymocytes, which can then progress through four stages of maturation (denoted DN1, DN2a/b, DN3a/b, and DN4 cells). T cell commitment occurs at the DN2a to DN2b developmental transitional stages (1–3), and there is a key checkpoint termed β-selection at the $CD25^{+}CD44^{-}$ DN3a to DN3b/4 maturation step, with gene rearrangement of the TCR β chain. Following β-selection, T cells further mature into $CD4^{+}CD8^{+}$ double-positive (DP) cells, which express both CD4 and CD8 (3). Some evidence suggests that dynamic changes in chromatin modifications and transcription are associated with T cell development (7), but it is not clear if there are genome-wide modifications in higher-order chromatin structures and whether such structures are required to establish T cell identity. GATA-3 is essential throughout the early T cell developmental stages, including for T cell commitment, β-selection, and $CD4^{+}$ cell fate choice during positive selection (8).

The IL-7/IL-7R axis plays major roles in the survival of DN thymocytes during early T-cell development (9, 10). IL-7-signaling activates major signaling pathways, including JAK1/JAK3-STAT5 and PI 3-kinase, and Y449 of the IL-7R is part of a YxxM motif and can mediate not only recruitment of STAT5 but also the p85 subunit of PI 3-kinase (11). IL-7-mediated signaling results in the induction of anti-apoptotic BCL-2 and MCL-1 proteins but the decreased expression of pro-apoptotic proteins (9), and STAT5 has been implicated in the regulation of expression of BCL-2 (12). IL-7-mediated STAT5 activation controls chromatin accessibility and rearrangement of the TCRγ locus (13, 14). In addition to its activation of STAT5, IL-7 was reported to activate NFATc1, with this serving as an alternative signaling pathway that cooperates with STAT5 to guide thymocyte development (15). Thus, IL-7-mediated transcriptional activation serves important roles in T cell development.

Recent studies have provided further insights into the mechanisms by which two TFs, TCF-1, and BCL11B, drive T cell differentiation by modifying the nuclear architecture to generate distinct chromatin landscapes (16, 17). Chromatin accessibility across distinct stages of T cell development was profiled using single-cell DNase-Seq (DNase I hypersensitive sites sequencing) (18, 19) and ATAC-Seq (Assay for Transposase Accessible Chromatin combined with DNA sequencing) (20, 21) to reveal that dynamic modifications in chromatin accessibility appeared genome-wide during T cell differentiation (16, 17). Strikingly, different stages of chromatin accessibility were observed as developing cells progress during T cell commitment (**Figure 1**). BCL11B, a critical regulator of T cell commitment, was found to play critical roles in maintaining higher-order chromatin structures and was associated with increased chromatin interactions during T cell lineage commitment (17). Furthermore, at early stages of T cell differentiation, TCF-1 was significantly enriched at accessible chromatin that was associated with T cell-lineage-specific gene loci (16). Mice deficient in *Tcf7*, which encodes TCF-1, cannot properly establish the open chromatin landscape of normal T cells, suggesting that the initiation of chromatin remodeling was TCF-1-dependent, and this was particularly evident at the *Bcl11b* locus (**Figure 1**).

PU.1 (encoded by *Spi1*/*Sfpi1*) was shown to function as a specialized nucleosome-binding transcription factor during the DN1-DN2 transition (22, 23), and this factor can bind to closed chromatin and rapidly open genomic sites. Specifically, ChIP-Seq (Chromatin Immunoprecipitation combined with high-throughput DNA sequencing) (24, 25) and ATAC-Seq analysis showed that the chromatin of selected regions is opened by PU.1 within 24 h (22, 23), suggesting that PU.1 acts as a "pioneer factor" to remodel chromatin structure during early T-cell development.

## TRANSCRIPTION FACTORS THAT MEDIATE CHROMATIN ACCESSIBILITY DURING T CELL DIFFERENTIATION

During $CD4^{+}$ T cell differentiation, a range of pioneer factors are activated to shape the epigenetic landscape and regulate chromatin accessibility for TFs (26–29). T cell activation requires antigen signaling via the TCR and co-stimulation with CD28, resulting in nuclear translocation of a number of TFs, including AP-1 and NFAT (**Figure 2**). Interestingly, BATF, a FOS-like AP-1 family transcription factor, and IRF4 were shown to function as pioneer factors that could regulate chromatin accessibility during differentiation of Th17 (30) and $CD8^{+}$ T cells (31).

Following TCR stimulation, cytokines including IL-2 and IL-21 are also produced and in turn activate and induce the nuclear translocation of STAT proteins (e.g., STAT5 and STAT3) through cytokine receptors IL-2R and IL-21R (**Figure 2**), and these factors collectively help to prepare the T cell chromatin landscape. Interestingly, both STAT dimers and tetramers can form, with STAT5 tetramers being critical for the normal development and expansion of key immune populations (32, 33). Cytokines secreted by immune cells can also drive T helper cell

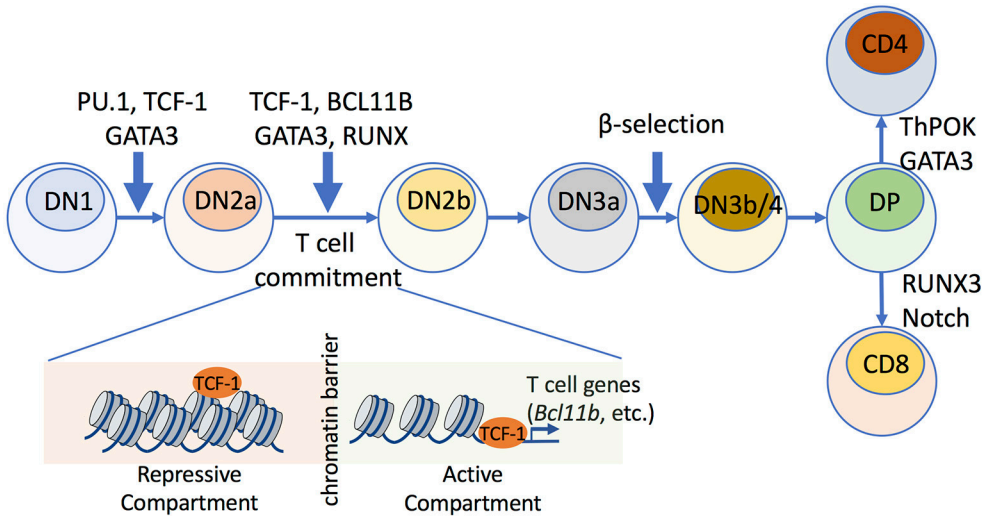


**FIGURE 1 |** Transcription factors that mediate chromatin accessibility during early thymic T cell development. Multiple TFs play roles in early stages of T cell maturation, which involve commitment of hematopoietic stem cells to T cell progenitors. The early DN stage consists of DN1, DN2a/b, DN3a/b, and DN4 cells. During T cell commitment, which occurs between the DN2a and DN2b stages, TCF-1 establishes chromatin accessibility and mediates compartment switch, where repressive compartments that harbor T cell-lineage-specific genes (e.g., *Bcl11b*) are switched to transcriptionally active compartments. TCF-1 upregulates the expression of BCL11B, which further remodels chromatin architecture and stabilizes the intra-TAD contacts within mature T cell subsets.

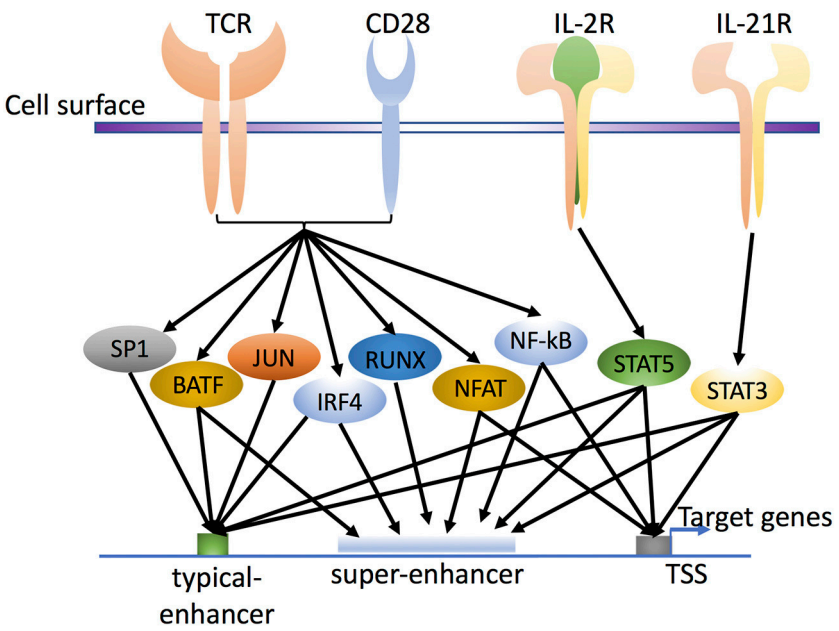


**FIGURE 2 |** Transcription factors in T cell activation and differentiation. T cell receptor (TCR) and CD28 signaling activate various pioneer factors, such as NF-$\kappa$B, NFAT, and AP-1 (FOS and JUN family proteins; the schematic shows BATF as the FOS-like AP-1 family protein). In addition, cytokine stimulation activates cytokine-specific TFs, such as IL-2-activated STAT5 and IL-21-activated STAT3, through their own cytokine receptors IL-2R and IL-21R, respectively. Together, these factors influence the enhancer landscape in a genome-wide fashion, with binding of TFs to typical-enhancers and/or super-enhancers to regulate the expression of target genes in T cells and influence cell differentiation and cell plasticity. Whereas typical enhancers span more limited regions, super-enhancers include groups, or clusters of enhancer elements, that span broader regions and are densely bound by transcriptional co-activators.

differentiation. For example, IL-12, IL-4, and IL-6 drive Th1, Th2, and Th17 differentiation, respectively, with critical roles for IL-2 in promoting (Th1, Th2, Th9, Treg) or inhibiting (Th17 or T follicular helper [Tfh] cell) differentiation and often opposing actions for IL-21 (34–37). All of these cytokines are 4-alpha helical bundle type 1 cytokines that use the JAK-STAT pathway

as a major signaling pathway to transduce extracellular cytokine signals into the cell and regulate expression of corresponding genes (37–39). Interestingly, of these cytokines, IL-2, IL-4, and IL-21 signal via receptors that belong to the common cytokine receptor γ chain ($\gamma_c$, also known as the IL-2 receptor γ chain IL-2Rγ, or CD132) family of cytokines (40, 41). During $CD4^+$ T cell differentiation, STATs can have major impact on the activation of lineage-specific enhancers and the suppression of enhancers associated with alternative cell fates. For example, STATs can shape the active enhancer landscape in Th1 and Th2 cells (42, 43) in the presence of different cytokine signals, with IL-12/STAT4 and IFN-γ/STAT1 driving Th1 and IL-4/STAT6 driving Th2 differentiation, respectively. In addition, however, IL-2 via STAT5 serves a key role and primes T cells for responsiveness to IL-12 and IL-4 and Th differentiation. For Th1 differentiation, IL-2 via STAT5 augments expression of IL-12Rβ2 and TBET (44) and for Th2 differentiation, it augments expression of IL-4Rα (45) and IL-4 (46–48), with IL-2-induced STAT5 kinetically binding earlier to the *Il4ra* than to the *Il4* locus. Interestingly, IL-2-activated STAT5 binding was shown to augment chromatin accessibility at the *Il4* locus (46). IL-2 via STAT5 also inhibits Th17 differentiation (49, 50), potentially by several mechanisms, including a direct IL-2-STAT5 competition with IL-6-STAT3 (49, 51), the inhibition by IL-2-STAT5 of gp130 expression and by IL-2-mediated induction of TBET, which interacts with RUNX1, potentially limiting the required RUNX1-RORγt interaction (44). Moreover, IL-2-STAT5 drives Th9 differentiation (52) and limits Tfh differentiation (53, 54) whereas, IL-21-STAT3 has an opposing effect (52, 55, 56). As compared to pioneer factors, cytokines that influence Th differentiation have less profound effects on the epigenetic landscape.

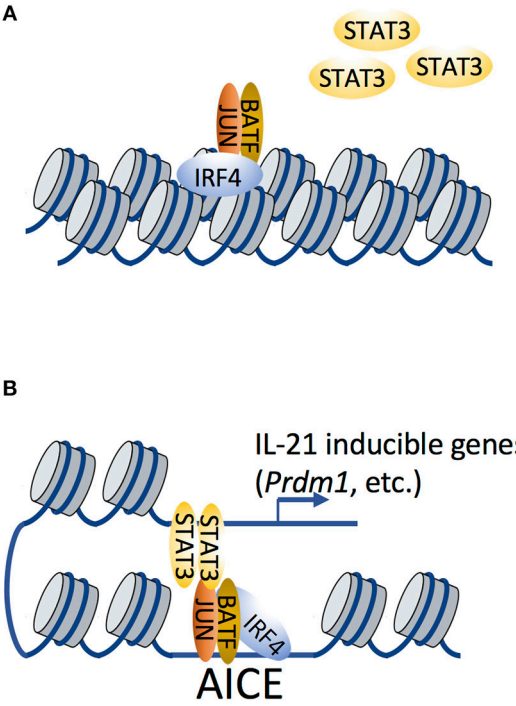


**FIGURE 3 |** IRF4 and BATF remodel the chromatin landscape to facilitate subsequent recruitment of transcription factor STAT3**. (A)** Cooperative binding of AP-1 (shown here as a BATF-JUN heterodimer; BATF in this setting is the FOS-like factor) and IRF4 function as pioneer factors to remodel the chromatin landscape, therefore affecting chromatin accessibility. **(B)** STAT3 is subsequently recruited by AP-1/IRF4 complexes (which recognize AP-1-IRF composite elements, AICEs) via possible STAT3-JUN interactions. Such STAT3/AP-1/IRF4 complexes have been shown to regulate the expression of IL-21-inducible genes.

## INTERPLAY OF CHROMATIN AND TRANSCRIPTION FACTORS AP-1, IRF4, AND STAT3 IN T CELLS

BATF and IRF4 were shown to functionally cooperate and recognize specific AP-1-IRF composite elements (AICEs) mainly in T cells and dendritic cells (57–59) and these factors cooperate to regulate chromatin accessibility during the differentiation of Th17 (30) and $CD8^+$ T (31) cells (**Figure 3**). FAIRE-Seq (Chromatin accessibility analysis using formaldehyde-assisted isolation of regulatory elements sequencing) (60) revealed that the loss of BATF or IRF4 in Th0 or Th17 cells had little if any effect on genomic loci already accessible in naive cells, but most loci with inducible accessibility exhibited marked reductions in *Batf*- or *Irf4*-deficient mice compared to wild-type cells, suggesting that IRF4 and BATF remodel the chromatin landscape and potentially facilitate subsequent recruitment of TFs involved in regulating expression of Th17-relevant genes (30). Enhancer occupancy by AP-1/IRF4 complexes correlates with sensitivity of gene expression in response to TCR signaling (61, 62), so that genes with low-affinity or high-affinity AICE-dependent enhancers are induced at lower or higher TCR signal strength, respectively. IRF4 alone was also shown to be induced in a manner dependent on TCR affinity, and as a dose-dependent regulator of the metabolic function of activated T cells (63). BATF is also a key regulator of early effector $CD8^+$ T cell differentiation (31), and BATF-deficient $CD8^+$ T cells are profoundly defective in their ability to undergo naive to effector differentiation and proliferative expansion. Moreover, BATF and IRF1 are induced early during *in vitro* regulatory T (Treg) cell differentiation and act as pioneer factors for the differentiation of type 1 Treg (Tr1) cells (64). BACH2, like AP-1 factors that contain a bZIP domain, can regulate $CD8^+$ T cell differentiation by controlling the access of AP-1 factors to enhancers, thus limiting the expression of TCR-driven genes by attenuating the availability of AP-1 sites to JUN family TFs (65, 66).

IRF4 often cooperates with STAT3 in modulating IL-21-dependent gene expression in Tfh and Th17 cells (30, 58, 67). Given that STAT3 can physically bind to c-JUN (68), it is reasonable to hypothesize that STAT3 can be recruited by BATF-JUN-IRF4 complexes via STAT3-JUN interactions (**Figure 3**). ChIP-Seq analysis revealed that IL-21-induced STAT3 binding was dramatically diminished in $Irf4^{-/-}$ $CD4^+$ T cells compared to WT cells (67), suggesting that it was IRF4-dependent. It is possible that STAT3 directly binds to IRF4, or that its binding is dependent on chromatin accessibility that is pre-patterned

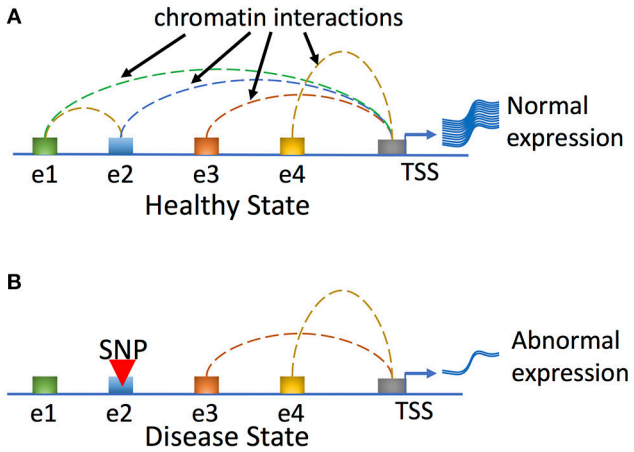


**FIGURE 4 |** Genetic variation can affect human disease phenotypes by disrupting regulatory networks mediated by long-range chromatin interactions. In the healthy state **(A)**, all regulatory enhancer elements (shown here for a hypothetical gene as elements e1–e4) are utilized and loop to the promoter to effect normal gene expression. In the disease state **(B)**, a hypothetical genetic variant (SNP) residing at e2 disrupts enhancer-promoter and enhancer-enhancer interactions and results in abnormal gene expression and disease phenotypes.

by IRF4 to facilitate the subsequent recruitment of STAT3 to AP-1. However, STAT3 binding motifs are not enriched in genomic proximity to AICEs (58, 69), suggesting that STAT3-IRF4 association may also occur via long-range chromatin interactions, a hypothesis that remains to be experimentally validated.

## T CELL RESPONSES TO DIFFERENT STIMULI INCLUDING IL-2 AND IL-21 AND THE ROLE OF SUPER-ENHANCERS

After antigen encounter, $CD4^+$ T cells are activated and secrete cytokines including IL-2 and IL-21, which regulate immune cell differentiation and effector functions by differentially activating specific STAT proteins that recognize and bind to γ-interferon-activated sequence (GAS) motifs. IL-2 potently activates STAT5, whereas IL-21 primarily activates STAT3. This differential STAT activation leads to differential gene expression by these cytokines. It is established that STAT proteins are critical components of cytokine-activated enhancers, but recently their roles related to super-enhancers (70) and their abilities to fine-tune gene expression (71) have been elucidated, with, for example, greater IL-2-inducibility of genes with STAT5-based super-enhancers, as compared to STAT5-based typical enhancers (71). As opposed to typical enhancers, where factor binding occurs in more limited regions, super-enhancers (also known as stretched or clustered enhancers) (70, 72, 73) represent groups of putative enhancers in close genomic proximity that span broader regions (**Figure 2**), are densely bound by transcriptional coactivators, and usually are associated with high levels of the active chromatin mark histone H3 lysine 27 acetylation (H3K27Ac). Although super-enhancers were originally recognized in the setting of master regulator genes (74) and genes associated with cell identity, STAT5- and STAT3-dependent super-enhancers have now also been shown to exist and to regulate gene expression in a cytokine- and context-specific manner (71). Chromatin interaction analysis using paired-end tag sequencing (ChIA-PET) (75, 76) revealed that IL-2–activated STAT5 can influence RNA Polymerase II (RNA Pol II)-based chromatin interactions, with looping anchor sites in proximity to STAT5 binding sites. Moreover, CRISPR–Cas9 (77, 78) genome editing was used to generate mutant mice in order to functionally analyze the STAT5-bound super-enhancer containing gene, *Il2ra*, *in vivo*. When three of the super-enhancer elements were separately deleted, each exhibited defective expression of IL-2Rα, indicating that each enhancer element contributed to IL-2-induced IL-2Rα expression and that these elements were not functionally redundant (71). These observations provide insights into the mechanism underlying the regulation of IL-2 target genes. Interestingly, IL-2-based super-enhancers included not only positive regulators of signaling, such as *Il2ra*, but also negative regulators such as SOCS family proteins (e.g., *Cish*), revealing that super-enhancers are critical for both the positive and negative regulation of IL-2 signaling (71).

Similar to the mouse *Il2ra* gene, human *IL2RA* also has a similar super-enhancer that is densely bound by STAT5, and some of the enhancer elements are highly conserved in both mouse and human, consistent with an evolutionarily conserved mode of gene regulation (71, 79, 80). Interestingly, tiled CRISPR activation (CRISPRa) (81) was used to identify several CRISPRa-responsive elements with chromatin features of stimulus-responsive enhancers, including an *IL2RA* enhancer that contains a non-coding autoimmunity risk variant (80) that is conserved between humans and mice. Mutating this element in mice did not completely block *Il2ra* gene expression but rather delayed gene activation in response to TCR stimulation, indicating that the kinetics of *Il2ra* gene expression are important. This mutation skewed polarization of naive T cells from Treg cells toward pro-inflammatory Th17 cells, which elucidates its role in autoimmune disease (80).

## ENHANCER-PROMOTER INTERACTIONS AND AUTOIMMUNE DISEASE-ASSOCIATED SNPs

Gene expression is regulated via complex interactions between promoters and long-range regulatory elements, and disruption of chromatin interactions by mutations (e.g., SNPs or INDELs) may result in altered target gene expression that leads to disease development (**Figure 4**). Another study correlated histone modification of H3K27ac with active enhancers and promoters and furthermore analyzed protein-centric chromatin interactions by utilizing HiChIP, chromatin immunoprecipitation (ChIP) and Hi-C assays (82). By generating enhancer–promoter contacts in primary naive $CD4^+$ T cells, Treg cells, and Th17 cells, chromatin loops were identified that were shared by all three cell types (82).

Strikingly, the majority of these chromatin interaction anchors were associated with enhancers or promoters. Furthermore, autoimmune disease–associated variants in intergenic regions could interact with multiple target genes, providing insights into the functional interrogation of disease associated genetic variants; however, further high-resolution chromatin interactions in various cell types are needed to better explain how connections between variants and genes can be translated into molecular and cellular functions.

## CONCLUDING COMMENTS

In summary, studies of the transcriptional and epigenetic regulation of T cells have identified several mechanisms of cross-regulation between TFs, chromatin modifiers, and the pre-existing chromatin landscape. The interactions between chromatin and TFs are influenced by a range of stimuli, including TCR and cytokine signals. Transcription factors are important for cell function, and they collaborate combinatorically with other factors to influence gene regulation. Their binding to DNA depends on epigenetic landscapes, and their function may depend on chromatin interactions to juxtapose distal regulatory elements with gene promoters. The ability of cytokine-activated proteins to modify nucleosome packing and influence histone modifications allows them to control developmental processes. The gene regulatory networks that determine T cell development are broad and involve chromatin accessibility, epigenetic status, and distant chromatin interactions in both time- and context-dependent manners. Our evolving understanding of gene regulatory networks will help to comprehensively link genetic variants to putative gene targets, furthering our understanding of molecular mechanisms for a range of immune diseases. Achieving a deeper understanding of the mechanisms involved has now been greatly facilitated by genetic manipulations including CRISPR/Cas9 gene editing but still awaits other advances, such as the ability to comprehensively study single cells in real time.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with the authors.

frontiers in Immunology

REVIEW
published: 12 December 2018
doi: 10.3389/fimmu.2018.02929

# Epigenetic and Transcriptional Regulation in the Induction, Maintenance, Heterogeneity, and Recall-Response of Effector and Memory Th2 Cells

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**Edited by:**
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**Specialty section:**
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

**Received:** 18 August 2018
**Accepted:** 29 November 2018
**Published:** 12 December 2018

**Citation:**
Onodera A, Kokubo K and Nakayama T (2018) Epigenetic and Transcriptional Regulation in the Induction, Maintenance, Heterogeneity, and Recall-Response of Effector and Memory Th2 Cells. Front. Immunol. 9:2929. doi: 10.3389/fimmu.2018.02929

Antigen-primed T cells respond to restimulation much faster than naïve T cells and form the cellular basis of immunological memory. The formation of memory Th2 cells starts when naïve CD4 T cells are transformed into effector Th2 cells and is completed after antigen clearance and a long-term resting phase accompanied by epigenetic changes in the Th2 signature genes. Memory Th2 cells maintain their functions and acquired heterogeneity through epigenetic machinery, on which the recall-response of memory Th2 cells is also dependent. We provide an overview of the epigenetics in the whole Th2 cell cycle, mainly focusing on two different histone lysine methyltransferase complexes: the Polycomb and Trithorax groups. We finally discuss the pathophysiology and potential therapeutic strategies for the treatment of Th2-mediated inflammatory diseases in mice and humans.

**Keywords: polycomb and trithorax, airway infiammation, pathogenic Th2 (Tpath2) cells, GATA3, allergic disease**

## INTRODUCTION

T cells experience several events before transforming into memory T cells: antigen priming, differentiation into certain functional distinct subsets, migration to inflammatory sites, exertion of the effector functions, and a long-term resting phase. Some of these events are unnecessary or may even be inadvisable for memory T cell formation. Antigen priming, however, is definitely essential for the formation of the immunological memory (1–4). During antigen priming, T cell receptor (TCR) signals induce epigenetic changes of the genes encoding lineage-specifying transcription factors and lineage-specific cytokines collaborating with signals from costimulatory molecules and cytokine receptors (5). After undergoing the above-described cellular events, T cells finally become memory T cells, in which the genes responsible for a rapid response to the same antigen are epigenetically poised for transcription. In this review, which focuses on Th2 cells, we discuss the epigenetic regulatory mechanisms underlying T cell-mediated immune responses beginning from the priming of naïve T cells and ending with the recall-response of memory T cells.

In contrast to innate immunity, acquired immunity recognizes non-self-peptide antigens through TCRs on naïve CD4 T cells, resulting in the functional differentiation of effector helper T (Th) cell subsets, including Th1, Th2, and Th17 cells (6). Each subset has its "working range" in immune response. For example, Th1 cells organize CD8 T cell-mediated cellular immunity against intracellular bacteria and viruses by producing IFNγ. However, Th1 responses are often associated with tissue-specific autoimmune diseases, including type 1 diabetes (7). Th2 cells produce IL-4, IL-5, and IL-13 (so-called Th2 cytokines) and play a role in immunity against extracellular parasites (1). Th2 cells also cause allergic diseases, including asthma, rhinitis, and atopic dermatitis. Th17 cells secrete IL-17 and are crucial for immunity against fungi; they are also involved in the pathogenesis of inflammatory bowel disease in collaboration with Th1 cells (8). The differentiation of each Th subset accompanies epigenetic changes in its specific genes (9). Thus, regulatory molecules in the epigenetic changes have received significant attention in the field of immunology. Histone modifications, DNA methylation, and non-coding RNA transcripts, such as microRNAs and long non-coding RNAs (lncRNAs), are now recognized as important epigenetic regulators (10–12). Various post-translational modifications of histone tails, which are tightly associated with gene expression, have been identified. The methylation of histone H3K27 is considered to be important for gene silencing and is catalyzed by Polycomb group (PcG) proteins (1, 13–15). PcG complexes were originally identified in Drosophila and are categorized into two basic types: Polycomb repressive complex (PRC) 1 and 2 (**Figure 1**). Enhancer of Zeste (EZH) 1 and 2, which methylate H3K27, are active subunits of PRC2. PRC1 recognizes and binds to H3K27 methylation and represses the target gene expression in collaboration with PRC2. Another subunit of PRC1, ring finger protein (RING1), possesses ubiquitin ligase activity for histone H2AK119. In contrast to H3K27 methylation—which is mediated by PcG proteins—H3K4 methylation, which is catalyzed by Trithorax (TrxG) proteins, is associated with a chromatin structure that permits transcription (**Figure 1**). In mammals, six H3K4 methylases have been identified and classified into three groups (15–17). The first group consists of mixed lineage leukemia (MLL)-1/2 and a specific component, Menin, which is encoded by the *MEN1* gene in humans, the mutation of which is often associated with multiple endocrine neoplasia type 1 (MEN1). The second group contains MLL-3/4 and H3K27 demethylase, UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome). The translocation or mutation of the genes encoding MLL proteins are frequently found in leukemia patients, indicating that appropriate control of the MLL functions is important for the homeostasis of hematopoiesis. The third group of H3K4 methylase complex is composed of SET1A/B and the unique subunit WDR82. TrxG proteins can both upregulate the expression of the target gene and keep it active, depending on their association partners or the epigenetic signatures of the target genes (18). The present review mainly focuses on the PcG- and TrxG-mediated epigenetic regulation of effector and memory Th2 cells, which have dual aspects in the immune system: protective and pathogenic.

# EPIGENETIC REGULATION IN THE INDUCTION OF TH2 CELL DIFFERENTIATION

## STAT6 Is Activated by IL-4 Signaling and Induces Epigenetic Changes of the *Gata3* Gene

Antigen recognition via TCR is an essential event for naïve CD4 T cells to initiate clonal expansion and differentiation into effector Th cell subsets, including Th2 cells. The TCR signaling pathway is known to turn on the activation switch of naïve CD4 T cells, whereas cytokines and their receptor signaling pathways direct the differentiation of naïve CD4 T cells toward each subset. Th2 differentiation is induced by IL-4 and its receptor signaling cascade, which finally phosphorylates STAT6. Phosphorylated STAT6 forms a dimer, moves into the nucleus, binds to the target genes, and controls their expression (19, 20). The most important target of STAT6 is the *Gata3* gene, which encodes a transcription factor, GATA3, the element responsible for the chromatin remodeling of Th2 cytokine gene loci. Actually, the direct binding of STAT6 is determined within the *Gata3* gene locus by both ChIP-seq and conventional ChIP assays (21, 22). IL-4 fails to upregulate the expression of *Gata3* without STAT6. Consequently, very few IL-4-producing Th2 cells can be generated from STAT6-deficient naïve CD4 T cells, even when cultured under Th2-inducing conditions. STAT6 also plays a role in the epigenetic regulation of the *Gata3* gene during Th2 cell differentiation (**Figure 2**). The *Gata3* gene is known to have two promoters: a proximal promoter and a distal promoter, the latter of which is located approximately 10 kilobases upstream of the transcription start site (TSS) (24). *Gata3* transcription is mainly dependent on the proximal promoter in both naïve CD4 T and Th2 cells, although qPCR (quantitative polymerase chain reaction) detected a small amount of transcripts driven by the distal promoter in Th2 cells (22, 25). A dramatic change in the epigenetic marks is observed between the distal and proximal promoters during Th2 cell differentiation. In naïve CD4 T cells, the binding of PcG proteins is detected in these regions. In contrast, TrxG proteins bind to the proximal promoter and its downstream region. Thus, the proximal promoter forms a boundary between the PcG-binding and TrxG-binding regions. During Th2 cell differentiation, PcG proteins disassociate from the region between the distal and proximal promoters, and the binding of TrxG proteins spreads into this region. Basically, histone modification patterns behave in a similar way. H3K27 is highly methylated in the region between the distal and proximal promoters in naïve CD4 T cells and demethylated during Th2 differentiation. H3K4me3, which is found at the proximal promoter and its downstream region in naïve CD4 T cells, spreads upstream. Thus, the exchange of PcG and TrxG at the region between the distal and proximal promoters of the *Gata3* gene is induced by STAT6 and defines the Th2 cell identity.

## Spatial Interplay Between the Polycomb and Trithorax Complexes

The *Gata3* gene is co-occupied by PcG and TrxG proteins and shows bivalency, with both H3K27me3 and H3K4me3 being

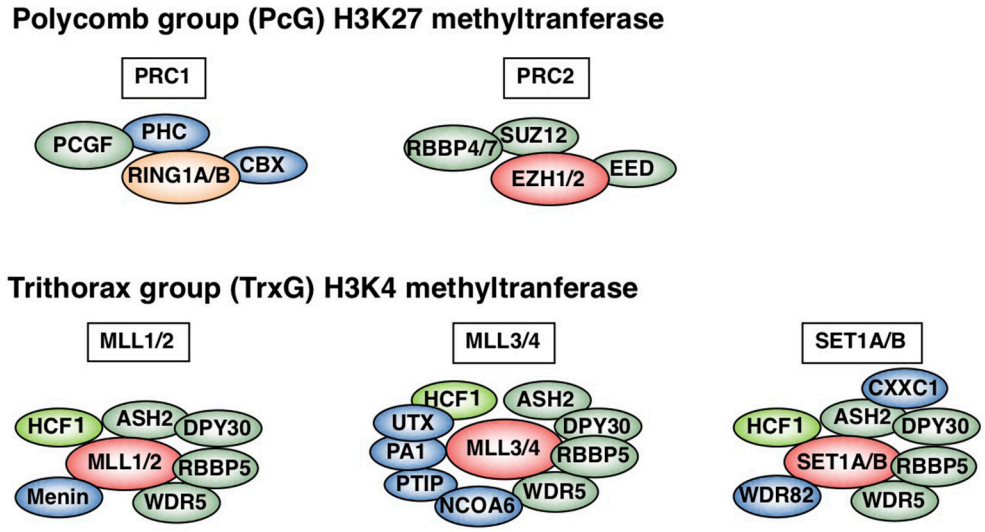


**FIGURE 1 |** Polycomb (PcG) and Trithorax (TrxG) complexes in mammals. Two basic types of Polycomb repressive complex 1 (PRC1) and PRC2 are shown (upper). Canonical PRC1 consists of four core subunits: RING1A/B, PCGF, CBX, and PHC (1, 15, 16). PCGF and RING1A/B, which ubiquitinate H2AK119, also compose non-canonical PRC1 (15). PCGF4 is also known as Bmi1. PRC2 consists of four core subunits: EZH1/2, EED, SUZ12, and RBBP4/7. The SET domain of EZH1/2 is responsible for PRC2 methylase activity. In contrast, mammalian cells have six H3K4 methylases: MLL1-4, SET1A, and SET1B (lower) (1, 15–17). All of these complexes share ASH2L, RBBP5, DPY30, WDR5, and HCF1, which is a substoichiometric component that is absent in some branches of the TrxG complexes (green) (17). Menin is a unique subunit of MLL1/2 complexes (blue). MLL3/4 complexes are uniquely associated with PTIP, PA1, UTX, and NCOA6, while SET1A/B complexes are specifically associated with WDR82 and CXXC1 (shown in blue). This figure was reproduced with permission provided by Annual Reviews copyright transfer agreement [originally published by Nakayama et al. (1)].

present at the same time in naïve CD4 T cells (26). The functions of these bivalent genes were originally analyzed in embryonic stem (ES) cells and are poorly understood in T cells (27). Furthermore, there are few reports on genes co-occupied by PcG and TrxG proteins. We therefore analyzed the features of the co-occupied genes in both ES and T cells. A substantial number of the co-occupied genes are found in ES cells, whereas only a few genes are co-occupied by PcG and TrxG proteins in T cells (23). The binding levels of PcG proteins and those of TrxG proteins are reciprocally correlated in both cell types. In this study, we also identified two binding patterns: "PcG bound upstream and TrxG bound downstream of the TSS," a pattern that was frequently observed in strongly expressed genes in T cells; and "PcG bound downstream and TrxG bound upstream of the TSS," a pattern that was frequently observed in weakly expressed genes in T cells (**Figure 2**) (23). Interestingly, the former gene group includes *Nfatc1*, *Fli1*, and *Gfi1,* which are important for the development and function of T cells (23). Thus, spatial interplay between the PcG and TrxG proteins may be a novel mechanism regulating the bivalent genes co-occupied by these two complexes. It has been proposed that PcG proteins maintain the *Gata3* expression at "an appropriate level in naïve CD4 T cells" based on observations in *Ezh2* knockout mice: CD4 T cells that lack *Ezh2* showed enhanced sensitivity to IL-4, increased *Gata3* expression, and Th2 cytokine hyper-production (1, 28). In contrast to the *Gata3* gene locus, the Ezh2 binding levels at Th2 cytokine gene loci were very low, suggesting that the Th2 cytokine expression is controlled though Ezh2-dependent repression of the *Gata3* gene.

## The GATA3-Dependent Epigenetic Regulation of Th2 Cytokines and Other Th2 Signature Genes

The GATA family transcription factors (GATA1-6) recognize the consensus DNA sequence WGATAR via one or two C2-C2-type zinc-finger motifs (29–31). Based on their expression patterns in the body, GATA1-3 are classified as hematopoietic factors, whereas GATA4-6 are recognized as endodermal factors. In the immune system, GATA3 is predominantly expressed in T cells and innate lymphoid cells (ILCs), including natural killer (NK) cells (32). Regarding T cells, GATA3 exercises important functions to go through the β-selection checkpoint during the CD4 versus CD8 lineage choice and it is indispensable for the development and maturation of CD4 single-positive (SP) thymocytes (33–36). One of the important roles of GATA3 in the thymus is regulating the expression of Th-POK, which is an essential transcription factor for CD4-SP T cell development (37, 38). Another role of GATA3 in the thymus is controlling a set of genes encoding TCR components, including *Cd3d* and *Cd3e* (37). Deletion of the *Gata3* gene results in the decreased expression of CD3 in double-positive (DP) T cells, indicating that GATA3-dependent TCR signal strength play an important role in thymocyte development (37). GATA3 is continuously expressed at a basal level in peripheral naïve CD4 T cells, until IL-4/IL-4 receptor signaling activates STAT6 and induces the upregulation of the mRNA expression of *Gata3* (39). The high-level expression of GATA3 has been proposed to induce histone H3K4 methylation and H3K9 acetylation in so-called Th2

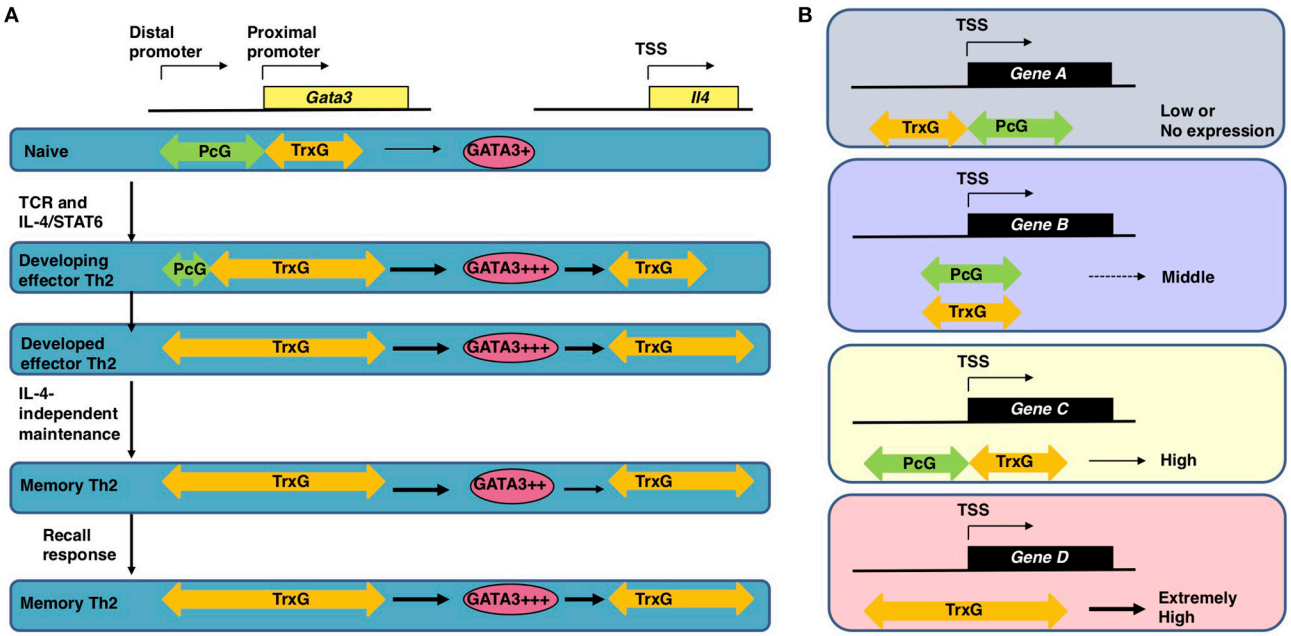


**FIGURE 2 | (A)** The epigenetic regulation of the induction, maintenance, and recall-response of effector and memory Th2 cells (1, 22). In naïve CD4 T cells, which express a moderate level of *Gata3* mRNA, the PcG proteins bind to the region between the distal and proximal promoter of the *Gata3* gene. After receiving signals via the T cell receptors (TCRs) in the presence of IL-4, activated STAT6 proteins bind to the *Gata3* gene locus, resulting in disassociation of the PcG complex and spreading of the TrxG complex binding to the region between the distal and proximal promoters. Extremely high *Gata3* mRNA expression levels are achieved "in an IL-4/STAT6-dependent but TrxG-independent manner" in developing Th2 cells (1, 22). During Th2 cell differentiation TrxG is also recruited to the *Il4* gene locus and induces H3K4 methylation and H3K9 acetylation in collaboration with GATA3 proteins. Once Th2 cells are differentiated, the TrxG protein binding is observed from the proximal promoter to intron 3 of the *Gata3* gene accompanied by a broad range of H3K9ac and H3K4me3. This TrxG binding pattern may be preserved during the process of generating memory Th2 cells from effector Th2 cells. The TrxG complex bound to the *Gata3* locus can maintain the strong expression of *Gata3* in memory Th2 cells where the transcription of *Gata3* is regulated "in an IL-4/STAT6-independent but TrxG-dependent manner" (1, 22). Note that in memory Th2 cells, the protein expression of GATA3 is slightly decreased in comparison to effector Th2 cells because GATA3 proteins are unstable in resting conditions. When memory Th2 cells reencounter their cognate antigens and a recall-response is induced, the GATA3 protein expression is immediately upregulated to produce large amounts of Th2 cytokines. TrxG proteins bound to the *Il4* gene also play an important role in the expression of IL-4 in memory Th2 cells. This figure was reproduced according to the permissions policy of Rockefeller University Press Journals © 2010 Onodera et al. **(B)** The spatial interplay between Polycomb (PcG) and Trithorax (TrxG) proteins. In CD4 T cells, the binding position of PcG and TrxG proteins relative to the transcription start site (TSS) of a gene is connected to its expression (1, 23). PcG and TrxG proteins show an exclusive binding pattern at the TSS of some genes: PcG is observed to bind downstream of the TSS while TrxG binds upstream of the TSS in genes with lower transcription levels (Gene A); in contrast, PcG is observed to bind upstream of the TSS while TrxG binds downstream of the TSS in genes with higher transcription levels (Gene C). When TrxG proteins bind throughout a gene, the gene shows an extremely high transcription level (Gene D). When PcG and TrxG proteins are bound in a similar position relative to the TSS, the transcription level of this gene is expected to be moderate (Gene B). This figure was reproduced according to the permissions policy of ASM Journals Copyright © Onodera et al. (23).

cytokine gene loci, which include the *Il4*, *Il5*, and *Il13* genes, during development of Th2 cells (40). These epigenetic changes play important roles in the formation of the accessible regions for transcription factor binding, which can be detected as DNase I hypersensitive (HS) sites. A recently developed technique, assay for transposase-accessible chromatin sequencing (ATAC-seq), has proven useful for analyzing these highly accessible regions (41). The enforced expression of GATA3 by a retroviral vector induces IL-4-producing Th2 cell differentiation, even if naïve CD4 T cells are cultured under Th1-inducing conditions, indicating that GATA3 is the necessary and sufficient master transcription factor for Th2 cell differentiation (39, 42, 43). The retroviral exogenous expression of GATA3 is shown to upregulate the endogenous GATA3 expression, and correspondingly, a single peak of GATA3 binding is detected in the *Gata3* gene and is located close to one of the STAT6 binding sites (44, 45). In addition, the GATA3 protein expression levels are tightly regulated by various posttranscriptional mechanisms in Th2 cells (46–48). "A conserved YxKxHxxxRP motif" in the C-terminal zinc finger domain of GATA3 protein has been shown to be critical for binding to DNA, inducing chromatin remodeling at Th2 cytokine gene loci, and exerting transcription factor activity (49). GATA3 is also known to be associated with some cofactors and to organize functionally distinct complexes (1). Fli1, an Ets family protein, is shown to colocalize with GATA3 and facilitate GATA3 functions (37). Chromodomain helicase DNA-binding protein 4 (Chd4) is proposed to interact with GATA3 and p300 and be involved in GATA3-dependent transcriptional activation (50). In contrast, Chd4 is also involved in GATA3-dependent gene silencing when interacting with GATA3 and nucleosome remodeling histone deacetylase (NuRD) (50). A recent study reported an interesting binding partner of GATA3,

Bcl11b, which plays an important role in limiting the Th2-related gene expression and suppressing the non-Th2 gene expression (51). It has been reported that several cis-regulatory elements (also known as locus control regions) at Th2 cytokine gene loci are also bound by GATA3. These regulatory elements include the conserved GATA response element (CGRE), the conserved non-coding sequence (CNS)-1, CNS-2, hypersensitive site HSVa, and HSII within the *Il4* gene (52–56). CGRE, which was originally identified in 2002 as a region containing four consensus GATA-binding sequences, overlaps with the previously identified HSI. This region is located 1.6 kilobases upstream of the TSS of the *Il13* gene (57). Correspondingly, strong GATA3 binding signals have been detected in the CGRE (37, 45, 58). Interestingly, the CGRE forms a boundary between hyper- and hypo-acetylated regions. This fact implies that GATA3 primarily binds to the CGRE and secondarily spreads histone hyperacetylation toward the 3′-end of the *Il13* gene (52). Indeed, the association of GATA3 with histone acetyltransferases CBP, p300, and RNA polymerase II is observed in this region (57, 59). Thus, the CGRE region may function as a regulatory element for chromatin remodeling at the *Il13* locus and subsequent mRNA expression of *Il13*. Notably, when Th2 cells are generated from naïve CD4 T cells of CGRE-deficient mice, the diminished IL-13 production but normal IL-4 or IL-5 production is observed, suggesting that a compensatory mechanism underlies the IL-4 and IL-5 production in the absence of this region (60). Genome-wide, GATA3 has been shown to regulate H3K4 methylation in enhancers, including these locus control regions; H3K4me2 levels are decreased in GATA3-deficient Th2 cells at non-promoter GATA3 biding sites (37).

In addition to epigenetic regulation, GATA3 is known to act as a transcription factor for the *Il5* and *Il13* genes: GATA3 directly binds to the promoters of these cytokine genes and induces transcription upon TCR restimulation (61–63). In fact, the decreased expression of *Il5* and *Il13* was observed in differentiated effector Th2 cells in which the *Gata3* gene was knocked down by siRNA just before TCR restimulation. Furthermore, other Th2 signature genes are transcriptionally regulated by GATA3 in effector Th2 cells (52). The expression of approximately half of the Th2-specific genes (16 out of 31) in effector Th2 cells was significantly reduced by *Gata3* siRNA knockdown; the *Tube1* gene was the only gene for which the expression was significantly increased, indicating that one of the major roles of GATA3 is the transcriptional activation of target genes (52, 58). In contrast, the transcription of other Th2-specific genes is not affected by *Gata3* siRNA knockdown. This fact implies that GATA3 is a master regulator for Th2 cytokine expression but not for all Th2 signature genes. A similar observation was reported in a study in which the expression of approximately half of a different set of Th2-specific genes (44 out of 90) was decreased in Th2 cells by *Gata3* knockout (37). The authors of that report noticed some interesting rules regarding GATA3-dependent transcriptional regulation. First, the genes positively regulated by GATA3 were found in the strongly expressed gene group while the genes negatively regulated by GATA3 were found in the weakly expressed gene group (37). Second, the authors argue that genes with higher numbers of GATA3 peaks tend to be affected by *Gata3* knockout. This appears to be true for genes both positively and negatively regulated by GATA3. Taken together, these findings suggest that the Th2-specific upregulation of GATA3 epigenetically and transcriptionally induces a set of Th2 signature genes as well as represses another set of genes that specifies other Th subsets. Approximately half of the Th2-specific genes are affected by *Gata3* knockdown or knockout, leaving the other half of Th2-specific genes intact.

### Epigenetic Mechanisms That Are Shared Between Th2 Cells and Other Conventional or Unconventional T Cells

DNA methylation is generally observed at cytosine of the CpG sequences in the genome. Dnmt1 is reported to be a maintenance enzyme responsible for converting hemi-methylated CpG into symmetrically methylated CpG after DNA replication (64). Genetic deletion of the *Dnmt1* gene results in the increased expression of both IL-4 and IFNγ in Th1 and Th2 cells and under unpolarizing conditions (65–67). Thus, Dnmt1-mediated gene silencing is important for preventing the excess production of these cytokines and modulating the proper differentiation of Th1 and Th2 cells. Th2 cells also share several molecular mechanisms with Th2-like unconventional T cells, including NKT2 cells (68, 69). In the absence of *Gata3*, a significant reduction in IL-4 production was observed in iNKT cells, indicating that GATA3 plays a crucial role in NKT2 cell development in the thymus (70). In addition, growth factor-independent-1 (Gfi-1) regulates the GATA3 protein expression in Th2 cells and iNKT cells. Gfi-1 knockout results in decreased IL-5 production and increased IFNγ production in Th2 cells, whereas both IFNγ-producing NKT1 and IL-4-producing NKT2 cells are abrogated in the absence of Gfi-1 in the thymus (46, 71). As described above, Th2 cell differentiation is considered to be controlled by both Th2-specific mechanisms and general epigenetic machineries shared with conventional and unconventional T cells.

## EPIGENETIC REGULATION IN THE MAINTENANCE OF THE MEMORY TH2 CELL FUNCTIONS

### Maintenance of the Memory Th2 Cell Function Depends on by Trithorax Molecules, MLL1, and Menin

Antigen-primed Th cells migrate to inflammatory sites in peripheral tissues and produce large amounts of effector cytokines when they reencounter their cognate antigens in order to eliminate these antigens. After antigen clearance, it is thought that most of these antigen-reactive effector Th cells die due to apoptosis in the contraction phase. However, some of the effector Th cells survive during the contraction phase, resulting in the generation of memory Th cells that can rapidly respond in cases of secondary antigen exposure (72). In general, CD4 T cells are thought to start acquiring the epigenetic signatures of memory Th cells from priming, which is almost established in differentiated Th subsets (5). The TrxG-binding pattern of the *Gata3* gene, which is established during Th2

cell differentiation, is basically maintained in memory Th2 cells (**Figure 2**). Memory Th2 cells are reported to maintain their Th2 signatures, specifically the Th2 cytokine production ability upon recall TCR stimulation and permissive histone modifications at the Th2 cytokine gene loci. These signatures are maintained by the high-level expression of GATA3 in an IL-4-independent manner (14, 59, 73–75). In addition, the expression of Th2 cytokine genes in memory Th2 cells depends on GATA3, since *Gata3* knockdown diminishes the transcription of these and other Th2-specific genes (52, 58). When TrxG proteins are genetically depleted, memory Th2 cells fail to maintain the *Gata3* expression and produce reduced amounts of Th2 cytokines after TCR stimulation due to the decreased methylation of H3K4 and the acetylation of H3K9. For example, the decreased expression of *Gata3* and impaired type 2 immune responses are observed in *Kmt2a*$^{+/-}$ (referred to as MLL1$^{+/-}$ elsewhere in this review) mice (76). Menin-deficient memory Th2 cells show a similar but milder phenotype (25). This is probably due to the redundancy of Menin, which is reported to only be included in the MLL1/2-bearing TrxG complex. In addition to the *Gata3* gene locus, permissive histone marks in Th2 cytokine gene loci are proposed to be maintained by MLL1 and Menin. In fact, the direct binding of MLL1 and Menin is detected at specific regions of Th2 cytokine gene loci as well as at the *Gata3* gene locus. Th2 cytokine production is dramatically reduced in MLL1$^{+/-}$ memory Th2 cells in concurrence with decreased levels of the permissive histone marks, including H3K9 acetylation and H3K4 methylation. Accordingly, MLL1$^{+/-}$ memory Th2 cells have a compromised ability to induce antigen-dependent allergic airway inflammation *in vivo* in comparison to wild-type control cells, suggesting a pathophysiological role of MLL1 in allergic diseases. Thus, TrxG molecules MLL1 and Menin epigenetically stabilize and maintain the *Gata3* mRNA expression in memory Th2 cells (14).

## The PcG Protein Bmi1 Regulates the Survival of Memory Th2 Cells

As described above, it is generally thought that some of the effector Th cells that survive after antigen clearance are a major source of memory Th cells. Thus, the mechanism underlying the survival of memory Th2 cells is an important issue to be addressed. It has been proposed that the PcG protein Bmi1 (also called Pcgf4) is responsible for the survival of memory Th2 cells as well as the self-renewal of hematopoietic stem cells (77). Indeed, a Bmi1-dependent (Bmi1$^{+/+}$, Bmi1$^{+/-}$, and Bmi1$^{-/-}$ were compared) decrease was observed in the numbers of memory Th2 cells. In hematopoietic stem cells, Bmi1 exerts its function via the repression of Ink4a/Arf, which are produced by different isoforms of the *Cdkn2a* gene (78, 79). However, the Bmi1-dependent repression of Noxa, which is encoded by the *Pmaip1* gene, is required to prevent apoptosis in memory Th2 cells (77, 80). Bmi1 binds to the CpG islands of the *Pmaip1* gene along with other PcG proteins (Ring1B and Suz12) and suppresses the gene expression via H3K27 methylation. In addition, Bmi1 recruits DNA methyl transferase 1 (Dnmt1) to preserve CpG methylation of the *Pmaip1* gene (77, 81). Thus, Bmi1 modulates the memory Th2 cell survival through the repression of the *Pmaip1* gene.

# THE HETEROGENEITY OF MEMORY TH2 CELLS

## The Identification of Pathogenic Th2 (Tpath2) Cells With Distinctive Epigenetic Modifications

Although we have described molecular mechanisms underlying the maintenance of the memory Th2 cell functions based on the analysis of the "bulk" cell population, recent advances in experimental techniques have enabled us to analyze the expression of proteins and transcripts at the "single cell" level *in vivo* (82). These analyses revealed that the cell populations (e.g., hematopoietic stem cell) in our body are much more heterogeneous than initially believed (83). The abovementioned memory Th2 cells also show heterogeneity and can be classified into subpopulations by the expression patterns of cell surface molecules, such as chemokine receptors and cell adhesion molecules. Among these subpopulations, we discovered that one population in which memory Th2 cells express low levels of both chemokine receptor CXCR3 and cell adhesion molecule CD62L (CD62L$^{lo}$CXCR3$^{lo}$) produces a large amount of IL-5, which is closely related to the pathogenesis of eosinophilic airway inflammation (84). The IL-5 secretion from CD62L$^{lo}$CXCR3$^{lo}$ memory Th2 cells is strictly regulated by histone modifications and the expression of the transcription factor Eomes. In this population, permissive histone modifications, including H3K4 trimethylation are observed at the promotor region of the *Il5* gene locus. Furthermore, the Eomes expression of CD62L$^{lo}$CXCR3$^{lo}$ memory Th2 cells is very low, which inhibits the binding of GATA3 to the *Il5* promotor and the subsequent *Il5* transcriptional induction in other populations. Thus, these cells are capable of producing a large amount of IL-5 in response to antigenic stimulation. CD62L$^{lo}$CXCR3$^{lo}$ memory Th2 cells, which produce large amounts of IL-5, recruit eosinophils to inflammatory tissues *in vivo* and are closely related to the pathogenicity of eosinophilic airway inflammation. Thus, we named these pathogenic memory Th2 (memory Tpath2) cells (84, 85). Another group reported that chemokine receptor CCR8-positive Th2 cells can produce large amounts of IL-5 and are involved in the pathogenicity of chronic atopic dermatitis in a mouse model (86). These reports raise the possibility that memory Tpath2 cells can be further classified into subpopulations with distinctive chromatin modifications that might be related to the pathogenicity of each disease.

## The Induction and Maintenance Mechanisms of Tpath2 Cells

How are Tpath2 cells that produce large amounts of IL-5 induced *in vivo*? It is proposed that epithelial cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which are released from the epithelial cells of the respiratory tract, play an important role in inducing Tpath2 cell differentiation (**Figure 3**). These cytokines have an "alarmin" function and induce an

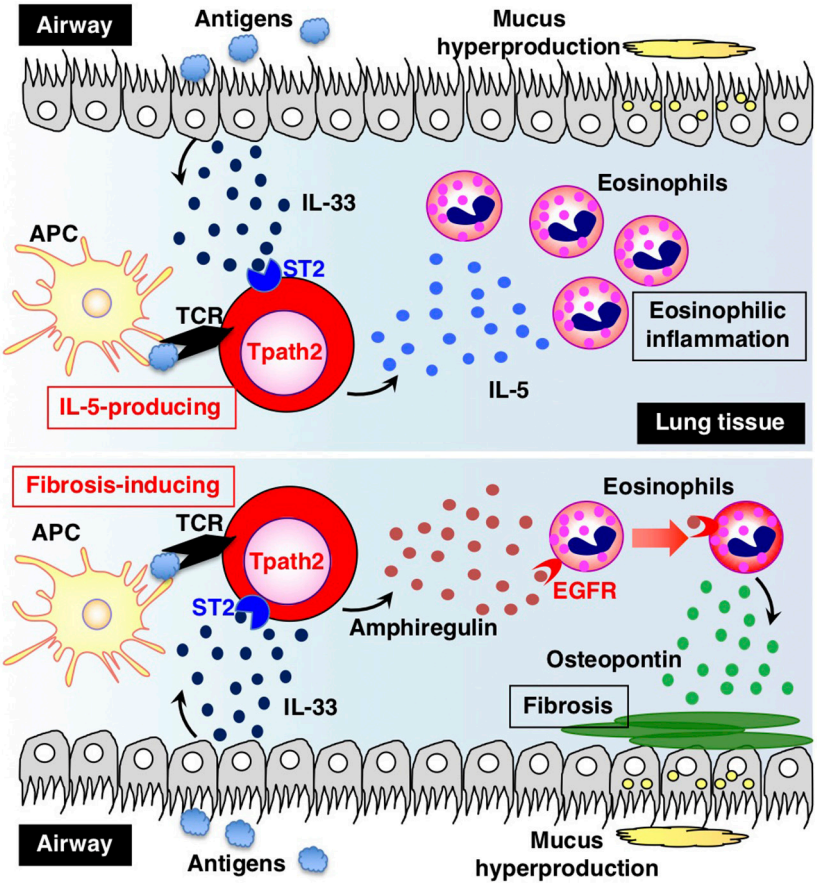


**FIGURE 3 |** The two types of pathogenic memory Th2 (Tpath2) cells. Antigens that are potentially associated with allergic reactions promote the secretion of IL-33 from airway epithelial cells. IL-33 binds to its receptor ST2 on memory Th2 cells and induces epigenetic changes of the *Il5* gene, resulting in the generation of IL-5-producing Tpath2 cells (upper) (1, 87). When Tpath2 cells reencounter their cognate antigens, these cells produce a large amount of IL-5, which exacerbates chronic eosinophilic inflammation in the lung. IL-33 also induces the production of fibrosis-inducing Tpath2 cells, which produce large amounts of Amphiregulin (lower) (88). Amphiregulin binds to the epidermal growth factor receptors (EGFRs) on eosinophils and induces Osteopontin secretion from the eosinophils, resulting in fibrosis in the lung tissue. This figure was reproduced according to the permissions policy of Cell Press journal [originally published by Morimoto et al. (88)].

inflammatory response in the mucosal membrane. Our study revealed that *in vivo*, memory Th2 cells express elevated levels of IL-33 receptor ST2 compared to differentiated effector Th2 cells *in vitro* (87). Indeed, IL-33 stimulation activates memory Th2 cells and induces a large amount of IL-5 production via chromatin remodeling at the *Il5* gene locus. Interestingly, IL-33 stimulation also induces chromatin remodeling at the *Il1rl1* gene locus, which encodes ST2, resulting in the increased expression of ST2 in memory Th2 cells. An RNA-seq analysis of gene expression patterns induced by IL-33 stimulation in memory Tpath2 cells identified other candidate molecules responsible for eosinophilic inflammation. Amphiregulin, which is encoded by the *Areg* gene, has been reported to be associated with tissue repair and fibrosis and was one of the candidates identified by this analysis (88). Fibrosis around the airway, which is often found in patients with chronic airway inflammation, is typically formed in an airway inflammation mouse model induced by house dust mite (HDM). Thus, we hypothesized that the IL-33-Amphreglin axis has a pathogenic function to induce fibrosis in airway inflammation. Indeed, IL-33 stimulation induced permissive histone modifications at the *Areg* gene locus *in vitro*. The deletion of the *Areg* gene resulted in the attenuation of the lung fibrosis induced by Tpath2 cells. Amphiregulin had a direct effect on epidermal growth factor receptors (EGFRs) on eosinophils, which causes them to produce Osteopontin, which induces fibrosis. Thus, a subpopulation of Tpath2 cells that produce Amphiregulin functions as "fibrosis inducing memory Tpath2 cells" (**Figure 3**). Although Tpath2 cells and ILC2 cells share some signatures, including the ability to produce IL-5, they differ in responsiveness to IL-33 stimulation. ILC2 cells can produce IL-5 in response to IL-33 stimulation whereas Tpath2 cells need TCR stimulation to produce IL-5. Dusp10, which is highly expressed in Tpath2 cells compared to ILC2 cells, was found to be involved in inhibiting IL-33-dependent IL-5 production in Tpath2 cells (89). Thus, the Dusp10-mediated suppression of IL-5 may explain the difference in responsiveness to IL-33 between Tpath2 and ILC2. Most recently, $CXCR6^{+}ST2^{+}$ memory Th2 cells have been found to exert a protective function

in immunity against helminth infection (90). This finding supports the hygiene hypothesis that lack of exposure to parasites increases susceptibility to allergic diseases: $ST2^+$ memory Th2 cells play a protective role against helminth infection but play a pathogenic role in allergic reactions in the absence of parasite infection.

In addition to IL-33, IL-7 also plays a role in the maintenance of memory Th2 cell functions in an ectopic lymphoid tissue called "inducible bronchus-associated lymphoid tissue" (iBALT) (91). The chronic inflammation caused by various factors such as infectious diseases, smoking, and collagen diseases is reported to induce the formation of iBALT in the lung (91). Notably, Thy1 (a cell surface molecule)-positive lymphatic endothelial cells produce IL-7 in the inflamed lung tissue and are essential for the formation of iBALT and memory Tpath2 cell maintenance in iBALT. More interestingly, Thy1-positive IL-7-producing lymphatic endothelial cells in iBALT also strongly express IL-33 and are implicated in the maintenance of the memory Tpath2 cell function in iBALT (91). Taken together, these findings suggest that memory Tpath2 cells develop from memory Th2 cells *in vivo* via epigenetic mechanisms in the presence of an environmental signal molecule (IL-33) and are maintained by receiving signals that are important for their functional maintenance and survival in the inflamed tissues microenvironment of iBALT, which is proposed to be an "inflammation niche."

## THE REGULATION OF THE RECALL-RESPONSES OF EFFECTOR AND MEMORY TH2 CELLS

### The Acute Immune Response in the Airway Mediated by Effector Th2 Cells Is Dependent on CD69 and its Ligand Myl9/12

Antigen-primed Th cells migrate to inflamed sites via the blood stream and infiltrate inflammatory tissues through vessels. Thus, migration into inflammatory tissues, where Th cells reencounter their cognate antigens, is important for Th cells to exert their effector functions in acute immune responses. In a recent study, we successfully identified myosin light chain (Myl9/12) as a functional ligand for CD69 and proposed a new migration mechanism that is dependent on interaction between CD69 and Myl9 (the "CD69-Myl9 system") (92, 93) (**Figure 4**). CD69 was originally identified as a molecule that is rapidly induced on T, B, and NK cells upon activation (93). CD69 is a type 2 cell membrane protein with a C-type lectin-like domain. TCR stimulation increases H3K4 methylation at the *Cd69* gene in naïve CD4 T cells, suggesting that the expression of CD69 is epigenetically regulated (94). More recently, CD69 has been found to be crucial for maturation of NKT2 cells in the thymus, where CD69 prevents immature precursors from exiting by suppressing the sphingosine-1-phosphate receptor 1 ($S1P_1$) expression (95). A number of studies have reported roles of CD69 in murine models of inflammatory diseases, including arthritis, airway inflammation, and dextran sulfate sodium (DSS)-induced colitis (96–98). However, the CD69 ligand had not been identified before our report on Myl9/12. We found that Myl9/12 molecules are released from platelets in inflammatory vessels and then form net-like structures (Myl9 nets) that help activated immune cells infiltrate the blood vessels and migrate into inflammatory tissues. Myl9/12 monoclonal antibody (Ab) treatment was proven to be effective in both OVA-induced and HDM-induced airway inflammation models. These results suggest that anti-Myl9/12 Abs-based antibody therapy may also be useful for severe steroid-resistant asthma treatment in humans, and humanized anti-Myl9/12 Abs that can be administered to humans are now being prepared.

### The Epigenetic Regulation of the Recall-Responses of Memory Th2 Cells by the TrxG Proteins MLL1 and Menin

As described in a previous section, TrxG proteins, such as MLL1 and Menin maintain H3K4 methylation of the *Gata3* and Th2 cytokine genes and are crucial for the rapid recall response of memory Th2 cells. Menin is also indispensable for the survival of memory Th2 cells because ablation of Menin is shown to significantly decrease the number of memory Th2 cells. However, even in an experimental setting where the same number of wild-type and Menin-deficient memory Th2 cells are transferred into congenic mice, which are challenged by OVA, the deletion of Menin attenuates airway inflammation, indicating that the Menin-dependent regulation of the Th2 signature genes is important for type 2 immune responses (25). TrxG proteins are involved in both pathogenic and protective immune responses. For example, MLL1 is reported to play a role in the anti-tumor immunity mediated by memory Th2 cells (99). Thus, TrxG proteins are required for both keeping the epigenetic states active in the Th2 signature genes and for preventing programmed cell death of memory Th2 cells, both of which are essential for a proper recall response to antigens.

Menin is also needed for the long-term maintenance of the Th2 cell identity and a proper response to antigen restimulation when Th2 cells are exposed to antigen multiple times *in vitro*. Th2 cells subjected to TCR stimulation multiple times are reported to produce higher levels of IL-5 and IL-13 *in vitro* than normal effector Th2 cells (25). In contrast, IL-4 production is slightly increased by multiple TCR stimulation, which is required for the complete demethylation of CpGs of the *Il4* gene (100). In these established Th2 cells, the deletion of Menin decreased the expression of Th2 signature genes, including the *Gata3* and Th2 cytokine genes (25). *In vivo*, multiple exposure to an antigen has a different effect: the pathophysiology of airway inflammation changes from Th2-mediated to Th1- and Th17-mediated inflammation. Th17-mediated airway inflammation is known to be associated with steroid-resistant asthma (101). Menin has been implicated in the pathogenesis of airway inflammation in a mouse model resembling steroid-resistant asthma (102). Menin also plays a role in the protective immune response to listeria infection in CD8 T cells (103). Thus, epigenetic regulation mediated by TrxG proteins is important for both pathogenic and protective immune responses.

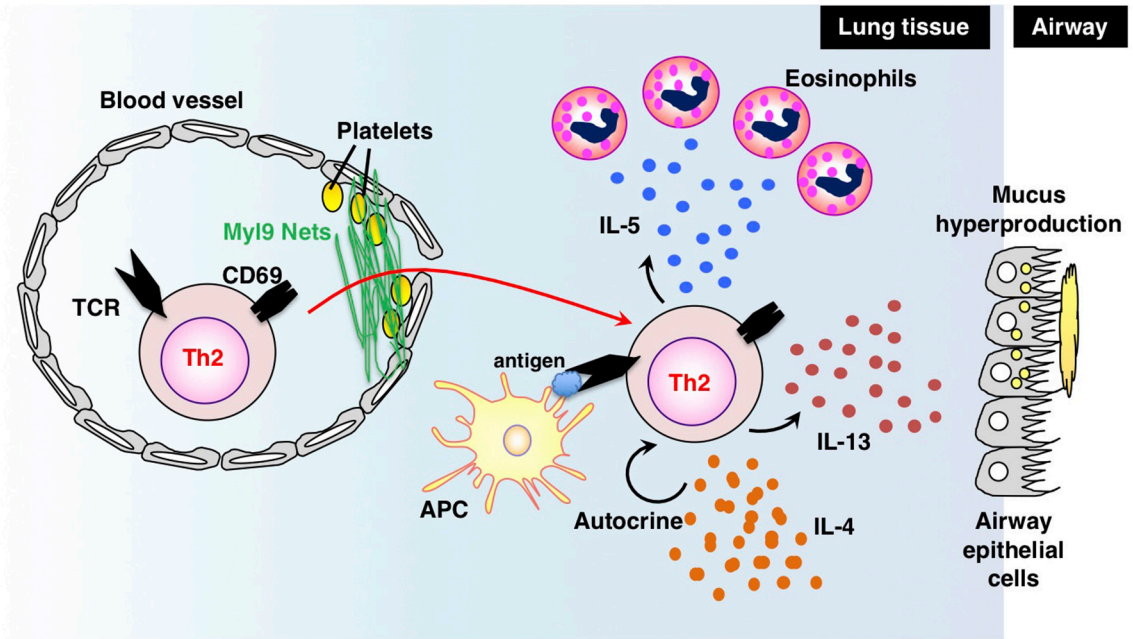


**FIGURE 4 |** The "CD69-Myl9 system." In the inflamed lung, platelet-derived Myl9 forms a net-like structure in association with platelets on the luminal surface of blood vessels (92, 93). CD69 molecules on effector Th2 cells interact with Myl9 nets and help Th2 cells infiltrate tissues. Infiltrating Th2 cells reencounter their cognate antigens and exert effector functions through the production of Th2 cytokines. IL-5 is known to recruit eosinophils whereas IL-13 promotes mucus hyperproduction from airway epithelial cells. IL-4 stimulates Th2 proliferation in an autocrine manner. This figure was reproduced according to the permissions policy of John Wiley & Sons publications [originally published by Kimura et al. (93)].

## The Involvement of Tpath2 Cells in Human Chronic Allergic Diseases

In previous sections, we focused on a mouse model of airway inflammation associated with Th2-mediated inflammatory diseases. In this section, we discuss the recent findings concerning human chronic allergic diseases, with a focus on chronic rhinosinusitis (CRS), which is one of the most common complications of bronchial asthma (1). CRS refers to a type of chronic upper respiratory tract inflammation that is characterized by the inflammation of the mucosa of the nasal and paranasal cavity and tissue remodeling. The pathogenesis of CRS and the process through which the inflammation of CRS develops are thought to be similar to those of bronchial asthma, which is caused by lower respiratory tract inflammation. CRS is categorized into two groups according to the presence or absence of nasal polyps (NPs): CRS without NPs (CRSsNPs) and CRS with NPs (CRSwNPs) (1). Polyps from CRSwNP patients usually contain large numbers of infiltrating eosinophils and are thought to be a local lesion of chronic eosinophilic inflammation. Thus, CRSwNPs is also called eosinophilic CRS (ECRS). The pathophysiology of ECRS is unclear at present; we analyzed polyp-infiltrating T cells and found that the polyps of eosinophilic rhinosinusitis patients contain large numbers of infiltrating memory CD4 T cells that secrete large amounts of IL-5 in response to IL-33 stimulation (87, 91). These memory CD4 T cells strongly express IL-17 receptor B (IL-17RB), which is a receptor of IL-25 and involved in IL-5 production in response to IL-25 stimulation (104). In addition, CD69-expressing T cells and Myl9 nets have been identified within the polyps, indicating that the "CD69-Myl9 system" plays a role in the pathogenesis of ECRS (92, 93). Consistent with the mouse model of airway inflammation, ectopic lymphoid tissues with Thy1-positive IL-7-producing lymphatic endothelial cells are formed in ECRS polyps (91). In addition, fibrosis is also observed in ECRS polyps. A further analysis revealed that memory Th2 cells that highly express the cell surface molecules CD161 and CRTH2 specifically produce IL-5 and Amphiregulin *in vivo* (88). Other research groups have reported that Tpath2 cells also contribute to allergic reactions in the gastrointestinal tract, such as human eosinophilic esophagitis and food allergy (105). In addition, it is reported that sublingual immunotherapy can reduce the number of Tpath2 cells in the peripheral blood of pollinosis patients (106). These results suggest that the IL-33-dependent induction of memory Tpath2 cells is closely associated with chronic inflammation in both humans and mice.

## CONCLUDING REMARKS AND OPEN QUESTIONS TO BE ADDRESSED

Extensive research on Th2 cells has shed light on the epigenetic regulation in the induction, maintenance, heterogeneity, and recall-response of memory T cells. For the induction of Th2 cells, STAT6 regulates epigenetic changes of the *Gata3* gene, resulting in the expression of extremely high levels of GATA3 proteins,

which control chromatin remodeling at Th2 cytokine gene loci. STAT6 and GATA3 also recruit TrxG H3K4 methylase proteins to the appropriate regions of the *Gata3* and Th2 cytokine gene loci, respectively. The recruited TrxG proteins are required for the maintenance of the high expression of the *Gata3* gene and the production of Th2 cytokines in memory Th2 cells upon secondary TCR stimulation, indicating that the recall-response of memory Th2 cells is also dependent on epigenetic machinery. Memory Th2 cells show heterogeneity and can be classified into subpopulations with distinctive epigenetic modifications. For example, $CD62L^{lo}CXCR3^{lo}$ Tpath2 cells produce a large amount of IL-5, whereas a subpopulation of the Tpath2 cells produces Amphiregulin and is involved in fibrosis in the airway of mice and humans. Taken together, these findings suggest that allergic airway inflammation is caused by a certain subpopulation of memory Th2 cells or a combination of subpopulations. The "pathogenic Th population disease induction model" we have proposed may thus explain the pathogenesis of allergic airway inflammation more accurately than the classical model, in which an imbalance in Th1/Th2 differentiation is proposed to be responsible for allergic disease (1, 85).

Various important and interesting questions remain to be addressed. The first question is how a small number of memory T cells are selected from a large number of effector T cells. Some reports show that effector T cells harboring TCRs with a low affinity to antigens are prone to survive and form a memory T cell population, while other reports argue that some naïve T cells are directly differentiated into memory precursor cells after antigen priming (107, 108). Another question is where memory T cells are located. Previously inflamed tissue, draining lymph nodes, other secondary lymphoid organs, the bone marrow, and the peripheral blood are potential locations (109). Regarding epigenetics, histone modifications and DNA methylation states are reported to be preserved from effector T cells to memory T cells (110). However, the extent to which the three-dimensional structures of the epigenome are maintained in memory T cells in comparison to effector T cells is not clear. For example, whether chromatin structures, interactions between enhancers and promoters and genomic locations in the nucleus are maintained, resolved, or renewed remains to be determined. Future mechanistic studies, including kinetic analyses of cell migration and cell-intrinsic changes will be needed to improve our understanding of memory T cell biology and epigenomics.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## ACKNOWLEDGMENTS

We thank Dr. Kiyoshi Hirahara for his helpful discussion on this manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT Japan) [Grants-in-Aid: for Scientific Research (S)#26221305, (C) #15K08522 and #18K07164] #221S0002, AMED-CREST, AMED (no.JP18gm1210003), and the Practical Research Project for Allergic Diseases and Immunology (Research on Allergic Diseases and Immunology) from AMED (no.JP18ek0410030). All animal experiments were approved by the ethics committee for animals at Chiba University. Informed consent was obtained from all ECRS patients who participated in this study, which was approved by the ethics committee of the Chiba University Graduate School of Medicine.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 13 December 2018
doi: 10.3389/fimmu.2018.02955

# The Role of Histone Methyltransferases and Long Non-coding RNAs in the Regulation of T Cell Fate Decisions

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***Specialty section:***
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *06 September 2018*
***Accepted:*** *30 November 2018*
***Published:*** *13 December 2018*

***Citation:***
*Gaballa JM, Braga Neto MB, Ramos GP, Bamidele AO, Gonzalez MM, Sagstetter MR, Sarmento OF and Faubion WA Jr (2018) The Role of Histone Methyltransferases and Long Non-coding RNAs in the Regulation of T Cell Fate Decisions. Front. Immunol. 9:2955. doi: 10.3389/fimmu.2018.02955*

T cell lineage decisions are critical for the development of proper immune responses to pathogens as well as important for the resolution of inflammatory responses. This differentiation process relies on a combination of intrinsic and extrinsic factors converging upon epigenetic regulation of transcriptional networks relevant to specific T cell lineages. As these biochemical modifications represent therapeutic opportunities in cancer biology and autoimmunity, implications of writers and readers of epigenetic marks to immune cell differentiation and function are highly relevant. Given the ready adoption of histone methyltransferase inhibitors in the clinic, we focus this review on the role of three histone modifying complexes: PRC-1, PRC-2, and G9A in modulating T cell fate decisions. Furthermore, we explore the role of long non-coding RNAs in regulating these processes, and discuss recent advances and challenges of implementing epigenetic therapies into clinical practice.

**Keywords: epigenetics, EZH2, G9a, long non-coding RNAs, PRC1, PRC2, T cell**

## BACKGROUND

The immune system comprises a large number of cell types that have the ability to respond to external environmental cues and adopt a wide variety of cell fates. These lineage decisions are critical for the development of proper immune responses to pathogens as well as resolution of inflammatory responses. As part of the adaptive immune system, T cells have the capacity to respond to the external environment by modulating the expression of lineage specific factors which are critical for protecting against a wide variety of pathogens. For the development of distinct T cell lineages, naive CD4+ T cells must convert the extrinsic instructions provided by encounters with antigen-presenting cells into cell-intrinsic changes (1). These intrinsic changes are largely facilitated by transcription factors that directly induce or repress gene networks and drive T cell differentiation (2). Emerging data demonstrates that lineage specific transcription factors recruit epigenetic complexes to regulate gene expression over multiple rounds of cell division, and their roles are indispensable for maintaining T cell homeostasis.

Deregulation of epigenetic pathways is a feature of many cancers, autoimmune diseases, and neurodegenerative disorders (3–5). The reversible nature of epigenetic modifications makes them attractive targets for pharmacological intervention, and indeed drugs targeting histone-modifying complexes, such as Enhancer of Zeste Homolog 2 (EZH2), are currently being evaluated in patients

for treatment of malignancy (6) and immune-mediated conditions (7, 8). While recent clinical trials have demonstrated a favorable safety profile of selective inhibition of EZH2 (6), a comprehensive understanding of the role that epigenetic modifying complexes play in the development and function of different immune cell types is relevant to the development and safety of epigenetic therapeutics. Here we review the role of three histone modifying complexes: PRC-1, PRC-2, and G9A in modulating T cell fate decisions. Furthermore, we explore the role of long non-coding RNAs in regulating these processes, and discuss recent advances and challenges associated with implementing epigenetic therapies in clinical practice.

## PRC1, PRC2, G9A, AND LONG NON-CODING RNAS

### PRC1

The Polycomb-Group proteins, Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2), mediate post-translational modifications (PTMs) of histones required for cell differentiation and development through the regulation of chromatin structure and gene expression. PRC1 is a multimeric protein complex containing the core proteins RING1A/B, and Polycomb-group ring finger (PCGF) proteins such as Bmi-1 (PCGF4) and Mel-18 (PCGF2). PRC1 functions to mono-ubiquitinate lysine 119 on histone H2A (H2AKub119), an epigenetic mark that is associated with transcriptional repression (9). Bmi-1 specifically is highly enriched in pericentric heterochromatin which is required for chromatin compaction and silencing (10). Although Ring1A/B is the catalytic subunit of PRC1, knockdown of Bmi-1 results in a significant loss of H2A ubiquitylation, demonstrating the important role that it plays in facilitating the enzymatic function of PRC1 (11). In the canonical or hierarchical model of Polycomb (PcG)-mediated transcription regulation, PRC1 is primarily described as the maintenance complex which silences target genes previously marked by the initiator complex, PRC2. More recently, a histone-independent role of Bmi-1 in driving NF-κB signaling has been reported (12). An interesting story is also evolving related to a PRC2-independent role for PRC1 in the maintenance of 3D genome structure through association with super-enhancers (13, 14). No immune cell specific data has yet emerged related to these exciting areas of investigation.

### PRC2

PRC2 modulates chromatin dynamics via the tri-methylation of lysine 27 on histone 3 (H3K27Me3), which is associated with transcriptional repression. EZH2, ubiquitously expressed by many mammalian cell-types, is the enzymatic subunit of PRC2 which contains other supporting non-catalytic proteins namely Suppressor of Zeste (SUZ12), embryonic ectoderm development (EED), Adipocyte Binding Protein 2 (AEBP2) and Retinoblastoma protein Associated protein 46 and 48 (RpAp46/48) (15). H3K27me3 recruits protein complexes involved in chromatin compaction and is associated with inactive genes (16). Histone-independent functions of PRC2 have also been reported to play important roles in regulating transcription factor stability and T cell receptor-mediated signaling (17–20). While EZH2 has a role in normal cellular and tissue function, studies involving EZH2 overexpression or genetic mutations show that EZH2 is critical in the development and progression of a variety of cancers (21–29). EZH2 is most frequently associated with the silencing of tumor suppressor genes, and decreased expression of PRC-target genes are associated with poor prognosis (30, 31). Thus, derepression of these genes using selective EZH2 enzymatic inhibitors or disruptors of PRC2 stability are likely to improve clinical outcomes, and are currently being explored in preclinical or clinical studies for cancer therapy (32–38).

### G9a

The histone methyltransferase G9a and the related G9a-like protein (GLP) form a heterodimeric complex to catalyze mono and di-methylation of lysine 9 on histone 3 (H3K9me1 & H3K9me2) at euchromatin *in vivo* (39). G9a and GLP are encoded by the *EHMT2* and *EHMT1* genes, respectively, both of which contain a SET domain necessary for the methylation of lysine residues. G9a has been shown to play a larger role in H3K9me2 methylation *in vivo,* but levels of H3K9me1 and H3K9me2 are severely reduced in both G9a and GLP knockout models (39). Furthermore, G9a has been shown to promote gene activation through a methyltransferase-independent fashion in different settings, including type II cytokine production in helper T cells, possibly by acting as a scaffold to recruit transcriptional machinery (40, 41). G9a/GLP-mediated H3K9me2 has been associated with cognition and adaptive behavior, germ cell development and meiosis, embryo development, cocaine-induced plasticity, tumor cell growth and metastasis, and more recently the immune response reviewed below (39, 42).

### Long Non-coding RNAs

Non-coding RNA's have emerged as an exciting new frontier of gene regulation in the immune system. It is now known that 75–90% of the human genome transcriptome is comprised of non-coding RNAs (43, 44). Long non-coding RNAs are defined as transcripts with minimal coding potential that are composed of more than 200 nucleotides; an arbitrary cutoff that distinguishes them from microRNAs (<200 nucleotides). Over 15,000 lncRNA genes have been annotated, although only 159 lncRNAs have known function[1,2] (45), highlighting a critical gap in knowledge in the field. They can be classified based on their position relative to protein coding genes as intergenic, intronic and antisense (46). Like mRNAs, long non-coding RNA's undergo transcription by RNA polymerase II, are 5′ capped, spliced and polyadenylated. However, distinct from mRNA, they lack canonical ORFs (and, therefore have minimal protein-coding potential), tend to be shorter in size, have lower expression levels, fewer exons and can localize to the nucleosome, chromatin or cytoplasm. For example, long intergenic non-coding RNAs localize primarily in the nucleus, in contrast to

[1] *GENCODE, v27 Release*. Available online at: https://www.gencodegenes.org/human/release_27.html

[2] *Long Non-coding RNA Database v2.,0 (lncRNAdb)*. Available online at: http://www.lncrnadb.org/

mRNAs which are primarily localized in the cytoplasm where they undergo translation (47). Furthermore, lncRNAs function by interacting with DNA, RNA, or proteins and the majority modulate transcription in *cis* (affecting nearby genes), although they can also modulate in *trans* (targeting distant genes), acting as scaffolds, molecular decoys and guides for epigenetic modifying complexes. Interestingly, lncRNAs can both activate and suppress target genes by a variety of mechanisms and are expressed in a cell-type and stage-specific manner (48, 49). They have been shown to play key roles in autoimmunity, cancer and infection (50–52). A recent comprehensive transcriptomic profiling of T cells demonstrated unique lncRNA signatures for specific T cell phenotypes signifying the relevance of lncRNA to cell and stage specific function (49). Thus, lncRNAs may represent exciting precise therapeutic targets.

## PRC1, PRC2, G9A, AND LNCRNAS IN THE ADAPTIVE IMMUNE SYSTEM

The development of T cells, an integral component of the adaptive immune system, occurs in the thymus where thymocytes mature into distinct T cell lineages defined by either CD4 or CD8 co-receptor expression. CD4+ T cells and CD8+ T cells are known to possess conventional alpha beta (αβ) T cell receptors (TCR), which recognize antigen-derived peptides bound by major histocompatibility complex (MHC) class II or I molecules, respectively. Upon antigen recognition and inflammatory environmental cues, naïve CD4+ T cells differentiate into distinct effector T helper (Th) subsets by expressing lineage-specific transcriptional programs. Th1, Th2, and Th17 cells mediate protective anti-pathogenic responses against bacteria and viruses via the secretion of distinct IFN-γ, IL-4, and IL-17 effector cytokines, respectively (53). Post-infection, Tregs, a regulatory component of the immune system, are recruited to inhibit effector T cell functions and reestablish homeostasis. Tregs can be generated from the thymus (natural Tregs) or induced in the periphery (pTreg) or *in vitro* (iTreg) from naïve CD4+ T cells via a FOXP3-driven transcriptome (54–56). Nonetheless, persistent activation of these effector T cell subsets has been associated with the pathogenesis of autoimmune disorders such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and psoriasis (57).

PRC1, PRC2, G9a, and a variety of lncRNAs influence T helper cell differentiation and maintenance by epigenetically regulating transcriptional programs associated with different T cell subsets. Given their significant influence in the pathogenicity of diseases as stated above, we focus here on the role of these molecules in the differentiation and maintenance of Th1, Th2, Treg, and Th17 phenotypes (**Figure 1**, **Table 1**).

### Treg/Th17

Treg and Th17 cells appear to share precursor lineage as demonstrated by *in vitro* study and murine lineage tracing experiments (77, 78). While TGFβ signaling is required for both effector cell types, IL-6 appears principally responsible for ultimate derivation of Th17 cells (79–81). Ultimately, lineage-specific transcription factors (FOXP3 and RORγt) drive the Treg or Th17 transcriptional program, respectively. FOXP3 and RORγt are known to reciprocally regulate one another, and the delicate balance between suppressive Tregs and effector Th17 cells has proven critical for maintaining immune homeostasis (78). Epigenetic modifying complexes, namely PRC2 and G9a, play key roles in orchestrating the Treg and Th17 transcriptional programs, and disruption of these epigenetic networks are characterized by the development of autoimmunity in murine models of human disease and human inflammatory bowel disease (66, 82, 83).

We and others have demonstrated that mice lacking EZH2 in natural FOXP3+ Tregs developed spontaneous multi-organ inflammation and were more susceptible to experimental models of autoimmunity (65, 66). In addition to decreased frequency of EZH2-depleted Tregs observed in certain murine tissues, DuPage et al. showed that EZH2 was required to promote the FOXP3-mediated gene repression program upon TCR activation as a number of FOXP3-bound genes were de-repressed in the absence of EZH2 (65). In support of the failure of EZH2-deleted Tregs to maintain the expression of Treg-specific signature genes, EZH2-deleted Tregs displayed impaired suppression of effector T cells *in vitro* (65, 66). Translating these findings from mice to human relevance, Crohn's disease (CD)-lamina propria CD4+ T cells were transcriptionally different from healthy controls (66). Specifically, normally repressed FOXP3-target genes were upregulated in CD CD4+ T cells and approximately 50% of these differentially expressed genes (DEGs) were EZH2 targets. Moreover, CD4+ T cells displayed a Th1/Th17 effector-like phenotype in contrast to that of healthy controls. Thus, loss of EZH2 function and consequently Treg dysfunction may drive pathophysiological mechanisms of particular autoimmune disorders.

In G9a deficient CD4+ T cells stimulated under Treg or Th17 promoting conditions, a significant increase in FOXP3-expressing and IL-17A-expressing cells is observed. In undifferentiated T cells, G9a normally functions as a mediator of H3K9me2 on loci associated with driving Treg and Th17 phenotypes (42). Loss of G9a-mediated H3K9me2 increases chromatin accessibility to transactivating factors and increases responsiveness to TGFβ (42). Much more work is required to define the molecular underpinnings of G9a's effects on Treg development, but some consistency is emerging regarding Th17 biology. G9a was shown to be recruited by RelB, a non-canonical NF-κB family member, to silence the *IL17A* locus and prevent Th17-mediated autoimmunity in an *in vivo* model of experimental autoimmune encephalomyelitis (EAE) (67). This work is consistent with effects seen in other T cell subsets, namely Th2 cells, in which loss of G9a leads to abnormal IL-17 expression (42). How these effects influence the balance between Treg and Th17 phenotypes is yet to be determined. Thus, G9a may become a viable target for therapeutic intervention of human Th17 mediated diseases.

Three lncRNAs (Flicr, Lnc-Smad-3, and LncEGFR) have been shown to influence Treg function. Flicr is selectively expressed in both human and mouse T regulatory cells and negatively

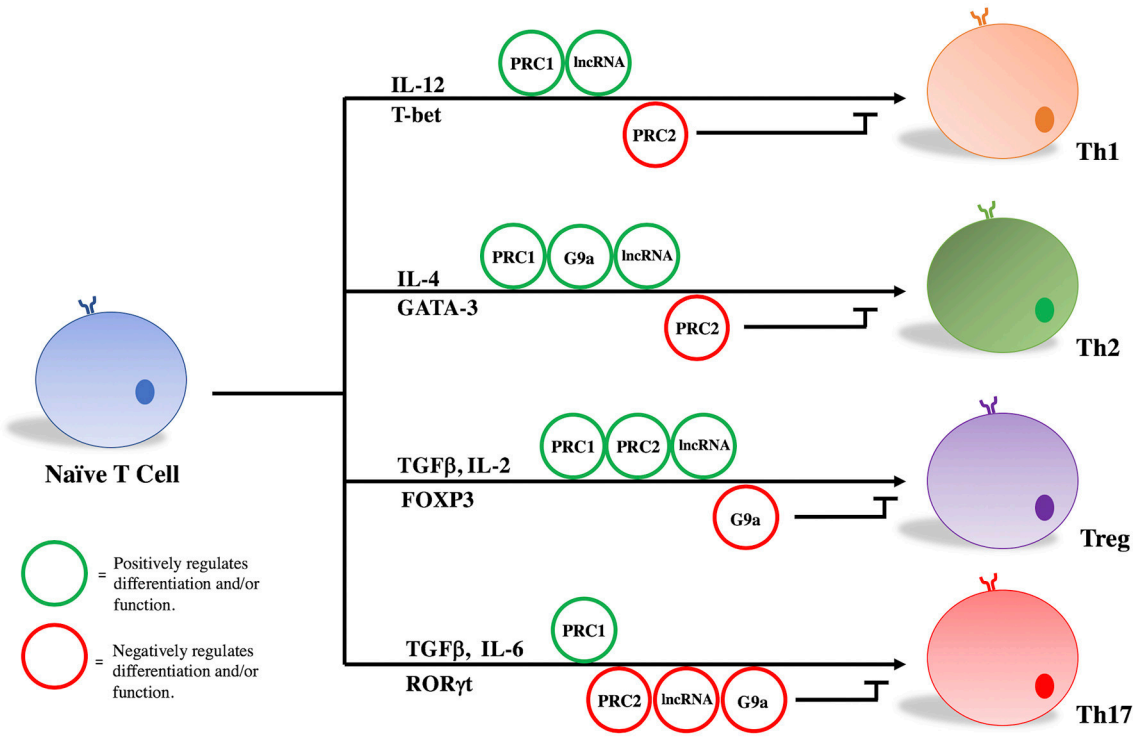


**FIGURE 1 |** PRC1, PRC2, G9a, and lncRNAs regulate T cell differentiation and function.

regulates FOXP3 in *cis* leading to decreased Treg function and heightened autoimmunity (74). Mechanistically, Flicr modifies chromatin accessibility in the FOXP3 locus, specifically non-coding sequence 3 (CNS3) and accessible region 5 (AR5), leading to decreased expression of FOXP3. *In vivo*, knockdown of Flicr decreased the incidence of autoimmune diabetes in mice (74).

Lnc-Smad-3 was recently shown to modulate TGFβ-mediated Treg polarization both in human and murine assays (75). Mechanistically, lnc-Smad3 prevents the histone deacetylase HDAC1 to bind to the SMAD3 promoter region, which renders the chromatin compact and inaccessible to Ash1l, an H3K4 methyltransferase that promotes SMAD3 activation and transcription. From a disease relevance standpoint, these results suggest a potential role for this long non-coding RNA in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis (75).

Lnc-EGFR was shown to stimulate Treg differentiation by a forward-feedback loop (51). Mechanistically, lnc-EGFR binds to EGFR using its R1 domain, preventing interaction with c-CBL and ubiquitination. In turn, EGFR activates ERK1/2 and AP-1, which then leads to increased expression of lnc-EGFR and FOXP3, perpetuating increased Treg differentiation. The authors found this to be a critical pathway for hepatocellular carcinoma (51).

LncRNA-1700040D17Rik was found to be deregulated in CD4+ cells derived from a mouse model of autoimmune encephalitis and have been shown to play a role in differentiation of Th17 cells. *In vitro*, overexpression of lncRNA-1700040D1Rik decreased expression of RORγt and IL-17 in Th17 cells, although the precise mechanism is yet to be known (76). These findings suggest a potential role for this long non-coding RNA in the pathogenesis of multiple sclerosis.

## Th1/Th2

Studies investigating the impact of G9a on Th1 biology have shown that the absence of G9a has little effect on Th1 responses *in vitro* nor *in vivo*, however, it is a critical component of the Th2 regulatory machinery (40). Lehnertz et al. demonstrated G9a to be necessary for expression of lineage-specific Th2-associated cytokines such as IL-4, and that loss of G9a in CD4+ T cells prevents Th2 cell differentiation. Mice with targeted CD4+ T cell deletions of G9a were susceptible to helminth infection by *Trichuris muris* due to the inability to express Th2-associated cytokines. Consistent with previous work (42), the absence of G9a in CD4+ T cells also resulted in the upregulation of IL-17A *in vivo*. Interestingly, whereas repression of IL-17A appears to be associated with G9a methyltransferase activity (42), Th2 gene regulation by G9a is independent of enzymatic activity, and thought to be related to G9a functioning as a scaffolding protein (40, 41).

The role of PRC1 in regulating T cell lineage fate decisions is best illustrated by the influence it has on the Th1/Th2 axis of development. Both Bmi1 (PCGF 4) and Mel-18 (PCGF 2) have been shown to physically interact with GATA3, a lineage specific transcription factor for Th2 differentiation, in a Ring finger dependent manner (59, 60). Mel-18 has been shown to

**TABLE 1 |** Roles of PRC1, PRC2, G9a, and annotated lncRNAs in the development and function of Th1, Th2, Treg, and Th17 cells.

| | Th1 | Th2 | Treg | Th17 |
|---|---|---|---|---|
| PRC1 | Absence of Bmi1 impacts Th1 generation and maintenance (58). | Regulates Th2 differentiation and cytokine expression (59, 60). Overexpression of Bmi-1 increases GATA3 expression and stability (59). Loss of Mel-18 impacts Th2 differentiation *in vivo* (60). | Maintains Treg signature gene expression (61). Inactivation leads to systemic immune mediated disease (61). | Knockdown of Mel-18 leads to decreased expression of *IL17A, IL17F,* and *RORC* (62). |
| PRC2 | Inhibits Th1 differentiation and cytokine production (63, 64). EZH2 deficiency enhances production of Th1 cytokines and increased T-bet expression (63, 64). | Inhibits Th2 differentiation and cytokine production (63, 64). EZH2 deficiency enhances production of Th2 cytokines and increased GATA3 expression (63, 64). | EZH2 is required to promote the FOXP3-mediated gene repression program following TCR stimulation (65). Loss of EZH2 in Tregs *in vivo* leads to multi-organ inflammation and increases susceptibility to experimental models of autoimmunity (65, 66). | EZH2-deficient naïve CD4+ T cells stimulated under Th17 polarizing conditions displayed enhanced production of IL-17 (63). |
| G9a | No evidence supports a role for G9a in Th1 biology. | Required for Th2-specific cytokine expression (40). Loss of G9a prevents Th2 differentiation and increases IL-17A expression (40, 42). | Absence of G9a in CD4+ T cells is associated with increased FOXP3 expression (42). G9a expression in CD4+ T cells is necessary for development of colitis in mice (42). | Absence of G9a in CD4+ T cells is associated with increased IL-17A expression *in vivo* and *in vitro* (42). Recruited by RelB to silence *IL17A* locus in mouse model of EAE (67). |
| LncRNA | Linc-MAF-4 promotes Th1 differentiation through silencing of Th2 transcription factor MAF (49, 68). IFNG-AS1 recruits H3-K4-methyltransferase to *Ifng* locus and is upregulated in response to Th1-polarizing cytokines (52, 69–71). | Th2-LCR-lncRNA recruits WDR5-containing complexes to Th2-specific cytokine loci facilitating their expression (72). LincR-CcR2-5′ AS interacts with GATA-3 to upregulate chemokine genes necessary for Th2 migration (73). | Flicr negatively regulates FOXP3 leading to decreased Treg function (74). Lnc-Smad-3 regulates TGFβ mediated Treg differentiation by interacting with HDAC1 (75). Lnc-EGFR promotes Treg differentiation through interactions with EGFR (51). | Overexpression of LncRNA-1700040D17Rik was associated with decreased expression of RORγt and IL-17 in Th17 cells (76). |

regulate *GATA3* transcription, and knockout of *mel-18* severely impacts Th2 differentiation *in vivo* (60). Bmi-1 regulates Th2 cell differentiation by acting as an inhibitor of GATA3 degradation and regulator of its stability. Bmi-1 overexpression in itself leads to an increase in GATA3 expression and an increase in Th2 cell differentiation under a Th2 specific cytokine milieu. Comparatively little data exist regarding the role of PRC1 in Th1 cell development/function; however adoptive transfer of CD4+ T cells from *Bmi1*$^{-/-}$ mice into nude mice showed impaired generation and maintenance of memory Th1 cells through Bmi1-mediated repression of *Noxa*, a pro-apoptotic gene (58).

The role of EZH2 in modulating effector T function was recently illuminated by Yang et al. who showed that EZH2-deficient naïve CD4+ T cells stimulated under Th1, Th2 or Th17 polarizing conditions displayed enhanced production of IFN-γ, IL-13 or IL-17 cytokines, respectively (63). Moreover, Tumes et al. also showed that EZH2 deficiency in naïve CD4+ T cells led to the upregulation of Th1 and Th2-associated cytokines with concomitant increase in lineage-specific transcription factors T-bet and Gata3, respectively (64). However, *in vivo* studies have revealed that EZH2 plays a dichotomous role in the differentiation and senescence of CD4+ T cells (63). For example, in an *in vivo* model of *Listeria monocytogenes* infection known to induce a Th1 response, CD4+ T-specific EZH2 deleted mice displayed impaired clearance of infection due to decreased survival of memory Th1 cells (84). Additionally, OVA-specific EZH2-deficient Th2 cells were pathogenic in a mouse model of allergic asthma due to an accumulated and exaggerated immune response from memory Th2 cells (64). Taken together, EZH2 inhibits effector cytokine production in naïve CD4+ T cells, and loss of EZH2 enhances differentiation to effector Th cells as well as effector Th cell plasticity. Based on evidence from *in vivo* studies in mice in the context of EZH2 deletion in T cells, effector Th cell dysfunction is consistent across all disease models, evidently through impaired clearance of pathogens or aggravated autoimmunity (potentiated tissue destruction). Additionally, H3K27me3-independent functions of EZH2 have been reported in T cells expressing conventional αβ-TCRs (17, 18). Vasanthakumar et al. demonstrated that EZH2 prevents NKT cell expansion through methylation, ubiquitination and subsequent degradation of the transcription factor promyelocytic leukemia zinc finger (PLZF) (17). *In vivo* studies have demonstrated that an increase in the frequency of NKT cells in the thymus and spleen occurs as a result of CD4+ T-specific EZH2 deletion, which may contribute to the perturbed immunity seen in murine studies previously mentioned (63, 64, 84).

Two lncRNAs, MAF-4, and IFNG-AS1 (also called NeST or Tmevpg1), have been shown to influence Th1 biology by

recruiting different epigenetic modifying complexes. Linc-MAF-4 is selectively expressed in Th1 cells and promotes Th1 differentiation through epigenetic silencing of the Th2 transcription factor MAF. Downregulation of linc-MAF-4 in human CD4+ cells skewed differentiation toward a Th2 phenotype. Mechanistically, linc-MAF-4 promotes a *cis* chromatin looping conformation, leading to the recruitment of chromatin remodelers EZH2 and LSD1 that place repressive H3K27me3 marks on the promoter region of MAF-4 silencing its expression (49). Recently, linc-MAF-4 was shown to be involved in the pathogenesis of multiple sclerosis by promoting Th1 cell differentiation (68). Thus, far, linc-MAF-4 has not been studied *in vivo*.

IFNG-AS1 is expressed in CD4+ Th1, CD8+, and natural killer cells (52, 69). It is upregulated in CD4+ cells in response to Th1-differentiating cytokine stimuli and plays a critical role in transcription of *Ifng*. This has been demonstrated both *in vitro* and *in vivo*. Mechanistically, it has been shown to recruit the H3K4-methyltranferase complex to the *Ifng* locus, leading to placement of activating marks at the promoter region. It has been associated with the pathogenesis of Hashimoto's thyroiditis (70), ulcerative colitis (71), and the immune response to viral infections *in vivo* (52).

Two lncRNAs, Th2-LCR-lncRNA and lincR-CcR2-5′AS, have been shown to influence the development and function of Th2 cells. Th2-LCR-lncRNA is selectively expressed in human Th2 cells and is transcribed in the RAD50 locus and epigenetically regulates expression of IL-4, IL-5 and IL-13 (72). Mechanistically, Th2-LCR-lncRNA recruits WDR5-containing complexes to targeted cytokine loci, enhancing transcription. Knockdown of human Th2-LCR-lncRNA *in vitro* causes major loss of expression of IL-4, IL-5 and IL-13 in Th2 cells through loss of H3K4me3 activating marks (72). Unfortunately, Th2-LCR-lncRNA is not conserved in mice, complicating *in vivo* studies.

LincR-CcR2-5′AS is selectively expressed in mouse Th2 cells and upregulates *CCR1*, *CCR2*, *CCR3* and *CCR5* chemokine genes in a GATA3-dependent fashion (73). Interestingly, knockdown of this lincRNA not only affected neighboring genes *CCR2* and *CCR3*, but also affected nearly 1,200 genes some of which were located in distant loci, suggesting it can act in both *cis* and *trans*. Although the precise mechanism is yet to be fully understood, *in vitro* knock down of lincR-CcR2-5′AS did not result in chromatin accessibility or modification of H3K4me3, suggesting that it does not act through recruitment of histone-modifying enzymes or chromatin structure modifications.

## FUTURE PERSPECTIVES: EPIGENETIC MODULATION OF T CELLS IN CLINICAL PRACTICE

Epigenetic mechanisms of disease are in theory inducible and reversible through environmental manipulation, however, some epigenetic features have been shown to be maintained after cellular division as a result of self-enforcing feedback mechanisms (85). The heritable, yet reversible nature of epigenetic therapy makes this a promising option for treatment. Persistence of epigenetic maintenance of engineered modifications has been shown to be stable up to 40 days post modification induction *in vivo* (86). Most epigenetic drugs currently in use inhibit DNA methyltransferase and histone deacetylase activity, and have been shown to reverse immune suppression and thus sensitize the host immune system in combination with anti-cancer therapies. Several anti-cancer mechanisms have been reported, such as enhancing antigen processing and presenting machinery pathways, inhibiting immune checkpoints, and enhancing chemokine production. For patients, there are three treatment options available: therapies reported to affect DNA methylation, inhibitors of histone post-translational modifications, and compounds interfering with non-coding RNA regulation (87). Repurposing drugs and screening for new compounds that display converse effects to treatment autoimmune disease is an exciting new option for autoimmune illnesses.

Distinct DNA methylation profiles have been demonstrated in CD8+ and CD4+ T cells isolated from patients experiencing autoimmune diseases (88–90). Epigenetic based therapeutics currently being employed for the clinic for non-inflammatory conditions, such as arrhythmias (procainamide), hypertension (hydralazine), and neoplasia (5-azacytidine), have been shown to induce auto-reactive pathology (7, 8). However, the 5-azacytidine derivative 5-aza-2'deoxycytidine, which is also a DNA methyltransferase inhibitor used in hematological malignancies, has been shown to have a positive outcome when administered in animal models of diabetes (91), colitis (92), multiple sclerosis (93), and graft-versus-host-disease (GvHD) (94). We need a better understanding of the implications of DNA methylation, the pharmokinetics of available compounds, and synergistic effects of combination therapy with immunomodulatory drugs already in practice for autoimmune diseases to allow us to develop and implement novel therapies. As of now, we are lacking a therapeutic arsenal to target global hypomethylation, which is most often associated with lymphocytes recovered from patients experiencing some of the most common autoimmune diseases.

The ubiquitous expression of EZH2 and the opposing role it plays in different cell-types makes EZH2 a delicate therapeutic target. Recent identification of PRC2- and H3K27me3-independent EZH2 functions in oncogenesis indicates that a complete suppression of all oncogenic functions of EZH2 is required to combat cancer. Anti-EZH2 therapy inhibits methylation at key repression/silencing associated histone marks, and these compounds have emerged as a promising therapy for cancer treatment, especially for B cell non-Hodgkin's lymphoma. However, we have observed that systemic anti-EZH2 therapy leads to mucosal hypersensitivity in mice. One complicating factor is that EZH2 is also utilized by PRC1 in the nucleus, therefore more study needs to be undertaken to dissect the specific roles these complexes play in inflammation before on can determine whether histone methyltransferase inhibitors can be co-opted for anti-inflammatory therapy. Of note, cytosolic forms of PRC2 have been shown in murine models to be necessary for TCR-mediated activation of signaling pathways that drive T cell proliferation and autoimmunity. Thus,

pharmacologic targeting of cytosolic PRC2 may represent a more precise therapeutic approach to suppressing autoimmunity caused by excessive T cell activation (19, 20).

From a translational standpoint, several studies have demonstrated that long non-coding RNAs can be used as biomarkers in malignancy and autoimmune diseases (95–97). Potential lncRNA-targeted therapeutic approaches include silencing by antisense base pairing (e.g., targeting lncUBE3ATS, which silences paternal UBE3A in Angelman's syndrome) or by targeting molecules that are necessary for lncRNA transcription, such as transcription factors (98, 99). The cell type specific expression of lncRNAs makes them excellent targets for therapeutic intervention, as off-target effects are minimized. One option being pursued in cancer therapies is to directly target HOTAIR; a primarily *trans*-acting long-non coding RNA that promotes gene silencing through recruitment of PRC2 and LSD1 complexes, resulting in trimethylation of H3K27 and demethylation of H3K4, respectively (100–102). Knocking down HOTAIR provides compelling evidence for therapeutic targeting in cancer. Arresting glioblastoma multiform cell migration and invasion through this approach is a case in point (103). To overcome the limitation of genetic targeting, peptide nucleic acids have been developed which disrupt complex function. This approach has had positive results in inhibiting NF-κB activity in addition to decreasing ovarian and breast cancer properties such as reduced tumor formation and survival (104). The potential for this approach in inflammatory diseases is still to be determined.

Precision medicine has brought about the advent of using CRISPR/Cas9 to target this gene editing tool to target epigenetic modifying enzymes to precise locus specific locations on the genome instead of the DNA endonucleases the technology originally utilized (105). This technique can be exploited to recruit enzymes that impact the methylation of the DNA, enzymes that post-translationally modify the histones, and proteins which interfere with non-coding RNA regulation. Further, it has been recently reported CRISPR/Cas9 technology can be rapidly delivered via a non-viral delivery technique capable of integrating large DNA sequences (106). These new developments will allow us flexible and precise epigenetic manipulation toward creating therapeutically epi-engineered primary human immune cells without the off-target effects associated with systemic epigenetic therapies.

## AUTHOR CONTRIBUTIONS

WF contributed conception and design of the manuscript. JG, MB, GR, AB, MG, MS, and OS wrote sections of the manuscript. JG wrote the first draft of the manuscript. All authors provided critical revision and final approval of the manuscript.

## FUNDING

Supported by grants 5R01AI089714-08, 5R01AI089714-08S1, 30DK084567, and CCFA #401661.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 12 February 2019
doi: 10.3389/fimmu.2019.00210

# TET Enzymes and 5hmC in Adaptive and Innate Immune Systems

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DNA methylation is an abundant and stable epigenetic modification that allows inheritance of information from parental to daughter cells. At active genomic regions, DNA methylation can be reversed by TET (Ten-eleven translocation) enzymes, which are responsible for fine-tuning methylation patterns. TET enzymes oxidize the methyl group of 5-methylcytosine (5mC) to yield 5-hydroxymethylcytosine (5hmC) and other oxidized methylcytosines, facilitating both passive and active demethylation. Increasing evidence has demonstrated the essential functions of TET enzymes in regulating gene expression, promoting cell differentiation, and suppressing tumor formation. In this review, we will focus on recent discoveries of the functions of TET enzymes in the development and function of lymphoid and myeloid cells. How TET activity can be modulated by metabolites, including vitamin C and 2-hydroxyglutarate, and its potential application in shaping the course of immune response will be discussed.

**Keywords: 5hmC, 5 hydroxymethylcytosine, ten eleven translocation (TET), DNA modification, epigenetics (methylation/demethylation), gene regulation and expression**

OPEN ACCESS

***Edited by:***
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***Reviewed by:***
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***Specialty section:***
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *24 October 2018*
***Accepted:*** *24 January 2019*
***Published:*** *12 February 2019*

***Citation:***
*Lio C-WJ and Rao A (2019) TET Enzymes and 5hmC in Adaptive and Innate Immune Systems. Front. Immunol. 10:210. doi: 10.3389/fimmu.2019.00210*

## INTRODUCTION

Cells rely on the proper propagation and preservation of epigenetic information in order to regulate gene expression appropriately. 5-methylcytosine (5mC), described as the 5th base of DNA, is a chemically stable modification that is one of the most reliable ways of transmitting epigenetic information. In most cells, 5mC is present primarily at symmetrically-methylated CG dinucleotides in DNA, although methylation of cytosines in other contexts (CH=CA, CT, CC) has been reported in stem cells and in neurons (1). During DNA replication, methylated CGs are replaced by unmodified cytosines in the newly synthesized DNA strand, and the resulting hemimethylated CGs are recognized by a complex of UHRF1 and the maintenance methyl-transferase DNMT1 (2–4). The remethylation of hemi-methylated CpGs in newly replicated DNA is complete within 20 min, accounting for the stable inheritance of DNA methylation (5). In contrast to DNMT1, which depends on 5mC deposition at CpG motifs for maintenance DNA methylation, the *de novo* methyltransferases DNMT3A and DNMT3B can methylate unmodified cytosines in both CG and CH sequence contexts. While the writers for DNA methylation (DNMTs) have been known for decades, how DNA methylation is removed remained unclear until the discovery of TET (Ten-Eleven Translocation) enzymes and their ability to oxidize 5mC to 5-hydroxymethyl-cytosine (5hmC) [(6); reviewed in (3, 4)].

5hmC, the so-called 6th base, is a stable epigenetic modification that accounts for 1–10% of 5mC depending on the cell type: ∼10% in embryonic stem cells (6) and as high as 40% in Purkinje neurons (7). While 5hmC or related modifications have been known to exist in simpler organisms

including T-even phages for more than half a century (8), it was not until 2009 that 5hmC was rediscovered in mammalian cells (6, 7). The mammalian enzymes responsible for generating this modification are the three TET dioxygenases (TET1, TET2, and TET3) that utilize the co-factors α-ketoglutarate (αKG), reduced iron ($Fe^{2+}$), and molecular oxygen to oxidize the methyl group at the 5 position of 5mC (6). TET proteins can be found in every metazoan organism that contains DNMTs, even simple organisms such as comb jellies (9–11).

Besides being a potential epigenetic mark, 5hmC is the key intermediate for TET-mediated active (replication-independent) and passive (replication-dependent) DNA demethylation (**Figure 1**). TET enzymes iteratively oxidize 5mC and 5hmC into other oxidized cytosines (oxi-mCs) including 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (12); in active DNA demethylation, 5fC and 5caC are recognized and excised by thymine DNA glycosylase (TDG), repaired by the base-excision repair system, and replaced by unmodified C, thus resulting in DNA demethylation (13). In replication-dependent passive DNA demethylation, the DNMT1/UHRF1 complex does not recognize hemi-modified CGs with 5hmC, 5fC, or 5caC and thus the cytosine on the newly synthesized DNA strand is not methylated (5, 14, 15). Thus, the interplay between DNMT and TET proteins sculpts the DNA methylation landscape and

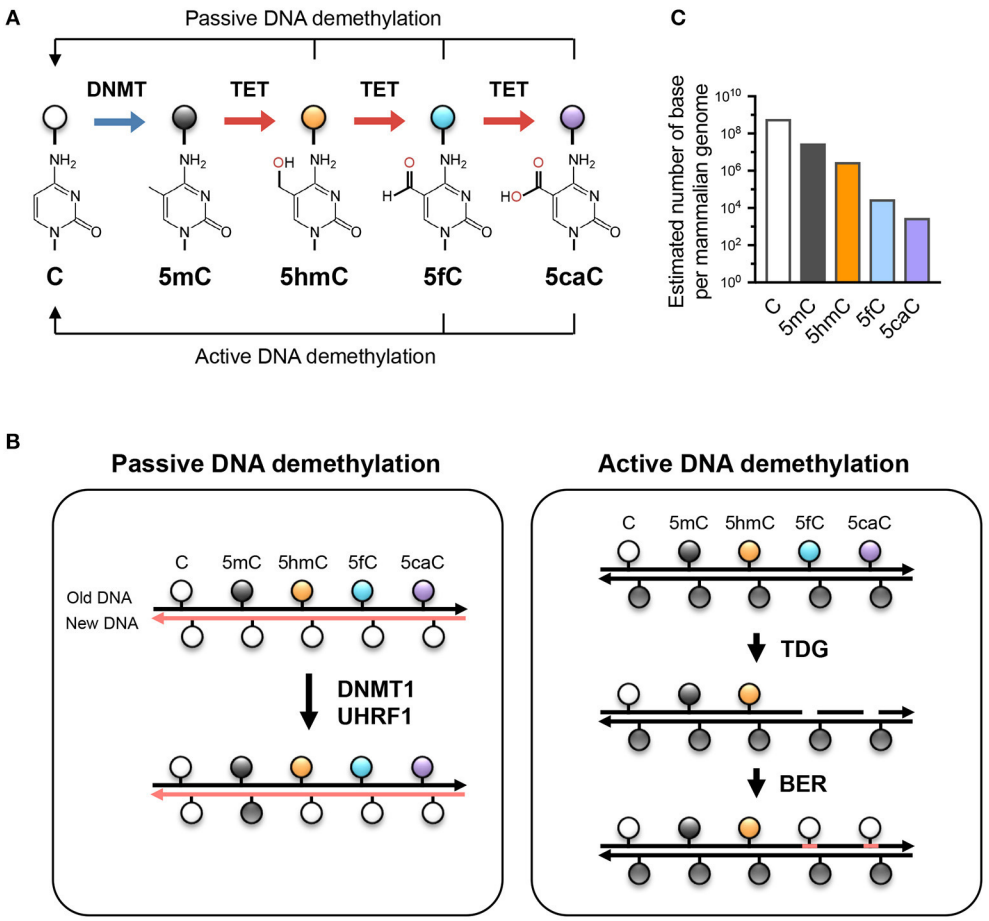


**FIGURE 1 |** TET-mediated DNA modifications and demethylation. **(A)** Unmodified cytosine (C) is methylated by DNA methyltransferases (DNMTs) at the 5 position to become 5-methylcytosine (5mC). TET proteins oxidize 5mC into 5-hydroxymethylcytosine (5hmC), a stable epigenetic mark, and subsequently to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). TET can demethylate DNA via replication-dependent (passive) or replication-independent (active) mechanisms. **(B)** Left, passive DNA demethylation. DNMT1/UHRF1 complex recognizes 5mC at the hemi-methylated CpG motif during DNA replication and methylates the unmodified cytosine on the newly synthesized DNA strand (left; pink strand). However, the oxidized methylcytosines 5hmC, 5fC, and 5caC (together, oxi-mC) are not recognized by DNMT1/UHRF1, resulting in unmodified cytosine on the new DNA strand. Further DNA replication in the presence of continuing TET activity will result in progressive dilution of 5mC in the daughter cells. *Right panel,* active DNA demethylation. While 5hmC is stable and persists in the genome, 5fC and 5caC can be recognized and excised by thymine DNA glycosylase (TDG), and the resulting abasic sites are repaired as unmodified C by base excision repair (BER). Other mechanisms (e.g., decarboxylation of 5caC) have been suggested but have not yet been proven to exist. **(C)** The approximate abundance of unmodified and modified cytosines in the haploid human/mouse genome. About 5% of cytosine is methylated (5mC); in most cells, the vast majority of 5mC is present at CG dinucleotides although it is low at CpG islands. 5hmC amounts to about 1-10% of 5mC (estimated at 10% here as in embryonic stem cells), while the levels of 5fC and 5caC are each about an order of magnitude lower than the previous oxidative modification. It is not known whether the low levels of 5fC and 5caC are due to features of TET enzymes that cause them to arrest at 5hmC, or to their continuing removal by TDG or other mechanisms.

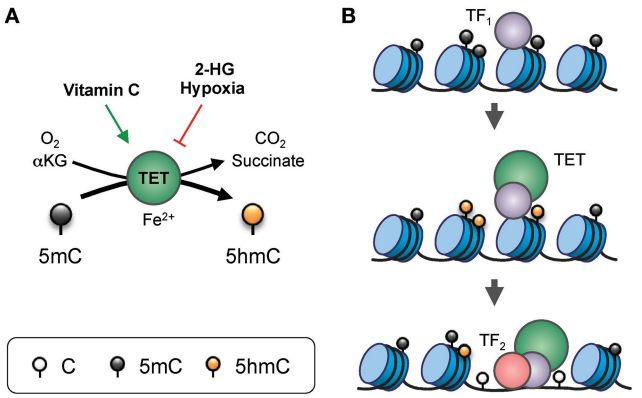


**FIGURE 2 |** Gene regulation by TET proteins. **(A)** Enzymatic activity of TET. TET proteins, with the co-factors $Fe^{2+}$ and α-ketoglutarate (αKG), use oxygen to oxidize 5mC into 5hmC, generating $CO_2$ and succinate as by-products. The enzymatic activity of TET can be modulated by additional factors. For instance, vitamin C (ascorbate) can enhance TET activity, potentially via reduction of the iron ion. On the other hand, the "oncometabolite" 2-hydroxyglutarate (2-HG), generated in acute myeloid leukemia and glioblastoma by recurrent dominant-active mutants of isocitrate dehydrogenase 1 or 2 (IDH1/2), inhibits TET activity. Furthermore, lack of oxygen in hypoxia also inhibits TET function. **(B)** Model of TET-mediated enhancer regulation. Prior to the commissioning of an enhancer, pioneer transcription factor (indicated as $TF_1$) binds to nucleosomal DNA and recruits TET which oxidizes the surrounding 5mC into 5hmC (and/or other oxi-mCs), facilitating DNA demethylation. TET proteins and, $TF_1$ promote enhancer accessibility by recruiting nucleosome remodeling complexes, thus allowing binding of secondary transcription factors (indicated here as $TF_2$) that are otherwise inhibited by DNA methylation or the presence of nucleosomes.

enables the flow of epigenetic information across cell generations.

DNA modification by TET proteins is essential for gene regulation (**Figure 2**). TET3 is expressed in the oocyte and the zygote; all three TET proteins are expressed in blastocysts; TET1 and TET2 are expressed in embryonic stem (ES) cells; and TET2 and TET3 are expressed ubiquitously in differentiated cells (3, 4). The three TET enzymes appear to have overlapping but distinct targets in the genome. For instance, in mouse ES cells, TET2 rather than TET1 is responsible for the vast majority of 5hmC generation, and TET1 preferentially facilitates promoter demethylation while TET2 and TET3 act on enhancers (16, 17). The longstanding association of high-level gene transcription with low levels of promoter methylation may be explained by TET-mediated conversion of 5mC to 5hmC at promoters, and subsequent DNA demethylation.

The genome-wide distribution of 5hmC reflects the strong association of TET enzymes with gene transcription. 5hmC is enriched at the most active enhancers and the gene bodies of the most highly transcribed genes (18). Moreover, multiple transcription factors important in cell differentiation and lineage specification, including NANOG, SALL4A, WT1, EBF1, PU.1, and E2A, have been shown to recruit TET proteins to specific genomic loci (primarily enhancers) for 5hmC modification, in most cases marking them for subsequent demethylation (19–24). As a result, TET function is particularly essential for gene transcription during cell activation and lineage specification, and deficiencies of TET protein expression or activity result in skewed or arrested cell differentiation in multiple lineages, including those in neural and hematopoietic systems (25–30).

TET loss-of-function is strongly connected to oncogenesis (31, 32). Especially in the hematopoietic system, arrested or skewed cell differentiation is often associated with cell transformation (22, 26). In humans, *TET2* is one of the most frequently mutated genes in hematopoietic cancers of both myeloid and lymphoid origin (26). Using mouse models, we and other groups have shown that deletion of *Tet2* alone, or deletion of both *Tet2* and *Tet3* (the two TET enzymes with the greatest overlap in expression and function), leads to myeloid or lymphoid expansion and the development of aggressive cancers with 100% penetrance (22, 25, 33). For instance, a striking example is the inducible deletion of both *Tet2* and *Tet3* in adult mice, which leads to acute myeloid leukemia with the mice succumbing as early as 3 weeks post-deletion (25). Since the role of TET proteins in malignancies has been reviewed extensively (26, 34–36), we will focus here on their roles in immune cell development and function. In the sections below, we outline our current understanding of the roles of TET proteins in regulating the adaptive and innate immune systems. The major findings are summarized in **Figures 3**, **4**.

## TET PROTEINS IN T AND B CELL DIFFERENTIATION AND FUNCTION

During development and immune responses, T and B cells continuously receive signals from antigen and cytokine receptors. These external signals converge and are interpreted by combinations of ubiquitously expressed and cell type-specific transcription factors, which function together with chromatin regulators to remodel the epigenome. The epigenetic changes associated with immune cell activation and differentiation include DNA and histone modifications, which allow information to be stored and/or inherited by daughter cells. As noted above, analyses of genome-wide 5hmC distribution reveal a close relationship between 5hmC and gene transcription. In thymic and peripheral T cell subsets, the level of 5hmC at gene bodies shows a striking positive correlation with the level of gene expression, as well as occupancy by RNA polymerase II and the level of H3K36me3 histone modification, an epigenetic mark reflective of RNA transcription into the gene body (17, 18, 50, 51). Similarly, in lymphoid cells, 5hmC showed a strong positive correlation with enhancer activity, denoted by the level of H3K27 acetylation (H3K27ac), suggesting that TET is important for regulating enhancer function (16, 18, 52). Indeed, recent studies of T and B cells from our lab (see below) and others demonstrated that one of the functions of TET proteins is to facilitate chromatin accessibility at enhancers (22, 28, 33, 37). TET-mediated conversion of 5mC to 5hmC potentially disrupts the binding of 5mC-binding proteins including MeCP2 and MBD (Methyl-CpG-binding domain) proteins, facilitating nucleosome remodeling and the binding of transcription factors (53, 54). These changes in the epigenetic status of enhancers

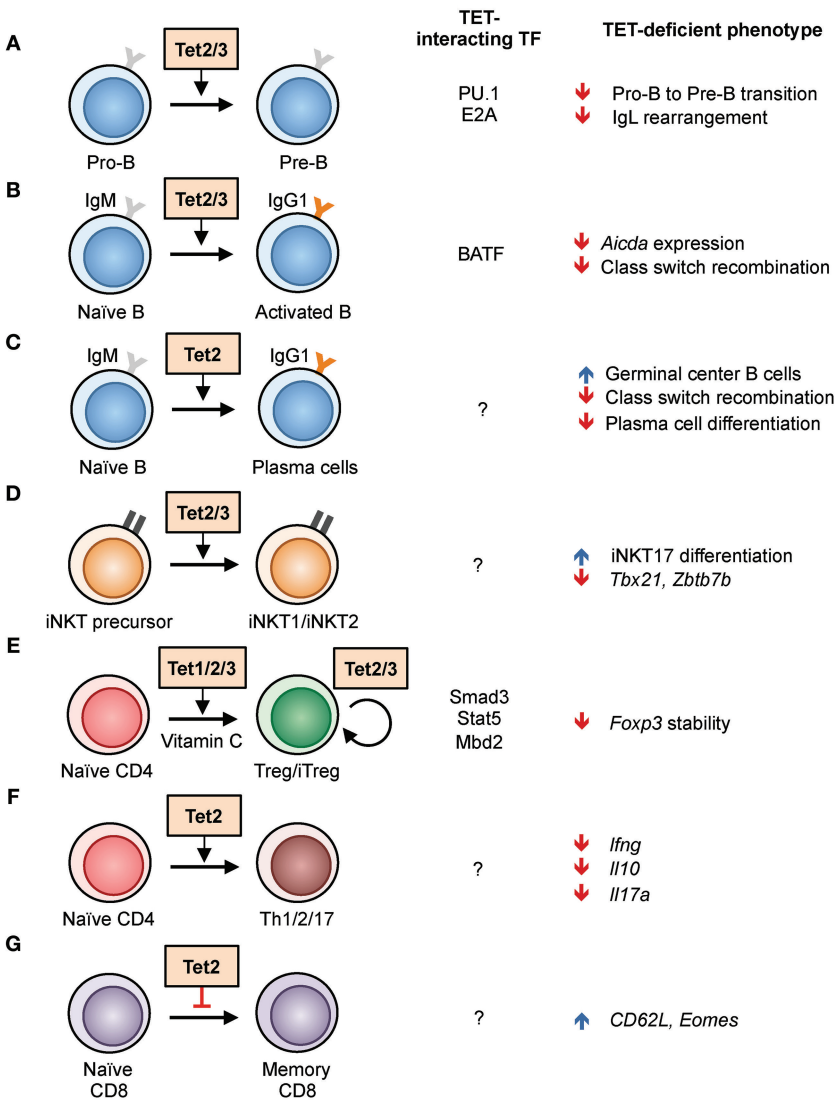


**FIGURE 3 |** Regulation of lymphoid development and function by TET proteins in the mouse. **(A–G)** List of known TET functions in lymphoid cells. The interacting transcription factors and the phenotypes found in *TET*-deficient mice are shown in the right columns. **(A)** The use of *Mb1-cre Tet2/3*-deficient mice showed that *Tet2* and *Tet3* regulate the pro-B to pre-B cell transition, in part by enhancing the rearrangement of immunoglobulin light chains (22, 37). **(B)** Acute deletion of *Tet2/3* using $Cre^{ERT2}$ in B cells resulted in decreased *Aicda* expression and thus class switch recombination (28). **(C)** Deletion of *Tet2* using *Vav-Cre* and *Cd19-Cre* resulted in hyperplasia of germinal center B cells. *Vav-Cre*-driven *Tet2* deletion resulted in decreased plasma cell differentiation (38). **(D)** *Cd4-cre Tet2/3*-deficient mice exhibited skewed differentiation toward iNKT17 cells, partly due to decreased expression of *Tbx21* and *Zbtb7b* expression, and a massive T-cell-receptor-dependent expansion of affected T cells (33). **(E)** Tet proteins facilitate the *in vitro* differentiation of naïve CD4 T cells to iTreg cells by demethylating *Foxp3* enhancer *CNS2*, a process enhanced by the presence of vitamin C. All three TET proteins have a role in stabilizing the expression of *Foxp3* in Treg cells *in vivo* (39, 40). **(F)** CD4 T cells from *Cd2-cre Tet2*-deficient mice showed impaired Th1, Th2, and Th17 differentiation and cytokine production (41). **(G)** Increased differentiation of CD8 memory cells from *Cd4-cre Tet2*-deficient mice in response to lymphocytic choriomeningitis virus infection (42).

are likely transmitted to daughter cells, thus facilitating the establishment of lineage identity.

## TET Proteins in B Cell Development and Function

From bone marrow progenitors to peripheral memory and plasma cells, the B cell genome undergoes progressive demethylation following differentiation (55). Furthermore, *TET2* is one of the most frequently mutated genes (6–12%) in diffuse large B cell lymphoma, a malignancy originating from germinal center B cells (56–58). These observations suggest that TET proteins play an important role in B cell biology. Indeed, when *Tet2* and *Tet3* were deleted in early B cells during bone marrow development using *Mb1-Cre*, B cell differentiation was arrested at the transition from the pro-B to the pre-B stage (22, 37) (**Figure 3A**). One function of these TET proteins during early B cell development is to regulate the arrangement

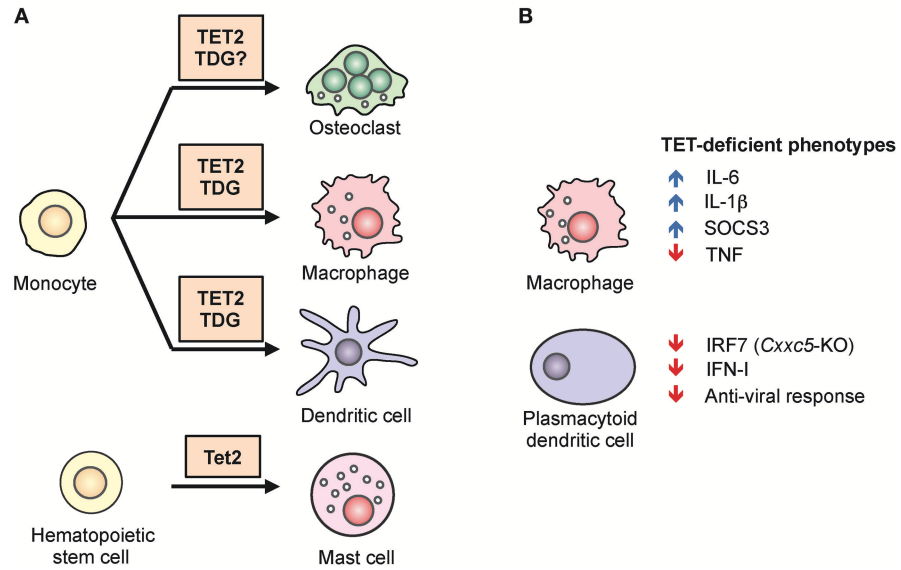


**FIGURE 4 |** The role of TET2 in myeloid differentiation and function. **(A)** TET2 regulates myeloid cell differentiation. TET2, together with the thymine DNA glycosylase (TDG), facilitates active DNA demethylation and promotes lineage-specific gene expression during the differentiation of osteoclasts, macrophages, and dendritic cells from human monocytes. In mice, TET2 is required for the differentiation of mast cells *in vitro* and *in vivo* (43, 44). **(B)** TET2 regulates the function of myeloid cells. In mouse and human macrophages, TET2 repressed expression of the pro-inflammatory cytokines IL-1β and IL-6 (45–48). TET2 was shown to associate with Iκbζ, bind to the *Il6* promoter, recruit HDAC2, and repress *Il6* expression (46). *Tet2*-deficient macrophages also expressed a high level of *Socs3* mRNA, which in normal mice was suggested to be demethylated by TET2 and subsequently degraded by ADAR1 (44). In plasmacytoid dendritic cells, CXXC5 recruited TET2 to an intragenic CpG island in *Irf7*, facilitating the demethylation and maintaining basal expression. As a result, loss of *Cxxc5*, and to a lesser extent *Tet2*, resulted in decreased levels of IRF7, decreased type I interferon (IFN-I) production, and decreased anti-viral responses (49).

of the Ig kappa (Igκ) light chain genes, that pairs with the rearranged Ig heavy chain to form the complete B cell receptor. TET proteins regulate Igκ rearrangement by oxidizing 5mC at Igκ enhancers and facilitating their DNA demethylation and chromatin accessibility.

Mechanistically, TET proteins appear to be recruited to enhancers by "pioneer" transcription factors, defined by their ability to associate with their binding motifs on nucleosome-bound DNA. Our data indicate that in pro-B cells, the pioneer transcription factor PU.1 binds to the Igκ enhancers prior to light chain rearrangement as a placeholder and recruits Tet proteins for DNA demethylation, facilitating the binding of additional B cell transcription factors including E2A (**Figure 3A**). Tet proteins also regulate the expression of IRF4 and IRF8, both of which are important for Igκ rearrangement (22). Similar to the expansion phenotype observed in T cells (described in more detail below), *Mb1-Cre Tet2/3*-deficient mice developed massive expansion of immature B cells resembling acute lymphoblastic lymphoma (22). Therefore, Tet proteins are essential for B cell development by controlling the expression of multiple key genes.

In mature B cells, TET proteins are important for the antibody response. Recently we showed that B cell activation induced Tet protein expression and changes in the genome-wide distribution of 5hmC (the hydroxymethylome): lipopolysaccharide (LPS) and IL-4 stimulation induced a progressive TET-dependent hydroxymethylation at ∼8,000 regions in the span of 3 days (28). Functionally, the two major members in naïve B cells TET2 and TET3 are crucial for antibody class switch recombination (CSR) (**Figure 3B**). Acute deletion of *Tet2* and *Tet3* by $Cre^{ERT2}$ resulted in an ∼50% decrease the expression of AID (Activation Induced Deaminase; encoded by *Aicda*), the critical enzyme for CSR; reconstitution of catalytically active AID in *Tet2/3*-deficient B cells restored CSR. Interestingly, the CSR phenotype is reminiscent of that resulting from *Aicda* haploinsufficiency (59, 60), suggesting that TET proteins are required for optimal expression of *Aicda*. Mechanistically, we showed that the transcription factor BATF recruits Tet proteins to the *Aicda* superenhancer, facilitating hydroxymethylation and chromatin accessibility of two *Tet*-responsive elements, *TetE1* and *TetE2*, within the superenhancer and augmenting the expression of *Aicda* (28) (**Figure 3B**).

Recently, *Vav-Cre* and *Cd19-Cre*, which are expressed in the entire hematopoietic system and during B cell development, respectively, were used to show that disruption of *Tet2* resulted in germinal center hyperplasia (38) (**Figure 3C**). However, germinal center B cells appeared to be normal in *Tet2* deletion driven by *Cγ1*-Cre, which is expressed in germinal center B cells. Consistent with our findings, *Tet2* was shown to be required for CSR and affinity maturation of antibody (**Figure 3C**). More importantly, TET2 positively regulated the expression of the transcription factor *Prdm1* (encoding BLIMP1), and plasma cell differentiation was impaired in *Tet2*-deficient mice. Interestingly, the gene signature of *TET2*-deficient DLBCL resembles that of cells with mutations in the histone acetyltransferase *CREBBP*,

suggesting that TET2 and CREBBP may cooperate to regulate enhancer H3K27 acetylation. Taken together, these observations demonstrate that TET proteins regulate multiple processes in B cells by preferentially strengthening the activity of enhancers, including individual enhancer elements located within super-enhancers (Igκ and *Aicda*) (61).

## TET Proteins in T Cell Development

*Tet2* and *Tet3* are expressed at higher levels than *Tet1* in thymocytes and peripheral T cells, and are responsible for the majority of 5hmC modification in these cells. Deletion of *Tet2* alone in the germline, in the hematopoietic system using *Cd2-cre*, or in T cells (*Cd4-cre*) did not lead to any obvious defect in T cell development (41, 42, 62), suggesting that *Tet3* was able to compensate for the loss of *Tet2*. Indeed, data from our lab showed that the deletion of both *Tet2* and *Tet3* in T cells using *Cd4-cre* caused a massive lymphoproliferative phenotype with enlarged spleen and lymph nodes, and the mice succumbed by 8 weeks of age (33). At 3–4 weeks of age, young *Tet2/3 Cd4-cre DKO* mice showed decreased thymic cellularity, a lower percentage of $CD4^+CD8^+$ double positive cells, and an increased percentage of $CD4^+$ and $CD8^+$ single positive cells, phenotypes reminiscent of thymic atrophy induced by stress or inflammation. Further examination showed that the expanded cells in the periphery were invariant natural killer T (iNKT or NKT) cells that expressed the transcription factor Rorγt and produced IL-17 (**Figure 3D**). These cells thus resemble the NKT17 subset, one of the three subsets of NKT cells besides NKT1 (T-bet-expressing) and NKT2 (Gata3-expressing). In contrast, NKT cells from wildtype mice are primarily of the NKT1 and NKT2 subsets (63).

Genome-wide analyses provided mechanistic explanations for the lineage skewing observed in *Tet2/3 Cd4-cre DKO* mice. Briefly, the profiles of transcriptome, whole-genome methylome, and chromatin accessibility showed that *Tet2/3* deficiency resulted in decreased expression of *Tbx21* (encoding T-bet) and *Zbtb7b* (encoding Th-POK), likely because of hypermethylation at the corresponding regulatory elements (**Figure 3D**). Both T-bet and Th-POK repress *Rorc* (encoding Rorγt) expression, thus the decreased levels of T-bet and Th-POK transcription factors in *Tet2/3*-deficient cells permitted increased Rorγt expression and skewed the cells to the NKT17 lineage (33). Interestingly, the *Tet2/3*-deficient iNKT cells were able to expand upon transfer to fully immunocompetent, wild-type (WT) but not *Cd1d*-deficient recipient mice (33), suggesting (i) that the expansion was secondary to recognition of "self" antigens presented by CD1d and (ii) that expansion was not suppressible by WT regulatory T (Treg) cells (see below). Together, these observations indicate that TET enzymes are important to maintain the proper expression of lineage-specifying transcription factors, and to limit the differentiation and proliferation of overly self-reactive cells including iNKT cells.

## Maintenance of $Foxp3^+$ Treg Cells Requires TET Proteins

TET enzymes are important for the homeostasis of T regulatory (Treg) cells, which are distinguished from other T cell lineages by their expression of the transcription factor FOXP3. In Treg cells, TET2 and TET3 are required for stable *Foxp3* expression through their ability to demethylate two intronic enhancers, termed conserved non-coding sequence (*CNS*) 1 and *CNS2* (39, 64) (**Figure 3E**). Bisulfite sequencing showed that the *Foxp3 CNS1* and *CNS2* enhancers were hypermethylated in Treg cells from *Tet2/3 Cd4-cre DKO* mice (39). Moreover, overexpression of the TET1 catalytic domain in CD4 cells induced to differentiate into *Foxp3*-expressing induced Treg cells (iTreg) *in vitro* and resulted in partial demethylation of *CNS2* (65), suggesting that TET enzymes may be in constant balance with the methylation machinery. Hypermethylation at *Foxp3 CNS2* was also observed in *Tet1/2*-deficient mice, suggesting all three Tet proteins may function redundantly in regulating *Foxp3* (40).

Several proteins have been identified to partner with TET proteins in regulating *Foxp3 CNS2*. For instance, loss of the DNA methyl-binding protein MBD2 also resulted in hypermethylation of *CNS2* (also termed TSDR), potentially because of decreased TET2 binding (66). How MBD2 cooperates with TET to demethylate *CNS2* remained to be determined. Besides MBD2, the transcription factors SMAD3 and STAT5, induced by TGFβ and IL-2 respectively, recruit TET proteins to *Foxp3 CNS2* and facilitate DNA demethylation (40). In addition, the level of TCR and cytokine stimulation has been linked to the degree of DNA demethylation at *Foxp3 CNS2* (67). Since there is only one functional allele of *Foxp3* per Treg cell, this observation implies that stronger a TCR stimulation might increase the probability of TET-mediated DNA demethylation at *Foxp3 CNS2* and concomitantly, the stability of *Foxp3* expression.

## TET Proteins Link Metabolism to Foxp3 Expression

The enzymatic activity of TET can be influenced by various factors, including the level of co-factors αKG, oxygen, and vitamin C (**Figure 2**). In a chemical screen using mouse embryonic stem cells, vitamin C was found to enhance the expression of gene expression in germ cells and ES cells by facilitating TET-mediated DNA demethylation at their promoters (68). Vitamin C treatment also facilitated TET-mediated demethylation of *Foxp3 CNS2* and stabilized *Foxp3* expression in differentiating induced Treg (iTreg) cells (**Figure 3E**). Inhibition of the vitamin C transporter by sulfinpyrazone confirmed the role of vitamin C and TET proteins in *CNS2* demethylation and the generation of peripheral Treg cells *in vivo* (69). In addition, vitamin C facilitated the conversion of mouse and human naïve CD4 T cells into iTreg cells induced by TGFβ and retinoic acid with improved stability and suppressive function (39). Besides vitamin C, another metabolite hydrogen sulfide ($H_2S$) was shown to be required for Treg cell differentiation, at least in part by increasing *Tet1* and *Tet2* expression (40).

TET activity can be inhibited by the "oncometabolite" 2-hydroxyglutarate (2-HG), a competitive inhibitor of αKG-dependent dioxygenases including TET (70, 71) (**Figure 2**). 2-HG is a normal metabolite that exists as two stereoisomers, R-2-HG and S-2-HG; the latter is considerably more potent at inhibiting TET activity (72). In the past few years, it has

become clear that 2-HG can be generated via multiple pathways; for instance, recurrent mutations of isocitrate dehydrogenase 1 and 2 (IDH1/2) give rise to dominant-active enzymes with the novel property of converting isocitrate to the R enantiomer of 2-HG (R-2-HG) (70, 71). A recent study identified a compound, (aminooxy)acetic acid (AOA), that is able to reprogram differentiating Th17 cells into *Foxp3*-expressing iTreg cells (73). Metabolic profiling identified the target of AOA in Th17 cells as GOT1 (glutamate oxaloacetate transaminase 1), an enzyme that catalyzes the conversion of glutamate to αKG. Th17 cells express a high level of GOT1 compared to iTreg cells, consistent with their elevated level of αKG. However, instead of facilitating the function of TET enzymes and other dioxygenases, the αKG is converted by wild-type IDH1/2 into R-2-HG, which inhibits TET activity, promotes increased methylation at *Foxp3 CNS2*, and represses *Foxp3* expression. By targeting GOT1, the small molecule AOA effectively decreased the intracellular level of R-2-HG and allowed TET proteins to demethylate *CNS2*, favoring differentiation to iTreg cells at the expense of the Th17 lineage (73). Therefore, these observations suggest that, besides conveying signals from cell surface receptors, TET proteins also integrate environmental cues into the epigenome.

## TET Proteins Regulate Peripheral T Cell Differentiation and Function

After stimulation and depending on the extracellular signal received, naïve CD4 T cells can differentiate into multiple lineages, including Th1, Th2, Th17, follicular T helper cells (Tfh), and Treg. Analysis of 5hmC distribution in peripheral T cells showed a positive correlation between gene expression level and 5hmC modification at gene bodies, including those of the lineage-specific transcription factor *Tbx21* and *Gata3* for Th1 and Th2 cells, respectively. This observation suggests that TET proteins may regulate the differentiation of peripheral T cells (18, 41). Similar lineage-specific 5hmC modifications during Th1 and Th2 polarization were also reported in human CD4 T cells (74). Indeed, *Tet2*-deficient murine CD4 T cells produced less IFNγ and IL-17 when polarized *in vitro* to Th1 and Th17, respectively (41) (**Figure 3F**). Compared to WT cells, adoptively transferred *Tet2*-deficient CD4 T cells were more pathogenic in an experimental autoimmune encephalomyelitis (EAE) model, and immunization with myelin oligodendrocyte glycoprotein (MOG) peptide induced significantly less IFNγ and IL-10 but a similar level of IL-17 (41). These observations reinforce the idea that Tet proteins are important for proper lineage differentiation and gene expression.

Analysis of *Tet2*-deficient (*Tet2*$^{fl/fl}$ *Cd4-cre*) CD8 T cells responding to infection with lymphocytic choriomeningitis virus (LCMV) showed increased LCMV gp33-specific memory precursor cells (KLRG1$^-$ CD127$^+$) and decreased short-lived effector cells (KLRG1$^+$ CD127$^-$) on day 8 post-infection (42) (**Figure 3G**). These memory-like cells expressed CD27, CD62L, and CXCR3, a phenotype similar to central memory cells, and persisted for at least 45 days post-infection with a higher level of Eomes compared to WT. Transfer of *Tet2*-deficient memory cells conferred better protection against gp33-expressing *Listeria monocytogenes* compared to WT memory cells, strongly suggesting that TET2 represses memory cell differentiation (42). In addition to TCR-induced TET protein expression (42), TET activity can also be modulated by physiologically produced 2-HG. CD8 T cells generate substantial levels of the potent 2-HG enantiomer "oncometabolite" S-2-HG as early as day two after TCR stimulation, coinciding with the decrease in 5hmC (75). Similar to genetic ablation of *Tet2*, S-2-HG treatment of CD8 T cells induced higher expression of Eomes and CD62L, markers for central memory cells. Surprisingly, OT-I CD8 T cells cultured in the presence of S-2-HG *in vitro* displayed enhanced survival and tumor clearance upon adoptive transfer *in vivo* (75), suggesting the effect of S-2-HG is long lasting by stably remodeling the epigenome.

In humans, TET loss-of-function was shown to have a major potentiating role in a case of cancer immunotherapy against B cell malignancy using T cells bearing the anti-CD19 chimeric antigen receptor (CAR). The patient bore a hypomorphic mutation in one allele of *TET2*, and the CAR lentivirus serendipitously became integrated into the other *TET2* allele. The resulting profound loss of function of *TET2* resulted in an almost monoclonal expansion of this particular CAR-T cell, and the patient went into complete remission (76). Thus the loss of *TET2* activity resulting from insertional mutation of one *TET2* allele due to lentiviral integration, combined with the preexisting hypomorphic mutation in the other *TET2* allele, led to superior anti-tumor function and again conferred a central memory phenotype on the expanded CAR-T cells. Together, these observations show that TET proteins are important in regulating peripheral T cell differentiation.

# TET PROTEINS IN MYELOID DIFFERENTIATION AND FUNCTION

## TET2 Regulates Myeloid Differentiation

*TET2* mutation has been closely linked to myeloid malignancies including myelodysplastic syndrome and acute myeloid leukemia in human (26). In mice, germline disruption of the *Tet2* gene decreased the global level of 5hmC in hematopoietic stem cells (HSCs), enhanced HSC survival and proliferation, inhibited T, B, and erythroid differentiation, and biased differentiation toward the myeloid lineage (62). Similarly, knockdown of *TET2* in human cord blood CD34$^+$ progenitor cells decreased total 5hmC in the cells and skewed their differentiation toward the granulomonocytic lineage, specifically monocytes, at the expense of both lymphoid and erythroid lineages (77). These and other studies suggested that, compared to other lineages, the myeloid lineage requires less reconfiguration of the DNA methylome during differentiation and therefore is relatively unaffected in the absence of TET2.

Beyond HSC, TET2 also regulates the differentiation of mast cells (**Figure 4A**). In a model of *in vitro* mast cell differentiation in which bone marrow progenitors were cultured with IL-3, loss of *Tet2* inhibited mast cell differentiation, decreased cytokine production, and induced aberrant hyperproliferation (43). Two major transcription factors involved in myeloid

development, C/EBPα and C/EBPε, were up-regulated in *Tet2*-deficient mast cells, and both contributed to the observed defect in differentiation. Similar to another observation in macrophages (see below), both catalytically active and inactive TET2 could partially rescue these phenotypic defects, suggesting that part of the function of TET2 is to maintain the structure of a repressive protein complex (43). *In vivo*, *Tet2* is important for the expansion of mast cells induced by parasites (44).

Human monocytes can differentiate into macrophages (MACs), dendritic cells (DCs), and osteoclasts (OCs) *in vitro* depending on cytokine signals, and the epigenetic regulation of this process has been studied extensively (78). During post-mitotic differentiation of DCs from monocytes, stimulation with cytokines GM-CSF and IL-4 induced DNA demethylation. Since these cells do not proliferate prior to differentiation, the mechanism of demethylation is assumed to involve an active replication-independent process. Similar observations were made during MAC and OC differentiation (20, 79–81). TET2-mediated oxidation of 5mC into 5hmC preceded and was required for DNA demethylation, which was accompanied by the presence of active histone modifications (H3K4me1, H3K4me3, H3/H4 acetylation) (**Figure 4A**). In general, the degree of DNA demethylation at distal elements or promoters showed a loose positive correlation with gene expression with numerous exceptions, suggesting that additional mechanisms contributed to gene regulation, such as H3K27 methylation by the polycomb complex (82). In monocyte to DC differentiation, IL-4-activated STAT6 induced TET2-dependent demethylation, and this was important for acquiring the proper cell identity and priming the expression of inducible genes (e.g., *IL1B*, *CCL20*) (81). During monocyte to OC or to MAC differentiation, the transcription factor PU.1 was found to associate with both hypo- and hypermethylated regions and to directly bind to TET2 as well as to the DNA methyltransferase DNMT3B (20). TET2 functioned together with thymine-DNA glycosylase (TDG), and to a lesser extent with activation-induced deaminase (AID), to hydroxymethylate and demethylate DNA, facilitating the establishment of cell-type-specific gene expression programs (83). The same study also showed that TET2 was responsible for recruiting the histone H3K4 methyltransferase SETD1A, and for increasing H3K4me3 modification at cell-type specific genes examined (83).

Together, these *in vitro* human studies showed that post-mitotic myeloid cells utilize TET2 and TDG for replication-independent, active DNA demethylation to establish cell-specific gene expression patterns or to prime gene for subsequent induction. Besides regulating lymphoid development, Tet proteins are required for the differentiation of multiple lineages of myeloid cells.

## TET Proteins Regulate Immune Responses by Myeloid Cells

One function of TET proteins in normal myeloid cells appears to be the repression of inflammatory gene expression (**Figure 4B**). For instance, *Tet2*-deficient macrophages and dendritic cells expressed a higher level of IL-6 in response to stimulation (45, 46). Mechanistically, TET2 was shown to associate with Iκbζ and bind to the *Il6* promoter, recruiting the histone deacetylase HDAC2 and repressing *Il6* expression. As discussed for mast cells above, the repression appeared to be independent of TET2 catalytic activity, suggesting that TET2 provided a structural scaffold for the formation of a repressive complex (46). Compared to WT controls, *Tet2*-deficient mice were more susceptible to endotoxin-induced septic shock and dextran sulfate sodium (DSS)-induced colitis, coincident with an increased IL-6 level (46).

TET proteins also repressed another inflammatory cytokine, IL-1β (47, 48). Moreover, loss of *Tet2* accelerated atherosclerosis development in a mouse model of low-density lipoprotein receptor (*Ldrl*) deficiency (47). *Tet2*-deficient macrophages increased IL-1β secretion via the NLRP3 inflammasome, the inhibition of which protects mice from atherosclerosis (47). Interestingly, IL-1R/MyD88 signaling was shown to induce Tet2 mRNA and protein expression in bone marrow-derived macrophages (84), suggesting a potential negative feedback loop controlling IL-1β expression by TET proteins. Lastly, TET2 facilitated immunosuppression by tumor-infiltrating myeloid cells in a melanoma model and loss of TET2 in myeloid cells inhibited melanoma growth *in vivo* (84), consistent with the role of TET proteins in suppressing inflammation in myeloid cells. TET proteins contribute to osteoclast differentiation and suppress inflammation, and osteoclast activation has been linked to rheumatoid arthritis (RA) (85), warranting further detailed investigation of the role of TET proteins in autoimmune and auto-inflammatory diseases.

TET proteins appear to have different functions in myeloid cells depending on the circumstances. For instance, TET proteins have been reported to promote myeloid immune responses and production of inflammatory cytokines rather than suppressing inflammation. Plasmacytoid dendritic cells (pDCs) are fast responders to infection and are able to produce a large quantity of type I interferon. This ability has previously been attributed to their high basal level of the transcription factor IRF7, the expression of which is regulated by an intronic CpG island (CGI) (49). TET2 is recruited to this locus by the zinc-finger protein CXXC5, and is required to maintain the demethylated status of the CGI (**Figure 4B**). As a result, mice deficient in *Cxxc5*, or to a lesser extent *Tet2*, were more vulnerable to infection by herpes simplex virus and vesicular stomatitis virus due to an impaired interferon response (49). Similarly, in a model of abdominal sepsis, *Tet2* deficiency was shown to reduce infection-induced myelopoiesis with a decreased level of TNFα and chemokines (44). The authors suggested that instead of oxidizing DNA, TET2 repressed *Socs3* expression by oxidizing methylcytosine in the 3′ untranslated region of *Socs3* RNA, thereby facilitating ADAR1-mediated destabilization of the mRNA in a manner independent of the normal RNA-editing function of ADAR1 (44) (**Figure 4B**). Although TET proteins are capable of oxidizing methylcytosine on RNA (86, 87), whether TETs can demethylate RNA (i.e., replace 5mC with unmodified C) is still an open question as neither passive nor active mechanisms for DNA demethylation would apply in RNA (**Figure 1**).

Finally, it is worth noting that the phenotypes in *Tet2*-deficient mice may be complicated due to environmental influences. Whole-body *Tet2* deficiency was shown to result in a compromised intestinal barrier, allowing bacteria to translocate from the intestinal lumen to internal organs and induce IL-6 production and inflammation; in turn, the pro-inflammatory signal facilitated pre-leukemic myeloproliferation (88). Therefore, depending on the microbiota at a given facility, *Tet2*-deficient mice may display differing basal levels of inflammation, a feature that may account for the variable reported phenotypes of different strains of *Tet2*-deficient mice (26). Since most *TET2* mutations in human are acquired somatically rather than through the germline, the extent to which inflammation plays a role in human myeloid neoplasms remains to be determined. Taken together, these studies provide clear evidence that TET proteins regulate innate immune responses in myeloid cells.

# OUR CURRENT UNDERSTANDING OF TET-MEDIATED GENE REGULATION

## TET Regulation of Transcription Factor Expression in Immune System

Transcription factors have emerged as one of the major targets of TET-mediated regulation. For instance, TET2 is important for inducing *Blimp1* expression in peripheral B cells by demethylating intronic CpGs (38). On the other hand, TET proteins may be required for repressing *BCL6* expression. In the human *BCL6* locus, DNA methylation of intragenic CpG islands at the first intron prevents CTCF binding and promotes *BCL6* expression. DNA demethylation at these CpG islands allowed CTCF binding, resulting in repressed *BCL6* expression (89). However, whether TET proteins regulate *BCL6* expression remains to be demonstrated.

Many loci encoding transcription factors are heavily hydroxymethylated, including *Tbx21, Zbtb7b,* and *Gata3* in iNKT and T cells (18, 33, 41). Loss of *Tet2* alone, however, has no significant effect on *Tbx21* expression in CD4 and CD8 T cells (41, 42). It is likely that other TET proteins such as TET3 can compensate, since *Tbx21* expression is decreased in iNKT cells that are deficient in both *Tet2* and *Tet3* (33). In contrast to *Tbx21* which is decreased in TET-deficient iNKT cells, loss of TET activity, either by gene targeting or inhibition by 2-HG, facilitates *Eomes* expression in iNKT and CD8 T cells (33, 42, 75). Whether TET proteins directly regulate *Tbx21* and *Eomes* expression by binding to regulatory elements in the *Tbx21* and *Eomes* loci remains to be determined.

## TET-Mediated Regulation of Enhancers

Consistent with the functions of TET proteins in gene regulation, enhancers are usually enriched in 5hmC. TET proteins can be recruited to specific regulatory elements through interaction with multiple transcription factors including NANOG, SALL4A, WT-1, PU.1, E2A, and EBF1 (19–24). The pleiotropic interaction between TET proteins and transcription factors is reminiscent of histone acetyltransferase p300, which interacts with hundreds of transcription factors (90). Once recruited to enhancers, TET proteins can oxidize 5mC into 5hmC, marking enhancers for DNA demethylation.

TET-dependent DNA modifications potentially affect gene expression via at least two non-mutually exclusive mechanisms. First, 5hmC, other oxi-mCs, and the ensuing DNA demethylation increase chromatin accessibility (22, 28, 33). In this scenario, unmodified C and oxi-mC potentially relieve the nucleosome rigidity caused by DNA methylation (91, 92); additionally, TET proteins may recruit nucleosome remodeling complexes to displace nucleosomes from enhancers. Second, TET-generated oxi-mC modifications may exert immediate effects on gene expression by modulating transcription factor binding, and TET proteins may also exert more long-term effects. Specifically, 5mC and oxi-mCs are known to modify the binding of several transcription factors with CG or TG dinucleotides in their recognition sequences (54). The methyl group of thymine is located at the 5th position, corresponding to the methyl group of 5mC. Thus, transcription factors with TG dinucleotides in their preferred binding sequences often also bind the same sequences with methylated CGs (93), and their DNA binding is likely to be modified by the presence of oxi-mCs. Other transcription factors, including WT1, can bind sequences containing 5caC in a CG context with higher affinity than the corresponding sequence with unmodified CG (94). The exact mechanisms of enhancer regulation by TET enzymes and oxi-mCs remain to be delineated.

## TET-Mediated DNA Oxidation and Demethylation

TET proteins can oxidize 5mC into oxi-mCs and mediate DNA demethylation. Depending on the conditions, TET2 can iteratively oxidize 5mC to 5hmC and then to all other oxidized cytosines in a single encounter (95). However, in the genome, most 5mC oxidation appears to pause at 5hmC and to a lesser extent 5fC (**Figure 1C**), a notion supported by mass spectrometric analyses showing that both 5hmC and 5fC are rather stable in cells (96, 97). It remains to be determined why 5hmC is the most abundant of the oxi-mCs. Two mechanisms (not mutually exclusive) may be involved: (i) TET-mediated oxidation preferentially arrests at 5hmC or 5fC; (ii) 5fC and 5caC, but not 5hmC, are continuously removed by TDG/ BER or by other mechanisms (**Figure 1B**). Regardless of the mechanism, the modified cytosines can facilitate active or passive demethylation and affect gene regulation. In addition, 5hmC may act as a bookmark to label CpG sites in *cis*-elements such as promoters, enhancers and insulators marked by CTCF binding (5, 98, 99) for subsequent demethylation upon cell division, thus affecting gene expression patterns in the daughter cells (a latent effect).

## Potential Co-transcriptional 5hmC Modification

5hmC distribution at gene bodies is positively correlated with gene expression levels, suggesting that TET activity is coupled to transcription by RNA polymerase II (RNA

pol II) (18). One of the possible links between TET and RNA pol II is via their mutual association with the histone H3K4 methyltransferase Set1/COMPASS complex (100). Another possible link between 5hmC and RNA transcription is via the gene body histone mark H3K36me3: the levels of 5hmC and H3K36me3 in gene bodies are positively correlated with one another and with gene expression. During transcription, the methyltransferase SETD2 associates with the phosphorylated C-terminal domain of RNA pol II and co-transcriptionally methylates H3K36 to yield H3K36me3 (94). H3K36me3 is subsequently recognized by the *de novo* DNA methyltransferases DNMT3B, and to a lesser extent DNMT3A, via the PWWP domain (101–103), mediating gene body DNA methylation. Since all three TET proteins have been shown to co-immunoprecipitate with the maintenance methyltransferase DNMT1, and all three DNMT proteins co-immunoprecipitate with TET2 (104), the extensive interaction between TET and DNMT may provide a possible mechanism for transcription-coupled 5hmC modification. The biological significance of gene body 5hmC modification remains to be determined.

### Potential Model for TET-Mediated Asymmetric Cell-Fate Decision

Hypothetically, it may also be possible to facilitate asymmetric gene regulation by engineering an asymmetric distribution of DNA methylation between two daughter cells via strand-biased 5hmC modifications. In one potential scenario, 5mC bases at CpG motifs on one strand at a given locus are preferentially oxidized by TET into 5hmC, while the complementary strand remains as 5mC (e.g., the template strand during transcription). As a result, after cell division, the CpG motifs at the locus in one of the daughter cell will remain methylated because the DNMT1/UHRF1 complex restores symmetrical methylation; the CpG motifs in the other daughter cell will contain 5hmC and unmodified C. This is an attractive putative mechanism by which TET enzymes could regulate cell fate decisions.

## HARNESSING THE POWER OF THE DARK SIDE FOR THE LIGHT SIDE

TET loss-of-function, either through genetic mutations or catalytic inhibition, has shown a strong causal relationship with multiple malignancies (31, 32). TET deficiency appears to enhance cell survival and increase "stemness," as in the case of *TET*-deficient HSCs which could be passaged for a much longer period of time *in vitro* and out-competed WT HSCs after transplantation *in vivo*. Interestingly, at least some of the phenotypes are reversible by re-introducing TET or enhancing the remaining TET activity by vitamin C (105), raising the possibility of temporarily inhibiting TET activity to enhance immune responses. In fact, two recent studies of human and mouse CD8 T cells provided supporting evidence for this approach. In both cases, *TET2*-deficiency facilitated the differentiation and expansion of CD8 T cells with central memory phenotype that could provide long-lasting protection against tumor and virus (discussed above). Using non-specific inhibitors such as the oncometabolite 2-HG or other TET-specific inhibitors that remain to be developed, it should be possible to inhibit TET activity and boost antigen-specific responses and immune cell expansion during vaccination or infusion of cancer-specific T cells. It would be of great interest to borrow the trick of losing TET function from cancer cells to arm immune cells with the superpower to fight against the cancer cells themselves and other pathogens.

## CONCLUDING REMARKS

TET proteins and 5hmC were identified/rediscovered almost 10 years ago. Numerous studies have shown their importance in gene regulation, tumor suppression, and cell differentiation. Yet, much remains to be learned about TET and 5hmC. For instance, how do TET enzymes suppress cancer progression? How does TET-mediated DNA modification affect cell identity? What is the relative contribution of enzymatic activity-dependent and –independent (structural) mechanisms to the functions of TET? Besides being intermediates for DNA demethylation, what is the function of 5hmC and other oxidized methylcytosines as potential epigenetic marks? Who are the "readers" of these epigenetic marks? Also, given their seemingly opposite functions, why do mutations of *Tet* and *Dnmt3a/b* result in similar phenotypes in hematopoiesis? Besides all these fundamental questions, modulating the activity of epigenetic regulating enzymes including TET proteins may provide a promising way to alter and to achieve the desired magnitude and direction of immune responses.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

This work was supported by NIH grants R35 CA210043, AI109842, AI128589 and Translation Research Project grants (6187-12 and 6464-15) from the Leukemia and Lymphoma Society (to AR). C-WL was supported by the Independent Investigator Fund (Kyowa Hakko Kirin/La Jolla Institute) and an Irvington Postdoctoral Fellowship from the Cancer Research Institute.

## ACKNOWLEDGMENTS

We would like to thank Dr. Xiaojing Yue for discussion and critical reading of the manuscript.

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**Conflict of Interest Statement:** AR is a member of the Scientific Advisory Board of Cambridge Epigenetix.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 20 February 2019
doi: 10.3389/fimmu.2019.00228

# Mechanisms of Action of Hematopoietic Transcription Factor PU.1 in Initiation of T-Cell Development

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OPEN ACCESS

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**Specialty section:**
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

**Received:** *23 November 2018*
**Accepted:** *28 January 2019*
**Published:** *20 February 2019*

**Citation:**
*Rothenberg EV, Hosokawa H and Ungerbäck J (2019) Mechanisms of Action of Hematopoietic Transcription Factor PU.1 in Initiation of T-Cell Development. Front. Immunol. 10:228. doi: 10.3389/fimmu.2019.00228*

PU.1 is an ETS-family transcription factor that plays a broad range of roles in hematopoiesis. A direct regulator of myeloid, dendritic-cell, and B cell functional programs, and a well-known antagonist of terminal erythroid cell differentiation, it is also expressed in the earliest stages of T-cell development of each cohort of intrathymic pro-T cells. Its expression in this context appears to give T-cell precursors initial, transient access to myeloid and dendritic cell developmental competence and therefore to represent a source of antagonism or delay of T-cell lineage commitment. However, it has remained uncertain until recently why T-cell development is also intensely dependent upon PU.1. Here, we review recent work that sheds light on the molecular biology of PU.1 action across the genome in pro-T cells and identifies the genes that depend on PU.1 for their correct regulation. This work indicates modes of chromatin engagement, pioneering, and cofactor recruitment ("coregulator theft") by PU.1 as well as gene network interactions that not only affect specific target genes but also have system-wide regulatory consequences, amplifying the impact of PU.1 beyond its own direct binding targets. The genes directly regulated by PU.1 also suggest a far-reaching transformation of cell biology and signaling potential between the early stages of T-cell development when PU.1 is expressed and when it is silenced. These cell-biological functions can be important to distinguish fetal from adult T-cell development and have the potential to illuminate aspects of thymic function that have so far remained the most mysterious.

**Keywords: transcription factor, developmental gene regulation, chromatin, T lymphocyte development, thymus, gene network, cell signaling, hematopoiesis**

## INTRODUCTION

### PU.1 Expression in Precursors of T Cells

PU.1, encoded by the *Spi1* gene, is an ETS-family transcription factor with multiple roles in hematopoiesis. It is a lineage-specifying transcription factor that positively regulates many genes in the macrophage, granulocyte, dendritic-cell and B-cell lineages. Expressed at highest levels in monocytes/macrophages, at low or moderate levels in B cells, and transiently in early erythroid precursors, its action is also important or indispensable for sustained generation of all known hematopoietic precursors that have lymphoid developmental potentials (1–9). Thus, B, NK, and T cell development are all affected by defects in PU.1 activity, despite partial complementation by

the related factor SpiB that is also activated in B-lineage precursors. Much is known about how PU.1 finds and binds to its sites in the DNA, typically (A/G)AGGAAGTG motifs [e.g., (10, 11)], and it is known to be able to bind either as a pioneer factor which displaces nucleosomes to open sites for other factors (12), or as a collaboration-dependent partner in binding complexes, either with activation-dependent factors like NF-κB or with lineage-defining partners like C/EBPα (or β) or IRF4/8 (13–15) [reviewed by (16–18)].

In myeloid, dendritic, and B lineage cells, PU.1 is a major contributor to the positive regulation of genes that establish lineage-specific identity (4, 17, 19). At the same time, PU.1 can work in an all-or-none gene network switch through mutual antagonism with GATA-1 (20–24), which has been much discussed as a possible mechanism for the irreversibility of erythro-myeloid lineage commitment [(25–29); but also see (30, 31)]. Nevertheless, the developmental scope of PU.1 activity is surprisingly broad, and one of its unexpected domains of action is in the early stages of T-cell development, in both the fetal and the postnatal mammalian thymus. To examine what it does in pro-T cells, this review focuses on recent data based on mouse T-cell development, mostly as it occurs in the postnatal thymus or from late fetal progenitors. The final section places these mechanisms in the context of the variants of T-cell development that characterize different ontogenic stages.

Most mature T cells do not express any detectable PU.1 protein or *Spi1* transcripts at all, and the T-cell developmental gene network sharply downregulates *Spi1* in precursors of αβ T cells before the expression of rearranged *Tcrb* genes, i.e., before any TCR-dependent steps of T cell development. However, the precursors that give rise to committed T cells express PU.1 at both RNA and protein levels for multiple cell divisions after these cells begin to differentiate in the thymus (32, 33). A summary of early T-cell developmental stages, is shown in **Figure 1**, with the approximate pattern of PU.1 expression marked. The downregulation of PU.1 occurs during the transition to commitment, between the DN2 (DN=double negative for CD4 and CD8, and Kit$^{+}$ CD44$^{+}$ CD25$^{+}$) and DN3 (DN, and Kit$^{low}$ CD44$^{low}$ CD25$^{+}$) stages. This expression timing relative to other developmentally regulated transcription factors is conserved between human and mouse (35, 36), and as in mouse (37), the downregulation of PU.1 is important to avoid malignancy in human T cells: a specifically aggressive class of human T-acute lymphoblastic leukemias results from translocations that promote abnormally sustained and elevated PU.1 expression (38). In the mouse, where lineage commitment has been studied in depth, there is good agreement between the cells' natural loss of access to the dendritic cell and granulocyte programs, on the one hand, and the timing of PU.1 downregulation, on the other hand (33, 39–42). This is part of a general downregulation of stem/progenitor associated regulatory genes ("phase 1 genes") (34, 43) and a major reorganization of active chromatin and chromatin interactions, genome-wide, that occurs during this transition (44). One important question is what role PU.1 itself may have in controlling the onset of this transformation.

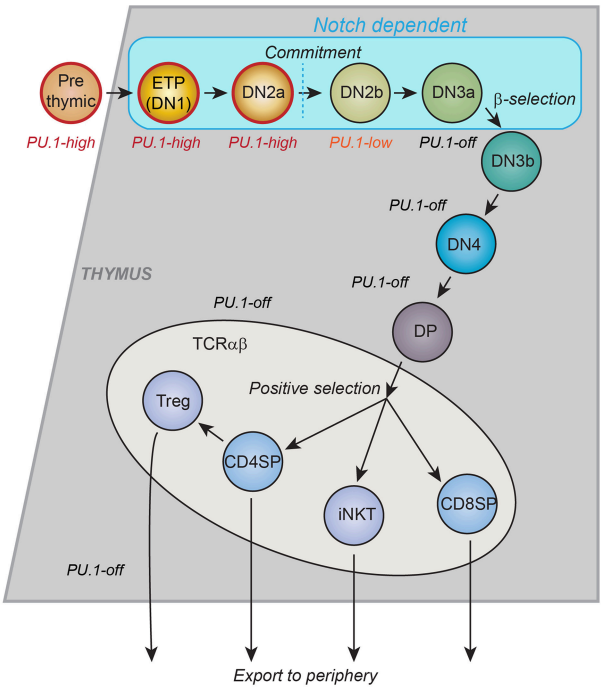


**FIGURE 1 |** Schematic of T-cell development in the thymus. Major landmarks for T cell developmental stages are CD4 and CD8. CD4$^{-}$ CD8$^{-}$: "DN"; CD4$^{+}$ CD8$^{+}$: "DP," CD4$^{+}$ CD8$^{-}$: "CD4SP"; CD4$^{-}$ CD8$^{+}$: "CD8SP." All events described in this review occur within the DN stages, which are divided by other markers. ETP: Kit$^{++}$ CD44$^{+}$ CD25$^{-}$; DN2a: Kit$^{++}$ CD44$^{+}$ CD25$^{+}$; DN2b: Kit$^{+}$ CD44$^{+}$ CD25$^{+}$; DN3a: Kit$^{-}$ CD44$^{-}$ CD25$^{+}$ CD28$^{-}$; DN3b: Kit$^{-}$ CD44$^{-}$ CD25$^{+}$ CD28$^{+}$; DN4: Kit$^{-}$ CD44$^{-}$ CD25$^{-}$ CD28$^{+}$. Stages up through DN3a do not depend on T-cell receptor gene rearrangement status and are called "Pro-T cells." Many cell cycles occur between the ETP stage and commitment, more in post-natal T cell development and fewer in fetal T-cell development. The trends in PU.1 expression, the timing of intrinsic cell commitment to the T-cell lineage, and the stages that depend on Notch signaling from the thymic microenvironment are shown. Gray or blue regions depict thymic cortex. Lighter region depicts thymic medulla, where final maturation of developing T cells takes place. CD4SP: maturing T helper cells. CD8SP: maturing T cytotoxic cells. Treg: thymically derived regulatory T cells. iNKT: Natural Killer T cells with invariant T cell receptors [Schematic adapted from Rothenberg et al. (34)].

## PU.1 as an Obstacle to T-Cell Lineage Commitment

The particular interest in PU.1 itself emerged from the hypothesis that it could well be responsible for maintaining the "bridge" to myeloid and dendritic alternative fates before commitment, because of its known roles in many of these alternatives (and in B cells) but not in T cells (45). This hypothesis was supported by the finding that re-expression of PU.1 after T-cell commitment turns on myeloid genes and readily transforms later pro-T cells into dendritic cells, macrophages, or promyelocytic-like cells (46–50). There is a very close relationship between the cells that naturally express PU.1 in the thymus and those that readily exhibit myeloid or dendritic potential in a variety of permissive cell transfer models, *in vivo* or *in vitro*. Whereas ETP and DN2 cells can generate myeloid cells if removed from the thymus, pro-T cells that have differentiated past the stage of PU.1 expression

in the thymus (i.e., from the DN2 stage to the DN3 stage or later) do not make myeloid cells under these conditions, and this difference between stages up to DN2 and stages from DN3 onward has been a highly consistent observation (33, 40, 41, 51–56). Why, then, do PU.1-expressing early T cell precursors within the thymus almost all go on to produce T cells, not myeloid cells, under normal *in vivo* conditions (57)? A potential explanation was provided by a key feature of the PU.1 effect: namely, that PU.1 actions are Notch-sensitive. Even artificially high-level PU.1 could only redirect the differentiation of the cells to myeloid or dendritic fates if Notch signaling were reduced (49, 50, 58). In primary fetal-derived pro-T cells and in a DN3-like cell line, the particular genes affected by a given, fixed level of PU.1 in the cells depended strongly on the strength of Notch signaling being induced in the cells at the time (58). Notch ligands are the most important of all the environmental signals that the thymus stroma provides to developing T cells, apparently across all vertebrates (59–61), and Notch activated target genes like *Hes1* are expressed throughout the pro-T cell phases (ETP to DN3), until T-cell receptor (TCR) gene rearrangement (62–64) [reviewed in (65)]. Thus, throughout the stages when PU.1 is expressed, the Notch signaling driven in the normal thymus environment could guarantee that PU.1 expression would confer only a potential for differentiation to alternative fates, which the cells would not actually follow unless the thymic environment were disrupted. The silencing of *Spi1* expression and permanent loss of PU.1 protein from the cells at a later stage of differentiation would then make their loss of myeloid potential unconditional.

The question raised by such results, however, was why PU.1 should continue to be expressed at all by cells once they entered the thymus. Population dynamic models imply that the stages when PU.1 is expressed occupy a minimum of 7–10 intrathymic cell divisions of pro-T cells (39, 66, 67). If PU.1 was evolutionarily selected to be expressed over such an extended period, it might be playing an important role in pro-T cells, and this could be despite or because of the Notch signaling conditions that were preventing it from diverting the cells to a non-T fate. The earliest stages of T cell development are not well understood, and it until recently it was not obvious what function could be important to the cells at this time *a priori,* other than proliferation. In the past 5 years, however, a detailed look at the molecular biology of PU.1 action on the genome in pro-T cells has revealed much about the ways that PU.1 works, the complex cell biology of the early precursor states, and previously under-appreciated principles of transcription factor systems operating in development.

# EFFECTS OF PU.1 LOSS ON T-CELL DEVELOPMENT: THE CELLULAR VIEW

## A Vital Role for PU.1 in Prethymic T-Cell Progenitors

Disruption of PU.1 has long been known to eliminate or greatly inhibit T-cell development, based on the dramatic phenotypes from the first lines of PU.1-knockout (*Spi1* knockout) mice with unconditional, germline mutations (68–71). The question has been how to interpret this severe effect, i.e., whether it is due to loss of a function within the T-cell program itself, or whether it simply reflects a loss of input cells to the pathway. One problem was originally the lethality of the hematopoietic phenotype (death either in late fetal development or immediately after birth), but even when conditional knockouts were developed (2, 72), this remained problematic. All the hematopoietic progenitors that generate either B or T cells appear to originate from PU.1-expressing, PU.1-dependent prethymic cells (2, 27); PU.1 is directly required to maintain the expression of the cytokine receptor Flt3 that is indispensable for progenitors with B and T cell potentials (73). Thus, in postnatal mice, although T cell development is much more severely affected by PU.1 deletion than neutrophil development (2), the effect could still be prethymic. In stark contrast, if *Spi1* is conditionally deleted in T-lineage cells only after the cells have passed the DN2 stage, there are very modest effects on T cells as a whole, apparently limited to selective reduction of IL-9-producing T-cells (74), and some loss of restraint on γδ T cells and T follicular helper cell activity (75, 76). Is PU.1 actually needed within the T-cell pathway for T-cell development at all, or is it simply needed to guarantee a supply of prethymic progenitors?

Addressing this question *in vivo* was handicapped by difficulties in the methods of inducing stage-specific *Spi1* deletion. The question about a transient role for PU.1, but one which might have strong effects on viability, makes it important to have high penetrance and high synchrony of deletion as well as fine developmental stage control, both of the deletion and through the analysis afterwards. The widely-used T-cell specific Cre expression constructs that might be appropriate for thymocyte analysis, pLck-Cre and CD4-Cre, actually begin to be expressed too late: pLck-Cre turns on just as PU.1 is turning off, and CD4-Cre is expressed even later, after the rearrangement of the first TCR genes. Constructs like Il7r-Cre or Rag1-Cre, which may have prethymic expession but are also expressed much more strongly during later pro-T cell stages, could make output cell phenotypes difficult to interpret because of uncertainty about when the deletion actually has become complete. Fortunately, pro-T-cell differentiation cultures on OP9-DL1 or OP9-DL4 stroma that constitively present Notch ligands (77, 78) are ideal for examining the stages relevant to PU.1 function, and a variety of efficient retroviral vectors can transduce the cells at these stages with high efficiency to introduce gain or loss of function agents. These systems have proven to be valuable tools not only for verifying the coarse-grained roles of PU.1 in pro-T cells, but also for investigation of their molecular mechanisms.

## PU.1 Promotes Proliferation While Slowing Differentiation of Pro-T Cells

PU.1 is indeed important within the T-cell program as well as before thymic entry, as shown by using *in vitro* differentiation to provide conditions where PU.1 could be removed acutely in a synchronized cohort of precursors and the fates of the cells could be monitored immediately afterwards. In these studies, floxed *Spi1* was disrupted in the input cells few days after T-lineage development had begun, using a Cre-encoding retroviral vector (79). The deletion of PU.1 reduced viable cell yield, but a co-transduced Bcl-xL transgene was

added with Cre to prevent specific effects on development and proliferation from being masked by cell death. Similar results were obtained independently using Cas9 plus *Spi1*-specific guide RNA to delete PU.1, and supporting cell viability with a Bcl2 transgene (80). In both experimental setups, PU.1 disruption reduced T-cell precursor proliferation substantially as compared to controls. PU.1-deficient cells underwent fewer cycles per unit time than controls both in ETP stage and in DN2a/2b stages (79), suggesting that even once the cells have begun to express definitive T-lineage markers, they need PU.1 to sustain optimal proliferation. However, of the cells that were generated from PU.1-disrupted precursors, a substantially larger fraction progressed to DN3 stage than in control cells, over the same length of absolute developmental time, suggesting that they were liberated from a differentiation constraint (79, 80).

Thus, endogenous PU.1 does have a functional role within early T-cell development. It slows developmental progression of pro-T cells even as it supports their early proliferation. While this may seem paradoxical, it could fit well with a role to build the size of the pre-selection pool of T-cell precursors before they progress to commitment and then TCR gene rearrangement, so as to maximize TCR gene rearrangement diversity in the population as a whole before selection occurs (66, 81). The effect of PU.1 on proliferation is conditional and dose-dependent, however. While added PU.1 can enhance pro-T cell proliferation in response to cytokine cocktails containing high levels of Stem Cell Factor (Kit ligand) and Flt3 ligand or myeloid growth factors (38, 50), it strongly inhibits the proliferation of pro-T cells under conditions that do not reward the cells for lineage switching (49). Such dose dependent effects are common for transcription factors as for signaling molecules, in part because high concentrations of these factors bind to inappropriate genomic sites, leading to off-target effects. The target genes stimulated by PU.1 include both pro-proliferative and G1-prolonging cell cycle effectors, whereas some important proliferative genes are repressed when PU.1 levels are high (82, 83). Thus, both too much and too little PU.1 can have negative impacts on proliferation of the cells within a similar developmental time window.

The *in vitro* assays used to define these roles (discussed in more depth in the next section) are powerful because of the easy accessibility of the developing cells during differentiation and because of the ability to follow differentiation of a synchronized cohort of cells in absolute time. As described below, however, the genes most sensitively regulated by PU.1 in developing T cells suggest that this factor may be important to endow cells with additional functions as well, functions that may only contribute to their development specifically in the thymus *in vivo*.

## DEFINING THE PU.1 REGULOME IN EARLY T-CELL PRECURSORS

### Cell Line and Primary-Cell Assay Systems for PU.1 Manipulation

To explain the roles of PU.1 in T cell development, it is crucial to take into account its developmental expression pattern. Its high expression in early-stage pro-T cells followed by downregulation during commitment means that its direct effects have to be correlated with developmental stage. Thus, any inferred role must be validated by developmental stage-dependence of putative target gene expression patterns or of chromatin features that characterize its binding sites. To look more closely at how PU.1 actually regulates specific target genes, acute gain and loss of function experiments are needed. Despite some overlap in occupancy, PU.1 binding sites and PU.1 binding partners are not the same in early T-cell precursors as in myeloid cells or B lineage cells (13, 80, 84, 85), a similar situation to its early role in erythroblasts (86). Therefore, these assay systems need to be based on pro-T cells (**Figure 2**). Exogenous PU.1 can easily be introduced into developing murine T-cell precursors using retroviral vectors for gain of function studies (46–50, 58, 80, 85). For loss of function, retrovirally transduced Cre can induce acute deletion in pro-T cells from *Spi1*$^{fl/fl}$ strain mice (79); and in Cas9-transgenic pro-T cells, retrovirally transduced guide RNAs (sg*Spi1*) can target rapid, biallelic disruption of the *Spi1* locus (80, 85). **Figure 2** introduces the way the primary-cell and cell-line models can be manipulated to relate experimental gain-of-function and loss-of-function PU.1 experiments to the normal dynamics of endogenous PU.1 expression.

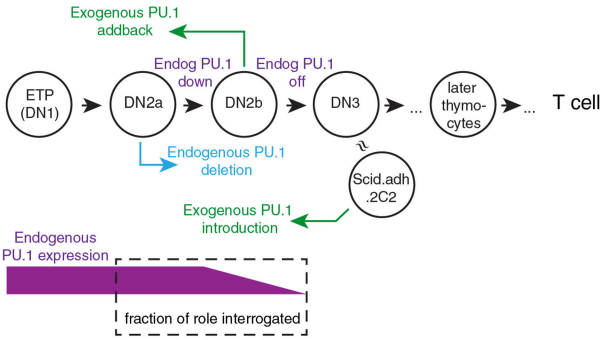


**FIGURE 2 |** Framework for experimental perturbation studies to define functions of PU.1 in early thymic development. Stages of cells are as in **Figure 1** (DN3: primarily DN3a). Exogenous PU.1 is added by retroviral transduction. Note the dependence of the PU.1 functions tested upon the timing of the experimental perturbation. Endogenous PU.1 can be deleted by Cas9 plus single-chain guide RNAs (sgRNA) against *Spi1*, or by introducing Cre into cells with floxed *Spi1* alleles. PU.1 can also be neutralized by adding a dominant negative construct. The DN2a-DN2b interval is accessible to experimental perturbation. The approximate developmental stage represented by the Scid.adh.2C2 cell line (see text) is also shown [Schematic adapted from Ungerbäck et al. (85)].

A very useful model cell line, Scid.adh.2C2, has made it possible to study PU.1 gain of function in a pro-T cell-like context (47). These cells are convenient because they are readily transfectable, retrovirally transducible, and fast-growing, so that cell numbers are not limiting and the developmental baseline is mostly static, all major advantages for genomic comparisons. These cells were a subclone derived from the Scid.adh cell line (87) and are similar to developmentally arrested versions of committed DN3 pro-T cells, lacking any expression of endogenous PU.1 (47). Despite being an immortal cell line, these cells are developmentally transformed by introduction

of exogenous PU.1. They respond in an all-or-none way to forced expression of PU.1, coordinately upregulating myeloid- or dendritic-cell associated genes and downregulating T-cell genes in a discrete fraction of the cells that increases with increasing levels of PU.1 (47, 58), resembling responses of primary fetal or postnatal pro-T cells (46–50, 58, 80, 85). The switch-like nature of this response was an important early clue to the regulatory circuit interaction between PU.1 and the Notch signaling pathway (49, 58).

Useful and informative as it is, this system is limited as a way to study the roles of endogenous PU.1 *in vivo*. The sites occupied by exogenous PU.1 in Scid.adh.2C2 cells overlap highly with the sites occupied by endogenous PU.1 in normal pro-T cells, but the match is by no means complete (85). Even with PU.1 transduction, Scid.adh.2C2 cells do not restore the full chromatin accessibility landscape of ETP and DN2a stage pro-T cells, and despite detectable upregulation of a few other early pro-T cell genes (e.g., *Bcl11a* and *Lyl1*), the transduced cells as a whole reactivate little of the program that forms the normal context for endogenous PU.1 activity in pro-T cells (58, 79, 85). Therefore, PU.1 has to be manipulated acutely in dynamically differentiating primary cells.

To focus the introduction of PU.1 into cells at a particular developmental stage, it has proven to be very useful to exploit the powerful *in vitro* T-cell development systems based on co-culture of primary-cell precursors on OP9-DLL1 (aka OP9-DL1) or OP9-DLL4 (OP9-DL4) stroma with IL-7 and Flt3L (78, 88), or similar systems using other stromal cell lines to express the Notch ligands DLL1 or DLL4. Either fetal-liver-derived precursors or adult bone marrow-derived precursors develop efficiently along the T cell lineage in these systems with strong proliferation through the stages around commitment, allowing the stages to be separated both by flow cytometric phenotypes and by absolute times of differentiation. In these open systems, the cells can be harvested easily at any time point, transduced with vectors, treated with drugs, and/or sorted, and then shifted to the same or a different culture condition for further development. These systems have been indispensable for deeper analysis of the molecular mechanisms that PU.1 uses to regulate development of pro-T cells. However, two issues have to be taken into account in these analyses, both arising from features that amplify the developmental impact of PU.1. These are reviewed in the next sections.

## Developmental Challenges: Implications of a Gene Regulatory Network Switch

The ideal conceptual framework of PU.1 gain of function experiments is to start with pro-T cells that have recently turned off their endogenous PU.1 expression and to assess how their developmental state is affected by re-introducing PU.1 expression, comparing the impact of exogenous PU.1 with the pre-commitment gene expression pattern. Ideally in this scenario, restoring PU.1 after commitment should promote some aspect(s) of retrograde differentiation. Both Scid.adh.2C2 and normal DN2b/DN3 pro-T cells make strong responses to forced expression of PU.1, as noted above, and often the response includes downregulation of multiple later T-cell differentiation genes. Does this shed light on PU.1's natural role in earlier T-cell development, or is it simply an inhibitory artifact of overexpression? Clues that the gain-of-function phenotype is linked with a genuine role of PU.1 in earlier T-cell development come from PU.1's (re-)activation of a group of genes that are specifically associated with the early progenitor state, including *Bcl11a*, *Mef2c*, *Hhex*, and *Lmo2* (58, 79, 85). Some of these are also upregulated in human T-acute lymphoblastic leukemias with highly expressed PU.1 fusions, as well (38).

However, the power of the response raises caveats about interpretation because of an important systemic feature of the PU.1 role in development. In primary pro-T cells and Scid.adh.2C2 cells, highly overexpressed PU.1 appears to inhibit Notch signaling, as measured by downregulation of Notch target genes and even *Notch1* itself. Whether cause or effect, this collapses the balance between Notch signaling and PU.1 activity that is fundamental to channel the natural role of PU.1 in early pro-T cells (see above) (49, 58). The most pronounced effects of PU.1 are thus a nonlinear response to PU.1 dosage mediated through a gene network switch (**Figure 3**), and this gene network switch underlies the stochastic, switch-like behavior of individual pro-T or Scid.adh.2C2 cells when forced to express high-level PU.1 (47, 58). Importantly, the combination of PU.1 with Notch signaling desensitization pushes the cells out of the T-cell program completely, rather than simply reversing their progression through the T-cell program. Instead of re-acquiring aspects of a progenitor-like state, the cells appear to trans-differentiate to a dendritic-cell or macrophage developmental program (48, 50, 58).

In newly-committed primary pro-T cells forced to express PU.1, the cells crossing this developmental boundary are seen to downregulate the Notch-dependent DN2/DN3 stage marker, CD25 (*Il2ra*), and often upregulate the myeloid-associated marker CD11b (Mac1; *Itgam*). The gene expression profiles of cells losing CD25 and upregulating CD11b are radically transformed from the state of newly-committed pro-T cells within 2 days after transduction, with widespread repression of T-lineage-affiliated transcription factor genes and Notch target genes as well as upregulation of multiple *Cebp* and *Irf* family transcription factor genes (**Figure 4**) (85). This response is quite different in gene expression pattern from retrograde differentiation to an ETP- or DN2a-like phenotype. In contrast, cells remaining within the T-cell pathway, continuing to express CD25 and remaining negative for CD11b, show relatively modest and selective changes in gene expression driven by upregulated PU.1, with minimal loss of T-cell regulatory gene expression (79, 85). Details of these transcriptome effects are discussed in a later section, but the point here is that they include qualitative as well as quantitative differences in the gene expression responses. The differences in average *Spi1* overexpression levels between cells making these two responses are only on the order of ∼2–3 fold (pink, dark red bars in **Figure 4**), so it is very likely that the additional changes in other regulatory genes contribute strongly to this global shift. Thus, the effect of PU.1 expression *per se* may be part of the normal T-cell program, but under high-level expression conditions it combines with additional, conditionally

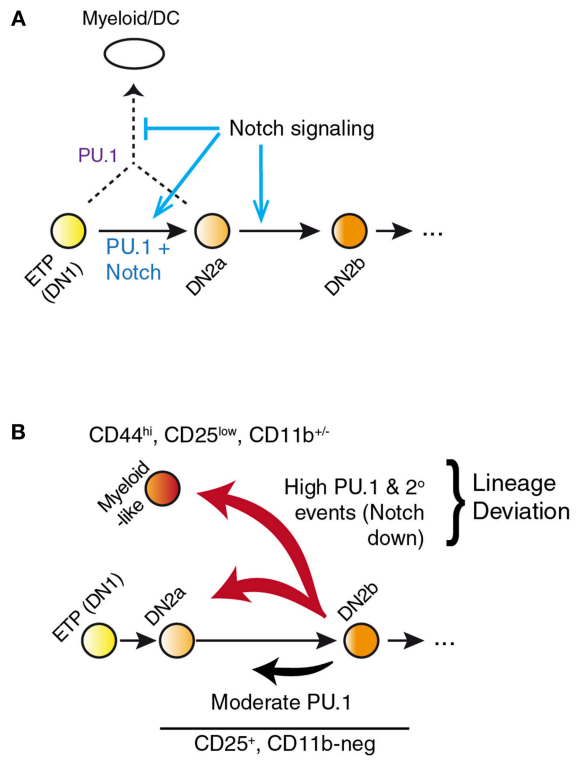


**FIGURE 3 |** PU.1 and Notch collectively determine T vs. myeloid lineage fates. **(A)** Notch signaling within the thymus normally constrains PU.1 effects to support early pro-T cell development while blocking alternative fates that PU.1 would otherwise promote. Notch signaling itself does not repress PU.1 expression; however, other transcription factors induced by Notch signaling eventually silence expression of *Spi1* during the DN2b stage. **(B)** Separation of the effects of PU.1 within the T-cell pathway from effects of PU.1 to promote lineage deviation, in PU.1 gain of function experiments. A fraction of cells expressing high levels of PU.1 shift to a myeloid-like state that can be phenotypically distinguished from cells remaining within the T-cell state. This distinction is necessary to relate gain-of-function effects of PU.1 in pro-T cells to effects of loss of endogenous PU.1 in perturbation experiments. Lineage deviation is associated with a broad loss of Notch signal response in the cells, suggesting that the constraint mechanism shown in A has been overwhelmed in these cells. Biochemical mechanisms of these effects remain to be fully defined. Schematic in **A**, adapted from (58); in **B**, adapted from (85).

induced mechanisms to produce a much broader spectrum of developmental effects that may not only be direct responses to PU.1 itself.

## Kinetic Challenges: Protein Half-Lives vs. Developmental Progression

Loss of function approaches are indispensable to confirm the roles of endogenous PU.1, especially in view of the potential for indirect effects in gain of function experiments, just described. Here, the challenge has been to find a way to remove or neutralize the endogenous factor quickly enough to see effects robustly, while keeping the controls and the experimental samples at comparable developmental stages. One problem is that the long half-life of PU.1 protein (82) can mask some loss effects at time points <2 days after deletion, while development of the pro-T cells can proceed to new stages if time windows are extended further. There are thus several problems with generating high-quality samples for analysis of transcriptome changes caused by PU.1 loss of function. Cre-dependent deletion of a loxP-flanked *Spi1* allele ($Spi1^{fl,fl}$) is asynchronous, and in an early T-cell population with mixed degrees of *Spi1* deletion, cells with inadequate PU.1 levels appear to be at a selective disadvantage, even *in vitro*. Ironically, because PU.1 protein can persist longer than a cell cycle (82), the very slowdown of cell division caused by deletion of *Spi1* (see above) can also interfere with the dilution needed to complete the clearance of the PU.1 protein. As a result of the enrichment of cells with undeleted alleles, and this persistence of pre-existing PU.1 protein even from the cells that have successfully deleted its coding gene, the effects on target gene regulation appear very weak at timepoints up to 2 days after PU.1 deletion, despite the fact that the reduced cell yields from the knockout cells show that PU.1 is biologically important (79). If timepoints are taken too long after deletion, the controls progress to the point when endogenous PU.1 is downregulated, so that any truly PU.1-dependent targets are expressed weakly in the controls, and comparisons with the knockout samples again lose statistical power. A very intriguing new prospect for fast antagonism of PU.1 activity is the discovery of small-molecule inhibitors, some of which are highly potent and specific at blocking PU.1 action in leukemia cells; however, these have not yet been tested for effects on normal T-cell development (89).

A relatively fast way to neutralize PU.1 protein activity directly has been to transduce the cells with a "dominant negative" obligate repressor derivative of PU.1, a fusion protein of the PU.1 DNA binding domain with the repression domain of *Drosophila melanogaster* Engrailed (PU.1-ENG), to compete for binding against endogenous PU.1 (79) (comparison with wildtype PU.1 shown in **Figure 5**). The obligate repressor should affect PU.1 positive regulation targets in the opposite direction from wildtype PU.1, and in theory should affect PU.1 negative regulation targets in the same direction, an "algebraic sign" distinction that could be used in principle to dissect indirect effects as well (79). This construct has been useful to reveal quick impacts on expression of positively regulated PU.1 target genes, many of which have been confirmed later by other approaches (85). For example, whereas PU.1 itself can upregulate progenitor-associated genes *Bcl11a*, *Lmo2*, *Mef2c*, and *Hhex* above their normal levels in DN2a and DN2b primary cells, PU.1-ENG can downregulate them (79). However, PU.1-ENG also has some spurious effects and cannot access closed chromatin sites as well as full-length PU.1 (79, 85).

Cas9-dependent acute deletion of the *Spi1* locus can be fast and highly efficient due to the availability of Cas9-transgenic mice (92) and vectors that can be used for high-level, synchronous expression of guide RNAs. However, deletion and clearance of PU.1 protein in this system still require analysis >2 days after introduction of the guide RNAs (80, 85), and the continuing developmental progression of both knockout samples and controls needs to be taken into account in interpreting the results. The strongest evidence for specific physiological PU.1 effects therefore comes from the consensus results from two or more of these perturbation systems. The highest confidence list

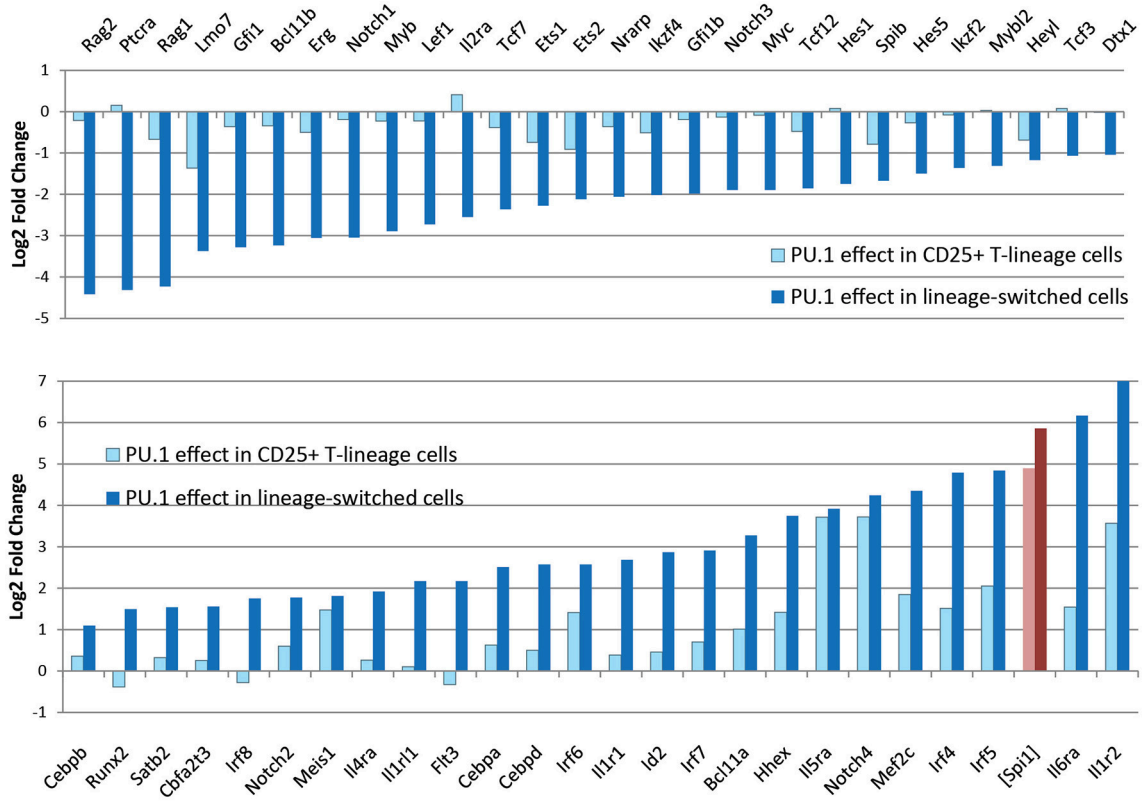


**FIGURE 4 |** Profound changes in regulatory gene expression distinguish PU.1-induced lineage deviation from PU.1 effects within the T-cell pathway. Charts show changes in expression of the indicated genes ($log_2$ Fold Change relative to controls) induced by introduction of PU.1 into post-commitment pro-T cells (DN2b-DN3). Panels compare effects on T-lineage regulatory genes **(top)** and non-T regulatory and signaling genes **(bottom)** between cells remaining within the T-lineage pathway (light blue bars) and cells undergoing lineage deviation (dark blue bars). Light, dark red bars show corresponding measured levels of exogenous PU.1 in these samples, as $log_2$ fold changes over controls, which have downregulated most of their endogenous PU.1 expression at this stage. Results are from Ungerbäck et al. (85).

of potential PU.1 target genes in pro-T cells could be defined as genes that responded reciprocally to gain and loss of PU.1 function within the same DN2a-DN2b developmental interval, and these genes are listed in **Table 1**. While this list underrepresents some PU.1 targets that are only expressed in ETP stage, rigorous definition of the genes that are directly regulated by PU.1 in pro-T cells has made it possible to investigate the range of mechanisms used by the PU.1 protein to exert these transcriptional effects.

# PU.1 ACTION ON THE GENOME VIA DIRECT BINDING

## PU.1 Protein Is Stable and Active Across the Genome in Early T Cells

Most of the initial hypotheses about PU.1's role in T-cell precursors were based on *Spi1* RNA expression patterns and on forced expression of exogenous PU.1 to supra-physiological levels (46, 48–50). With the advent of ChIP-seq data, though, it was confirmed that endogenous, naturally expressed PU.1 is indeed a prominent actor across the genome in T-cell precursors before commitment. PU.1 was found binding to >30,000 genomic sites in these cells at the earliest stages (84), and intracellular protein staining confirmed that some PU.1 expression is still detectable at later stages, in the same individual cells that go through T-cell commitment (marked by activation of the *Bcl11b* gene) (33, 93). In addition, although the RNA transcript levels are modest in absolute terms, the impact of PU.1 on the cells can be magnified by the high stability of PU.1 protein (82). Although PU.1 occupancy of genomic sites declines as development proceeds, PU.1 occupancy is still detectable through T-lineage commitment at ∼5,000 sites before disappearing (84).

## PU.1 Binding Site Characteristics

The sites where PU.1 binds are enriched for open chromatin as defined by DNase accessibility or ATAC-seq [assay of transposase-accessible chromatin (94)], and circumstantial evidence suggests that PU.1 is a major factor at those sites that change activity during commitment. PU.1 recognition motifs are the most highly enriched of all defined motifs at sites that start out highly accessible in early pro-T cell stages, when PU.1 is present, and lose accessibility during commitment, i.e., as PU.1 levels decline (44, 85, 95). PU.1 itself is functionally important for the open status of these chromatin sites in the

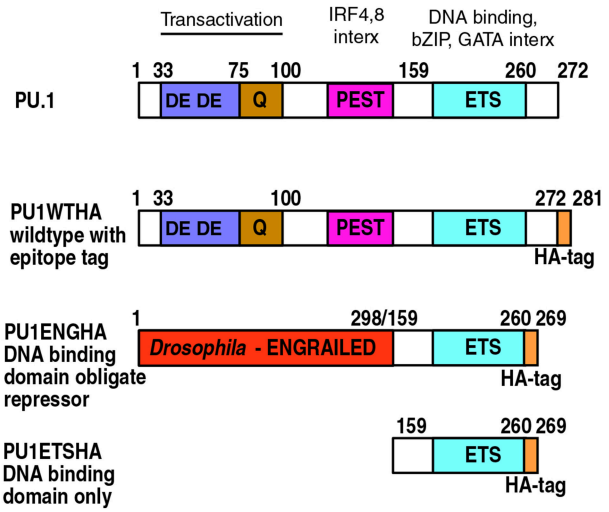


**FIGURE 5 |** PU.1 structure and derivatives of PU.1 used for functional analysis. Domain boundaries within the amino acid sequence of murine PU.1 are depicted with their associated functions indicated at the top (4, 18, 90, 91). Epitope-tagged wildtype PU.1 (PU1WTHA) and two epitope-tagged, modified constructs are shown (PU1ENGHA, PU1ETSHA); these are used to interrupt endogenous PU.1 activity (79, 82). PU1ENGHA and PU1ETSHA have a full DNA binding domain and efficiently enter open chromatin, but are deficient in entering closed chromatin (85). DE: Acidic residue-rich transactivation domain. Q: Glutamine-rich transactivation domain. PEST: Proline, Glutamate, Serine and Threonine-rich domain, site of IRF4 and IRF8 interaction (interx). Note that in PU.1 this "PEST" domain does not make the protein unstable. ETS: E-twenty-six proto-oncogene homology domain, the DNA binding domain of PU.1. This is also the region that interacts with basic leucine zipper (bZIP) factors such as Jun and C/EBP factors, and GATA family factors.

early stages, for many of these sites in fact do not remain as open if the PU.1 is removed acutely from primary pro-T cells by Cas9-mediated deletion (85). This is consistent with PU.1's activity as a site-specific chromatin opening factor in B cells (96, 97), with the ability of PU.1 to eject nucleosomes from sites where it binds in macrophage lines (12), and with its ability to cause rapid increases in ATAC accessibility at the sites it occupies when introduced into Scid.adh.2C2 cells (85). While PU.1 binds at both promoters and non-promoter sites, the evidence from both gain and loss of function studies shows that PU.1 is most associated with chromatin accessibility when it is binding at non-promoter sequences, within introns of genes or in intergenic regions. As described in detail below, such sites, where PU.1 itself is important to maintain chromatin accessibility, are the ones most often linked to genes that are positively regulated in their expression by PU.1 (85). Thus, PU.1 action to keep sites open in chromatin may be an important way that it promotes transcriptional activation in pro-T cells.

Because PU.1 mediates different effects in the rather different regulatory contexts of B, dendritic, myeloid, erythroid progenitors and pro-T cells, an important question is how much of PU.1's binding choice hierarchy is dependent on the prior epigenetic history of cells. PU.1 cannot enter all genomic sites. Notably, PU.1 appears to be excluded from genomic regions

**TABLE 1 |** High confidence targets of PU.1 regulation in pro-T cells.

| Consensus PU.1-repressed target genes | Consensus PU.1-activated target genes |
|---|---|
| 2900079G21Rik | 3110043O21Rik |
| Ablim1 | 5430427O19Rik |
| Adamts9 | 9930111J21Rik1 |
| Adora2a | 9930111J21Rik2 |
| Arsi | Abcb1b |
| Bcl2 | Acer3 |
| Ccnd3 | Actn1 |
| Cd247 | Actr2 |
| Cd28 | Acy1 |
| Cdc25b | Adam11 |
| Cecr5 | Adam15 |
| Clec2i | Adap1 |
| Clic5 | Adgre1 |
| Csrnp1 | Adrbk2 |
| Cx3cr1 | Alcam |
| Cxcr5 | Alox5ap |
| Cxxc5 | Anks3 |
| Dgka | Antxr2 |
| E2f2 | Ap1s3 |
| Eng | Apbb1ip |
| Epcam | Aqp9 |
| Fam160a2 | Arhgap6 |
| Gbp10 | Arhgef40 |
| Gbp11 | Atp13a2 |
| Gbp4 | Atp6v0a1 |
| Gbp6 | Avpi1 |
| Gimap1 | B3gnt7 |
| Gimap4 | BC035044 |
| Gimap6 | Bcat2 |
| Gimap8 | Bex6 |
| Gimap9 | Bloc1s2 |
| Hdac4 | Bri3bp |
| Hid1 | Btk |
| Hsd11b1 | Cacnb2 |
| Il12rb1 | Ccdc180 |
| Ilvbl | Ccl9 |
| Irak3 | Ccnd1 |
| Itk | Cd180 |
| Jph2 | Cd300a |
| Lmo7 | Cd300lf |
| Lztfl1 | Cd33 |
| Mbp | Cd34 |
| Mir1903 | Cd44 |
| Neil1 | Cdh1 |
| Nipal1 | Clec10a |
| Pik3ip1 | Col9a3 |
| Pitpnc1 | Coro2a |
| Pitpnm2 | Cotl1 |
| Ppm1h | Creg1 |
| Prf1 | Crtac1 |

*(Continued)*

**TABLE 1 |** Continued

| Consensus PU.1-repressed target genes | Consensus PU.1-activated target genes |
|---|---|
| Ptprf | Csf2rb2 |
| Rab27a | Csgalnact2 |
| Rdh10 | Ctbp2 |
| Repin1 | Cyp4f18 |
| Selplg | Dnase2a |
| Sh2d5 | Dock5 |
| Sh3bp5 | Dstyk |
| Slc11a2 | Ebi3 |
| Slc12a7 | Entpd1 |
| Slc27a1 | Erlin1 |
| Sox13 | Erp29 |
| Spata6 | Fam101b |
| Spib | Fam217b |
| Spo11 | Fam49a |
| Spry1 | Fcgr2b |
| Ssbp2 | Ffar2 |
| Sstr2 | Fgd2 |
| Tas1r1 | Fgr |
| Tecpr1 | Fh1 |
| Tlr12 | Fig4 |
| Tmc8 | Flnb |
| Tnfsf11 | Gapt |
| Tox2 | Gfod1 |
| Traf3ip2 | Gm16712 |
| Trat1 | Gm16897 |
| Trp53inp1 | Gm2a |
| Tspan13 | Gng10 |
| Tspan32 | Gng2 |
| Utrn | Gns |
| Wnt5b | Gpx1 |
| | Gucy1a3 |
| | Gusb |
| | Haao |
| | Hbb-b1 |
| | Hbb-b2 |
| | Hbb-bs |
| | Hbb-bt |
| | Hck |
| | Hfe |
| | Hpse |
| | Hsd17b6 |
| | Idh2 |
| | Il12rb2 |
| | Il13ra1 |
| | Il1r2 |
| | Il5ra |
| | Inpp5j |
| | Irf5 |
| | Irf6 |
| | Itgad |
| | Itgam |
| | Itgax |
| | Jak2 |
| | Kcnk12 |
| | Kcnk6 |
| | Khdc1a |
| | Khdc1c |
| | Klhl18 |
| | Kmo |
| | Krt80 |
| | Lair1 |
| | Lmo1 |
| | Lpcat2 |
| | Lrba |
| | Lrrc25 |
| | Lrrc75a |
| | Lst1 |
| | Ltb4r1 |
| | Ltbr |
| | Lyn |
| | March1 |
| | Matk |
| | Mb21d1 |
| | Mef2c |
| | Megf8 |
| | Met |
| | Mfsd12 |
| | Myo1f |
| | Naaa |
| | Nccrp1 |
| | Ncf1 |
| | Ncf2 |
| | Ndst1 |
| | Ndufb8 |
| | Nedd9 |
| | Neurl3 |
| | Nfam1 |
| | Nlrc4 |
| | Nlrp10 |
| | Nlrp1b |
| | Nod2 |
| | Npl |
| | Nuak2 |
| | Oas1a |
| | Oas2 |
| | Ogfrl1 |
| | P2ry13 |
| | P2ry14 |
| | Padi2 |
| | Pak1 |

*(Continued)*

**TABLE 1** | Continued

| Consensus PU.1-repressed target genes | Consensus PU.1-activated target genes |
|---|---|
| | Pdxk |
| | Phactr2 |
| | Pik3ap1 |
| | Pik3r6 |
| | Piwil2 |
| | Pla2g4a |
| | Plac8 |
| | Pld4 |
| | Plek |
| | Plxnd1 |
| | Pmvk |
| | Pqlc1 |
| | Prex1 |
| | Prkcd |
| | Prtn3 |
| | Ptpn6 |
| | Ptpre |
| | Rab31 |
| | Ralb |
| | Rcn3 |
| | Relt |
| | Rgs18 |
| | Rnf149 |
| | Rogdi |
| | Rufy1 |
| | Samhd1 |
| | Sema3c |
| | Serpina3g |
| | Sh2b2 |
| | Sh3pxd2a |
| | Siglece |
| | Siglecf |
| | Siglecg |
| | Skap2 |
| | Sla |
| | Slc16a7 |
| | Slc35d3 |
| | Slc8a1 |
| | Snx10 |
| | Sorl1 |
| | Spi1 |
| | Stx7 |
| | Susd3 |
| | Svip |
| | Syk |
| | Tbc1d24 |
| | Tbxas1 |
| | Tdrd7 |
| | Tgm1 |
| | Themis2 |
| | Tlr9 |
| | Tmc5 |
| | Tmem51 |
| | Tmprss3 |
| | Tnni2 |
| | Tor3a |
| | Trim55 |
| | Trmt2a |
| | Trpm2 |
| | Tyrobp |
| | Ufsp2 |
| | Unc93b1 |
| | Vamp8 |
| | Vps18 |
| | Wdfy4 |
| | Ywhag |
| | Zc3h12d |
| | Zfp385a |
| | Zfp52 |

*This table lists high-confidence PU.1-regulated genes which show reciprocal responses in the loss of function and gain of function perturbations of PU.1. The lists give the three-way intersection of genes affected in loss of function, and (reciprocally) in gain of function within cells remaining $CD25^+$, and in gain of function within cells becoming $CD25^-$ $CD44^+$. Consensus PU.1-repressed target genes: genes with expression that goes down in PU.1-transduced DN2b with or without lineage diversion ($CD25^+$ or $CD44^+$ cells), and also increases in normal DN2a cells after deletion of endogenous PU1. Consensus PU.1-activated target genes: genes with expression that increases in PU.1-transduced DN2b with or without lineage diversion, and also goes down in normal DN2a cells after deletion of endogenous PU.1. Data compiled from Ungerbäck et al. (85).*

that are packaged in Polycomb Repressive Complex 2-modified chromatin, as marked by trimethylation of Histone H3 Lysine 27 (H3K27me3) (84). However, approximately half of the PU.1 occupancy sites in early pro-T cells appear to be relatively "inaccessible" in chromatin by the criterion of ATAC-seq at the stages when PU.1 is seen to be binding there, showing that PU.1 binding can occur without opening the chromatin. These sites in closed chromatin have particularly high-quality matches to the consensus PU.1 binding position weight matrix (85), which several lines of evidence show to be a good indicator of PU.1 binding affinity (11, 85). This suggests that closed chromatin may be less permissive to PU.1 binding than open chromatin, so that only high-affinity site recognition allows binding in closed regions. However, this stringent specificity criterion also shows that these are not "off-target" sites: the tradeoff between site accessibility and the affinity of binding needed for occupancy indicates that PU.1 itself is identifying these sites in closed chromatin to establish its occupancy. By the ability to enter closed chromatin at its own high-affinity sites, and by its functional role in controlling chromatin accessibility at other sites, PU.1 meets the criteria for "pioneer" factor activity in early pro-T cells (98), and the scope of its binding suggests a broad role in genomic architecture of these cells.

Still to be determined are the rules determining when PU.1 binding to closed chromatin results in opening of the closed site. A priori, one could imagine that PU.1 establishes occupancy using its DNA-binding domain and then uses its protein-interaction domains to recruit chromatin modifying complexes (**Figure 5**, diagram of structures). Many of the known protein-protein interactions between PU.1 and other transcription factors on the DNA are also mediated through parts of the DNA binding domain, consistent with a compartmentalized role of this domain of the protein for binding site choice [reviewed in (18)]. However, PU.1 also contains non-DNA binding domains, acidic and glutamine-rich "transactivation" domains and an IRF4/8 binding domain, that are also clearly implicated in PU.1 function, as selective deletions of these domains greatly reduce PU.1 developmental impacts (24, 47, 99). Recent evidence has pointed to another role of the non-DNA binding domains of PU.1, a function needed for PU.1 to enter closed chromatin. Genome-wide, exogenously introduced full-length PU.1 and the isolated PU.1 binding domain establish quantitatively similar patterns of occupancy at open sites and especially at open promoters all across the genome. However, they show a marked difference in binding between open and closed chromatin, especially at non-promoter sites. Full-length PU.1 binds closed sites nearly as well as open sites, whereas the isolated PU.1 DNA binding domain binds open sites as well as full-length but selectively fails to bind at closed sites (85) (**Figure 6**). This suggests that an additional process beyond simple DNA sequence recognition is required to establish PU.1 occupancy in closed chromatin, even without an overt change in chromatin accessibility as measured by ATAC-seq. A similar mode of action has already been described for a separate, non-DNA binding domain to enable EBF1 entry into closed chromatin (100). It will be interesting to see if this is a general feature of pioneer transcription factors.

# PU.1 ACTION VIA COFACTOR RECRUITMENT

## Direct Gene Regulation by PU.1: Activation by Distal Enhancer Engagement and Opening

The major problem with connecting PU.1 binding to PU.1 regulatory function is that PU.1 binds to too many genomic sites in ETP and DN2a pro-T cells (84). It shows high fidelity in terms of sequence recognition, but its binding is not confined to functionally responsive target genes. It is found at a large fraction of open, accessible chromatin elements during the stages when it is expressed, and often bound at promoters as well as distal elements. However, only a minority of the genes linked to its binding sites change expression at all across the developmental interval when PU.1 goes from full expression to silence (84). Much of PU.1 binding in pro-T cells thus appears to be either functionally redundant or opportunistic. Identifying PU.1's functional mechanisms of target gene regulation has required a way to link an experimentally inducible *change* in PU.1 binding at a given site with the rapid, measurable *change* in expression of the target gene linked to that site. This is considerably easier to do in a gain of function format than in loss of function, as an epitope-tagged exogenous PU.1 construct can be introduced with fast kinetics and its newly established binding tested for association with local gene expression responses (85). Note that the gain-of-function experimental design makes it necessary to use another criterion to screen out genes that are only indirectly affected, as described above. For this reason, in our recent study (85) only cells that remained $CD25^{+}$ $CD11b^{-}$ (see above) were used for ChIP-seq analysis of exogenous PU.1 binding.

The results showed that PU.1 exerts its main functional regulatory impacts in pro-T cells via non-promoter sites, and especially via sites that are normally developmentally changing in chromatin accessibility (85) (**Figure 6**). In the aggregate, most of the responses of genes linked directly to PU.1 binding sites were positive; direct repression targets were much rarer. Genes responding to the addition of exogenous PU.1 usually had the exogenous PU.1 binding to distal (intronic or neighboring intergenic) sites, whereas genes that had PU.1 binding only to their promoter regions usually did not change expression at all. In the "blank slate" background of the Scid.adh.2C2 cell line, exogenous PU.1 binding opened chromatin at its non-promoter sites within 2 h, increasing the "activating" H3K27Ac marks at these sites a few hours later, and the linked genes were predominantly upregulated within 8–24 h. The genomic sites that were most highly associated with these responses in primary pro-T cells were developmentally dynamic in chromatin accessibility: normally open in early stages of T-cell development (endogenous PU.1-expressing) but closed once the cells went through commitment (endogenous PU.1-low or negative). Thus, the sites in pro-T cells with the strongest sensitivity to exogenous PU.1 for transcriptional impact were also sites where endogenous PU.1 might be important for maintaining chromatin accessibility.

## The Problem of Pro-T Cell Gene Repression by PU.1

The impact of PU.1 on pro-T cell gene expression overall is at odds with the biochemical and genomic evidence for its mode of action in one respect: PU.1 introduced into primary pro-T cells or Scid.adh.2C2 cells causes downregulation of many T-cell genes, especially those associated with Notch signaling and TCR gene rearrangement after commitment. This response is fast, reducing existing transcript pools for many repressed genes even before most positively regulated PU.1 target genes are seen to be turned on (80). However, the local impact of PU.1 binding is strongly biased toward activation of genes linked to the binding sites. While much of the data showing T-lineage affiliated gene downregulation comes from forced PU.1 re-expression or overexpression experiments, and might therefore be a high-dose artifact, it is important to note that the developmental speed-up observed in primary pro-T cells when PU.1 is knocked out also points to a normal PU.1 role as a brake on developmental progression (79). Thus, to account for PU.1's overall role, some explanation for the repressive outcomes is essential.

There is a long history of research on PU.1 as a repressor of genes associated with non-myeloid pathways, especially in

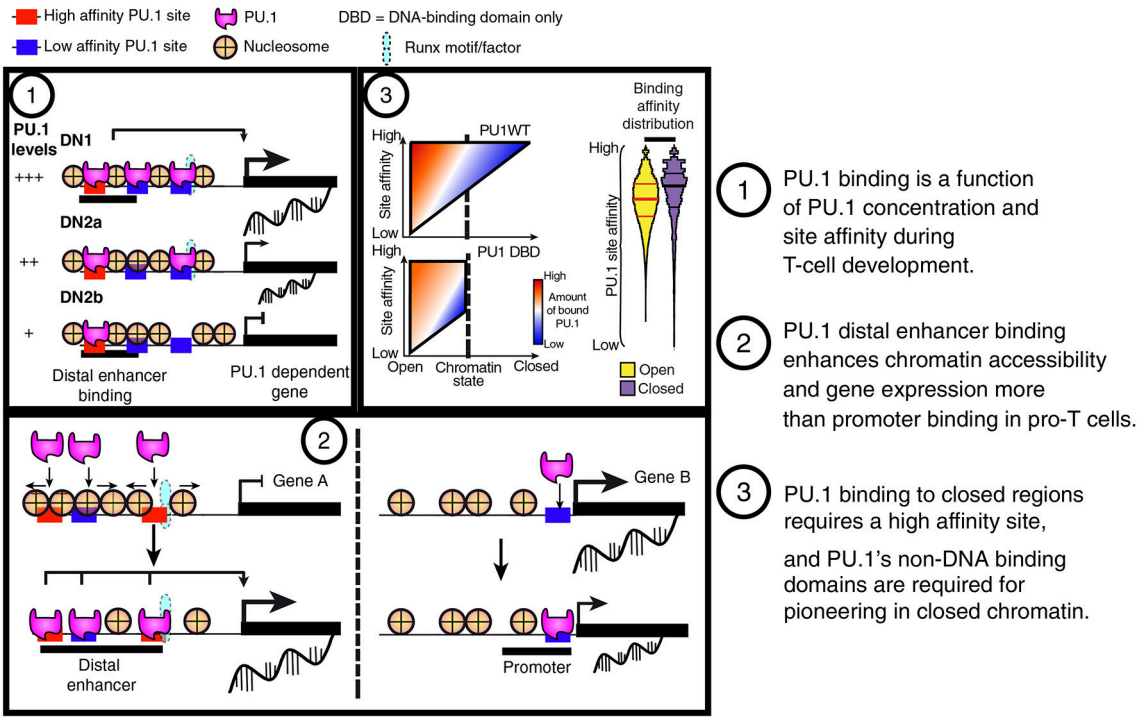


**FIGURE 6 |** Summary of PU.1 binding features in pro-T cells. (1) PU.1 binding is preferentially retained at high-affinity sites as pro-T cells progressively reduce their PU.1 levels. This feature indicates that PU.1 primarily uses mass action (concentration × affinity) to determine its genomic site choices in these cells. (2) PU.1 works as a positive regulator in pro-T cells primarily by binding and controlling accessibility of sites distal to the transcriptional start sites, not at promoters. (3) The binding profiles of full-length PU.1 (PU1WT) show a tradeoff between binding site affinity and binding site accessibility in chromatin; however, constructs with the PU.1 DNA binding domain but lacking the transactivation domains (PU1 DBD) are poor at engaging sites in closed chromatin no matter how high their potential affinities.

the context of PU.1—GATA-1 antagonism in hematopoiesis [reviewed in (4, 7, 25)]. At high levels, PU.1 has been found to block DNA binding by GATA-1 (23), while at lower levels it is reported to antagonize GATA-1-mediated transactivation by forming complexes with it that recruit Rb through the PU.1 acidic transactivation domain (101). It is also reported to act as a repressor by direct recruitment of Dnmt3b (102). In most of these cases, PU.1 is observed to bind directly to the regulatory DNA of its repression targets (86, 103). However, in the pro-T cells, the genes that are repressed when exogenous PU.1 is introduced are not necessarily linked to the sites that the exogenous PU.1 actually binds. In fact, results with the PU.1-ENG obligate repressor construct implied that some kind of indirect effect must be involved: while the obligate repressor downregulated genes that are positive regulatory targets of wildtype PU.1, it actually upregulated many genes that wildtype PU.1 represses, completely inconsistent with a direct repression mechanism (79).

To date, three mechanisms appear to be involved. First, as noted above, high-dose PU.1 can inhibit expression of multiple Notch target genes and *Notch1* itself (58, 85). It is possible that the fast downregulation of Notch response genes, including *Hes1, Nrarp, Dtx1, Lef1*, and *Il2ra,* by overexpressed PU.1 is due to the loss of positive Notch signaling input rather than to a gene-specific mechanism. This Notch-inhibitory mechanism is not operating in cells that remain within the T-cell pathway, but it becomes prominent in cells that PU.1 causes to transdifferentiate, and would be expected to affect all T-cell genes that use Notch signaling as an obligate positive input, whether or not PU.1 binds them directly. A related scenario in which PU.1 could interfere with a T-lineage specific positive regulatory input might be through repression of GATA-3 by PU.1, by analogy with the cross-inhibition of PU.1 and GATA-1. However, in pro T cells, both PU.1 and GATA-3 are active together and both functionally important throughout the ETP to DN2a stages (79, 104), and there is more evidence for GATA-3 repression of PU.1 than for PU.1 repression of GATA-3 (58, 79, 104, 105). However, GATA-3 function also may become a casualty of PU.1 action when Notch signaling is inhibited (58).

Second, in pro-T cells forced to express PU.1, those that make the lineage jump (i.e. lose CD25, gain CD11b) not only silence *Notch1* but also activate myeloid regulatory genes (85). They also begin to express multiple transcription factors of the Egr and IRF families, and in the case of primary cells, they also upregulate C/EBP family factors. These factors probably contribute independently to the repression of pro-T cell genes. Egr2, for example, can collaborate with PU.1 in positive regulation when co-bound with it (106), but has also been implicated as a PU.1-stimulated repressor of the *mir17∼92* complex (107). Although not required for *Notch1* repression in Scid.adh.2C2 cells (58), when activated in primary cells,

C/EBPα itself can also repress *Notch1* (50). PU.1 does not strongly upregulate transcription factors annotated as repressors, however, in cells remaining within the T-cell program (see below). Therefore, this indirect repressive activity, too, would only be deployed under conditions of lineage shift.

The third mechanism that could play a role in repression within the T-cell program comes from PU.1's own ability to recruit other transcription factors to collaborate with it at PU.1 binding sites. This is a hallmark of pioneering activity in developmental gene regulation (98, 108), but in this case it exposes a particularly intricate post-transcriptional relationship between PU.1 and the factors required for progression of the T-cell program.

## System Consequences of Cofactor Recruitment: Repression by Theft

PU.1 is a powerful organizer of the occupancy patterns of other transcription factors genome-wide. PU.1 binding shifts the disposition of other factors in the cell across the genome, even when their own expression levels and total numbers of binding sites remain essentially unchanged (80). The *positive* regulatory significance of these kinds of shifts is well established; many factors recruit others to collaborate with them in functional complexes at active enhancers [e.g., reviews by (17, 109–111)], and PU.1 is known to establish preferential binding sites for multiple other transcription factors in myeloid cells. However, in this case the positive impact is coupled with a *negative* regulatory consequence, via action at a distance (**Figure 7**). For PU.1 in pro-T cells, IRF and C/EBP family partners are mostly not available, but a key positive regulatory partner is Runx1 (previously known as AML1 or CBFα2), which has long been known to interact with PU.1 (and C/EBPα) to form a functional complex at its myeloid positive regulatory target sites (9, 112, 113). In pro-T cells, PU.1 binding sites in open chromatin genome-wide are highly enriched for Runx motifs, raising the possibility that Runx factors assist in the chromatin opening process (85), and proteomic analysis provides support for a strong representation of Runx1 in PU.1-containing complexes formed in the pro-T cell like Scid.adh.2C2 cell line (80). However, Runx1 also has sites at a large fraction of all enhancers active in the cells without PU.1 expression (**Figure 7**).

When epitope-tagged PU.1 was introduced into Scid.adh.2C2 cells and the complexes were isolated for proteomic analysis, much enrichment was seen for SWI/SNF complex components as well as some other chromatin modifiers (80). The preponderance of SWI/SNF complex interactions was consistent with the evidence that PU.1 usually acts as an activator. Based on the longstanding literature of PU.1–GATA factor antagonism through protein interaction (20–23), GATA-3 was expected to be present as well, and it was detectably enriched over background in these complexes. However, by far the most highly enriched sequence-specific transcription factor proteins interacting with PU.1 in these cells were Rest and Runx1 (80). Runx1 was of particular interest because of its sequence motif enrichment at PU.1 sites. Although Runx1 can act as a global chromatin accessibility organizer (114), PU.1 itself does not depend on Runx1 for establishing permissive sites for its binding, even in the "blank slate" context of the Scid.adh.2C2 cells (85). However, PU.1 strongly affected the sites where Runx1 bound, resulting in a dramatic shift in Runx1 binding site choices in tests of gain of PU.1 function (80). Supporting the physiological relevance of this mechanism, many of the same genomic sites where Runx1 was shifted by PU.1 in Scid.adh.2C2 cells underwent the reverse changes in Runx1 occupancy in normal primary pro-T cells, as they progressed from PU.1-high to PU.1-low developmental stages.

As expected, PU.1 recruits Runx1 to sites where Runx1 exerts measurable functional collaboration with PU.1, mostly to help in the positive regulation of PU.1 targets (80). However, the aspect of this redistribution that is most notable is that Runx1 is depleted in the process from alternative sites, and the sites that it abandons are themselves highly functional sites. The analysis is somewhat complicated by the fact that many developmentally important genes are linked with multiple Runx1 and/or PU.1 binding sites, only some of which gain or lose Runx1 occupancy. However, focusing on those genes that have Runx1 binding sites but not PU.1 binding sites, the genes that "lose" Runx1 binding when PU.1 is expressed clearly include a large fraction that depend quantitatively on Runx1 for their own expression. These genes show weak downregulation when Runx1 is disrupted by Cas9 and they show stronger downregulation when Runx1 is neutralized by a Runx1 dominant negative construct (80). Thus, the competition for Runx1 protein by PU.1 directly causes coupled positive and negative regulation, to cause a switch-like alteration in genome-wide cell state (**Figure 7**).

Three features of this mechanism are noteworthy (80). First, PU.1 does not appear to bind, even transiently, at the sites from which Runx1 is lost: Runx1 is competitively redistributed, but is not displaced. Thus, the PU.1 effect differs from "squelching" or other negative regulatory mechanisms where transcription factors are expelled by chromatin closing (115, 116). Second, one might expect that the Runx sites available for redistribution could have been vulnerable to dissociation because they were marginal quality binding sites in the first place; however, motif analysis shows that many of the Runx occupancy sites that are emptied when PU.1 is in the cell are high quality Runx sites in the upper half of the position weight matrix score distribution (80). Considered only as Runx sites, they are likely to be much higher affinity than the ones to which Runx1 moves, to occupy together with PU.1. Thus, the ternary (or higher-order) complexes nucleated by PU.1 are more favored binding sites for Runx1 when PU.1 is present than functionally relevant, high-quality Runx sites elsewhere. Finally, it is clear that this is a system-level mechanism. It is the limited pool of Runx1 operationally available for action across the genome that makes the impact of PU.1 a "zero-sum" outcome. Thus, the regulated level of Runx1 protein contributes to the switch-like impact made by the developmental shift from high-level PU.1 to PU.1 shutoff. However, given the high frequency of Runx factor utilization at multiple lymphoid enhancer sites, this kind of mechanism can propagate local PU.1 impacts to a much broader genomic scale.

The "theft" mechanism of repression by partner factor redistribution is not unique to the PU.1-Runx1 pair. PU.1

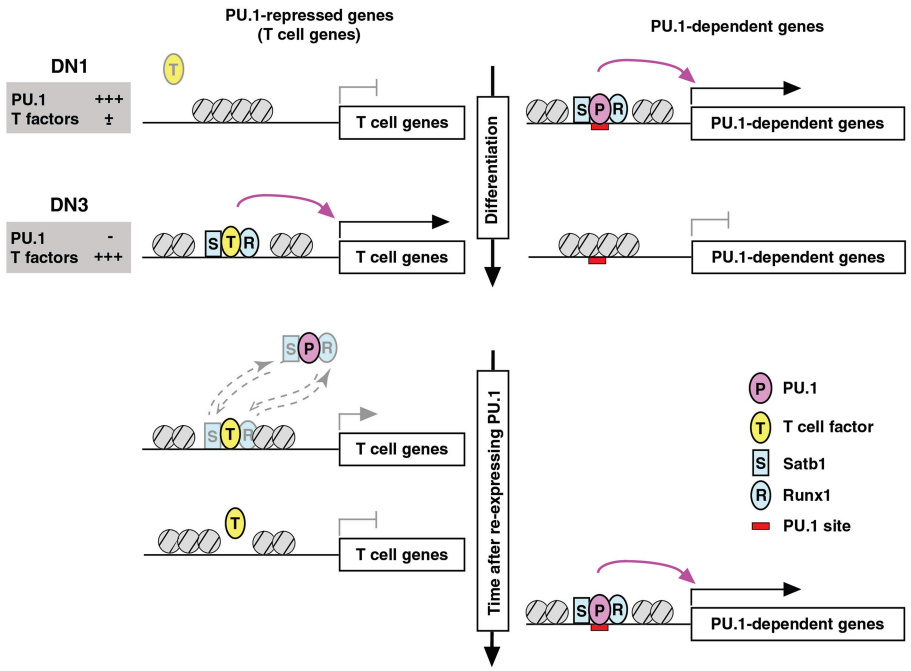


**FIGURE 7 |** Repression by PU.1 can be caused by redistribution of a limiting co-regulator (co-regulator theft). Schematic depicts the complementary changes in factor binding patterns as well as in transcriptional activity of genes that are normally PU.1-dependent **(right)** or PU.1-inhibited **(left)**, across the developmental stages when PU.1 goes from high to low **(top)** and then in an experimental condition when PU.1 is re-introduced into committed cells that have already turned off endogenous PU.1 **(bottom)**. The figure shows that the redistribution of partner factors Runx1 (R) and Satb1 (S) by recruitment to PU.1 (P) binding sites occurs at the expense of sites that these factors would otherwise occupy together with other T-cell factors (T). Broken-line arrows **(lower left)** indicate that redistribution probably involves the dynamic equilibrium of binding of these factors between genomic sites that are differentially preferred in the presence and absence of PU.1. In at least some cases, the "theft" of the cofactors also results in relative closing of the chromatin at sites from which these cofactors are removed. Schematic modified from Hosokawa et al. (80).

has an even stronger effect on binding site choice of Satb1, another transcription factor that is expressed throughout early T-cell development, and GATA-3 also shifts, when PU.1 is added, to occupy sites together with PU.1 (80). Although Satb1 in DN2-DN3 stages appears to have weaker effects on gene expression than Runx1, the PU.1-repressed genes that appear to be responding to Satb1 loss are different from those that are most dependent on Runx1, broadening the full impact of this mechanism (80). A very similar phenomenon has been reported earlier by Jenner and colleagues for the effect of T-bet on GATA-3 in establishing the Th1 cell program (117, 118). Thus, "partner factor theft" can be an integral part of the machinery for program choice operated by lineage-determining transcription factors.

# THE PU.1 REGULOME IN EARLY PRO-T CELLS AND ITS PHYSIOLOGICAL ROLES

## PU.1 Target Genes: Gene Network Roles and Developmental Timing

While indirect regulation plays a large role in its developmental impact, the target genes that PU.1 directly regulates are ultimately crucial for understanding what this factor contributes to the T-cell program. PU.1's action as a positive regulator implies that most of its direct target genes should be expressed in a pattern concordant with its own expression. Indeed, PU.1-activated target genes are preferentially expressed in the earliest stages of T-cell development (examples shown in **Figure 8**). Many of them are expressed also in at least one of the other contexts where PU.1 is active: in myeloid and dendritic lineage cells, in B lineage cells, and particularly also in multipotent progenitor cells (**Figure 8**). Among the smaller number of genes that appear to be directly repressed by PU.1, most are specific for later stages of T-cell development. These patterns reinforce the case for PU.1's impact in shaping the developmental timecourse of gene expression in pro-T cells.

Developmentally potent transcription factors often transform a cell's identity by positively or negatively regulating the expression of other transcription factors. As noted above, PU.1 can have this effect on early pro-T cells when it is overexpressed and the cells switch to a non-T cell lineage program. But to what extent does PU.1 control the expression of other transcription factors within the T-cell program? It has become clear that the progression of cells through T-cell commitment involves the ordered downregulation of a substantial set of progenitor-specific transcription factors, called "Phase 1" factors in this context, concomitant with the upregulation of T-lineage affiliated factors (34, 65, 84, 121, 122). PU.1 itself is downregulated at stage when multiple other Phase 1 factors are downregulated, and a key question is whether the withdrawal of positive PU.1 input plays a role in the downregulation of these progenitor factors. With respect to PU.1-mediated repression, some T-cell factors are

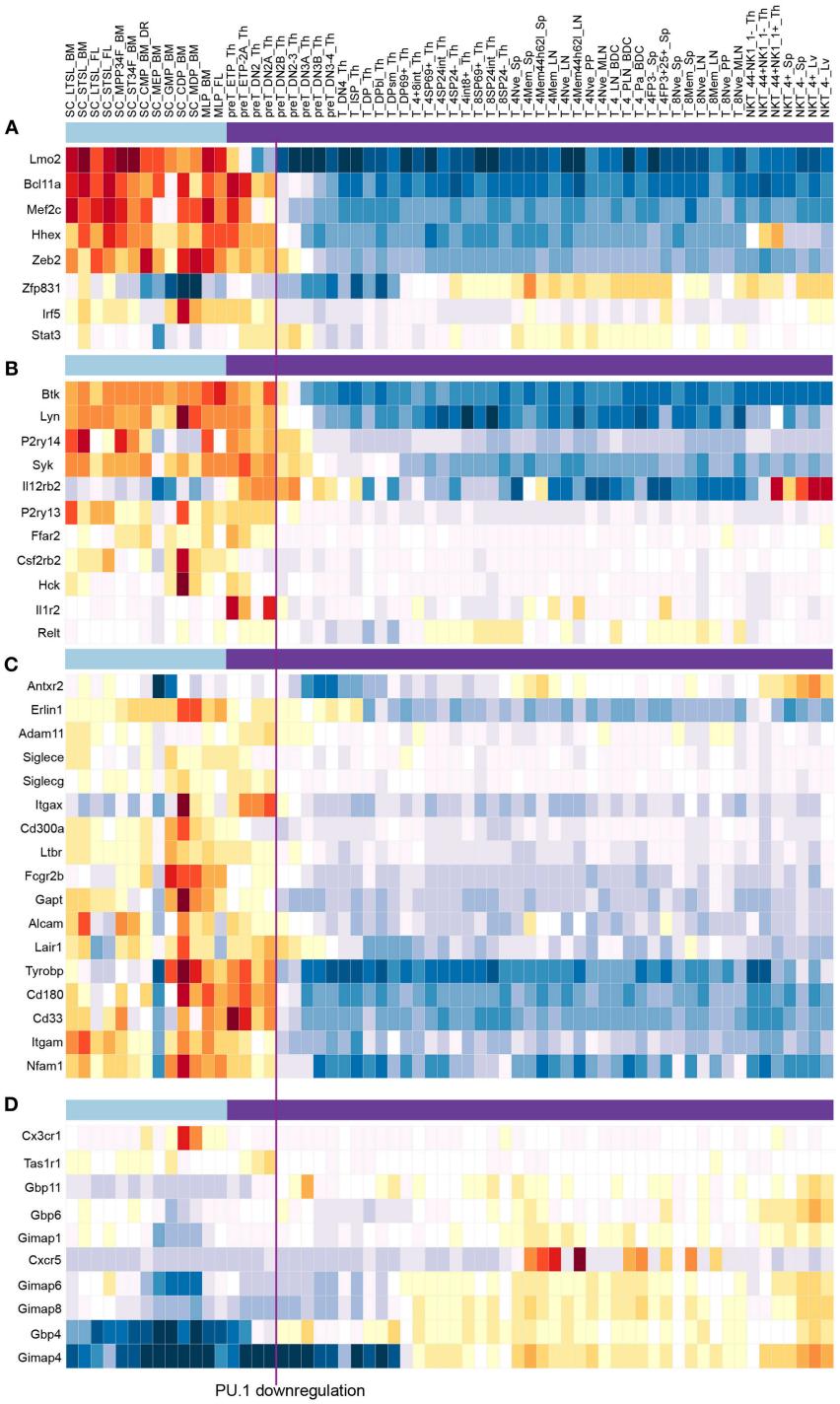


**FIGURE 8 |** PU.1 globally regulates genes involved in multiple signaling and cell biological properties as well as other "phase 1" transcription factors across the stages when PU.1 is expressed. Summary of normal expression patterns of representative groups of PU.1 regulated genes is shown, illustrated using the ImmGen (119) (www.immgen.org) "My Gene Set" browser (http://rstats.immgen.org/MyGeneSet_New/index.html; Microarray V1). Natural levels of expression are shown in different "Stem and Progenitor" cell sets (120) (under light blue bar) and in successive stages of "αβ T cell" development (121) (under purple bar), where the color scale represents z score (warm colors, high expression; cold colors, low expression). Vertical line between "preT_DN2A_Th" and "preT_DN2B_Th" relates these stages to the timing of commitment, when PU.1 levels decline. **(A)** Genes encoding transcription factors activated by PU.1 in multiple tests (58, 79, 85). **(B)** Representative genes activated by PU.1 that encode tyrosine protein kinases, cytokine receptors, and G protein coupled receptors, from **Table 1** [data from Ungerbäck et al. (85)]. **(C)** Genes activated by PU.1 that encode additional cell surface molecules, signaling receptors and adhesion molecules, from **Table 1**. **(D)** Genes repressed by PU.1, encoding chemokine receptors and G-protein coupled receptors, from **Table 1**. Functional clusters used in this summary were as defined by DAVID Gene Functional Classification tool (DAVID 6.8) (https://david.ncifcrf.gov/gene2gene.jsp).

already upregulated while PU.1 is still highly expressed (GATA-3, TCF-1, and the Notch target Hes1), but others are upregulated only during the period when PU.1 declines (Bcl11b, Ets1, Lef1) and could, in principle, have their expression timing affected by PU.1 negative regulation. To what extent does PU.1 actually control these gene expression patterns under the circumstances of actual pro-T cell development, i.e. with strong Notch signaling that prevents lineage switching?

Data from multiple studies show that PU.1 regulates a subset of developmental control genes but is not alone in its actions. Varied PU.1 gain and loss of function perturbations in the DN2-DN3 stages show that PU.1 does provide positive input into a discrete subset of Phase 1 regulatory genes, with *Mef2c*, *Lmo2*, *Bcl11a*, and often also *Hhex* responding over a range of different tests (58, 79, 80, 85). Consistent with an evolutionarily conserved program in regulating these genes, the developmental expression patterns of these genes and PU.1 (*Spi1*) in human pro-T cells (Thy1—Thy4) are similar to their patterns in murine pro-T cells (35). PU.1 is not uniquely responsible for the Phase 1 gene expression pattern, however, for other Phase 1 genes are either unaffected or moderately inhibited by PU.1, as discussed elsewhere (79, 123). Additional factors also probably collaborate with PU.1 to fine-tune the responses of *Mef2c* and *Lmo2*, for they are already declining by the end of ETP stage, multiple cell cycles before DN2b stage when PU.1 itself declines (65, 67, 84, 121, 124) (**Figure 8A**). Thus, PU.1 is likely to be one of several important positive regulators for these genes. PU.1 can indeed have negative regulatory effects on some of the T-cell factors that are upregulated during commitment, but these effects are greatly limited when the analysis is confined to PU.1 activities within the T-cell program. *Hes1*, *Tcf12* (HEB), *Ets1*, and *Lef1* are strongly affected in cells making a lineage switch, but none of these are measurably repressed in cells within the T-cell path. The cell cycle-regulatory locus *E2f2,* which is also upregulated during commitment, is rare among transcription factor coding genes in that it does appear to be under active repression by PU.1 until the transition to commitment. Thus, within the T-cell pathway, PU.1 has a specific role in promoting maintenance of certain Phase 1 regulatory genes before commitment, but little role in repressing T-cell differentiation regulators directly.

## PU.1 as a Choreographer of Thymocyte Cell Biology

An important result from the genome-wide analysis of PU.1 target genes has been recognition of the major gene sets that it does actively control in early pro-T cells. The number of high confidence PU.1 target genes within the T-cell pathway that code for transcription factor genes is low (**Figure 8A**). In contrast, Gene Ontology and Pathway analyses as well as simple gene lists reveal that PU.1 directly controls major systems of cytokine receptors, chemokine receptors, tyrosine protein kinases, G-protein receptor signaling molecules, and adhesion or cytoskeletal system molecules (**Table 2**) (85). These directly regulated targets, some of them studied little, if at all, in T-cell development to date, might have a transformative impact on the cell biology of the developing lymphocytes between the stages when PU.1 is present and when it is shut off.

Potentially important clues to PU.1 roles are the prominence among positively regulated PU.1 targets of genes encoding specific cytokine receptors not yet studied in T-cell biology (e.g., Pdgfrb); multiple protein tyrosine kinases (Btk, Syk, Hck, Lyn); and G-protein coupled receptors (Ffar2, P2ry13, P2ry14) and G protein signaling mediators (Gng2 and Rgs18); while PU.1 represses other G protein signaling mediators (Gimap and Gbp family members). In addition, PU.1 directly promotes expression of cell surface molecules (CD33, CD34, CD44) used as markers for stages in early T-cell development, but which *in vivo* work to mediate environmental interactions, and it drives expression of adhesion molecules (integrins and Siglecs) as well as cytoskeletal components such as Coro2a and Myo1f. Representative samples of the expression patterns of such genes are shown in **Figures 8B–D**. The result is that not only signaling capability but basic properties of adhesion, motility and chemoresponsiveness of the cells can be under PU.1 control in the early stages of T-cell development. While these effects are not seen as direct transcriptional regulation of other transcription factor coding loci, such target genes should have numerous impacts on activation pathways in the cells that induce transcriptional as well as migratory responses to environmental signals.

The PU.1-high stages of thymocyte development are relatively obscure in the context of the whole thymus, yet their accurate regulation is crucial for establishment of immune system homeostasis and avoidance of leukemia (125, 126). These stages span multiple cell cycles *in vivo* and *in vitro* (33, 39, 67, 124). However, cells in these stages are hard to visualize in the intact thymus, as only a few cells per day are granted regulated entry into the thymic antechamber (127), then migrate slowly through the cortex, dispersing among a vast excess of more advanced T-cell precursors, as they begin to differentiate toward commitment (128). In postnatal mice, the entry point is thought to be formed by specialized endothelial cells at the cortical/medullary border of the thymus (129). Following an unknown triggering signal, after a variable delay (125), the cells in each cohort then begin to migrate centrifugally toward the outer thymic cortex, and cell surface marker expression patterns imply that it is somewhere midway in the course of this migration that the individual cells undergo lineage commitment [reviewed by (60, 130)]. Because of the extreme rarity of these very immature cells relative to the later-stage thymocytes at any given time, they were almost impossible to study in depth before the development of *in vitro* culture systems (88), which have continued to be informative to the present. However, the types of genes positively regulated by PU.1 are overwhelmingly in categories likely to be involved in mediating the interaction of the cells with very specific environments. The tests of PU.1 function in these early pro-T cells that have been done so far present the cells with Notch ligands and cytokines, but could be fundamentally lacking in other molecules presented by the normal thymic environment. It will be of great interest to discover which anatomical subdomains of the thymus actually supply the molecules that interact with the potentially important receptors and adhesion molecules that

**TABLE 2 |** Gene ontology and pathway classifications of genes regulated by PU.1 in pro-T cells.

**(A) Genes upregulated by PU.1 in CD25$^+$ cells with gain of function, downregulated with sgRNA, relative to all genes expressed in cells**

| GO biological process complete (top 21) | Fold enrichment | Adjusted *P*-value |
|---|---|---|
| Peptidyl-tyrosine phosphorylation (GO:0018108) | 7.18 | 2.40E-02 |
| Myeloid leukocyte activation (GO:0002274) | 7.05 | 9.14E-03 |
| Reactive oxygen species metabolic process (GO:0072593) | 6.97 | 3.05E-02 |
| Regulated exocytosis (GO:0045055) | 6.87 | 3.43E-02 |
| Peptidyl-tyrosine modification (GO:0018212) | 6.87 | 3.43E-02 |
| Exocytosis (GO:0006887) | 5.11 | 1.14E-02 |
| Immune response-activating signal transduction (GO:0002757) | 4.99 | 1.46E-02 |
| Inflammatory response (GO:0006954) | 4.76 | 7.00E-06 |
| Immune response-regulating signaling pathway (GO:0002764) | 4.74 | 2.53E-02 |
| Activation of immune response (GO:0002253) | 4.63 | 6.83E-03 |
| Myeloid cell differentiation (GO:0030099) | 4.55 | 3.80E-03 |
| Positive regulation of protein secretion (GO:0050714) | 4.21 | 2.11E-02 |
| Regulation of body fluid levels (GO:0050878) | 4.21 | 4.39E-02 |
| Positive regulation of peptide secretion (GO:0002793) | 4.15 | 1.23E-02 |
| Adaptive immune response (GO:0002250) | 4.1 | 2.92E-02 |
| Positive regulation of defense response (GO:0031349) | 4.07 | 1.59E-02 |
| Regulation of MAP kinase activity (GO:0043405) | 3.95 | 2.32E-02 |
| Defense response to other organism (GO:0098542) | 3.93 | 3.28E-03 |
| Regulation of inflammatory response (GO:0050727) | 3.87 | 2.96E-02 |
| Immune effector process (GO:0002252) | 3.74 | 5.31E-04 |
| Innate immune response (GO:0045087) | 3.73 | 3.02E-04 |

**(B) Genes downregulated by PU.1 in CD25$^+$ cells with gain of function, upregulated with sgRNA, relative to all genes expressed in cells**

| GO biological process complete | Fold enrichment | Adjusted *P*-value |
|---|---|---|
| Defense response to protozoan (GO:0042832) | 27.62 | 1.72E-02 |
| Response to protozoan (GO:0001562) | 25.11 | 2.57E-02 |
| Cell activation (GO:0001775) | 4.44 | 2.43E-03 |
| Immune response (GO:0006955) | 3.55 | 6.41E-03 |
| Immune system process (GO:0002376) | 2.86 | 2.26E-04 |
| Cellular response to stimulus (GO:0051716) | 1.75 | 4.20E-02 |

*(Continued)*

**TABLE 2 |** Continued

**(C) Genes upregulated by PU.1 in CD44$^+$ CD25- cells with gain of function, downregulated with sgRNA, relative to all genes expressed in cells**

| GO biological process complete (top 21) | Fold enrichment | Adjusted *P*-value |
|---|---|---|
| Regulation of coagulation (GO:0050818) | 8.85 | 5.55E-03 |
| Regulation of blood coagulation (GO:0030193) | 8.58 | 2.49E-02 |
| Regulation of hemostasis (GO:1900046) | 8.36 | 2.98E-02 |
| Positive regulation of inflammatory response (GO:0050729) | 7.17 | 3.76E-05 |
| Myeloid leukocyte activation (GO:0002274) | 6.03 | 1.61E-02 |
| Regulated exocytosis (GO:0045055) | 5.93 | 4.94E-02 |
| Defense response to bacterium (GO:0042742) | 5.16 | 5.18E-03 |
| Positive regulation of stress-activated protein kinase signaling cascade (GO:0070304) | 5.04 | 2.95E-03 |
| Inflammatory response (GO:0006954) | 4.98 | 5.37E-09 |
| Positive regulation of stress-activated MAPK cascade (GO:0032874) | 4.77 | 1.29E-02 |
| Regulation of body fluid levels (GO:0050878) | 4.62 | 1.78E-04 |
| Leukocyte activation involved in immune response (GO:0002366) | 4.53 | 4.90E-02 |
| Positive regulation of defense response (GO:0031349) | 4.51 | 2.71E-05 |
| Positive regulation of MAP kinase activity (GO:0043406) | 4.42 | 6.86E-03 |
| Exocytosis (GO:0006887) | 4.29 | 4.18E-02 |
| Regulation of inflammatory response (GO:0050727) | 4.29 | 6.51E-05 |
| Positive regulation of response to external stimulus (GO:0032103) | 4.09 | 2.38E-03 |
| Regulation of MAP kinase activity (GO:0043405) | 4.01 | 8.19E-04 |
| Activation of immune response (GO:0002253) | 3.85 | 3.77E-02 |
| Positive regulation of protein serine/threonine kinase activity (GO:0071902) | 3.8 | 1.28E-02 |
| Defense response to other organism (GO:0098542) | 3.7 | 8.48E-04 |

**(D) Genes downregulated by PU.1 in CD44$^+$ CD25- cells with gain of function, upregulated with sgRNA, relative to all genes expressed in cells**

| GO biological process complete | Fold enrichment | Adjusted *P*-value |
|---|---|---|
| T cell activation (GO:0042110) | 4.77 | 3.82E-04 |
| Lymphocyte activation (GO:0046649) | 4.51 | 7.36E-07 |
| Cell-cell adhesion (GO:0098609) | 4.22 | 9.44E-03 |
| Cell activation (GO:0001775) | 4.19 | 1.78E-08 |
| Lymphocyte differentiation (GO:0030098) | 4.18 | 1.07E-02 |
| Leukocyte activation (GO:0045321) | 4.12 | 1.14E-06 |
| Regulation of cell-cell adhesion (GO:0022407) | 3.98 | 6.49E-04 |

*(Continued)*

**TABLE 2 |** Continued

**(D) Genes downregulated by PU.1 in CD44$^+$ CD25- cells with gain of function, upregulated with sgRNA, relative to all genes expressed in cells**

| GO biological process complete | Fold enrichment | Adjusted *P*-value |
|---|---|---|
| Leukocyte differentiation (GO:0002521) | 3.52 | 2.88E-02 |
| Positive regulation of cell adhesion (GO:0045785) | 3.45 | 2.10E-02 |
| Regulation of defense response (GO:0031347) | 3.11 | 1.15E-02 |
| Regulation of cell adhesion (GO:0030155) | 3.09 | 1.01E-03 |
| Biological adhesion (GO:0022610) | 2.9 | 2.29E-02 |
| Positive regulation of transcription by RNA polymerase II (GO:0045944) | 2.46 | 2.21E-03 |
| Regulation of immune system process (GO:0002682) | 2.35 | 3.85E-03 |
| Immune system process (GO:0002376) | 2.3 | 3.44E-05 |
| Regulation of multicellular organismal process (GO:0051239) | 1.74 | 3.15E-02 |
| Positive regulation of biological process (GO:0048518) | 1.48 | 6.54E-03 |

*The table shows PANTHER Overrepresentation Analysis (www.geneontology.org) of categories of genes upregulated or downregulated by PU.1. In each case, responding genes were defined by reciprocal changes in expression in PU.1 gain of function and PU.1 loss of function experiments in the DN2a-DN2b interval as in* ***Table 1****. Whereas* ***Table 1*** *shows the three-way intersection of genes affected in loss of function, in gain of function for cells remaining CD25$^+$, and in gain of function for cells becoming CD25$^-$ CD44$^+$, here the effects of the gain of function perturbations were separated to allow comparison of results from cells remaining in the T-cell pathway (CD25$^+$) with results from cells likely deviating toward another fate (CD44$^+$). Database for comparison was all genes expressed in control DN2 cells. Statistical results shown are for a Fisher Test with Bonferroni correction for multiple sample testing. The PANTHER Overrepresentation Test version was released 2018-10-10 using the GO Ontology database released 2018-10-08. For PU.1 activated genes, only the top 21 enriched categories are shown.*

PU.1 enables the cells to express, and what responses they trigger in these earliest T-cell precursors.

## PU.1 AND THE REGULATION OF LYMPHOID DEVELOPMENT IN ONTOGENY

The studies reviewed throughout this paper have characterized the roles of PU.1 in T-cell development in the young postnatal mouse or in late fetal life. Very recent work has now placed these roles of PU.1 into a wider developmental perspective.

The cells used for *in vitro* differentiation as well as *in vivo* analysis in the work reviewed above have all been derived from waves of hematopoiesis that begin with definitive hematopoietic stem cells, which first appear in the mouse fetal liver by about day 11.5 of gestation and may be followed by additional stem-cell waves through the end of gestation (day 20) (131). Thus, postnatal thymocytes and any *in vitro* differentiation cultures seeded with cells from bone marrow or fetal liver from E15 onward are likely to come from true stem cell origins. However, there are earlier hematopoietic progenitors in the embryo that derive from yolk sac, cells with varied developmental potentials but without true stem-cell self-renewal. The first wave of T cell development in the fetus is now thought to arise from these non-stem-cell precursors in the yolk sac (132, 133). It has long been recognized that the earliest fetal thymocytes are different from later waves of developing thymocytes in terms of their abilities to generate particular classes of TCRγδ cells (134) and in terms of their extremely fast differentiation kinetics, both *in vivo* and in fetal thymic organ culture or stromal coculture systems (104, 133, 135). This is now understood to be intrinsically programmed (136) and due to an altered pathway of differentiation in the first-wave cells, which results in T-cell lineage commitment even before entry into the thymus (137–139).

Remarkable differences have been reported between genetic requirements for T cell development derived from earlier and later waves of prethymic progenitors. For example, the crucial T-lineage transcription factor TCF-1, which plays roles in numerous phases of thymocyte development (95, 140–145), is essential to maintain adult T-cell production but dispensable in fetal and early postnatal T cell development (146). A wave of fetal T cell development can also, apparently, be generated without PU.1 (71). In the case of PU.1, the change in its role occurs within fetal life, and this has now been sharply situated in the transition from precociously committed "first-wave" precursors to precursors that enter the thymus while still multipotent (147). ETPs derived from these precursors naturally express lower levels of PU.1 than adult ETPs, but they are almost unchanged in their ability to generate early fetal T cells when the level of PU.1 is reduced still further (∼5 fold) by deletion of the major upstream regulatory element of PU.1 (147). In contrast, the same five-fold diminished level of PU.1 sharply degrades the ability of later fetal hematopoietic stem and progenitor cells to generate T cells at all, *in vivo* or *in vitro*, with functional and phenotypic defects evident in the mutants in both multipotent progenitors and newly-entered intrathymic ETPs, as early as in the late fetus. This difference in PU.1 dependence accompanies a subtly different T-cell developmental program. Gene expression differences have been noted between the normal first-wave fetal and adult pro-T cells in the thymus at corresponding stages which indicate that the fetal program drives accelerated development (148, 149). Montecino-Rodriguez et al. point out that these differences conspicuously include reduced initial expression of multiple PU.1-dependent genes in the fetal cells (147). Thus, not only is the first-wave fetal program less dependent on PU.1, but also it may rely on relatively low PU.1 activity for its very distinctiveness. These results therefore support a role for PU.1 in delaying differentiation in order to allow more extended proliferation before commitment, showing how the importance of this role is ontogenically scaled to the needs of the developing organism.

The first-wave precursor cells, also uniquely, enter the thymus by a different route than all subsequent waves. Instead of entering through the blood vessels near the cortical-medullary junction, these early cells migrate through cervical-region mesenchyme to the thymic anlage before it is vascularized. The thymus does not yet have a capsule to present a physical barrier, and the first-wave cells enter directly through the future outer cortex. Thus, they may not use the same interactions with basement membrane, endothelial cells, or chemokine gradients as any future wave of thymic precursors. Not only are these cells intrinsically programmed to cut short the stages supported by PU.1-dependent transcriptional regulators, but also they

can dispense with many of the cell biological tools that PU.1 may provide to later-wave successors to navigate the adult or late-fetal thymus.

## CONCLUSIONS AND FUTURE QUESTIONS

PU.1 is a broad regulator of the properties of the cells that first enter the thymus, and it helps to determine their proliferation and rate of progression to commitment after they arrive. While dysregulated PU.1 can cause trans-differentiation to myeloid or dendritic-cell fates, endogenous PU.1 normally plays a protracted role within early T-cell development. Its target genes are occasionally repressed but mostly activated by PU.1 binding, and they confer on the cells distinctive stage-specific transcription factor expression patterns as well as a rich array of stage-specific cell biological features that await proper functional analysis. This positive regulatory role is one result of PU.1's strong pattern of binding across the genome, its prominent occupancy of open chromatin sites, and the evidence that it helps to maintain the open chromatin states at bound regulatory sites as long as it is expressed. The number of genes that respond quickly to changes in PU.1 activity may only account for a minority of all the genomic sites where PU.1 is found engaged; at other sites, its role could be structural or redundant with other factors. However, it is clear that PU.1 also affects the activity of certain genes that it does not bind to directly, via creating preferential interaction sites for other factors that can deplete the regulatory elements of those factors' alternative target genes. Through chromatin state placeholding and "coregulator theft" as well as through its own direct transcriptional activities, PU.1 pervades the regulatory state of early T cells as long as it is expressed.

This phase comes to an end when other transcription factors finally accumulate to the point where they can shut PU.1 off. The best current candidates for this silencing activity include GATA-3 (104, 105), TCF-1 or LEF-1 (37), and especially Runx1 (80, 150–152), probably working in a dose-dependent combination, although the mechanism through which they finally achieve the ability to repress PU.1 has not yet been reported. Importantly, the duration of the PU.1 activity phase is regulated to vary among different ontogenic waves of T cell development. It probably extends for over 10 days for the thymocytes in young adult mice (153), where it is crucial for successful T-cell generation (2, 147). In contrast, for many first-wave fetal thymocytes it may last only a day or two, and is mostly or entirely dispensable (71, 147). This indicates that the specific constellation of functions that PU.1 serves in T-cell development is a module within the larger T-cell developmental program that can be deployed optionally to serve a particular role. Perhaps it is more important for scaling the population dynamics of T-cell production as the animal finishes gestation and grows, or for promoting accurate migration through distinct thymic microenvironments, than for making T cell precursors *per se*.

The pioneering role of PU.1 on the genome raises fascinating questions for future study that connect mechanism with developmental lineage selection. Hematopoietic progenitors express PU.1 before they enter the thymus, but the pattern of its occupancy is not well defined at that stage, so the onset of PU.1's pioneering activity in precursors that will eventually generate T cells is not easy to study. The mechanisms discussed in this review show that it establishes a pre-pattern that can influence the binding of the other transcription factors expressed in the cell throughout multiple cell cycles in the thymus. It is not clear, though, how this particular pre-pattern is set, to be distinguished from PU.1 binding patterns in B cells and myeloid cells (84). The question could be linked with the deeper mystery of the factors involved in designating some multipotent precursors to enter the thymus in the first place, as opposed to remaining in the bone marrow for programming into B cells, natural killer cells, or innate lymphoid cells. So far the innate lymphoid cell developmental program in particular appears to resemble the intrathymic T-cell program in many respects (154–157), enough to raise the question of what makes T-cell precursors wait to activate genes like *Tcf7* and *Gata3* until they reach the thymus. Is PU.1 part of the answer? The system-wide impact of PU.1 on other factors suggests that in scenarios where PU.1 is absent, the same T-cell transcription factors might initially choose different binding sites. Indeed, pro-T cells that have PU.1 acutely deleted at an early stage do not only differentiate faster along the T lineage; they also tend to shift to a natural killer-like program more readily than controls (79). Thus, activity of PU.1 may be important, also, to block certain alternative differentiation paths for pro-T cells. In the end, is T-cell lineage fidelity itself partly a legacy of PU.1's transient role?

## AUTHOR CONTRIBUTIONS

ER wrote the paper, contributed to ideas in the review, and directed research that led to this review. HH and JU carried out research that led to this review, contributed to ideas in the review, provided some figures and edited the paper.

## ACKNOWLEDGMENTS

We thank members of the Rothenberg lab and members of the laboratories of Mikael Sigvardsson, Tomoaki Tanaka, and Barbara Wold for stimulating discussions. The authors' own research on this subject was supported by fellowships from the Swedish Research Council (JU) and the Manpei Suzuki Diabetes Foundation (HH), by grants from the USPHS, R01AI095943 and R01HD076915 (ER), and by the Albert Billings Ruddock Professorship (ER).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 03 April 2019
doi: 10.3389/fimmu.2019.00688

# Control of Intra-Thymic αβ T Cell Selection and Maturation by H3K27 Methylation and Demethylation

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In addition to transcription factor binding, the dynamics of DNA modifications (methylation) and chromatin structure are essential contributors to the control of transcription in eukaryotes. Research in the past few years has emphasized the importance of histone H3 methylation at lysine 27 for lineage specific gene repression, demonstrated that deposition of this mark at specific genes is subject to differentiation-induced changes during development, and identified enzymatic activities, methyl transferases and demethylases, that control these changes. The present review discusses the importance of these mechanisms during intrathymic αβ T cell selection and late differentiation.

**Keywords: thymus, T cell development, histone methylation, histone demethyalse, polycomb, H3K27 methylation**

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***Edited by:***
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***Specialty section:***
*This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *12 October 2018*
***Accepted:*** *13 March 2019*
***Published:*** *03 April 2019*

***Citation:***
*Bosselut R (2019) Control of Intra-Thymic αβ T Cell Selection and Maturation by H3K27 Methylation and Demethylation. Front. Immunol. 10:688. doi: 10.3389/fimmu.2019.00688*

## INTRODUCTION: CHROMATIN AND CONTROL OF GENE EXPRESSION

Pioneering studies in prokaryotes have led to the paradigm that adjusting gene transcription in response to environmental signals involves transcription factors, proteins that bind specific DNA sequences (cis-regulatory elements) close to the transcription start site. Such binding promotes DNA-templated RNA synthesis by the RNA polymerase. The same paradigm governs the control of transcription in eukaryotic cells, with added layers of complexity at virtually every step, including the multiplicity of RNA polymerases, the functional overlap among trans-acting factors, and the unsuspected promiscuity of transcription factors with cis-regulatory elements. Typical genes are controlled by multiple, often tissue-specific cis-regulatory elements, potentially distantly located relative to the transcription start site. Such elements are bound by transcription factor assemblies which themselves typically recruit cofactor complexes that mediate their action on the polymerase complex.

In addition, eukaryotes use two important layers of controls of gene expression, DNA methylation and chromatin dynamics. Eukaryotic DNA is methylated on cytosines located upstream of a guanine, and stretches of such palindromic CpG dinucleotides (called CpG islands) are frequently found in cis-regulatory elements. Their methylation status is inversely correlated with gene expression (1). The impact of CpG methylation is not limited to transcriptional silencing, as it affects transcription factor binding, positively or negatively depending on the transcription factor and target sequence (2). Additionally, eukaryotic DNA is packaged into nucleosomes and higher-order nucleosome-based structures referred to as chromatin, in which DNA is tightly associated with histones, thereby restraining its accessibility to transcription factors or to the polymerase machinery. Such packaging is dynamic and subject to two sets of modifications. First, "chromatin remodeling," performed by energy-dependent enzymatic complexes, changes the

position of nucleosomes over DNA; this process is essential to "open" specific regulatory sequences for transcription factor binding or polymerase recruitment (3). Second, histone molecules themselves are subject to covalent modifications, including acetylation, methylation, and ubiquitination (4). Many of these modifications occur on specific amino-acid residues within the amino-terminal "tail" of histone molecules, that is not tightly associated with DNA. Through their combinatorial effect, these modifications constitute a high-order "code," that has a broad impact on chromatin structure and gene expression (5, 6). Covalent modifications are "written" (added) or "erased" (by catalytic removal) by specific enzymatic complexes, and recruit "reader" protein complexes that affect transcription.

Specific histone modifications are associated with specific gene expression states or regulatory regions (4). Acetylation of histone H3 on lysines 9 or 27 (H3K27Ac or H3K9Ac) is preferentially found at enhancers or promoters of expressed genes. Similarly, methylation of H3 lysine 4 is associated with active enhancers (H3K4 mono- or di-methylation) or found at the promoter of actively transcribed genes (H3K4Me3). In contrast, H3 K9 methylation, and in particular tri-methylation, is associated with heterochromatin formation. This review focuses on the methylation of H3 lysine 27 (H3K27Me3), which has attracted much interest because of its association with lineage-specific gene repression and because its impact on transcription is in large part mediated through its interactions with Polycomb Repressive Complexes (PRC), which were initially identified as controllers of homeotic gene expression in Drosophila (7, 8).

There is compelling evidence that changes in H3 K27 methylation are not simply associated with gene expression status, but have a causative role in setting gene transcription levels (9–11). However, it has been difficult to quantify the actual contribution of this mechanism because chromatin modifications and sequence-specific transcription factors serve cooperatively to control transcription, and because these mechanisms mutually affect each other with multiple examples of interactions between transcription factors and H3K27Me3 writer, eraser or reader complexes (12, 13). Additionally, the genetic tools available for such studies, i.e., inactivation of chromatin modifiers, methyl-transferases and demethylases for H3K27Me3, by definition have a broad impact on the transcriptome, complicating mechanistic studies. The present review will discuss how these mechanisms control H3K27Me3 homeostasis in the thymus and contribute to the development of αβ T cells.

## αβ T Cell Development

### Early Stages

T cell development in the thymus is a multi-step process combining cell proliferation, differentiation and survival-selection events (14). As a result, it has attracted interest not only because of the essential role of T cells in immune responses, but also because it is one of the few developmental processes that is amenable to both genetic and functional studies after the completion of embryonic development. Two main lineages of T cells can be separated based on the composition of their heterodimeric antigenic receptor: αβ and γδ T cells, respectively expressing TCRα and TCRβ, or TCRγ and TCRδ chains. All T cells derive from bone marrow precursors, and their development can be divided into three schematic steps: (i) T cell lineage commitment, common to both αβ and γδ lineages (15–17), (ii) antigen receptor gene rearrangement and commitment to either of the two main T cell lineages (αβ vs. γδ) (18, 19), and (iii) selection-maturation of αβ- and γδ-committed T cells. This review will focus on the selection and maturation of αβ lineage T cells (20), a process involving acquisition of long-term survival, choice of either of the two main lineages of αβ T cell, defined by the expression of CD4 and CD8 surface molecules (14), and intrathymic migration events that culminate in the egress of mature thymocytes to the blood circulation and their entry in secondary lymphoid organs.

### Conventional T Cell Differentiation From Early αβ Lineage Precursors

The earliest αβ lineage-committed thymocytes have successfully rearranged one of their TCRβ-encoding genes and express neither CD4 nor CD8 coreceptors ("double-negative" [DN] thymocytes) (**Figure 1**). After they have up-regulated both molecules (and are thus called "double-positive" [DP]), these cells rearrange their TCRα genes, allowing the surface expression of TCRαβ complexes which "probe" the set of MHC peptide complexes expressed by thymic epithelial cells (22). In the absence of productive MHC-peptide interactions (and therefore signaling though their TCR), these short-lived cells undergo programmed cell death in the thymic cortex within 3 days of their generation (23). In contrast, thymocytes that express an αβ TCR with appropriate affinity for MHC peptide complexes are rescued from cell death, a process referred to as positive selection (24–27); positive selection is closely associated (and possibly mechanistically linked) to the termination of TCRα gene rearrangement and changes in chemokine receptor expression that will eventually lead DP thymocytes from the cortex to the thymic medulla (28). Of note, cells with high avidity for MHC peptide complexes are either targeted for activation-induced cell death ("negative selection" by deletion) or diverted to alternate developmental fates, most notably differentiation into regulatory T cells with suppressive activity (29–31). Although the latter processes are critical for immune tolerance, they have not been shown to be affected by H3K27 methylation and will not be further discussed below.

Positively selected DP thymocytes differentiate into either CD4- or CD8-lineage T cells, defined by the cessation of either CD8 or CD4 expression and accompanied by "pre-programming" for helper vs. cytotoxic functions, respectively (32–34) (**Figure 1**). The "choice" of lineage is determined by the cell's MHC specificity, so that thymocytes that recognize MHC-II bound peptides become $CD4^+$ T cells, whereas those recognizing MHC-I-bound peptides become $CD8^+$ T cells (35). This process involves multiple transcription factors, including two with lineage specific expression, the zinc finger molecule Thpok in $CD4^+$ thymocytes and Runx3 in $CD8^+$ thymocytes (36–39). Following their CD4-CD8 differentiation, differentiating αβ lineage thymocytes undergo terminal maturation, including expression of surface receptors enabling their migration to secondary lymphoid organs after thymus exit, and of

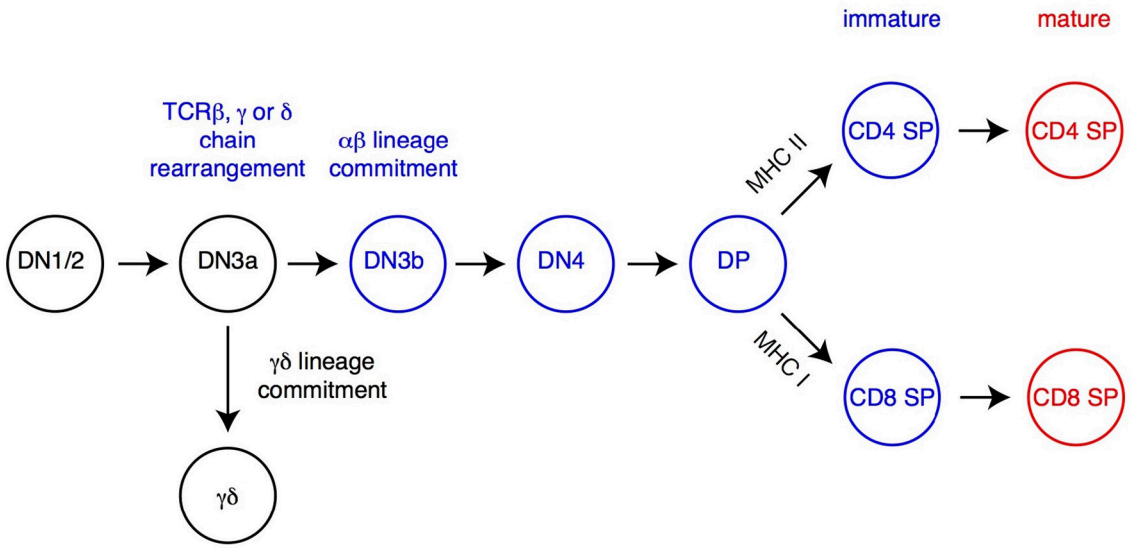


**FIGURE 1 |** Overview of T cell development. The most immature T cell precursors ($CD4^-CD8^-$DN thymocytes), $CD4^+CD8^+$ DP and CD4 and CD8 SP thymocytes are depicted. Relevant DN thymocyte subsets (defined on expression of CD25 and CD44) are shown (14, 21). Commitment to either αβ or γδ lineage occurs after successful rearrangement, at the early DN3 stage (DN3a, characterized by low expression of surface markers CD27 and CD28), of the corresponding TCR chains (TCRβ for αβ lineage cells, TCRγ and TCRδ for γδ lineage cells). DP thymocytes signaled by MHC-I or MHC-II-associated peptides undergo positive selection, differentiate into the CD8 or CD4 lineage (respectively) and complete their maturation before leaving the thymus. Cells signaled by high-affinity ligands are either deleted (negative selection, not depicted) or directed toward specific fates, including iNK T cells (**Figure 2**) or regulatory T cells (Treg, not depicted).

S1pr1, a sphingosine phosphate receptor needed for thymic egress (40, 41).

The differentiation of DP thymocytes into mature T cells involves extensive changes in gene expression (42), accompanied by modifications of the chromatin landscape (43–45). Unlike in many other differentiation processes, αβ lineage thymocytes do not divide during their intrathymic differentiation into mature T cells (23, 46). Thus, changes to the chromatin landscape cannot be mediated by "dilution" of chromatin marks but must be implemented by active mechanisms that remove or add chromatin marks on relevant genes.

## Innate-Like αβ T Cells Undergo Effector Differentiation in the Thymus

In addition to classical MHC-I or MHC-II molecules, DP thymocytes can be signaled by MHC-like molecules and differentiate into "innate-like" or "non-conventional" αβ T cells, which acquire effector functions during their intrathymic differentiation. By far the best characterized among these cells are invariant natural killer (iNK) T cells, which recognize lipids bound to CD1d molecules (47–49). In mice, most iNK T cells express a TCR including a specific Vα14 Jα18 TCRα chain paired to a restricted set of TCRVβ chains; such type I iNK T cells react with CD1d-bound α-galactosyl ceramide (αGalCer), and can be identified through their binding to a tetramerized version of this complex (**Figure 2**). In contrast, type II iNK T cells, while also CD1d-restricted, do not bind CD1d-αGalCer, and do not express the canonical Vα14 Jα18 chain (50, 51).

Regardless of the ligand they recognize, iNK T cells differ from conventional T cells in multiple respects (**Figure 2**). They

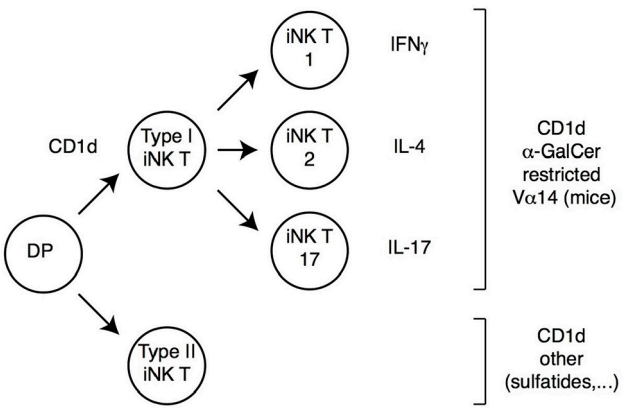


**FIGURE 2 |** iNK T cell subsets. DP thymocytes signaled by CD1d-bound lipids differentiate into iNK T cells. Most iNK T cells (Type I iNK T cells) express a Vα14 invariant TCRα chain (and exhibit a reduced TCRβ chain diversity), and recognize CD1d-bound α-Galactosyl-Ceramide (αGalCer). These cells undergo functional differentiation in the thymus (requiring the transcription factor PLZF) into IFNγ, IL-4 or IL-17-expressing effector fates (therefore referred to as iNK T1, T2, or T17, respectively). A smaller subset of CD1d-signaled cells (Type II iNK T cells) does not carry the prototypical Vα14 chain and does not recognize αGalCer; these cells also undergo functional differentiation, although specific cytokine expression patterns are not as extensively characterized as for Type I iNK T cells.

are selected in the thymus by CD1d molecules expressed by DP thymocytes (unlike conventional thymocytes which are selected by MHC-I or MHC-II molecules expressed by the thymic epithelium), and their development requires homotypic

interaction between SLAM-family receptors expressed on both the CD1d-presenting cell and the CD1d-signaled differentiating iNK thymocyte (47). As a result of these signals, iNK T precursors up-regulate the zinc finger transcription factor PLZF, and undergo intrathymic proliferation and effector differentiation (52–55). The resulting mature iNK thymocytes acquire differentiation programs and cytokine production pattern typical of Th1, Th2 or Th17 effector T cells; they express the corresponding fate-specific transcription factors (T-bet, Gata3 and RORγt, respectively) and are thus called NKT1, NKT2, and NKT17 cells (54); note that this "functional" classification is unrelated to the aforementioned distinction between type I and type II iNK T cells, which refers to ligand specificity. The acquisition of effector functions by iNK T cells in the thymus contrasts with the vast majority of conventional thymocytes, which do not acquire effector properties during their development and leave the thymus as "naïve" T cells. Importantly, analyses in recombinant mice have shown that PLZF is both necessary and sufficient for the implementation of the NK T effector program, and the control of PLZF expression and function is therefore a critical factor in iNK T differentiation. Last, most iNK T cells colonize effector sites in tissues rather than secondary lymphoid organs, most prominently the liver and gut mucosa, where they contribute to the recognition of CD1d-bound microbial metabolites (48).

## Enzymatic Activities Carrying H3K27 Methylation and Demethylation

### H3 K27 Methylation and Methyl Transferases

Nucleosomes carrying trimethylated H3K27 are preferentially located at and near promoters of silent genes (7, 56–58). There is evidence that H3K27Me3 actually contributes to transcriptional repression, mostly by recruiting Polycomb-repressive complex 1 (PRC1), which is considered as the main H3K27Me3 "reader." Recruitment is mediated by direct binding of H3K27Me3 to PRC1 Cbx subunits (8, 12, 59), although recent studies have highlighted the role of long non-coding RNAs in modulating these interactions and PRC1 functions (60–62). When recruited to chromatin, other PRC1 subunits repress transcription, notably by promoting histone H2A ubiquitination (63). Additionally, the methylation of H3 K27 prevents its acetylation and thereby indirectly contributes to transcriptional repression. Polycomb-repressive complexes 2 (PRC2) "write" the H3K27Me3 modification, through their catalytic components Ezh1 or Ezh2 methyl transferases (7, 64). Both Ezh2 and components of PRC1 are critical at multiple stages of immune cell development and responses, highlighting the importance of H3K27 methylation for cell homeostasis and differentiation (65–72).

### H3K27Me3 Demethylases

Conversely, H3K27Me3 can be "erased" by catalytic demethylation (into di- and monomethyl forms) by Jmjd3 and Utx demethylases. These enzymes belong to a large family defined by the presence of a complex catalytic domain, called JmjC (73–77). Their demethylase activity requires oxygen and α-ketoglutarate, and is therefore controlled by the cell metabolic status. The protein sequences of Jmjd3 and Utx are largely unrelated outside of their JmjC domain, suggesting that these molecules have unique, and potentially non-redundant, demethylase-independent activities. *In vitro* analyses suggest a strict correspondence between Jmjd3 and Utx catalytic activities and H3K27Me3 demethylation. That is, both molecules are highly specific for H3K27Me3, relative to other methylated histone residues (78–83), whereas most other JmjC-based demethylases have no significant *in vitro* activity on H3K27Me3.

Importantly, both H3K27 methyl-transferases and H3K27Me3 demethylases have histone-independent activities. Ezh2 methylates non-histone substrates, including cytosolic factors controlling actin polymerization and TCR signaling (66, 72). It was also reported to methylate and promote the degradation of the transcription factor PLZF needed for iNK T cell differentiation (84, 85). Jmjd3 and Utx have demethylase-independent activities and are notably part of KTM2 complexes (also called MLL), which are found at the promoter of active genes (86) and include H3 Lysine 4 histone methyl transferases (hence the KTM name). Both Jmjd3 and Utx were reported to associate with specific (and distinct) KTM2 complexes (87, 88), in which they may serve a structural (scaffold-like) role, or promote association with transcriptional regulators. In addition, Jmjd3 and Utx interact with Brg1-based chromatin remodeling complexes (89), which displace nucleosomes over the DNA (3) and have notably been implicated in the control of *Cd4* and *Cd8* expression and T cell development (90, 91). For Jmjd3, this association is independent of its demethylase activity (89) and has been reported to be important for the function of the transcription factor T-bet during the differentiation of activated $CD4^+$ T cells into Th1 effectors (92).

### H3K27Me3 Erasers: Do They Matter?

Early studies of H3K27Me3 homeostasis raised a puzzling paradox. They found that disruption of Polycomb genes (writers or readers) has a strong impact on cell differentiation and function in multiple experimental systems, including in ES cells and embryonic development, tumor development, and early hematopoiesis (93–96). This is in line with experiments in Drosophila and analyses of tumor-specific mutations in pediatric glioblastoma, which indicate that H3K27 trimethylation causes, rather than results from, transcriptional repression (10, 11). In contrast, and unexpectedly, disrupting H3K27Me3 erasing, by impairing catalytic demethylation, showed a much lesser impact. While germline Utx disruption arrests embryonic development at the time of organogenesis, this involves demethylase-independent activities of Utx, as shown by analyses of mutant mice expressing a catalytically inactive version of the protein (97–100). Germline disruption of Jmjd3, or disruption of Jmjd3 and Utx demethylase activity, are compatible with the development of most organs and systems, although it results in death of newborn mice due to the impaired development of the brain center controlling respiratory rhythm (101–103).

A tentative explanation for this apparent paradox is that "dilution" of H3K27Me3 marks at each cell division could make Jmjd3 and Utx demethylase, but not demethylase-independent, activities dispensable during differentiation processes associated with cell proliferation. In antigen-activated mature T cells,

which extensively proliferate, such "dilution" could account for the limited effect of Utx disruption on H3K27Me3 distribution during the differentiation of follicular helper T cells (104). However, other observations challenge the idea that "dilution" can efficiently clear the mark. Jmjd3 disruption increased H3K27Me3 levels at more than 2,500 genes during the differentiation of Th1 effector $CD4^+$ T cells (105), which is also accompanied by proliferation. Additionally, catalytic demethylation serves important functions *in vivo*, as it mediates in part the activity of Jmjd3 in macrophage effector differentiation (101) or in the development of the brain respiratory center (102), and of Utx in somatic cell reprogramming (106). As detailed below, studies of Jmjd3 and Utx functions in developing T cells shed light on this question.

## Role of H3 K27 Methyl Transferases and H3K27Me3 Demethylases During T Cell Development

Analyses of genomic H3K27Me3 deposition by chromatin immunoprecipitation followed by deep-sequencing (ChIPseq) suggested that this modification was important for transcriptomic changes during late αβ T cell differentiation (43, 44). Changes (increase or decrease) in H3K27 tri-methylation were detected at hundreds of promoters during the differentiation of DP into CD4 SP thymocytes (43, 44). Of specific interest were the almost complete removal of the mark at the genes encoding the CD4-differentiating transcription factor Thpok (38, 39), the S1pr1 receptor required for thymic egress (40), and the transcription factor Klf2, involved in the terminal maturation of SP thymocytes and S1pr1 expression (107). Conversely, increased H3K27Me3 decoration was observed at genes silenced during αβ T cell differentiation, including those encoding the recombinases Rag1 and Rag2. These changes in H3K27 methylation raised the possibility that mutations in Ezh1 and Ezh2 methyl transferases, or in Jmjd3 and Utx demethylases, would affect positive selection and the subsequent differentiation of αβ T cells in the thymus.

Experimental assessments of these predictions have produced mixed results. Deletion of Ezh2, the predominant H3K27 methyltransferase in the T cell lineage, has no reported impact on the differentiation of SP from DP thymocytes, unlike at earlier stages of T cell development, during the differentiation of iNK T cells, or in mature T cells (66–71, 108). This unexpected result does not imply that H3K27 methylation is not important for transcriptomic changes during the DP-SP transition, as the lack of an effect in DP thymocytes may reflect the potential functional overlap with Ezh1, highlighted in other developmental studies (64, 109–111) or the extended half-life of Ezh2 or H3K27Me3 molecules. A recent report pointed out to mechanisms controlling the stability of Ezh2 in activated T cells (112); future studies will address if it is controlled in developing thymocytes as well.

The reciprocal experiment, namely deletion of Jmjd3 or Utx targeted to DP thymocytes, showed at first glance similar results as mice lacking either or both enzymes had CD4 and CD8 SP thymocytes and T cells (44, 101, 105). However, a detailed analysis showed that both enzymes are important for late T cell differentiation (44): Jmjd3 and Utx double-deficient mice had increased numbers of mature CD4 and CD8 SP thymocytes but reduced numbers of peripheral T cells; inactivation of either enzyme resulted in more limited effects, more pronounced for Jmjd3 than for Utx, consistent with functional overlap. Gene expression analyses and reconstitution experiments showed that these enzymes were needed for the expression of S1pr1, the sphingosine receptor required for thymic egress (40), and that this requirement accounted at least in part for their impact on late T cell differentiation (**Figure 3**) (44). Although the impact of Jmjd3 and Utx double-disruption on S1pr1 expression and T cell development was limited in animals expressing a diverse endogenous TCR repertoire, it resulted in an almost complete developmental block at the SP thymocyte stage in transgenic mice in which thymocytes all expressed a single TCR specificity, or when the development of mutant thymocytes was assessed in mixed bone marrow chimera, where they developed in competition with wild-type control cells. These findings indicated that loss of Jmjd3 and Utx activities can be compensated, in part, by changes in the repertoire of thymocytes completing their differentiation, and therefore suggested that H3K27Me3 demethylases contribute to gene expression in coordination with signals coming from TCR engagement.

Analyzing the impact of these enzymes on H3K27 methylation status and the transcriptome gave unexpected results. Even though DP and SP thymocytes are non-dividing cells, the inactivation of Jmjd3 and Utx had a highly specific impact on H3K27Me3 distribution (44). Unlike in a study of Jmjd3-deficient effector T cell differentiation (105), double-deficient thymocytes showed no general trend toward increased H3 K27 tri-methylation, whether at promoters or in non-promoter regions. Rather, H3K27Me3 density was significantly enhanced at fewer than 1% of loci (44), many of which were genes at which H3K27Me3 was normally removed during the DP to the CD4 SP transition, including *S1pr1* (**Figure 3**). This indicated a role of Jmjd3 and Utx in the dynamics of differentiation-induced H3K27Me3 erasing, rather than in its steady-state homeostasis. Intriguingly, deletion of Jmjd3 and Utx failed to affect H3K27Me3 erasing at a subset of promoters induced in differentiating αβ lineage thymocytes and at which H3K27Me3 is normally removed, including that of the gene encoding Thpok (44). The latter was in line with the lack of an effect of Jmjd3 and Utx on the differentiation of CD4 SP thymocytes and Thpok expression, and suggested that additional mechanisms contribute to H3K27Me3 removal. Similarly, the differentiation of MHC I-signaled thymocytes into the $CD8^+$ was not affected by the double disruption of Jmjd3 and Utx (although the terminal maturation of CD8 SP cells was impaired to an extent similar to that of their CD4 SP counterparts).

Aside from *S1pr1*, the impact on the transcriptome of differentiating SP thymocytes was limited to a small number of genes, many of which were normally up-regulated during the terminal differentiation of SP thymocytes, including *Klf2* (44). Expression of most of these genes was reduced by the double disruption, suggesting that the impact of Jmjd3 and Utx on gene

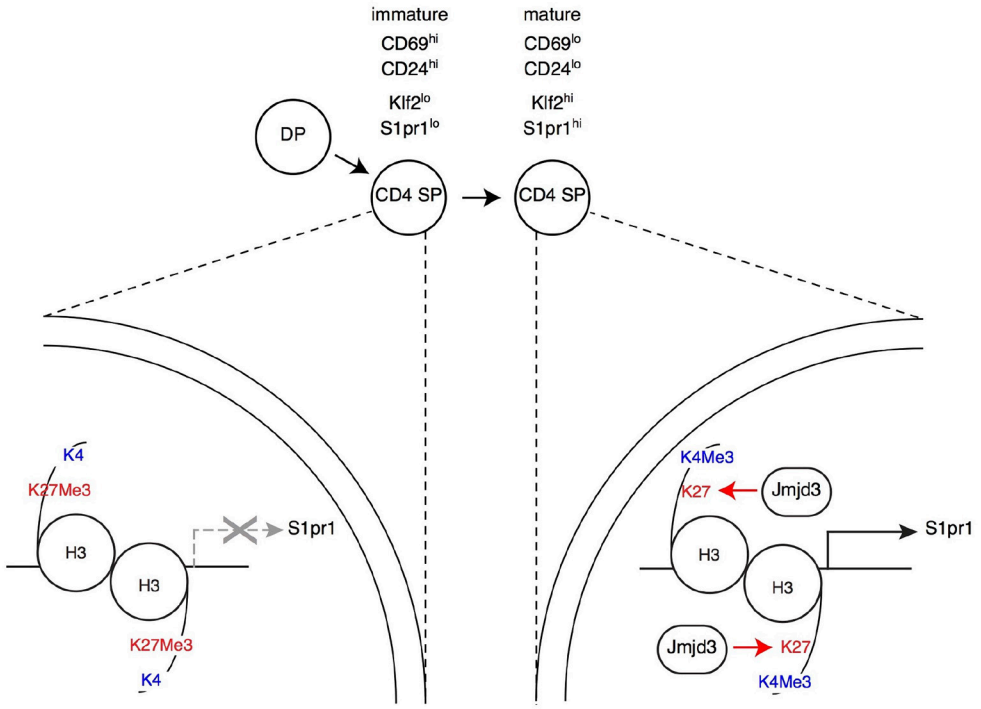


**FIGURE 3 |** Impact of H3K27Me3 demethylation on late thymocyte differentiation Immature SP thymocytes (characterized by their expression of surface markers CD69 and CD24, as indicated) have low expression of the surface receptor S1pr1 (needed for thymic egress) and of the transcription factor Klf2 (needed for S1pr1 expression). Expression of both genes increases in mature SP thymocytes, allowing their export to the bloodstream and secondary lymphoid organs. In immature SP cells, the promoters of *S1pr1* and *Klf2* are enriched in the repressive H3K27Me3 mark, whereas the "active" H3K4Me3 mark is absent (**left**, depicted here for *S1pr1*). Thymocyte maturation is accompanied by an inversion of this pattern at both genes **(right)**. The H3K27Me3 demethylase Jmjd3 (with functional overlap with the related protein Utx, not depicted) is needed to "erase" the H3K27Me3 mark at *S1pr1*, for *S1pr1* expression and for thymic egress. Note that Jmjd3 is expressed at similar levels in both mature and immature SP cells (not shown in the latter for simplicity), suggesting that it is recruited to target genes through interactions with sequence-specific transcription factors.

expression was mostly stimulating (in line with their "erasing" the repressive H3K27Me3 mark) (44).

## H3 K27 Methylation and iNK T Cell Development

Although the development of iNK and conventional T cells differs in important respects, both subsets differentiate from DP thymocytes upon engagement of their TCR by intrathymic ligands. Nonetheless, because of developmental steps unique to iNK T cells, disruption of H3 K27 methylation or demethylation has specific effects on their differentiation (summarized in **Supplementary Table 1**). Initial hints came from analyses of Ezh2-deficient thymocytes (70). Although it has no detectable effect on the development of conventional T cells, Ezh2 disruption in DP thymocytes results in increased numbers of iNK T cells, an effect particularly pronounced on IL-4-producing NKT2 cells and associated with increased PLZF expression.

Most remarkably, Ezh2 disruption "uncouples" iNK T cell differentiation from TCR specificity. Normally, PZLF expression and the acquisition of effector functions are characteristic of CD1d-restricted NK T cells, and of related "innate" T cells subsets restricted by non-classical MHC or MHC-like molecules, including mucosal-associated invariant T (MAIT) cells (113). Unexpectedly, Ezh2 deletion resulted in the appearance of large populations of T cells expressing PLZF, producing effector cytokines (including IL-4 and IFNγ), but without detectable binding to αGalCer-CD1d complexes and therefore distinct from type I iNK T cells (70). Additional lines of evidence supported the conclusions that these "NK T wannabe" are not type II NK T cells. Unlike type II NK T cells (50), they express a diverse TCR repertoire characteristic of conventional T cells, and they could develop in mice expressing an MHC II-restricted transgenic TCR specific for ovalbumin, which normally directs the differentiation of conventional $CD4^+$ T cells. In line with their expression of PLZF, Ezh2-deficient NK T cell "wannabes" had no H3K27Me3 accumulation at the promoter of the gene encoding this factor, unlike conventional T cells (70). Thus, these experiments indicated that H3K27Me3 methylation restrains PLZF expression and effector differentiation to CD1d-restricted T cells and other subsets of innate T cells.

Studies of histone demethylase functions provided a mirror image of these findings. In contrast to their selective impact on late thymic maturation in conventional thymocytes, Utx and to a lesser extent Jmjd3 were found to be important for multiple aspects of iNK T cell development (13, 70, 114). Inactivation of both enzymes causes a broad block in the development of

iNK T cells in the thymus, with a similar impact on liver iNK T populations. The block is contemporary with the up-regulation of PLZF and the acquisition of effector functions. However, there is no evidence that Utx is needed for PLZF up-regulation. Rather, it seems important to enforce the PLZF-mediated transcriptomic program characteristic of iNK T cell differentiation; consistent with this idea, Utx binds to PLZF molecules in iNK T cells (13). Of note, it is possible that additional mechanisms mediate the impact of Utx and Jmjd3 on iNK T cells, as the developmental block in Utx-deficient iNK T cells was more marked for T-bet-expressing and IFNγ-producing NKT1 cells than for the NKT2 and NKT17 subsets. Future studies will address these questions.

### Mechanistic Considerations

An important question raised by these observations is whether the impact of Jmjd3 and Utx on T cell development is mediated by their catalytic demethylase activity, since it is dispensable in embryonic development (97–100, 103). Multiple lines of evidence point to the importance of catalytic demethylation in developing T cells. Initial insight came from comparisons of female and male mice, because the gene encoding Utx (*Kdm6a*) is located on chromosome X. Accordingly, female cells carry (and express) two *Kdm6a* alleles; in contrast male cells express Utx from their single *Kdm6a* allele and the Y chromosome-located *Uty* gene, encoding the Utx-related protein Uty. Although lacking demethylase activity, Uty is functionally redundant with Utx during the development of male mice (103). In contrast, the impact of Jmjd3 and Utx disruption on conventional CD4 SP thymocyte maturation is the same in female and male cells (44). This indicates that demethylase-dead Uty is insufficient to promote thymocyte development, and therefore supports the idea that H3K27Me3 demethylase activity is required.

Three results from analyses in iNK T cells corroborate this conclusion. First, as in conventional thymocytes, Uty failed to rescue the defect caused by Utx disruption (114). Second, the combined deletion of Utx and Ezh2 resulted in a milder defect in iNK T differentiation, suggesting that the two proteins have opposite effects on a common target (114). Last, retroviral transduction "rescue" experiments directly demonstrated that a mutant of Utx lacking catalytic activity failed to restore iNK T cell differentiation from Utx-deficient thymocytes, unlike wild-type Utx (13).

Studies in thymocytes also raised the intriguing possibility that demethylase and demethylase-independent functions synergize for optimal gene expression. In mature conventional thymocytes, *S1pr1* gene expression depends both on H3K27Me3 demethylase activity (44) and on Ptip1 (115) an Utx-associated component of KTM2 complexes (87), suggesting that Utx could contribute to both functions. In differentiating iNK T cells, it was reported that Utx affects the chromatin accessibility of super-enhancers (chromosomal regions associating multiple enhancer elements and operationally defined by continuous high density stretches of H3K27Ac in ChIPseq experiments) and therefore presumably their activation (13). Indeed, Utx promoted expression of genes located near Utx-dependent super-enhancers. These results support the idea that Utx, through recruitment to gene regulatory regions by sequence-specific transcription factors (including PLZF in iNK T cells) contributes to enhancer activation.

## CONCLUDING REMARKS AND PERSPECTIVES

The work summarized in this review highlights the importance of H3 K27 methylation in the development and function of T cells. Analyses of its function during cell differentiation face numerous challenges, including (i) the genome-wide deposition of the mark and its implied pleiotropic impact, (ii) the multiplicity of protein and protein complexes involved in the "writing," "reading," and "erasing" of the mark, with various degree of functional overlap, and (iii) the multifunctional nature of many components, and specifically H3K27Me3 demethylases. Nevertheless, studies over the past few years have brought important clarifications on the function of this mark in T cell development, both on its impact on the transcriptome of differentiating cells and its biological consequences, and on the mechanisms that underpin this impact.

Several important questions remain to be addressed. In particular, while it is clear that complete disruption of PRC1 activity (through inactivation of both Ezh1 and Ezh2, or of the non-redundant component Suz12) abrogates H3 K27 methylation and results in a major disruption of cell homeostasis and differentiation, the consequences of the double Jmjd3-Utx disruption are less striking, both on H3K27Me3 and developmental fates. At the gene level, evidence in non-dividing thymocytes that H3K27Me3 is "erased" despite Jmjd3 and Utx disruption (e.g., at the gene encoding Thpok) (44) indicates the involvement of additional mechanisms. While the involvement of other JmjC-family enzymes in H3K27Me3 demethylation cannot be excluded, there is little supporting evidence at present (74). Only Kdm4 family members have been reported to act on H3K27Me3 (116), and their actual activity remains to be clarified (117). Of note, the fact that Jmjd3 and Utx are required for H3K27Me3 clearance at other promoters (e.g., *S1pr1*) indicates that such effects would be gene specific. A distinct and tantalizing possibility is that, even in non-dividing cells, H3K27Me3 is functionally erased by nucleosome replacement rather than (or in addition to) catalytic demethylation. Replacement mechanisms (118) deposit nucleosomes containing the H3 variant H3.3 at actively transcribed genes (119–121) and could therefore "erase" the H3K27Me3 mark if such newly deposited nucleosomes contained un-methylated H3.3.

It will be important to integrate the dynamics of H3 K27 methylation in the broader context of epigenetic control of gene expression. Much progress has been made understanding the mutual relationships of activating and repressive histone marks. H3 K27 methylation and acetylation are biochemically mutually exclusive, and accordingly exert opposite effects on gene expression. More strikingly, evidence is accumulating that H3 K4 and K27 methylations, which are typically found in active vs. silent genes or enhancers, respectively, are the end products of enzymatic complexes that coordinate writing of one mark with erasure of the functionally opposite mark. That is, Ktm2/MLL complexes associate both an H3 K4 methyl transferase and

H3K27Me3 demethylases, whereas PRC2 complexes associate H3 K27 methyl transferase activity and H3K4Me3 demethylases of the Jarid1-RBP2-Kdm5 family (62, 122, 123).

How these activities integrate with the other key histone repressive mark, H3 K9 methylation, has been addressed in various experimental systems (124) but remains to be explored in T cells. While H3K9Me3 has been traditionally associated with constitutive heterochromatin, there is ample evidence that H3 K9 methyl transferases contribute to the control of lineage-specific gene expression, including those involved in T cell development and function (125–128). Additional data suggest that PRC2 and H3 K9 methyl transferase complexes could share components, including Jarid2 (or Jumonji, the founding member of the JmjC family), which was shown to restrain PLZF expression in and iNK T differentiation of thymocytes and to promote H3 K9 but not K27 trimethylation at the promoter of the gene encoding PLZF (129).

Last, histone modifications are super-imposed on the dynamics of DNA methylation, which was the first epigenetic modifications identified in developing T cells at the *Cd4* and *Cd8* loci. T cell development is accompanied by reduced methylation at CpG islands in both loci following commitment to the αβ lineage and onset of CD4 and CD8 expression, followed by partial, lineage specific, remethylation of the silenced coreceptor gene (130). More recent studies have pointed to the importance of DNA methylation in the maintenance of *Cd4* silencing in mature $CD8^+$ T cells, suggesting a yet to be determined coupling between the mechanisms writing the methyl mark (presumably involving DNA methyl transferases Dnmt3 isoforms) and those ensuring the active repression of *Cd4* in CD8-differentiating thymocytes (131).

Conversely, work in the past few years has identified a complex mechanism erasing cytosine methylation, without actual catalytic demethylation, initiated by oxidization of methyl cytosine catalyzed by Tet1, Tet2, and Tet3 enzymes (of the ten-eleven-translocation family) (132, 133). Although the full impact of Tet enzymes on the development of conventional αβ T cells remains to be elucidated, they are essential to restrain the activation of iNK T cells (134, 135). While the current evidence indicates an impact on cell proliferation, deletion of Tet enzymes also impaired the differentiation of NKT1 cells, suggesting an additional impact on cell differentiation. Thus, it will be important to understand the respective contributions of DNA methylation and H3 K27 trimethylation in the control of T cell homeostasis and function, especially in the light of studies suggesting that DNA methylation antagonizes H3K27Me3 deposition (124).

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

## ACKNOWLEDGMENTS

I thank Laura Chopp and Melanie Vacchio for critical reading of the manuscript and Avinash Bhandoola and Paul Love for useful discussions. Research work in the author's laboratory is supported by the Intramural Research Program of the National Cancer Institute, Center for Cancer Research, National Institutes of Health.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00688/full#supplementary-material

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 26 April 2019
doi: 10.3389/fimmu.2019.00922

# A Review in Research Progress Concerning m6A Methylation and Immunoregulation

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Over 100 types of cellular RNA modifications have been identified in both coding and a variety of non-coding RNAs. N6-methyladenosine (m6A) is the most prevalent and abundant post-transcriptional RNA modification on eukaryote mRNA, and its biological functions are mediated by special binding proteins (i.e., methyltransferases, demethylases, and effectors) that recognize this modification. The presence of m6A on transcripts contributes to diverse fundamental cellular functions, such as pre-mRNA splicing, nuclear transport, stability, translation, and microRNA biogenesis, implying an association with numerous human diseases. This review principally summarizes recent progress in the study of m6A methylation mechanisms and relevant roles they play in immunoregulation.

**Keywords: N6-methyladenosine, binding proteins, mechanisms, immunoregulation, cellular functions**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology*

***Received:*** *01 October 2018*
***Accepted:*** *10 April 2019*
***Published:*** *26 April 2019*

***Citation:***
*Zhang C, Fu J and Zhou Y (2019) A Review in Research Progress Concerning m6A Methylation and Immunoregulation. Front. Immunol. 10:922. doi: 10.3389/fimmu.2019.00922*

## INTRODUCTION

In 1970, m6A was first recognized as an abundant nucleotide modification in eukaryotic messenger RNA (1), by far the most prevalently known of over 100 kinds of RNA modifications identified in various classes of RNAs, including mRNA, rRNA, tRNA, snRNA, microRNA (miRNA), and long non-coding RNA (lncRNA) (2). m6A is present in 0.1–0.4% of all adenosines in global cellular RNAs and accounts for ∼50% of all methylated ribonucleotides (3). However, little was known about its potential functional significance and extent transcript identities until very recently. m6A occurs primarily in two consensus sequence motifs, G m6A C (∼70%) and A m6A C (∼30%) (4, 5). Long internal exons, locations upstream of stop codons, and the 3′-UTR of mRNA are preferred modification sites for m6A, implying roles involving translational control, influencing affinities of RNA binding proteins or unique m6A-derived transcriptome topology (6–9).

The discovery of proteins involved in m6A regulation has been among the most significant achievements in this area of study, elucidating their roles as "writers" (m6A methyltransferases), "erasers" (m6A demethyltransferases), and "readers" (effectors recognizing m6A) (10). Methyltransferase like-3 (METTL3, also known as MT-A70) was among the first of all identified core writer components, responsible for installing m6A on RNA (11), Other core enzyme components such as METTL14 (12, 13), in addition to accessory components including Wilms' Tumor 1-associating Protein (WTAP) (12, 14), KIAA1429 (15), RNA binding motif protein 15 (RBM15), RBM15 paralog (RBM15B) (16), and zinc finger protein 217 (ZFP217) (17) have also been studied. The METTL3 complex acts at the consensus RRACH motif (R=A or G, H=A, C, or U) (18). METTL3 or METTL14 depletion reduces the ratio of m6A/A, while knockdown of WTAP decreases amounts of METTL3 complex bound to RNA, implying that WTAP may be responsible for the recruitment of RNAs (12, 14). m6A demethylase fat mass and obesity-associated (FTO) protein

was the first recognized "eraser" enzyme that reverses RNA modification and controls cellular homeostasis (19). ALKB homolog 5 (ALKBH5) was also recognized as a demethylase involved in alkylated DNA repair (20). Three "reader" proteins that can directly interact with m6A sites via their YTH domains have been discovered to date. YTH domain-containing family (YTHDF) proteins 1 and 3 promote translation of m6A-modified mRNA via interaction with translation initiation factors (21, 22), whereas YTHDF2 promotes RNA degradation via recruitment of m6A modified mRNA to nuclear processing bodies (P bodies) (23).

m6A as an mRNA modification that is abundant in some viruses and nearly all eukaryotes (2). A variety of cytopathologic processes involving nuclear RNA export, splicing, mRNA stability, circRNA translation, miRNA biogenesis, and lncRNA metabolism have recently been linked to aberrant levels of m6A (**Figure 1**) (24–26). In addition, m6A modification has been associated with numerous physiological and pathological phenomena, including obesity, immunoregulation, yeast meiosis, plant development, and carcinogenesis (2, 27).

This review summarizes the most recent progress in research concerning m6A and analyzes newly identified roles this modification plays in the regulation of gene expression and immune responses.

## m6A WRITERS, ERASERS, AND READERS

### Writers

A methyltransferase complex within the nuclear speckle, mainly consisting of METTL3 and METTL14, installs m6A modification on distinct target RNAs via the methyl groups of S-adenosylmethionine (SAM) transferase (11, 12). Two subunits constitute an m6A methyltransferase complex: MT-A (200-kDa) and MT-B (800-kDa). METTLE3 (or MT-A70), a 70-kDa protein, was first identified in 1997 and possesses a SAM-binding domain and a DPPW motif (Asp-Pro-Pro-Trp). It serves as a catalytic subunit and constituent of a 200-kDa methyltransferase complex isolated from the nuclear extract of HeLa cells (11). Knockdown of METTL3 leads to decreased m6A levels and concomitant apoptosis of human HeLa and HepG2 cells (12). The other core writer component, METTL14, harbors two conserved functional domains, a SAM-binding domain and an EPPL motif (Glu-Pro-Pro-Leu) involved in catalyzing the methylation reaction, an additional N-terminal coiled-coil domain for mediating protein–protein interaction, and a G-rich sequence at the C-terminal end (10). METTL3-METTL14, in a ratio of 1:1, form a stable heterodimer (1 MDa) localized at nuclear speckles (28). In addition, METTL14 supports METTL3 in recognizing special RNA substrates. The heterodimer preferentially methylates RNA substrates exhibiting a consensus GGACU domain and contains a moderate preference for substrates with less structure (29). WTAP, a splicing factor, acts as the third crucial component of the writer. Although it does not possess any recognizable domains or motifs, WTAP binds to the METTL3-METTL14 heterodimer and abundantly regulates m6A deposition inside cells (12). WTAP may mediate the position of the heterodimer on nuclear speckles and recruit target RNA for m6A modification (14), thus indirectly enhancing the catalytic capacity of the writer. WTAP may also recruit unknown factors to bind to the methyltransferase complex and modulate methylation.

### Erasers

FTO is the first discovered demethylase that removes methyl groups from m6A (19), indicating that m6A is a dynamically reversible RNA modification. FTO modulates alternative splicing of RUNX1T1, which is involved in adipogenesis (30), as well as the 3′-end mRNA processing in 293T cells (31). ALKBH5 is the second identified demethylase and exhibits distinct physiological functions (20). An ALKB domain is commonly situated in the middle regions of FTO and ALKBH5, consisting of two active motifs termed as HXDXnH and RXXXXXR (X = any amino acid), which binds to Fe(II), as well as α-ketogluterate (α-KG) and substrate, respectively. Compared to ALKBH5, a distinctive fold mediates protein interaction at the C-terminal end of FTO. The N-terminus of ALKBH5 is characterized by an additional A-rich motif responsible for localizing ALKBH5 at nuclear speckles (10). FTO and ALKBH5 have substantial tissue-specificity and diverse intracellular localization. FTO has been reported to be highly abundant in adipose and cerebral tissue, while ALKBH5 has been reported to be primarily expressed in the testes (20). Thus, demethylation in some tissues may be performed solely by either FTO or ALKBH5.

### Readers

While writer proteins install m6A at a specific domain on target RNA, altering its secondary or tertiary structure (32), another class of proteins, termed readers, recognize, and preferentially bind the RNA to confer its fate and regulate downstream functions. An RNA-pull down assay initially revealed that YTH domain-containing family proteins YTHDF1-3 rich in the P/Q/N motif were discovered in mammalian cells to be m6A readers that recognize the consensus sequence G[G > A]m6ACU (7, 23). Among this protein family, YTHDF2 has the strongest affinity to localize P bodies via its P/Q/N motif where the concentration of mRNA turnover factors in facilitating RNA degradation (23). Notably, YTHDF2 recruits the deadenylase complex CCR4-NOT via the YTHDF2 N-terminus and mediates RNA degradation in mammalian cells (33). YTHDF1 was found to interact with translation initiation factors to evoke m6A-containing mRNA translation (21). YTHDC1, a YTH domain-containing protein, was subsequently validated as a nuclear m6A reader and showed almost completely overlapping sites with m6A in nuclear RNAs. YTHDC1 recruits the serine and arginine-rich splicing factor 3 (SRSF3), restricts exon-skipping factor SRSF10 binding, and promotes exon inclusion (34). YTHDC1 also plays a critical role in pre-mRNA processing in the oocyte nucleus via interaction with the pre-mRNA 3′-end processing factors CPSF6, SRSF3, and SRSF7 (35). A nuclear m6A reader has been identified recently within the pre-mRNA consensus motif RRACH that destabilizes the stem structure, enabling the U-tract motif to become exposed as a single-strand and become more accessible for heterogeneous nuclear ribonucleoprotein (hnRNP) HNRNPC binding, altering alternative splicing of target RNA (36). HNRNPG is another

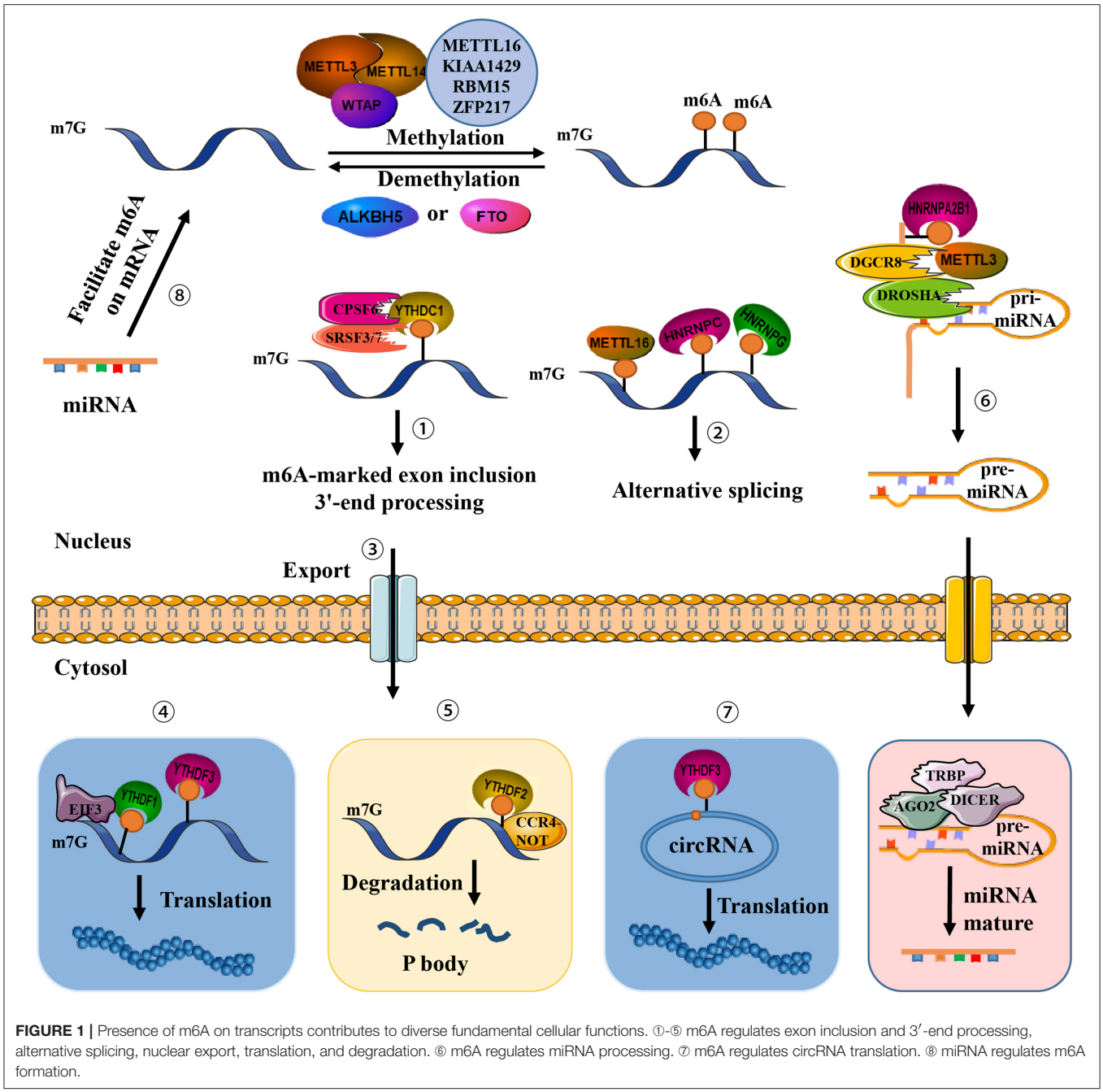


**FIGURE 1 |** Presence of m6A on transcripts contributes to diverse fundamental cellular functions. ①-⑤ m6A regulates exon inclusion and 3′-end processing, alternative splicing, nuclear export, translation, and degradation. ⑥ m6A regulates miRNA processing. ⑦ m6A regulates circRNA translation. ⑧ miRNA regulates m6A formation.

critical protein containing a low-complexity domain at its C-terminus. Arg-Gly-Gly repeat sequences bind directly m6A sites and alter the expression and alternative splicing pattern of target mRNA (37). Additionally, the hnRNP family protein HNRNPA2B1 binds to the m6A RGAC motif in a subset of primary miRNA transcripts, recruits a microprocessor complex to facilitate miRNA processing, and elicits alternative splicing effects similar to those of METTL (37, 38).

A number of m6A readers have been recently identified in addition to the aforementioned proteins. FMR1, a protein that contains a RGG domain, three KH domains, and two Agenet domains at its N-terminus, preferentially recognizes the sequence GGm6ACU via RGG domain binding and represses translation by stalling ribosomal translocation (39, 40). Further data revealed that a class of proteins termed m6A-repelled proteins preferentially bind only to unmodified mRNA. The stress granule proteins G3BP1 and G3BP2 are reported to be the most forceful of the repelled proteins. G3BP1 consistently interacts with the GGACU but not the GGm6ACU motif to stabilize target RNA (39, 41). Interestingly, METTL16 serves as a both m6A writer and reader of U6 snRNA, and is concerned with mRNA splicing (42). METTL16-dependent sites are mainly located in introns or exon-intron boundaries, unlike common m6A sites.

## RESEARCH TECHNIQUES FOR M6A

### m6A Seq and MeRIP-Seq

The profile of m6A sites throughout the transcriptome remained unclear until two independent sequencing methods, m6A Seq and MeRIP-Seq (m6A-specific methylated RNA immunoprecipitation (IP) with next-generation sequencing), were established in 2012 (7, 9). In brief, the mRNA is first randomly fragmented into approximately 100 nt prior to IP using m6A-specific antibody. Then, RNA-seq is applied to the RNA pool, in which m6A-tagged RNA fragments are enriched. These two methods revealed m6A to be a pervasive and dynamically reversible modification, particularly enriched in 3′-UTR regions and near mRNA stop codons. It was also reported that m6A sites in some RNAs have very high levels of conservation between human and mouse transcriptomes. Although the aforementioned research methods are easily and effectively applied toward the field of epitranscriptomics, a resolution of approximately 200 nt is impractical to precisely identify m6A positions.

### PA-m6A Seq

PA-m6A-seq (photo-crosslinking-assisted m6A sequencing) has recently been developed as one of two UV-induced RNA-antibody crosslinking strategies (43). It significantly improves resolution on the basis of m6A-seq/MeRIP-seq. Addition of 4-thiouridine (4SU) into medium results in it embedding into RNA. This is followed by anti-m6A antibody IP with subsequent m6A-containing RNA crosslinking using 365-nm UV light. Afterwards, RNase T1 digests the crosslinked RNA to about 30 nt, allowing for efficient sequencing. Within approximately 23 nt, single consensus methylation sequences can be determined by PA-m6A-seq at single-base resolution. However, m6A sites cannot be detected if the distance between them and those of 4SU incorporation is too great. This UV crosslinking strategy effectively provides insight into m6A-containing RNA and RNA-binding proteins.

### m6A-CLIP/IP and miCLIP

Additional UV crosslinking strategies are known as m6A-CLIP/IP and miCLIP (m6A individual-nucleotide-resolution crosslinking and immunoprecipitation) (8, 44). Under 254-nm UV light, crosslinking can be brought about between RNA fragments and anti-m6A antibodies. The crosslinked fragments are subsequently retrieved by applying proteinase K and reverse transcription, thereby leading to highly specific mapping of mutation or truncation profiles to precise m6A sites at single-nucleotide resolution throughout the transcriptome.

### SCARLET and m6A-LAIC-seq

To determine the m6A status of a random mRNA or lncRNA from a total RNA pool rather than from purified distinct RNAs and quantify m6A stoichiometry at specific locations, SCARLET (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography) was developed in 2013 (45). To achieve site-specific cleavage, RNase H is added to the total RNA sample followed with radiolabeling using $^{32}$P. Labeled RNA fragments are then splint-ligated to DNA oligonucleotides by DNA ligase. All RNAs are subsequently digested completely with RNases T1/A, but $^{32}$P-labeled sites remain protected from digestion. Finally, after gel purification and nuclease P1 digestion, samples are analyzed using thin-layer chromatography (TLC). Although SCARLET can precisely determine m6A modification sites at single-nucleotide resolution, it is incapable of use in high throughput screening and is time-consuming as a whole (2).

A recently established m6A-LAIC-seq (m6A-level and isoform-characterization sequencing) method can also quantify m6A presence, even on a transcriptome-wide level (46). First, full-length RNA IP is performed employing excess anti m6A antibody. The sample is next treated with External RNA Controls Consortium (ERCC), and the internal standards are supernatant (m6A-negative fraction) and eluate (m6A-positive fraction). In the two pools, an ERCC-normalized RNA enrichment ratio quantifies m6A modification of each gene, followed by library construction and sequencing. m6A-LAIC-seq was found to indicate that under 50% m6A modification levels exist in a majority of genes, and that 3′-ends of RNA molecules containing m6A would become shorter due to proximal alternative polyadenylation sites.

Identifying precise m6A sites in RNA transcripts is a critical step toward comprehending the biological functions of this modification. Although existing methods have been adopted widely in multiple areas and resulted in some achievements, challenges remain at both single-base and quantitative sequencing levels (47). For example, how to relatively distinguish the m6A levels of different sites in a common transcript, and the establish of new methods getting rid of m6A-special antibody will make perfect sense.

## m6A MODIFICATION AND NCRNA

Identification of m6A methyltransferases, demethylases, and effectors have revealed that m6A modification is critical throughout the whole RNA life cycle, including pri-mRNA splicing, mRNA nuclear transport, molecular stability, translation, and subcellular localization (48), additionally involved in miRNA biogenesis, lncRNA processing, and circRNA functions. m6A modification is especially abundant in circRNA, and only a single m6A residue is sufficient to drive circRNA translation via recruitment of YTHDF3 and translation initiation factors eIF4G2 and eIF3A. Hundreds of endogenous circRNAs thus possess translation potential (**Table 1**). Of note, m6A modification is reversely regulated by miRNA. Via sequence pairing with mRNA containing miRNA targeting sites, miRNA modulates METTL3 binding to target RNA, resulting in an increase of m6A modifications. Mouse embryonic fibroblasts are thus reprogrammed into pluripotent stem cells (18).

## PHYSIOLOGICAL AND PATHOLOGICAL IMMUNE FUNCTIONS

m6A modification is necessary in the biogenesis and functions of RNA. Modification aberrancies have been associated with various pathophysiologies. Recently, m6A modification has been recognized as crucial regulator in T cell homeostasis

**TABLE 1 |** m6A affects ncRNA genes.

| m6A targets | Writers/effectors | Mechanisms /functions | References |
|---|---|---|---|
| pri-miRNA | METTL3/HNRNPA2B1 | Recruiting DGCR8 to pri-miRNA transcripts to facilitate miRNA processing | (38, 49) |
| lnc-XIST | RBM15/RBM15B/ YTHDC1 | Responsible for XIST-mediated transcriptional repression | (16) |
| lnc-MALAT1 | HNRNPG | Binding to the m6A-modified purine-rich hairpin to regulate splicing and gene expression | (37) |
| circRNA | YTHDF3 | YTHDF3 recruits eIF4G2 and eIF3A driving translation of circRNA containing m6A modifications | (26) |
| circRNA | YTHDF2 | mediating circRNA stability yet independently accelerates circRNA degradation | (50) |

and the immune response to bacterial or viral infection. Selectively altered m6A levels along with other types of immunotherapies may be efficient management strategies in a variety of immunological diseases.

## m6A Methylation and T Cell Homeostasis

The suppressor of cytokine signaling (SOCS) protein family encodes inhibitory proteins involved in JAK-STAT signaling, including SOCS1, SOCS3, and CISH, playing a vital role in T cell proliferation and differentiation (51, 52). In naive T cells, which are induced by "gatekeeper" IL-7 stimulation as well-known immediate-early genes, SOCS genes control IL-7 signal and play critical roles in adaptive immunity (51, 52). All three SOCS genes were found to undergo m6A modification via the RNA-IP assay. The modification induces mRNA degradation of SOCS genes that initiates naïve T cells re-programming for proliferation and differentiation. IL-7/JAK signaling is activated *in vitro* and *in vivo* via relieving the inhibition on IL-7-STAT5 signaling (**Figure 2A**) by an evolutionarily-conserved m6A-dependent mechanism (53). This modification is likely a crucial factor in the regulation of immune homeostasis and the mitigation of various autoimmune diseases.

Regulatory T cells (Tregs) are a crucial specialized T cell lineage, and are involved in reducing inflammation and immunosuppression (54). Chronic intestinal inflammation in METTL3 knockout mice has occurred when the mice reach at least 3 months of age. Co-culture assay of naïve $CD4^+$ T cells and Tregs with m6A KO revealed naïve T cells to exert more rapid proliferative influences due to a complete lack of suppressive function for Tregs (**Figure 2A**) (55). In $CD4^+$ T cells, m6A modification is indeed enriched at the GG/AACA/U domain at 3′-UTR and at 5′-UTR of SOCS genes. Decreased m6A modification enhances the mRNA stability of SOCS genes, thereby blocking transduction of cytokine signaling in the IL2-STAT5 pathway (55). As this pathway is critically essential for the suppressive function and stability of Tregs (54), m6A levels are considerably responsible in controlling naïve T cells homeostasis.

## m6A Methylation and Inflammatory Response

Dental pulp inflammation, which can progress to pulp necrosis and periapical diseases, is characterized by a partial accumulation of inflammatory mediators and is a typical inflammatory disease (56, 57). In pulpal and periapical diseases, it is recognized that bacterial infection is a major pathogenic factor (58). Recent findings indicate that in LPS-treated human dental pulp cells (HDPCs), METTL3 expression and m6A modification levels are up-regulated instead of METTL14, FTO, and ALKBH5. Moreover, METTL3 knockdown decreases the expression of LPS-induced inflammatory cytokines, including IL-6, IL-8, GRO, Gro-α and RANTES. At the same time, NF-κB and MAPK signaling pathway activation is suppressed (58). MyD88 exists in two forms (MyD88L and MyD88S). MyD88Land TRIF pathway activate the innate immune response by transducing TLR signals, whereas MyD88S inhibits the response (59). Further research revealed that m6A inhibition significantly increases MyD88S mRNA levels, suggesting that m6A mediates alternative splicing of MyD88 and mediates the LPS-induced inflammatory reaction in HDPCs (**Figure 2B**) (58). Whether or not m6A also regulates TRIF signaling remains unclear.

## m6A Methylation and Antiviral Immunity

Influenza virus and Rous sarcoma virus were previously reported to produce viral transcripts with m6A modifications. At an antiviral innate state, RNAs containing m6A modifications is unable to stimulate RIG-I-mediated antiviral signaling and induce interferon expression (60). Further research has suggested that m6A modification is involved in the export and translation of signaling molecules, including MAVS, TRAF3, and TRAF6, thus regulating interferon production in the antiviral innate immune response (**Figure 2C**) (61). The DEAD-box (DDX) helicase family contains 12 conservative domains, many of which have been identified vital in the recognition of viral nucleic acids and regulation of downstream pathways (62–65). DDX46 was recently found to negatively regulate innate antiviral transcripts via recruitment of ALKBH5. This results in reduced m6A levels on MAVS, TRAF3, and TRAF6 RNA and prevents their transport from nucleus into cytoplasm, thus reducing their translation. Moreover, the target mRNA CCGGUU motif is responsible for the effects DDX46 exerts on the antiviral innate immune response by decreasing the production of type I interferons (61). DDX3 also interacts with ALKBH5, the only protein among the identified methyltransferases and demethylases as partnering with DDX3 via the ATP-binding domain of DDX3 and the DSBH domain of ALKBH5. DDX3 is involved in diverse biological processes via the interactions between its different domains and many distinct proteins (66). Interestingly, recent article revealed a seemingly opposing mechanism of m6A in type I interferon response to the herpesvirus human cytomegalovirus (HCMV) infection. m6A level is dramatically upregulated in primary human foreskin fibroblasts infected by HCMV, and required for viral propagation (67). Following infection in METTL3-depleted cells, decrease of m6A modification results

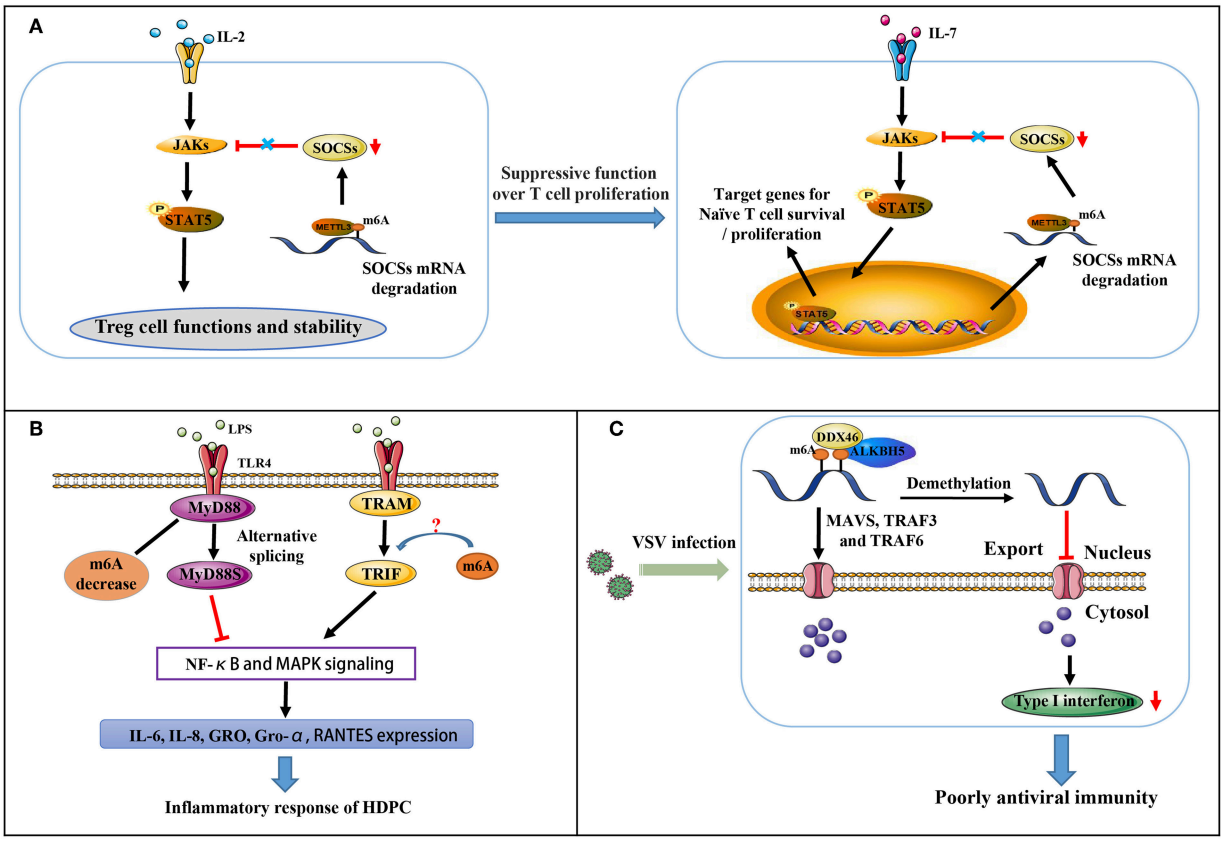


**FIGURE 2 |** Partial immune mechanisms regulated by m6A modification. **(A)** m6A methylation regulates the suppressive function of Tregs on naïve T cells. **(B)** m6A mediates MyD88 alternative splicing that is responsible for LPS-induced inflammatory reactivity in HDPCs. **(C)** m6A represses type I interferon production in an innate antiviral state.

in enhanced mRNA stability of IFNB and sustained IFN-β production, the main type I interferon in human non-immune cells, thus triggering a stronger antiviral response to block HCMV growth. Three putative adenosines proximal to stop codon are mechanistically direct targets of m6A, responsible for IFNB mRNA stability. It is probable that for different viruses, the contribution of m6A machinery for immune response may vary even adverse.

Human $CD4^+$ T cells infected by HIV-1 can trigger a massive m6A increase in both T cells and HIV-1 mRNA (68). In the CDS and UTR sequences of HIV-1 mRNA, additional splice junctions and splicing regulatory domains have been identified to possess 14 methylation peaks. Gene ontology analysis has revealed that 56 host genes with special m6A modification are active in viral infection. Silencing of METTL3/METTL14 or AKBH5 either decreases or increases HIV-1 replication, respectively. Mechanically, the formation of the HIV-1 Rev-RRE (Rev response element) complex is enhanced by the A7883 site methylation in the stem loop II region of RRE RNA. This complex thereby influences HIV-1 replication and nuclear export (68). Furthermore, one third of the 56 genes which encode diverse functional proteins, including MOGS, TRAF2, and HSPA1A, have been linked with HIV-1 replication. It thus seems that altered host genes modified with m6A are involved in the antiviral T cell immune response due to regulation of RNA expression and biological metabolism.

Kaposi's sarcoma-associated herpesvirus (KSHV) has been reported as the leading cause of cancer in AIDS patients, resulting in both primary effusion lymphoma and the lymphoproliferative disorders multicentric Castleman's disease (69). In KSHV-infected renal carcinoma cell line iSLK.219 treated with doxycycline, the level of total m6A was found to be markedly increased, and about one third of KSHV transcripts with m6A modification were found to contribute to KSHV gene expression (70). METTL3 and YTHDF2 are involved in triggering production of virion in cells infected by KSHV. ORF50 protein, a major viral transcriptional trans-activator, upon depletion of METTL3 or YTHDF2 is initially significantly reduced and unable to expedite the expression of KSHV lytic genes. However, ORF50 expression is independent of KSHV infection. m6A initially promotes ORF50 mRNA abundance, but knockdown of YTHDF2 or METTL3 leads to a subsequent negative feedback on the ORF50 promoter. Furthermore, in a KSHV-infected iSLK.BAC16 cell line, ORF50 protein expression is inverted following METTL3 or YTHDF2 depletion, suggesting that m6A variably functions in a cell-specific way. Additionally, m6A regulates newly transcribed viral RNA splicing, stability, and protein translation to control viral lytic gene expression

and KSHV replication. Blockade of an event regulated by m6A modification decreases viral protein expression and halts virion production (71). Moreover, m6A also plays important roles in latent and lytic KSHV replication as well as KSHV-induced oncogenesis (72). m6A may be a novel, effective target in the management of KSHV infection.

In 1979, m6A residues were identified on the polyomavirus simian virus 40 (SV40) (73). Overexpression of YTHDF2 substantially enhances SV40 replication in the SV40-permissive cell line BSC40 (74). PA-m6A-seq has revealed that SV40 possesses an early region where two m6A clusters are located, and a late region that possesses 11. Instead of detectably affecting SV40 late transcript alternative splicing, m6A present on VP1 ORF principally increases VP1 expression by expediting translation. By decreasing the formation of SAM without affecting mRNA capping, 3-deazaadenosine (DAA), as an inhibitor of methylation, reduces overall m6A modification (75). Subsequent depletion of m6A via DAA treatment inhibits SV40 replication. Drugs that alter m6A modification levels as needed might be in a position to repress replication of different kinds of pathogenic viruses, in particular of those causing acute infections.

## m6A Methylation and Antitumor Immune Response

Tumor neoantigens are important for generating spontaneous antitumor immunity and for valuating clinical responses to immunotherapies (76, 77). Recent article indicates that m6A-modificated mRNAs encoding lysosomal cathepsins are recognized by YTHDF1 in dendritic cells (DCs), subsequently the binding of YTHDF1 facilitates translation of cathepsins, suppressing the cross-priming ability of DCs. Loss of Ythdf1 inhibits tumors growth and host survival in mice model, owing to elevated infiltration of neoantigen-specific $CD8^+$ T cells in tumors. Consistently, DC-specific Ythdf1 depletion enhances the cross-presentation of tumor antigens and the cross-priming of $CD8^+$ T cells *in vivo*. Furthermore, given that Ythdf1 depletion promotes IFNγ production, followed by PD-L1 increase in $CD8^+$ T cells (78), combining PD-L1 checkpoint inhibitor with Ythdf1 depletion shows stronger therapeutic efficacy. In combination with burgeoning checkpoint blockade strategy, YTHDF1 could be a potential new therapeutic target for immunotherapy.

# CONCLUSION AND PERSPECTIVES

Being exquisitely regulated by "writers," "erasers," and "readers," additional repelled proteins or miRNAs, m6A modification relates to nearly any step of mRNA metabolism, as well as ncRNA processing and circRNA translation. There is compelling evidence suggesting that m6A modification is especially critical in a variety of pathologic and physiologic immune responses including T cell homeostasis and differentiation, inflammation, and type I interferon production. Further results have indicated that aberrancies of interferon and Th17 frequencies in systemic lupus erythematosus (SLE) patients may be caused by relevant genes changes for the altered m6A levels (79, 80). Whether functional dysregulations observed in SLE are associated with altered m6A modification warrants future exploration (81). Furthermore, m6A controls stem cell self-renewal, differentiation and pluripotency, additional critical functions in metabolism, as well as metastasis in many cancers (82), including acute myeloid leukemia, glioblastoma, breast cancer and lung carcinoma. Altered m6A modification might thus be an effective therapeutic target in preventing and treating human diseases. m6A exerts a variety of functions that result in alterations of particular functional proteins with m6A on mRNAs.

Though the field of m6A modification has become increasingly attractive, serious challenges in research remain. More advanced technology will be needed for quantification of m6A modification on a transcriptome-wide level and identification of precise m6A sites. How one of the members of writers, readers, and erasers performs its separate functions and interacts with one another remains to be elucidated. It is likely that either FTO or ALKBH5 performs the sole demethylation in different tissues as the presence of one has been found to indicate absence of the other. More biochemical participants in the machinery involved in m6A modification remain to be uncovered, and whether the diverse functions of m6A work in concert or are antagonistic in cellular biological processes remains to be researched in detail. We anticipate that subsequent focus on researching m6A modification in the setting of physiological and pathological processes will enrich our knowledge concerning a variety of conditions, contribute to the advancement of the biological sciences and provide us with novel therapeutic strategies.

# AUTHOR CONTRIBUTIONS

CZ the first author, contributed to collection of references and manuscript preparation. JF and YZ contributed to the modification of the manuscript.

# FUNDING

This work was supported by grants from the National Key R&D Program of China (2016YFC1305102), National Natural Science Foundation of China (81671561), 1000 Young Talents Plan Program of China, Initial Funding for New PI, Fudan Children's Hospital and Fudan University, the International Joint Laboratory Program of National Children's Medical Center (EK1125180109) and Shanghai Municipal Planning Commission of Science and Research Fund (201740065 to YZ). Shanghai Pujiang Program 16PJ1401600 (to JF).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 12 June 2019
doi: 10.3389/fimmu.2019.01351

# Lysine-Specific Histone Demethylase 1A Regulates Macrophage Polarization and Checkpoint Molecules in the Tumor Microenvironment of Triple-Negative Breast Cancer

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Macrophages play an important role in regulating the tumor microenvironment (TME). Here we show that classical (M1) macrophage polarization reduced expression of LSD1, nuclear REST corepressor 1 (CoREST), and the zinc finger protein SNAIL. The LSD1 inhibitor phenelzine targeted both the flavin adenine dinucleotide (FAD) and CoREST binding domains of LSD1, unlike the LSD1 inhibitor GSK2879552, which only targeted the FAD domain. Phenelzine treatment reduced nuclear demethylase activity and increased transcription and expression of M1-like signatures both *in vitro* and in a murine triple-negative breast cancer model. Overall, the LSD1 inhibitors phenelzine and GSK2879552 are useful tools for dissecting the contribution of LSD1 demethylase activity and the nuclear LSD1-CoREST complex to switching macrophage polarization programs. These findings suggest that inhibitors must have dual FAD and CoREST targeting abilities to successfully initiate or prime macrophages toward an anti-tumor M1-like phenotype in triple-negative breast cancer.

**Keywords: macrophage polarization, LSD1, CoREST, breast cancer, epigenetics, tumor microenvironment, tumor associated macrophages**

OPEN ACCESS

***Edited by:***
*Dinah S. Singer, National Cancer Institute (NCI), United States*

***Reviewed by:***
*Panagiotis F. Christopoulos, Oslo University Hospital, Norway*
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***Specialty section:***
*This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology*

***Received:*** *11 January 2019*
***Accepted:*** *28 May 2019*
***Published:*** *12 June 2019*

***Citation:***
*Tan AHY, Tu W, McCuaig R, Hardy K, Donovan T, Tsimbalyuk S, Forwood JK and Rao S (2019) Lysine-Specific Histone Demethylase 1A Regulates Macrophage Polarization and Checkpoint Molecules in the Tumor Microenvironment of Triple-Negative Breast Cancer. Front. Immunol. 10:1351. doi: 10.3389/fimmu.2019.01351*

## INTRODUCTION

Breast cancer is the most common female cancer worldwide (1). The triple-negative subtype of breast cancer (TNBC) accounts for 15–20% of cases (2, 3) and is characterized by an absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) expression (3–5). TNBC patients have a worse prognosis than patients with other breast cancer subtypes, not least because they do not have the targets and so do not respond to hormonal or HER2-targeting therapies. Various novel treatments have been trialed in patients with TNBC, but standard chemotherapy regimens remain the standard of care (5–7).

TNBCs have a particularly high immune cell infiltrate compared to other breast cancer subtypes, but these immune cells are often functionally impaired (8, 9). The tumor microenvironment (TME) of any cancer contains a complex mixture of immune cells with both pro- and anti-tumor properties. Tumor-associated macrophages (TAMs) are a major immune cell subset in the TME, where they exist along a phenotypic spectrum from classically (M1) to alternatively (M2) activated (10, 11). Lipopolysaccharide (LPS) and IFN-γ-induced M1 macrophages secrete pro-inflammatory cytokines and reactive oxygen/nitrogen species that contribute to tumor cell cytotoxicity. Conversely, IL-4- and IL-13-induced M2 macrophages produce anti-inflammatory cytokines that can suppress other immune cells in the TME and promote tumor progression (12–14).

Epigenetic programming plays a significant role in regulating macrophage polarization and can be manipulated using various inhibitors (15). Numerous epigenetic enzymes control DNA methylation, histone methylation, and histone acetylation [see reviews in (16–18)]. Lysine-specific demethylase 1 (LSD1) is a H3K4 and H3K9 demethylase essential for myeloid cell differentiation (19), reactivating key immune checkpoint regulators, producing cytotoxic T cell chemokines (20), and preventing IL6 silencing in LPS-tolerant macrophages (21). We previously showed that immune-incompetent mice treated with the LSD1 inhibitor phenelzine had a higher proportion of M1-like macrophages in the TME of xenografts (22). We also showed that LSD1 is critical for reprogramming cancer stem cell (CSC)-inducible gene signatures and directly regulates distinct CSC genes implicated in breast cancer metastasis by tethering to their promoter regions (22).

Here we show that M1 (IFN-γ + LPS) or M2 (IL-4) macrophages differentially express LSD1 and nuclear serine 111 phosphorylated LSD1 (LSD1-s111p). LSD1 and LSD1-s111p downregulation in the M1 (IFN-γ + LPS) phenotype correlates with decreased nuclear activity and increased expression of histone H3 lysine 4 dimethylation (H3K4me2) and histone H3 lysine 9 dimethylation (H3K9me2) marks and decreased interactions with nuclear REST corepressor 1 (CoREST) and zinc finger protein SNAI1 (SNAIL) complexes. Phenelzine treatment mimics the phenotype of these M1 (IFN-γ + LPS) polarized cells by disrupting the LSD1-CoREST complex unlike the catalytic inhibitor GSK2879552. Thus, showing the importance of targeting the LSD1-CoREST complex to epigenetically prime macrophages toward an M1-like phenotype. *In vivo*, LSD1 inhibition by phenelzine primes TAMs to express M1-like gene that displayed both common and unique pathways to the chemotherapeutic protein-bound paclitaxel (Abraxane). Phenelzine treatment also led to a higher proportion of macrophages expressing M1 like protein (iNOS, CD86 and PD-L1) in formalin-fixed paraffin embedded (FFPE) tissue sections of tumors from a murine model of triple negative breast cancer (TNBC). Collectively, our data show for the first time that LSD1 inhibitors that target the LSD1 FAD and disrupt the LSD1-CoREST complex leading to a destabilization of LSD1 can epigenetically prime macrophages toward a M1-like phenotype in the TME, and future immunomodulatory drug development must take LSD1 FAD and LSD-CoREST complex into account to improve efficacy.

# MATERIALS AND METHODS

## Cell Culture

RAW264.7 cells (ATCC TIB-71) were cultured in high-glucose DMEM with 2 mM L-glutamine, 1 x penicillin-streptomycin-neomycin (PSN) (Gibco, Thermo Fisher Scientific, Waltham, MA), and 10% heat-inactivated fetal calf serum (FCS). 4T1 cells (ATCC CRL-2539) were cultured in DMEM with 2 mM L-glutamine, PSN, and 10% heat-inactivated FBS. Transfection reactions were performed with 10 nM mouse LSD1 siRNA (sc-60971) and mock siRNA (sc-37007) (Santa Cruz Biotechnology Inc., Dallas, TX) using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA).

## *In vitro* Macrophage Polarization

RAW264.7 cells were seeded into 6- or 12-well plates 24 h before polarizing macrophages. M1 (IFN-γ + LPS) classical activation was induced by adding 100 ng/ml lipopolysaccharide (LPS) and 20 ng/ml IFN-γ, and M2 (IL-4) alternative activation was induced by adding 20 ng/ml IL-4 for 24 h. Phenelzine and GSK2879552 (GSK) were added at 500 μM for 24 h.

## RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from RAW264.7 cells using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. RNA was measured using the Nanodrop spectrophotometer (Thermo Fisher Scientific) and reverse transcribed into cDNA using the SuperScript VILO cDNA synthesis kit using the manufacturer's protocols. TaqMan quantitative real-time PCR was performed with the following mouse TaqMan probes: *Nos2* (Mm00440502_m1), *Gpr18* (Mm02620895_s1), *IL6* (Mm00446190_m1), *Fpr2* (Mm00484464_s1), *IL12b* (Mm00434174_m1), *ILb* (Mm00434228_m1), *CCR7* (Mm01301785_m1), *Myc* (Mm00487804_m1), *Egr2* (Mm00456650_m1), *Arg1* (Mm00475988_m1), *Mrc1* (Mm00485148_m1), *Mgl2* (Mm00460844_m1), *Pdcd1* (Mm01285676_m1), *CD274* (Mm03048248_m1), *Pdcd1lg2* (Mm00451734_m1), KDM1A (Mm01181029_m1), and *Gapdh* (Mm99999915_g1). DNA from formaldehyde-assisted isolation of regulatory elements (FAIRE) was quantified by SYBR real-time PCR with the primer set listed in **Supplementary Table 1**. qPCR data were normalized to *Gapdh* loading control.

## Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE)

FAIRE samples were prepared as outlined in Simon et al. (23). Briefly, cells were cross-linked with 1% formaldehyde and lysed. The cell lysates were sonicated to yield an average DNA fragment distribution of ∼200–500 bp. A 50 μl aliquot of fragmented DNA (total input control DNA) was reverse cross-linked at 65°C followed by phenol-chloroform extraction. The remaining

sonicated DNA (FAIRE DNA) was directly isolated by phenol-chloroform extraction and purified using the Zymo-SpinTM I kit (Zymo Research, Irvine, CA).

## Animal Studies

Five-week-old female BALB/c mice were obtained from the Animal Resources Center (ARC), Perth, and allowed to acclimatize for 1 week in the containment suites at The John Curtin School of Medical Research (JCSMR). All experimental procedures were performed in accordance with the guidelines and regulations approved by the Australian National University Animal Experimentation Ethics Committee (ANU AEEC). Mice were shaved at the site of inoculation the day before subcutaneous injection with $2 \times 10^5$ 4T1 cells in 50 µl PBS into the right mammary gland. Treatment was started at day 12 post inoculation, when tumors reach approximately 50 $mm^3$. Tumors were measured using external calipers and volumes calculated using a modified ellipsoidal formula ½ ($a/b^2$), where $a$ = longest diameter and $b$ = shortest diameter. Mice were treated with Abraxane (30 mg/kg) and PD1 (10 mg/kg) every 5 days (twice) and phenelzine (40 mg/kg) daily. All treatments were given intraperitoneally in PBS. Tumors were collected on day 27 post-inoculation of 4T1 cells for flow cytometry, macrophage enrichment for NanoString, and immunofluorescence microscopy.

## Tumor Dissociation Protocol

4T1 tumors were harvested in cold DMEM supplemented with 2.5% FCS before being finely cut using surgical scalpels and enzymatically dissociated using collagenase type 4 (Worthington Biochemical Corp. Lakewood, NJ) at a concentration of 1 mg collagenase / 1 g of tumor at 37°C for 1 h. Dissociated cells were then passed through a 0.2 µM filter before downstream assays.

## Flow Cytometry

Single cell suspensions were prepared as in the tumor dissociation protocol. Non-specific labeling was blocked using anti-CD16/32 (Fc block; BD Biosciences, Franklin Lakes, NJ) before specific labeling. BD Horizon fixable viability stain 780 was used to distinguish live and dead cells. Tumor cells were stained with antibodies targeting F4/80 PE, CD206 APC, and Ly6C Brilliant Violet 421 (all from BioLegend, San Diego, CA). Sample acquisition was performed with the BD LSR II cytometer and results analyzed with FlowJo software.

## Macrophage Enrichment and NanoString nCounter Protocol

Single cell suspensions were magnetically labeled with anti-F4/80 microbeads UltraPure (Miltenyi Biotec, Bergisch Gladbach, Germany) in MACS running buffer. Macrophages were then positively isolated using the autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocols. Enriched cells were then snap frozen and RNA isolated using the RNeasy Mini kit (Qiagen). Samples were analyzed using the NanoString platform according to the manufacturer's procedures. Briefly, 100 ng of RNA was hybridized with the mouse myeloid innate immunity panel codeset for 18 h at 65°C. Samples were then loaded onto the chip via the nCounter prep station and data acquired using the nCounter Digital Analyzer. Data analysis was performed using nSolver Analysis Software. The Benjamini-Yekutelli method was used to calculate the false discovery rate (FDR) (24).

## Immunofluorescence

Cells were cultured for 24 h on sterilized coverslips and then for a further 24 h after treatment with either complete medium, 100 ng/mL LPS and 20 ng/mL IFN-γ, 20 ng/mL IL-4, 500 µM phenelzine, or 500 µM GSK to form the treatment groups: control, M1 (IFN-γ + LPS), M2 (IL-4), phenelzine, and GSK, respectively.

After culturing, cells were fixed with 3.7% paraformaldehyde and permeabilized using 2% Triton X-100 solution. Cells were then blocked using 1% bovine serum albumin (BSA) and probed with rabbit-LSD1p (ABE1462, EMD Millipore, Burlington, MA), mouse-H3K9me2 (ab1220, Abcam, Cambridge, UK), goat-H3K4me2 (ab11946, Abcam), mouse-CD38 (102716, BioLegend), and goat-SNAIL1 (sc-10433, Santa Cruz Biotechnology) followed by visualization with corresponding secondary antibodies (all Thermo Fisher Scientific): anti-rabbit (A21206 and A10042), anti-mouse (A10037), and anti-goat (A21082 and A11055) conjugated to either Alexa Fluor 488, or 568, or 633. Coverslips were mounted onto glass microscope slides using SlowFade™ Diamond Antifade Mountant with DAPI.

Formalin-fixed, paraffin-embedded melanoma primary tumor biopsies were processed in the BondRX for OPAL staining (Perkin-Elmer, Waltham, MA) using the instrument protocol: ER1 for 20 min at 100°C with Epitope Retrieval Solution (pH6 Citrate-based retrieval solution) followed by probing with primary antibodies to F4/80 (ab100790, Abcam), iNOS (ab115819, Abcam), CD86 (ab213044, Abcam) and PD-L1 (ab2386097, Abcam) (for the M1 panel) or F4/80, EGR2 (ab90518, Abcam), CD206 (ab64693, Abcam) and PD-L2 (PAB12986, Abnova) (for the M2 panel). Primary antibodies were visualized with an Opal Kit 520, 570, 650, and 690. Coverslips were mounted on glass microscope slides with ProLong Clear Antifade reagent (Life Technologies, Carlsbad, CA). Opal kits used: 7-color automation kit (NEL801001KT) and the 4-color automation kit (NEL820001KT).

Slides were observed under a Leica DMi8 inverted microscope running Leica Application Suite X software. Multiple images were taken at various positions on the slide using a 100x oil immersion lens. Images were analyzed using ImageJ software, with the fluorescence intensity measured from a minimum of 20 cells and an average total fluorescence of either the nucleus or cytoplasm reported. Background fluorescence was measured and subtracted from all results.

For high-throughput microscopy, protein targets were localized by confocal laser scanning microscopy. Single 0.5 µm sections were obtained using an Olympus-ASI automated microscope with 100x oil immersion lens running ASI software. The final image was obtained by employing a high throughput automated stage with ASI spectral capture software. Digital images were analyzed using automated ASI software (Applied

Spectral Imaging, Carlsbad, CA) to automatically determine the distribution and intensities with automatic thresholding and background correction of either the average nuclear fluorescent intensity (NFI) and average or whole cell total fluorescent intensity (TFI). The plot-profile feature of ImageJ was used to plot the fluorescence signal intensity along a single line spanning the nucleus ($n = 5$ lines per nucleus, 5 individual cells) using the average fluorescent signal intensity for the indicated pair of antibodies plotted for each point on the line with SE. Signal was plotted to compare how the signals for each antibody varied compared to the opposite antibody. For each plot-profile, the PCC was determined in ImageJ. PCC indicates the strength of relationship between the two fluorochrome signals for at least 20 individual cells ± SE. Colors from representative images correspond to plot-profiles.

## LSD1 Activity Assay

Nuclear extracts were prepared as previously described from cells, and 5 μg of protein/well in triplicate was used to measure LSD1 demethylase activity using the Abnova LSD1 Demethylase Activity/Inhibition assay kit (Abnova, Taipei City, Taiwan) according to the manufacturer's protocol.

## X-Ray Crystallography

Recombinant human LSD1 encoding residues 173-830 was expressed in *E. coli* BL21(DE3)pLysS using auto-induction medium (Studier, 2005) from the pMSCG21 expression vector. Cells were grown at 25°C, harvested by centrifugation, and resuspended in His buffer A (50 mM phosphate buffer, 300 M NaCl, and 20 mM imidazole). Resuspended cells were lysed by two repetitive freeze-thaw cycles and the cell lysate clarified by centrifugation. The soluble cell lysate was filtered using a 0.45 μm low protein binding filter and injected onto a 5 mL Ni-Sepharose HisTrap HP column equilibrated with His buffer A. Following sample injection, the column was washed with 15 column volumes of His buffer A, then eluted with His buffer A containing 500 mM imidazole. The purified protein was purified further by size exclusion chromatography and applied to a pre-equilibrated Superdex 200 26/60 size exclusion column. The protein was concentrated to 17 mg/ml using an Amicon ultracentrifugal device with a 10 kDa molecular weight cut off, aliquoted and stored at −80°C. The LSD1 protein was screened for conditions that induce crystals, with diffraction quality crystals obtained in 2–15% PEG 3350, ammonium citrate pH 6.5 and pH 7.0. A 10-molar excess of phenelzine sulfate or GSK2879552 was added to the LSD1 prior to crystallization, with a notable change in color from yellow to pale yellow/clear.

All X-ray diffraction data were collected on the MX1 crystallography beamline at the Australian Synchrotron. Images were indexed and integrated in iMosfilm (25), and data merged and scaled in Aimless (26). The number of molecules within the asymmetric unit was estimated based on the Matthews coefficient (VM) and the predicted molecular weight of the protein (27, 28). Model building and refinement was performed using COOT (29) and REFMAC (30).

## Bioinformatic Analysis

Promoter and enhancer analysis was performed on significant genes ($p < 0.05$, False Discovery Rate (FDR) $< 0.15$) up or down-regulated by phenelzine from the NanoString nCounter assay. Benjamini-Yekutieli false discovery rate method was used to calculate the FDR (24). Enhancer regions are from **Supplementary Table 1** in Ostuni et al. (31). Raw data was downloaded from GEO, GSE38377, GSE91009 and GSE78873 and adapter trimmed and mapped to mm9 using Trimmomatic (32) and Bowtie2 (33) in Galaxy. CpG and GC, and histone levels were calculated with HOMER (34). Counts for promoters [were 1 kb ± around the transcription starts site (TSS)], and enhancer regions (using the given range). Accessibility and histone levels for stimulated and non-stimulated cells were equalized to the mean promoter values for all RefSeq genes. Bedtools (35) was used to detect which enhancers were within 10 kb of the gene TSS. Welch two sample *t*-test and boxplots were performed in R.

## Statistics

All statistical comparisons between sample groups were calculated using the two-tailed non-parametric Mann-Whitney test (GraphPad Prism, San Diego, CA) unless otherwise indicated. Where applicable, statistical significance is denoted by $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, $^{***}P \leq 0.0005$ and $^{****}P \leq 0.0001$. Data are expressed as mean ± SE.

# RESULTS

## Phenelzine and GSK2879552 Modify the LSD1 Flavin Adenine Dinucleotide (FAD) Cofactor

We previously showed that LSD1 modulates epithelial to mesenchymal transition in CSCs and that LSD1 inhibition promotes an M1-type response in an immune-deficient mouse cancer xenograft model (22). Here we aimed to further characterize the effect of LSD1 inhibition on macrophage polarization utilizing two different LSD1 inhibitors, phenelzine, and GSK2879552 (GSK).

Phenelzine and GSK are monoamine oxidases (MAOs) and effective LSD1 inhibitors (36, 37), probably via modification of the flavin adenine dinucleotide (FAD) cofactor (38, 39). We first confirmed whether the inhibitors act via a FAD-dependent mechanism by crystallizing LSD1 in the presence and absence of phenelzine and GSK. The gross crystal morphology was markedly different: yellow in the absence of inhibitor and translucent in the presence of phenelzine or GSK (**Figure 1A**). However, the crystals diffracted to similar resolution and belonged to the same space group (**Supplementary Table 2**), each containing one LSD1 molecule in the asymmetric unit.

In the absence of inhibitor, there was clear density corresponding to FAD (**Figure 1A**). In the presence of phenelzine, there was clear positive density at the central nitrogen of the flavin moiety on FAD, consistent with a previously determined structure of human MAO-B in the presence of phenelzine (PDB 2VRM). This is also consistent with the observed color change, since the flavin moiety confers

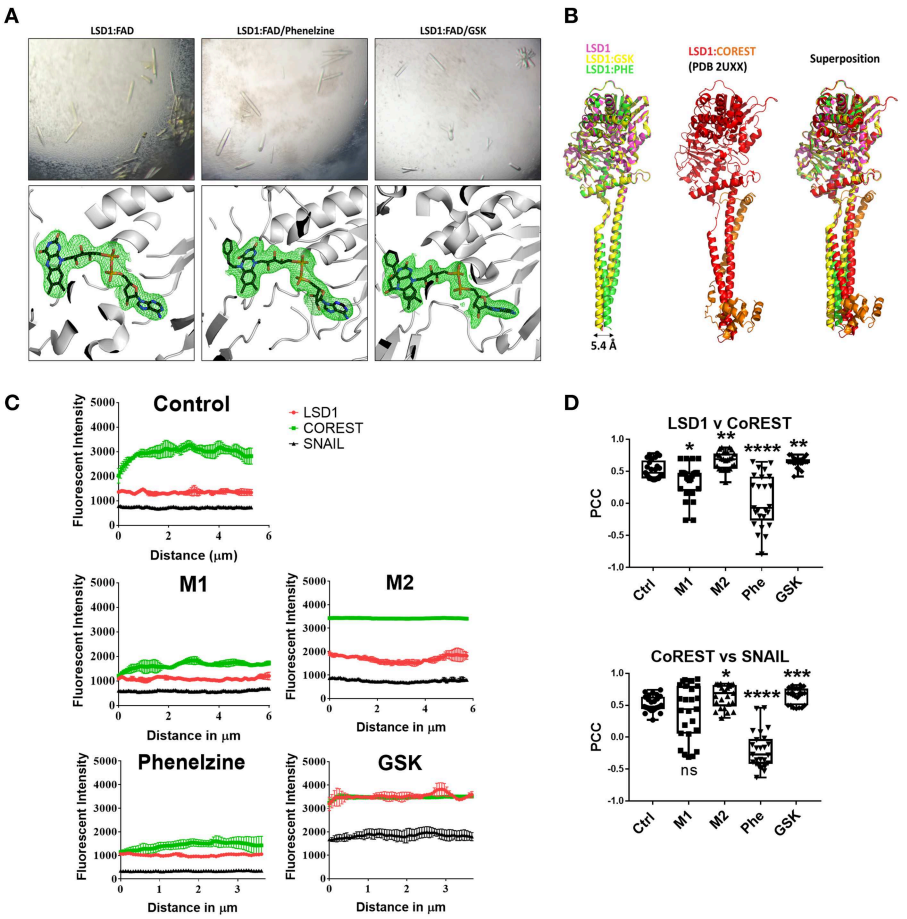


**FIGURE 1 |** Phenelzine targets the FAD domain of LSD1 and potentially disrupts the LSD1/CoREST axis resulting in destabilization of LSD1 and its nuclear activity. **(A)** LSD1 protein crystals (top panel) grown in the absence (left) and presence of phenelzine (middle) and GSK2879552 (right). In the absence of inhibitors, strong density (bottom panels) was observed consistent with the FAD cofactor (shown as sticks colored with carbons black, nitrogen blue, oxygen red, and phosphate orange). The map is a simulated annealed omit map for FAD contoured at 2.5 sigma. LSD1 is colored gray and shown in cartoon mode. The FAD modifications by phenelzine (middle) and GSK (right) are supported by strong density, with corresponding maps and colors as per LSD1:FAD. **(B)** Structural superposition of LSD1 in the absence and presence of phenelzine and GSK. The structures solved in this study (left panel), LSD1 alone (pink) (PDB 6NQM), LSD1:GSK (yellow) (PDB 6NQU), and LSD1:phenelzine (green) (PDB 6NR5) are represented in cartoon mode. These structures are superimposed in the left panel, showing a high degree of structural homology in the LSD1 catalytic domain for all three structures. LSD1 alone and LSD1:GSK also show high structural conservation in the alpha-helical tails; however, LSD1:phenelzine has a 5.4 Å displacement in this region. This region is important for CoREST binding, as shown in the middle panel (PDB 2UXX). Superposition of all structures in the left and middle panels is shown on the right, highlighting that CoREST binding is mediated by the correct position of these domains. All images were generated in Pymol. **(C)** The plot-profile feature of ImageJ was used to plot the fluorescence signal intensity along a single line spanning the nucleus ($n = 5$ lines per nucleus, 5 individual cells) using the average fluorescent signal intensity for the indicated pair of antibodies plotted for each point on the line with SE. **(D)** Pearson's correlation coefficient (PCC) indicating the colocalization of LSD1/CoREST and CoREST/SNAIL. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ Mann–Whitney *t*-test.

these spectral properties (40). Similarly, LSD1 protein crystals grown in the presence of GSK exhibited clear additional positive density at the flavin moiety, consistent with a previous crystal structure of LSD1 bound to a GSK analog (PDB 2UXX) (41). Both inhibitors appear to act via similar mechanisms and modify the FAD cofactor at the flavin moiety.

The inhibitor-bound structures were superimposed and compared with the native LSD1 structure to examine whether these inhibitors resulted in any other observable structural changes. Phenelzine resulted in a small 5.4Å shift in the long alpha-helical tails of LSD1 (residues 415–514) (PDB 6NR5) that was not present in LSD1 (PDB 6NQM) or LSD1:GSK2879552 crystals (PDB 6NQU). This region mediates CoREST binding, making it possible that phenelzine-induced structural changes in this region may also affect LSD1 activity outside the catalytic region (**Figure 1B**).

## LSD1 Inhibitors Differentially Target or Disrupt the LSD1/CoREST Complex in Macrophages

Given that phenelzine-induced structural changes in the CoREST region (**Figure 1B**) may affect LSD1 activity outside the catalytic region, we examined the impact of phenelzine on

the LSD1/CoREST complex and its impact on macrophage polarization by comparing unpolarized, M1 or M2 polarized, and phenelzine- or GSK-treated RAW264.7 cells *in vitro* by high-resolution fluorescent confocal microscopy (**Figure 1C**). Phenelzine treatment or M1 polarization with IFN-γ and LPS significantly reduced the number and nuclear expression of CoREST, LSD1, and SNAIL in RAW264.7 macrophages (**Figure 1C**). The nuclear to cytoplasmic ratio of LSD1 (Fn/c) was almost equal, suggesting downregulation of LSD1 in both the cytoplasmic and nuclear compartments on phenelzine treatment (**Supplementary Figure 1**). Conversely, treatment with either GSK or M2 polarization with IL-4 had the opposite effect, with enhanced cell number and expression intensity of CoREST and LSD1 and an overall increase in expression of CoREST, LSD1, and SNAIL in both the cytoplasmic and nuclear compartments. However, in this case, the Fn/c of LSD1 was clearly nuclear biased in the control group and increased further by M2 polarization or GSK treatment, perhaps by stabilizing LSD1 in the nucleus and enhancing expression (**Figure 1C**).

Phenelzine treatment abrogated co-localization of LSD1 and CoREST or SNAIL and CoREST as indicated by a strong negative PCC score in the phenelzine-treated samples (**Figure 1D**). GSK induced the opposite, with LSD1 and CoREST or SNAIL and CoREST strongly co-localizing with a positive PCC score (**Figure 1D**).

Overall, these data suggest that M1 (IFN-γ + LPS) polarization destabilizes and globally reduces LSD1, SNAIL, and CoREST expression, the overall cell population expressing these markers, and CoREST/LSD1 and CoREST/SNAIL complexes. Cells treated with phenelzine mimics this phenotype, impacting on both the FAD and CoREST domains of LSD1. Conversely, GSK or M2 (IL-4) polarization stabilizes and induces nuclear LSD1 and CoREST expression by enhancing their spatial co-localization. This suggests that overall that phenelzine inhibition aligns with M1 (IFN-γ + LPS)—macrophage polarization in the context of inhibiting both the catalytic FAD and nuclear CoREST domain of LSD1 Nuclear LSD1 activity can determine the macrophage phenotype.

To address the impact of LSD1 on macrophage polarization, we employed high-throughput, ASI Digital Pathology Platform which allows both the quantification of immuno-fluorescent intensity and population distribution of stained cells using proprietary algorithms developed in partnership with ASI:Metagene using automatic autofluorescence correction with automatic signal intensity and cell detection to detect up to 6 colors plus DAPI. This system was employed to analyze both the expression and population distribution of the M1 marker CD38 and M2 marker EGR2 in RAW264.7 mouse macrophages treated with phenelzine, GSK, or cytokine-induced M1 (IFN-γ + LPS) or M2 (IL-4) phenotypes. Treatment with M1 (IFN-γ + LPS) or phenelzine reduced expression of EGR2 (an M2 marker) and the overall percentage of cells positive for EGR2 in F4/80$^+$ RAW264.7 cells (**Figure 2A**), whereas induction with M2 (IL-4) or treatment with GSK induced expression of EGR2 and increased the percentage of EGR2$^+$ cells (**Figure 2A**). Conversely, phenelzine treatment or M1 (IFN-γ + LPS) induction increased expression of the M1 marker CD38 and increased the proportion of CD38$^+$ cells (**Figure 2A**), and M2 (IL-4) polarization and GSK treatment significantly reduced both expression of CD38 and the percentage of CD38$^+$ cells. Thus, different LSD1 inhibitors have different and opposing effects on macrophage polarization.

We previously reported the importance of nuclear LSD1 phosphorylation at serine 111 (LSD1-s111p) in both CSCs and macrophages within the TME (22). We also showed that LSD1 inhibition significantly reduces LSD1-s111p and the transcription factor SNAIL expression in circulating tumor cells (CTCs) (22). We therefore sought to determine the impact of these LSD1 inhibitors on LSD1 activity and how M1 (IFN-γ + LPS) or M2 (IL-4) macrophage polarization affects the nuclear distribution of LSD1-s111p using high-resolution immunofluorescent microscopy for LSD1-s111p, histones modifications H3K9Me2 and H3K4me2, and SNAIL in RAW264.7 macrophage nuclei. H3K4 and H3K9 are direct LSD1 targets, and overexpression of the transcription factor SNAIL is associated with M2-like macrophage polarization (19, 42). Phenelzine treatment significantly reduced nuclear LSD1-s111p levels to like those seen in M1-polarized macrophages and enhanced levels of H3K9me2 and H3K4me2 (**Supplementary Figure 1**). Conversely, there was increased LSD1-s111p and decreased H3K9me2 and H3k4me2 levels in M2 and GSK-treated cells (**Supplementary Figure 1**). Phenelzine also reduced LSD1 nuclear enzymatic activity. Polarizing cells toward an M1 phenotype using IFN-γ and LPS also reduced nuclear LSD1 activity compared to controls (**Figure 2B**). However, treatment of cells with IL-4 (M2) and GSK did not inhibit the nuclear activity of LSD1 (**Figure 2B**). Interestingly, we also observed similar morphological changes between IFN-γ + LPS and phenelzine treatment after 7 days (phenelzine and media changed every 2 days) (**Figure 2C**).

Therefore, phenelzine can target nuclear LSD1 activity and have a role in initiating/priming macrophage polarization that is likely a pre-requisite for initiating phagocytosis.

## Phenelzine Treatment Can Reprogram Macrophages to Exhibit M1-Like Gene Signatures With PD1, PD-L1, and PD-L2 Checkpoint Expression

Given the similarities between M1 (IFN-γ + LPS)-polarized macrophages and macrophages treated with phenelzine, we next determined whether LSD1 inhibition with phenelzine and GSK mimic polarized macrophage gene signatures. The gene expression of phenelzine-treated cells was similar to the M1 phenotype (*Nos2, Gpr18, IL6, Fpr2, IL12b, Il1b*, and *Ccr7*) (12) induced by IFN-γ and LPS (**Figure 3A**). This corresponded to increased accessibility at the promoter (**Figure 3C**) and enhancer (**Figure 3D**) regions of those M1-like genes in the M1 (IFN-γ + LPS) or phenelzine treated RAW264.7 cells. In addition, genes associated with the M2 phenotype (*Myc, Egr2, Arg1, Mrc1*, and *Mgl2*) were expressed at much lower levels compared to cells polarized toward an M2 phenotype using IL-4 (**Figure 3B**). GSK-induced gene signatures, on the other hand, did not show a similar correlation with M1-polarized cells (**Figures 3A,B**).

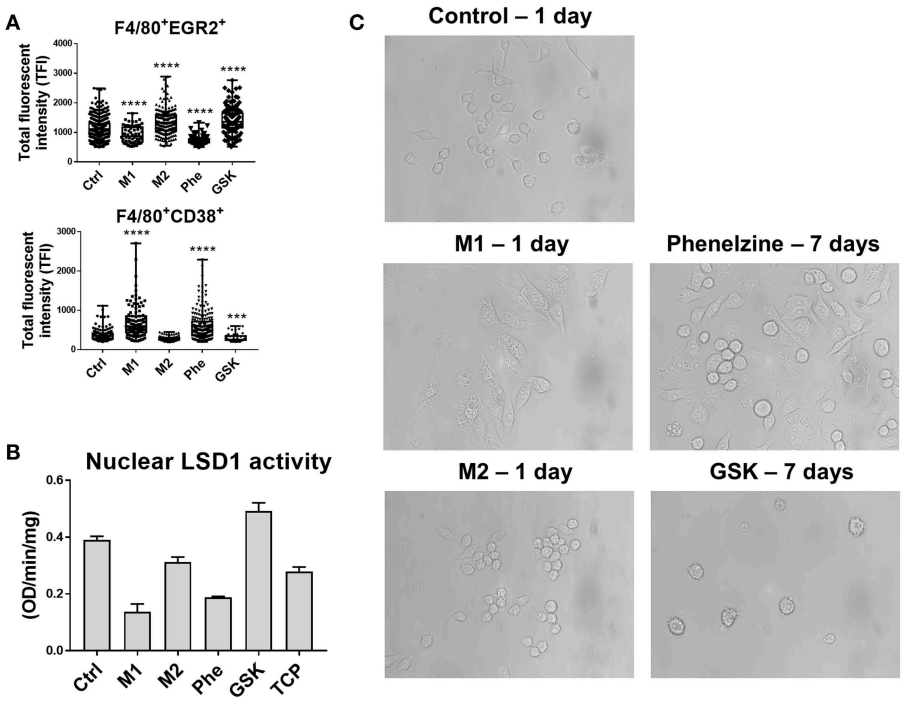


**FIGURE 2 |** Phenelzine or M1 polarization upregulates M1 protein CD38 and targets the nuclear activity of LSD1. RAW264.7 cells were treated with LPS + IFN-γ, IL-4, or 500 μM phenelzine or GSK for 24 h. Protein targets **(A)** EGR2 and CD38 in F4/80$^{+}$ cells were localized by confocal laser scanning microscopy. Single 0.5 μm optical sections were obtained using an Olympus-ASI automated microscope with 100x oil immersion lens running ASI software. The final image was obtained by employing a high-throughput automated stage with ASI spectral capture software. Digital images were analyzed using automated ASI software to determine the distribution and intensities automatically with automatic thresholding and background correction. Graphs represent either a dot plot of the individual cell intensities or the average TFI ($n$ = 2,000 cells). **(B)** LSD1 activity assay on nuclear extracts of RAW264.7 cells either untreated, M1/M2 polarized, or treated with phenelzine, GSK, or LSD1 inhibitor tranylcypromine. **(C)** Images of RAW264.7 cells treated with vehicle control, LPS + IFN-γ (M1), and IL-4 (M2) for 24 h. Phenelzine and GSK treated cells did not show morphology changes in 24 h (data not shown). In comparison, cells were treated with Phenelzine or GSK for 7 days.

In order to confirm these effects were due to LSD1 inhibition, we knocked-down LSD1 in RAW264.7 cells with siRNAs. This resulted in a 40% inhibition of LSD1 gene expression (**Figure 3E**) and an increase in key M1 markers such as *Nos2* and *Il-6*, a decrease in M2 markers *Egr2* and no change in *Mrc1* (CD206) (**Figure 3E**).

Therefore, inhibiting the catalytic FAD and nuclear CoREST domain of LSD1 with phenelzine can upregulate M1-associated genes and decrease M2-associated genes, while inhibition of the FAD domain (GSK) alone does not. This indicates an important role for both the FAD domain of LSD1 and its stabilization by CoREST in regulating genes associated with M1 macrophages.

Targeting the PD1-PD-L1 axis is an effective therapeutic approach in cancer, and macrophages express these checkpoint molecules (43, 44). Unpolarized and M1 (IFN-γ + LPS)- and M2 (IL-4)-polarized RAW264.7 cells express different levels of PD1, PD-L1, and PD-L2, so given the effect of LSD1 inhibition on macrophage polarization, we also wanted to determine the effect of LSD1 inhibition on the PD1-PDL1/2 axis.

*Pd1* expression was generally lower in all treated cells compared to controls (**Figure 3F**), with M1 (IFN-γ + LPS)-polarized cells expressing the lowest *Pd1* (**Figure 3F**), Interestingly, we observe high enrichment of *Pd-l1* (approximately 450-fold) and *Pd-l2* (approximately 3-fold) in M1 (IFN-γ + LPS) and M2 (IL-4)-polarized cells, respectively compared to control (**Figure 3F**). Phenelzine-treated macrophages displayed similar *Pd-l1* and *Pd-l2* expression to M1 (IFN-γ + LPS)-polarized cells except for *Pd1* (**Figure 3F**). GSK treatment, however, mimicked an M2 (IL-4)-type expression pattern of *Pd-l1* but not *Pd1* and *Pd-l2* (**Figure 3F**).

These data suggest that macrophage polarization may contribute to *Pd-l1* and *Pd-l2* expression at both the gene and protein level, with M1 (IFN-γ + LPS)-polarized cells expressing higher *Pd-l1* levels and M2 (IL-4)-polarized cells expressing higher *Pd-l2* levels. Phenelzine treatment appears to mimic this M1(IFN-γ + LPS)-like checkpoint protein expression but GSK induces greater variability, suggesting that other post-translational mechanisms may be involved.

## Phenelzine Treatment Can Produce More Favorable Macrophage Signatures in the TME That Mimic Those Seen With Protein-Bound Paclitaxel (Abraxane) and PD1-Based Immunotherapy

We next sought to determine if phenelzine treatment also reprograms macrophages in the TME of cancers in mice.

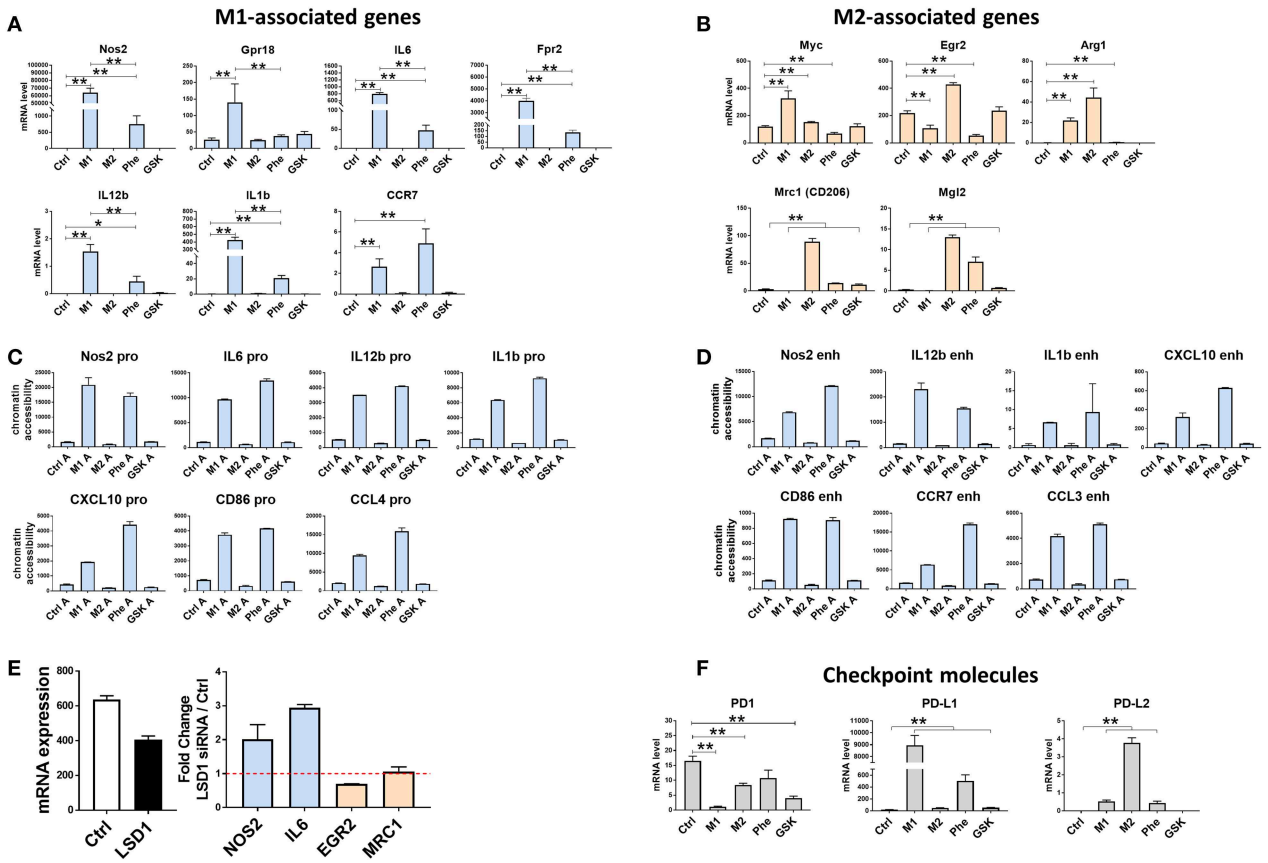


**FIGURE 3 |** LSD1 can regulate genes associated with macrophage polarization toward an M1 phenotype and checkpoint molecules. RAW264.7 cells were untreated or treated with LPS + IFN-γ (M1), IL-4 (M2), or 500 μM of phenelzine or GSK for 24 h. Quantitative real-time PCR of genes associated with **(A)** M1-associated genes and **(B)** M2-associated genes were used to compare different treatment groups. Graphs are represented as mRNA levels normalized to GAPDH. Graphs show means ± SE (n = 3). $^{*}p < 0.05$, $^{**}p < 0.005$ Mann-Whitney t-test. **(C)** and **(D)** shows chromatin accessibility of genes in the promoter **(C)** and enchancer **(D)** regions of associated genes using quantitative real-time PCR using FAIRE samples. **(E)** 10nM LSD1 siRNA transfected cells and **(F)** checkpoint molecules and were used to compare different treatment groups.

Since chemotherapy is the standard of care for breast cancer patients and given our results on PD1 expression in response to phenelzine treatment, we also treated syngeneic TNBC 4T1 mice with protein-bound paclitaxel (Abraxane) and PD1 immunotherapy (**Figure 4A**). Phenelzine, Abraxane and PD1 reduced tumor volumes (**Figure 4B**) compared to controls, however, this difference was not significant.

There were no significant differences in total F4/80$^{+}$ macrophages between treatment groups (**Figure 4C**). However, all three treatments induced significantly lower proportions of inflammatory (F4/80$^{+}$Ly6C$^{+}$) and M2-like macrophages (F4/80$^{+}$CD206$^{+}$) (**Figure 4C**). We next quantified F4/80 and M1-like markers (iNOS, CD86 and PD-L1) (**Figure 4D**) or M2-like markers (EGR2, CD206, and PD-L2) (**Figure 4E**) in tissue sections from individual tumors using the high-throughput, ASI Digital Pathology Platform as described above in section Phenelzine Treatment Can Reprogram Macrophages to Exhibit M1-Like Gene Signatures With PD1, PD-L1, and PD-L2 Checkpoint Expression (**Figures 4F,G**). There was a significant increase of F4/80 macrophages expressing three M1-like markers with phenelzine and PD1 treatment alone compared to control and Abraxane treated mice (**Figure 4F**). Further, there was a significant decrease in F4/80 macrophages expressing all three M2-like markers with phenelzine and PD1 treatment compared to control and Abraxane treated mice (**Figure 4G**). Interestingly, treatment with Abraxane alone decreased the number of macrophages expressing the M1-like markers and significantly increased the number of macrophages expressing M2-like markers (**Figures 4F,G**).

The macrophages were then analyzed for innate immunity pathways (770 genes, 19 default pathway annotations) using the NanoString platform. Phenelzine, Abraxane, and PD1 all modulated key M1 gene signatures compared to macrophages from control mice (**Figures 5A,B**), although PD1-related changes were non-significant.

Macrophages are professional antigen-presenting cells and express various co-stimulatory molecules that help with antigen presentation to T cells via MHC class II (45, 46). Phenelzine or Abraxane upregulated some MHC II genes, positive co-stimulatory genes such as *Cd80*, *Cd86*, *Cd40*, and *Icos-l*,

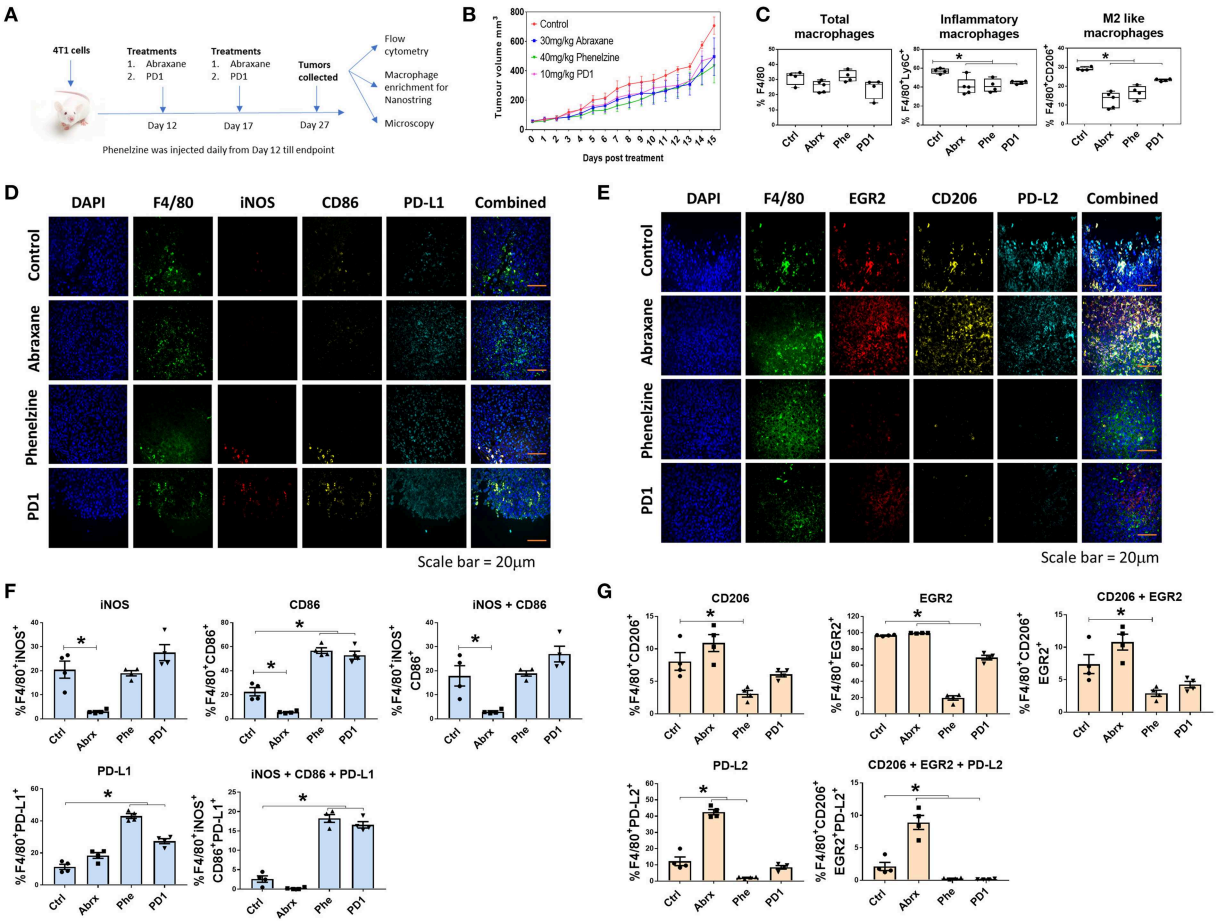


**FIGURE 4 |** Phenelzine treatment polarizes macrophages in the tumor microenvironment toward an M1 phenotype. **(A)** Treatment regime using the BALB/c 4T1 breast cancer model. **(B)** Tumor volumes of mice treated with vehicle control, Abraxane, Phenelzine or PD1 ($n = 4/5$). **(C)** Flow cytometry for total macrophages, inflammatory macrophages, and M2-like macrophages in the TME. *$p < 0.05$, Mann–Whitney $t$-test ($n = 4/5$). Representative images of **(D)** M1 and **(E)** M2 staining of FFPE tumor tissues in 4T1 mouse model. **(F)** Sections of primary 4T1 tumors were fixed and IF microscopy performed probing with M1 focused primary antibodies to F4/80, iNOS, CD86, and PDL1 with DAPI (green = F4/80 red = iNOS, yellow = CD86, cyan = PDL1, blue = DAPI). The population % of F4/80 cells positive for iNOS, CD86 and PDL1 was measured using ASI's mIF system. Representative images for each dataset are shown. Graphs plots represent the % population ($n \geq 500$ cells profiled per a group, $n = 4$ mice). **(G)** Section of primary 4T1 tumors were fixed and IF microscopy performed probing with M2 focused primary antibodies to F4/80, EGR2, CD206, and PDL2 with DAPI (green = F4/80 red = EGR2, yellow = CD206, cyan = PDL2, blue = DAPI). The population % of F4/80 cells positive for EGR2, CD206, and PDL2 was measured using ASI's mIF system. Representative images for each dataset are shown. Graphs plots represent the % population ($n \geq 500$ cells profiled per a group, $n = 4$ mice).

and downregulated negative regulators such as *B7-H3* in macrophages in the TME (**Figure 5C**). Genes were also upregulated in a subset of $CD169^{+}$ macrophages (**Figure 5D**). Therefore, phenelzine had a significant impact on the genetic reprogramming of macrophages toward a more M1-like phenotype in the TME in mice.

## The Macrophage Post-translational Modification Landscape of Genes Up-Regulated in Phenelzine

We next determined how phenelzine treatment affected macrophage gene expressions measured using the NanoString platform (FDR < 0.15) by overlaying these data with published epigenomic data.

The 178 genes up-regulated by phenelzine had promoters (±1 kb TSS) with significantly ($p < 0.01$) less CpG and GC content than either the "unchanged" or down-regulated genes (**Figure 6A**). The 38 down-regulated gene promoters had significantly ($p < 0.01$) more CpG and GC content than unchanged gene promoters (**Figure 6A**).

Up-regulated gene promoters had significantly less H3K4me3 ($p < 0.05$) but not H3K27ac and H3K4me1 than the down-regulated gene promoters, in resting RAW264.7 cells (**Figure 6B**) (47). Importantly the up-regulated gene promoters had less H3K27me3 than the unchanged genes, suggesting their non-maximal gene expression is not due to H3K27me3-mediated repression (**Figure 6B**).

Further, the up-regulated promoters were less accessible in both NS and 6 h LPS-stimulated bone marrow derived

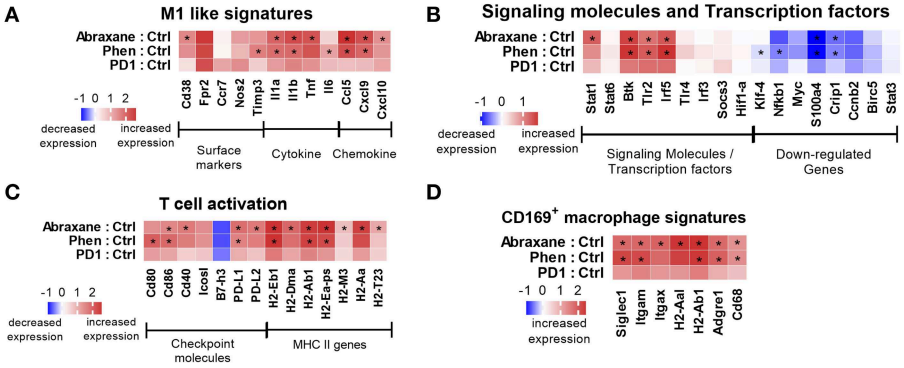


**FIGURE 5 |** Nanostring counts (log2 fold-change) from RNA isolated from macrophages in the TME for **(A)** M1 phenotypic signatures and pathways, **(B)** M1 signaling molecules and transcription factors, **(C)** T cell activation gene signatures, and **(D)** CD169$^{+}$ macrophage gene signatures. *Indicates Benjamini–Yekutieli false discovery rate value $< 0.05$.

macrophages (BMDMs) than the down-regulated genes but were more accessible than the unchanged genes (**Figure 6C**) (48).

The up-regulated promoters had significantly higher H3K4me1, H3K27ac, and H3K4me3 levels than the unchanged genes in BMDMs (31) and H3K4me1 and H3K27ac levels significantly increased (1.6-fold and 2.3-fold respectively) after 24 h LPS stimulation (**Figure 6D**).

Using enhancer regions identified in Ostuni et al. (31), we determined how many of the up- and down- regulated genes had enhancers within 10 kb of their TSS. Significantly more of the up-regulated genes had constitutive or constitutive but not steady (24 h_CONST) macrophage enhancers than all the entire set of genes. This is consistent with our chromatin accessibility profiles in **Figures 3C,D**.

## LSD1 Inhibition and Chemotherapy Target the Hippo and Wnt Signaling Pathways

Using the NanoString platform, there was higher differential expression (DE) of NanoString default geneset annotations between mice treated with Abraxane and phenelzine than to control (orange) than with PD1 (blue) (**Figure 7A**, undirected). The complement activation, interferon and chemokine signaling, T-cell activation and checkpoint signaling, Th1 activation, antigen presentation, and TLR signaling pathways had higher DE (orange) (**Figure 7A**). Phenelzine showed similar upregulation of pathways as macrophages from Abraxane-treated mice (**Figure 7A**, directed). When the gene signatures were annotated using KEGG, Abraxane-, and phenelzine-treated groups upregulated genes associated with the Hippo signaling pathway and downregulated genes associated with the Wnt signaling pathway (**Figure 7B**). Phenelzine treatment also upregulated genes linked to the Ras signaling pathway, distinct from Abraxane- and PD1-treated mice (**Figure 7B**).

We next determined which genes were specific to Abraxane and phenelzine treatments. The treatments shared 93 gene signatures, 50 specific to Abraxane and 48 specific to phenelzine (**Figure 7C**). The top six NanoString default pathways specific to Abraxane or phenelzine are shown in **Figure 7C**. Abraxane treatment seemed to have a greater impact on genes associated with antigen presentation that phenelzine (**Figures 7C,D**), while phenelzine had a greater effect on genes associated with extracellular matrix remodeling and metabolism (**Figures 7C,D**).

Although macrophages isolated from the TME of mice treated with Abraxane and phenelzine showed similar gene expression changes such as upregulation of M1-like genes and pathways, these two different treatments also target specific pathways: antigen presentation in the case of Abraxane-treated mice and ECM remodeling and metabolism in phenelzine-treated mice.

# DISCUSSION

Macrophages form a large component of the TME and may have anti- or pro-tumorigenic properties, making them a viable target for cancer immunotherapy. Macrophages are broadly described as M1 (classical) or M2 (alternative) depending on their activation, although this is known to represent a phenotypic spectrum. Here we examined the effects of epigenetic inhibition of LSD1 on macrophage phenotype *in vitro* and *in vivo* using two different LSD1 inhibitors: GSK, which only binds to the FAD domain, and phenelzine, which can bind to the FAD domain and disrupt the LSD1-CoREST complex. Using these inhibitors, we show for the first time a potential role for the FAD and LSD1-CoREST complex in mediating downstream gene signatures to generate an M1-like macrophage phenotype *in vitro* and in the TME of mouse triple-negative breast cancers.

To understand how LSD1 inhibition could affect macrophage polarization, we utilized two LSD1 inhibitors, GSK and phenelzine. While both inhibitors bound to the FAD domain, phenelzine but not GSK induced small structural changes in the CoREST binding region of LSD1, which has been shown to be important for LSD1's activity and stability (49). Phenelzine disrupted co-expression of nuclear LSD1 and CoREST and SNAIL and CoREST. Interestingly, when cells were polarized toward an M1 phenotype using IFN-γ and LPS, there was similar downregulation of nuclear LSD1 and CoREST, but this did not occur in M2 polarization with IL-4 or with GSK treatment. Therefore, phenelzine may play a dual role

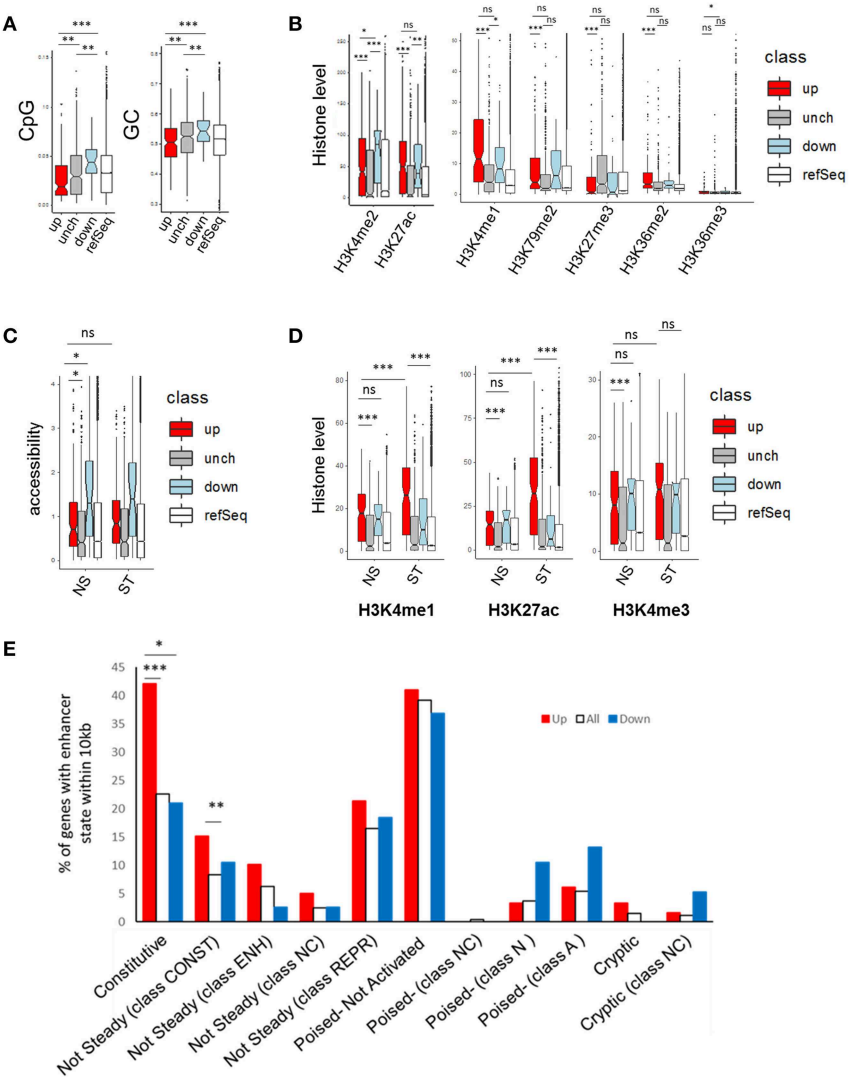


**FIGURE 6 |** CpG content, GC content, histone marks, and accessibility of genes in the NanoString panel. **(A)** CpG and GC content, **(B,D)** histone levels and **(C)** accessibility of genes ± 1 kb from transcription start site (TSS) of NanoString genes up-regulated, downregulated (FDR < 0.15), or unchanged (FDR > 0.15) and genes from refSeq in RAW264.7 cells (47). **(C)** shows accessibility of Nanostring genes against gene sets from bone marrow derived macrophages (BMDMs) with or without 6 h of LPS stimulation (48). **(E)** Levels of up/down regulated NanoString genes that had enhancers within 10 kb of their TSS from BMDMs with or without 24 h of LPS stimulation (31). A *t*-test with unequal variance (Welch two sample *t*-test) was used with $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

by disrupting the LSD1-CoREST complex and potentially its stability and activity, while GSK can only bind the FAD domain. The LSD1-CoREST complex also appears to have a role in repressing the M1 macrophage phenotype, because there was similar downregulation of LSD1, CoREST, and SNAIL expression when the cells were polarized toward an M1 phenotype using IFN-γ and LPS. However, this was not observed when cells were polarized to an M2 phenotype or treated with GSK, which only targets the FAD domain. This highlights the importance of targeting both the FAD and CoREST domains of LSD1 to reprogram macrophages toward an M1-like phenotype for therapeutic benefit.

The LSD1-CoREST complex has been shown to promote demethylation of nucleosomal histone 3 lysine 4 (H3K4) (49, 50) and histone 3 lysine 9 (H3K9) (51). To determine if LSD1 demethylase activity participates in macrophage polarization, we used immunofluorescence microscopy to show that cells treated with phenelzine had higher expression of histone H3K9me2 and H3K4me2, which are direct targets of LSD1. Macrophages polarized to an M1 phenotype using IFN-γ and LPS showed similar higher expression of these histone markers, with the opposite true in cells polarized to an M2 phenotype or treated with GSK. We also measured nuclear LSD1 demethylase activity in our *in vitro* model and showed that phenelzine, tranylcypromine (an LSD1 inhibitor), and macrophages polarized with IFN-γ and LPS inhibited LSD1 activity compared to control cells, whereas treatment with GSK or M2 polarization had little effect on nuclear LSD1 activity.

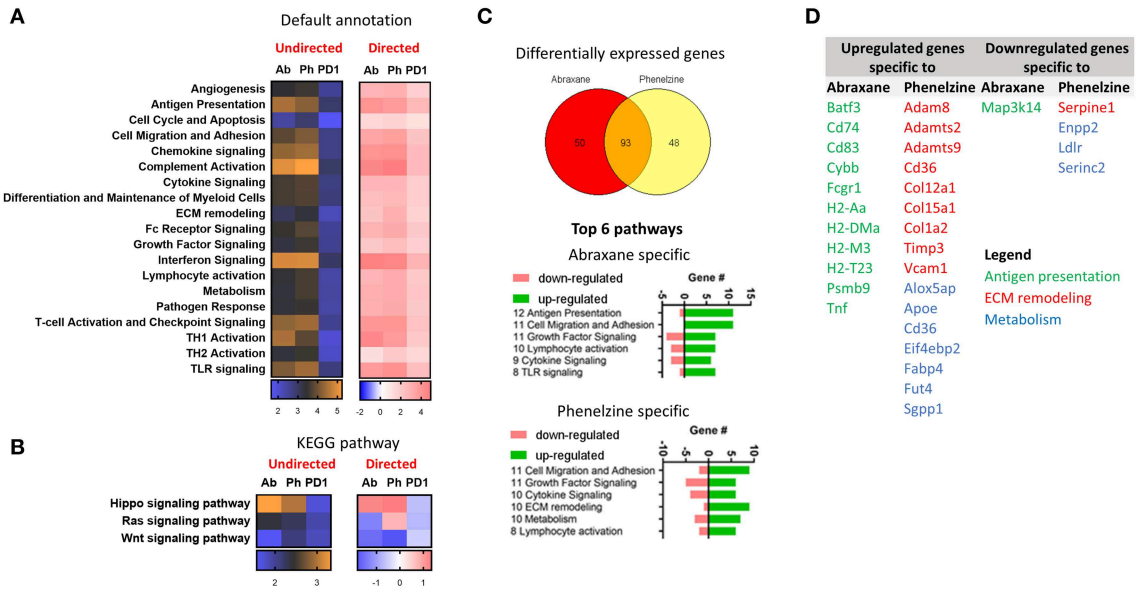


**FIGURE 7 |** Phenelzine affects Hippo, Wnt, and Ras signaling pathways and genes associated with ECM remodeling and metabolism. **(A)** Heatmap displaying the undirected and directed global significance score statistics using the default and **(B)** KEGG pathway annotations. Undirected scores measure the extent of differential expression of a geneset's genes against control ignoring whether each gene within the set is up- or downregulated. Orange denotes genesets whose genes exhibit extensive differential expression against control, and blue denotes genesets with less differential expression. Directed scores measure the extent to which a geneset is up- or downregulated compared to control. Red denotes genesets that show extensive overexpression and blue denotes genesets with extensive underexpression. **(C)** Venn diagram indicating genes that are differentially expressed with a false discovery rate of < 0.05 (Benjamini-Yekutieli) and top 6 pathways that those genes are fall under. **(D)** Table showing the upregulated and downregulated genes that are specific to Abraxane and phenelzine.

This could potentially be due to several factors; for example, LSD1-CoREST complex disruption by phenelzine or IFN-γ and LPS, could destabilize the LSD1 protein in addition to inhibiting the FAD enzymatic domain. Signaling through the IFN-γ receptor by IFN-γ and toll-like receptors (TLR) by LPS could also impede LSD1 demethylase activity on H3K4me2 and H3K9me2. Interestingly, we also observed that RAW264.7 cells had similar cell morphology after 7 days of treatment to cells treated with IFN-γ and LPS (M1). This did not occur after 24 h of treatment (data not shown), suggesting that phenelzine might prime the macrophages to differentiate into a similar morphology to M1 treated cells. As predicted, the expression of nuclear phosphorylated LSD1 at serine 111 (LSD1-s111p) is lower in macrophages polarized to M1 (IFN-γ + LPS) or treated with phenelzine. This most likely the result of the loss of LSD1 due to the destabilization of the LSD1-CoREST complex.

We also showed that M1 (IFN-γ + LPS)-polarized and phenelzine-treated macrophages downregulate expression of the transcription factor SNAIL, and previous work has shown that SNAIL knockdown in human THP-1 macrophages and breast cancer cells promotes M1 polarization both *in vitro* and *in vivo* (42, 52). Therefore, inhibition of the demethylase activity of LSD1 using phenelzine, which targets both the FAD and CoREST domains, could play a role in M1 polarization, either directly or indirectly through the transcription factor SNAIL. Intriguingly, we have previously shown that this nuclear phosphorylated form of LSD1-s111p is mediated by protein kinase-C theta (PKC-θ) in cancer stem cells (CSCs) (22, 53). PKC-θ has been reported to regulate various genes in T cells (54) and promotes a potent pro-inflammatory macrophage phenotype (55). However, this latter study may not have examined the nuclear role of PKC-θ, so it could be possible that in the context of LSD1-s111p, the nuclear role of PKC-θ is distinct from its cytoplasmic role as previously shown in CSCs (22). We have also previously shown that nuclear PKC-θ can regulate microRNAs in T cells (56). Therefore, it would be interesting to explore the nuclear role of PKC-θ and its ability to mediate the M1 phenotype via LSD1.

The classically activated M1 (IFN-γ + LPS) phenotype has been shown to have anti-tumorigenic properties. We found that the gene signatures of RAW264.7 mouse macrophages inhibited with phenelzine mimicked the M1-like signatures of macrophages classically activated with IFN-γ and LPS. It has previously been shown that the increase in demethylase Jumonji domain containing 3 (Jmjd3) contributes to the decrease in H3K27me2/3 and transcriptional activation of specific M2 marker genes such as *Chi3l3, Retnla*, and *Arg1* (57). Our results show that LSD1, another demethylase, might play a role in regulating macrophage polarization toward an M1 phenotype. Of therapeutic relevance, we also discovered that PD1, PD-L1, and PD-L2 might also change when macrophages polarize, with unpolarized cells expressing PD1, M1 cells expressing PD-L1, and M2-polarized cells expressing PD-L2. Therefore, these immune checkpoint proteins might be useful M1 or M2 biomarkers.

We hypothesized that treatment of tumor-bearing mice with phenelzine could alter the TAMs in the TME. Using a TNBC syngeneic mouse model, we showed that LSD1 inhibition slightly reduced tumor volume and epigenetically reprogrammed TAMs to a more anti-tumor phenotype. While there was no change in the total F4/80$^+$-expressing macrophage population, there was a significant reduction in both inflammatory (Ly6C$^+$) and M2-like macrophages (CD206$^+$). We postulated that since there were no significant changes in the total macrophage population, phenelzine treatment reprogrammed the macrophages already present in the tumor toward an anti-tumor phenotype. Interestingly, this effect was also seen with Abraxane and anti-PD1 antibody, suggesting that phenelzine alone was able to contribute to this reprogramming at the gene level. Our tissue section of mice tumors showed that phenelzine and PD1 treated mice tumors contained more macrophages expressing M1-like markers (iNOS, CD86, and PD-L1) and lower proportions of M2-like markers (CD206, EGR2, and PD-L2) suggesting that phenelzine and PD1 treatment favors a M1-like phenotype in the TME.

To further characterize the TAMs in phenelzine-treated mice, we used the NanoString platform to show that they altered expression of genes related to an M1 phenotype such as *Il1a, Il1b, Il6, Ccl5, Cxcl9*, and *Cxcl10* (58–62). There was also increased expression of *Stat1* and decreased expression of *Stat3*, which are associated with M1 and M2 polarization, respectively (58, 63, 64). Macrophages from phenelzine-treated mice also showed a significant decrease in the NFκB1 transcription factor compared to control. It has been shown that blocking NFκB signaling can switch TAMs to an M1-like phenotype (65) and that p50 overexpression in TAMs inhibits M1 anti-tumor resistance (66). It is known that the NFκB signaling pathway activation through TLRs induces M1 macrophage polarization and subsequent pro-inflammatory effects through the p65 phosphorylation and IκB (67–69), so it would be interesting to determine whether the TLR4/NFκB signaling is affected by phenelzine treatment. Phenelzine treatment also significantly reduced KLF4 expression in macrophages isolated from the TME, with KLF4 previously shown to be reduced in M1 macrophages and robustly induced in M2 macrophages (70) via the RORα (71) and IRF4 axes (72). We also saw a significant increase in IRF5 expression, another M1-associated protein, on phenelzine treatment (63, 73, 74) and Btk was similarly increased; Btk inhibition with ibrutinib impairs M1 polarization (75, 76). Phenelzine-treated macrophages also significantly increased TIMP3, which is a potent tumor angiogenesis and growth inhibitor (77–79). We have previously shown using LSD1 chromatin immunoprecipitation (ChIP) sequencing that LSD1 can directly or indirectly execute genome-wide EMT via target transcription factors (22). Therefore, it is interesting to observe common mechanisms affecting gene regulation in CSCs and M1 polarization.

Of note, phenelzine-treated macrophages had similar features to the CD169+ macrophages that dominate anti-tumor immunity via cross-presentation to cytotoxic T lymphocytes (80–84). LSD1 inhibition also upregulated checkpoint molecules such as CD80/86 and MHC class II genes and downregulated negative regulators such as B7-H3. Consistent with our *in vitro* polarization studies, phenelzine significantly upregulated PD-L1. Although PD-L1 is usually an inhibitory signal, it was upregulated when macrophages were polarized toward an M1 phenotype, and a similar trend was also seen in macrophages isolated from Abraxane- and PD1-treated mice.

Our *in silico* analysis also showed that genes upregulated by phenelzine treatment had promoters with significantly less CpG and GC content compared to "unchanged" or

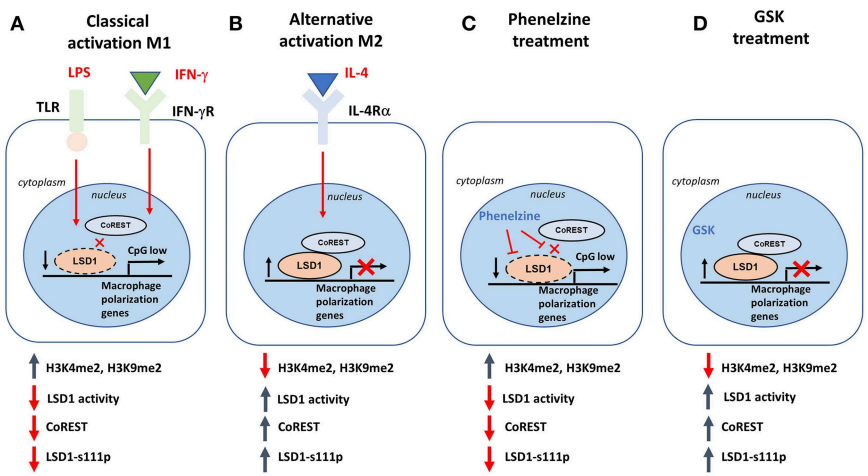


**FIGURE 8 |** Putative model of how LSD1 can reprogram macrophage polarization. **(A)** When macrophages are stimulated with LPS and IFN-γ (classical activation; M1), disruption of CoREST destabilizes LSD1, which leads to LSD1 losing its repressive role in regulating M1-associated genes. It also increases LSD1 demethylase activity. **(B)** When macrophages are stimulated with IL-4 (alternative activation; M2), CoREST is not affected, resulting in stable expression of LSD1p and LSD1 maintaining its repressive role in regulating M1-associated genes. It also decreases LSD1 demethylase activity. **(C)** LSD1 inhibition using phenelzine can target both LSD1p and CoREST, mimicking a similar response to M1 polarization while **(D)** GSK was not able to achieve the same result because it did not disrupt the LSD1/CoREST complex.

downregulated genes. Conversely, downregulated gene promoters had significantly more CpG and GC content. A direct repressive role for LSD1 for the M1 genes is more likely to be due to its demethylation of H3K4. High H3K4 methylation is associated with increased DNA accessibility at promoters and enhancer regions] and the phenelzine responsive gene promoters are initially less accessible and more tilted toward a lower H3K4 methylation state than the down-regulated genes. In LPS activated cells the H3K4 methylation levels at the phenelzine responsive gene promoters increases. We also show that phenelzine treatments increases accessibility at both the promoters and nearby enhancers which is mostly likely linked to increased methylation of the surrounding histones. H3K4 methylation is dependent, not only on demethylases but also on methylases like MLL1 and SET1 (85). MLL1 contains a CpG binding domain, and SET1 binds an accessory protein with one (85). It is possible that the H3K4 methylation levels of CpG low promoters are more dependent on the levels and activity of demethylases, while CpG high promoters are more dependent on levels and activities of methylases.

When examining pathway changes, phenelzine inhibition increased genes associated with the Hippo and Ras pathways but decreased genes associated with the Wnt pathway. Upregulated Hippo signaling sequesters β-catenin in the cytoplasm via YAP/TAZ, negatively regulating the Wnt pathway (86). Wnt/β-catenin signaling is activated via c-myc during monocyte to macrophage differentiation and M2 polarization (87). Active Wnt signaling is also implicated in macrophage-associated angiogenesis and tumor invasion (88–90). Therefore, LSD1 can play a role in regulating genes associated with both pathways, and LSD1 inhibition by phenelzine may be able to reduce M2 macrophage polarization as well as macrophage-associated angiogenesis and tumor invasion. Interestingly, we also observed the upregulation of genes associated with Ras signaling, a commonly dysregulated pathway in various cancer types that regulates cell growth, survival, proliferation, and apoptosis (91–93). How LSD1/CoREST destabilization in M1 macrophages upregulates Ras signaling would be worthy of further study.

Overall, Abraxane treatment affected genes associated with antigen presentation, whereas phenelzine affected genes associated with extracellular matrix (ECM) remodeling and metabolism. Therefore, LSD1 can modulate genes associated with ECM remodeling and metabolism, both important components of the TME (94–97). Since, ECM remodeling is mainly associated with M2 macrophages, it is plausible that phenelzine also impacts genes associated with M2 macrophages. Further studies should investigate whether this has a positive or negative functional impact in the context of TNBC. Macrophage function and polarization are also closely associated with metabolic functions, with the M1 inflammatory phenotype heavily dependent on glycolysis and M2 alternatively activated macrophages relying on oxidative phosphorylation [extensively reviewed in (98, 99)]. Since phenelzine treatment affects genes associated with metabolism, there may be the potential to epigenetically prime macrophages by modulating LSD1.

We have previously shown that LSD1 could target gene induction programs promoting epithelial to mesenchymal transition (EMT) and cancer stem cells (CSC) and that inhibition of LSD1 suppresses chemotherapy-induced EMT and cancer associated fibroblasts (CAFs) (22). However, it is also important to note that phenelzine could potentially affect other tumor infiltrating subsets of cells in the TME such as effector T-cells and myeloid-derived suppressor cells. This is beyond the scope of this study; however, it would be a very interesting area worth exploring.

Overall, our data proposes a model in which LSD1 poises M1-selective gene signatures in naïve macrophages by tethering to the epigenome of such genes, similar to in CSCs (22). LSD1 globally decorates the epigenetic landscape of M1 gene signatures in naïve macrophages with H3K4 and H3K9 methylation marks. Following activation of M1 signaling pathways, nuclear LSD1 activity is rapidly reduced due to the disassembly of the LSD1-CoREST complex, leading to destabilization of the nuclear LSD1 pool (**Figure 8**). This primes the epigenome of M1-inducible genes, leading to their expression. In parallel, M2 gene activation increases nuclear LSD1 activity and LSD1-CoREST, in turn maintaining repression of M1 genes and skewing induction of the M2 gene signature (**Figure 8**). Sequential ChIP and co-immunoprecipitation studies will be required to unravel the in-depth molecular signatures underlying LSD1's contribution to the M1/M2 phenotypes. Priming by phenelzine alone may not be sufficient to polarize macrophages toward a M1 phenotype, and further studies are needed to establish which combinatorial therapies optimally enhance the phenotypes observed in this study.

In conclusion, the LSD1 inhibitors phenelzine and GSK are useful tools for studying the catalytic and non-catalytic role of LSD1. These inhibitors have allowed us to dissect the contribution of LSD1 enzymatic activity and the nuclear LSD1-CoREST complex on M1/M2 phenotype switching. These effects were replicated *in vitro* and *in vivo*. Inhibitors with dual FAD and CoREST-targeting abilities could be important for reprogramming macrophages and potentially initiate an anti-tumor M1-like phenotype in TNBC and other cancers.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, Australian National University Animal Experimentation Ethics Committee. The protocol was approved by the Australian National University Animal Experimentation Ethics Committee.

## AUTHOR CONTRIBUTIONS

AT, WT, RM, JF, and SR designed the experiments. AT, WT, RM, TD, ST, KH, and JK performed the experiments and analyzed the data. AT and SR wrote the manuscript. SR conceived the study.

## FUNDING

This work was supported by the National Health and Medical Research Council (Grant ID APP1068065 and GNT1105747) (CIA SR).

## ACKNOWLEDGMENTS

We would like to thank ASI-Metagene and Nir Katzir with his assistance in setting up and developing the algorithms and high-throughput microscopy workflow for the ASI platform. We would like to thank Jenny Dunn with her assistance in running some of the quantitative real time PCR.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01351/full#supplementary-material

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**Conflict of Interest Statement:** In accordance with NHMRC guidelines and our ethical obligations as researchers, we report that the University of Canberra, SR, RM, and AT have a financial interest in EpiAxis Therapeutics Pty Ltd. SR is also Chief Scientific Officer of EpiAxis Therapeutics Pty Ltd. We have in place a plan for managing any potential conflicts arising from that involvement.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 14 June 2019
doi: 10.3389/fimmu.2019.01355

# Transcriptional Regulation in the Immune System: One Cell at a Time

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Transcriptional regulation of cells in the immune system must be strictly controlled at multiple levels to ensure that a proper immune response is elicited only when required. Analysis in bulk, or ensemble of cells, provides a wealth of important information leading to a better understanding of the various molecular steps and mechanisms involved in regulating gene expression in immune cells. However, given the substantial heterogeneity of these cells, it is imperative now to decipher these mechanisms at a single cell level. Here I bring together several recent examples to review our understanding of transcriptional regulation of the immune system via single cell analysis and to further illustrate the immense power of such analyses to interrogate immune cell heterogeneity.

**Keywords: transcriptional regulation, gene expression, B cells, single cell analysis, immunity**

OPEN ACCESS

***Edited by:***
*Keiko Ozato,*
*National Institutes of Health (NIH), United States*

***Reviewed by:***
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***Specialty section:***
*This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology*

***Received:*** *08 April 2019*
***Accepted:*** *29 May 2019*
***Published:*** *14 June 2019*

***Citation:***
*Roy AL (2019) Transcriptional Regulation in the Immune System: One Cell at a Time. Front. Immunol. 10:1355. doi: 10.3389/fimmu.2019.01355*

## INTRODUCTION

Given that transcriptional regulation plays a critical role in mounting a proper immune response, control of gene expression in various cells of the immune (both adaptive and innate) system at transcriptional level has been studied for decades, which has provided important and fundamental information regarding various control mechanisms as well as identified crucial factors that are necessary for transcriptionally regulating the expression of genes required for mounting appropriate immune responses (1–4). Combining these *ex vivo* studies with *in vivo* studies, primarily through murine models, enabled us to decipher in exquisite details, both molecular mechanisms and physiological steps involved in transducing immune signals to elicit correct immune responses at the right time (1–4). Collectively, these studies have provided insights into the logic that dictates how the adaptive and innate arms of the immune system differ with respect to regulating specific genes at the level of structural and functional folding of the chromatin domains, epigenetic regulations, long-range interactions that bring promoter regions and regulatory enhancers in proximity, specific transcription factors that are necessary for lineage commitment and differentiation, and non-coding RNAs that play pivotal roles in immunity (5, 6). However, while the reductionist approaches of studying regulation of individual genes and gene clusters in a given cell were necessary, they were insufficient because such mechanisms in isolated and/or cultured cells could not lead to a systems level view of gene regulation. The advent of next generation sequencing allowed probing global regulatory processes and genome-wide changes in gene expression during immune responses simultaneously in multiple cell types.

In animal tissue, neighboring cells that are apparently identical turn out to exhibit important differences when significant depth of analysis was achieved via single cell techniques. Originally, single cell techniques were applied in situations where biological sample was limiting. But now, given the high throughput technologies that are at our disposal, profiling hundreds of thousands of heterogeneous cells within a population is possible with relative ease (5, 6). With all these remarkable technological advances in studying cellular heterogeneity and discovering rare cell

populations via single cell analysis in animal tissues/organs, the question might still be asked whether we really need to understand human biology at single cell resolution. After all, the human body has been defined over centuries by anatomical landmarks, tissue and organ distributions. The answer might lie in the fact that the bewildering cellular heterogeneity in humans often dictates the diseased states and their origins and subsequent treatment. For instance, two apparently "identical" cells in the same organ might behave differently to therapeutic intervention depending on their molecular and functional states. Hence, a "shotgun" approach to treat all neighboring cells in a given tissue might not be necessary or achieve the precision that we strive to attain in modern medicine. Given these considerations, it is no wonder that the precise anatomical landmarks are insufficient and that molecular and positional information of tissue and organ-resident cells must be understood in greater depth to define the human body and its associated maladies (7).

Despite significant technological advances, our understanding of the gene regulation in the immune system still remains incomplete because there is substantial heterogeneity in the cells constituting the system. Immune cells are diverse with respect to developmental stages, function and cell types (e.g., adaptive vs. innate immune cells) as well as location (e.g., primary vs. secondary lymphoid organs) in addition to circulating immune cells through peripheral blood and lymphatic systems (5, 6). Moreover, the function of primary immune cells, apparently of the same lineage, also frequently depends on their interactions with the secondary non-immune cell types and tissues. An added layer of complexity for specific identification of immune cells is introduced by their clonality: they express signature surface immune receptors with distinct genetic diversity that might functionally respond differently to a distinct set of ligands (6). Due to these complexities and the fact that apparently identical immune cells can function at different locations in the body depending on the nature of the requisite immune response, it is imperative that they be profiled at high resolution to determine if indeed they arise from the same origin and consequently might respond similarly during an immune response (6). Here I outline a few recent studies to illustrate the lessons learned from single cell approaches in immune cells and how they often fill gaps of our understanding of the immune system gathered from ensemble and organismal level analysis. Because single cell analysis is still largely limited to transcriptomic analyses (e.g., Single cell RNA-seq, scRNA-seq), these studies illustrate the immense power but also limitations of such analyses. scRNA-seq has been used to identify and classify cell types. Furthermore, it has also been used to characterize rare cell types and analyze variation of gene expression across distinct cell populations based on their steady state RNA levels. However, the dynamics of precise cellular states that are often transient in nature are more difficult to assess simply based on transcriptomic studies (8). But recent developments in imaging (e.g., single molecule Fluorescent *in situ* Hybridization, FISH), proteomics (with CyTOF and MIBI-TOF) and genomics (e.g., LIANTI) provide substantial hope that these additional methods reporting functional states of a cell would complement RNA-seq to identify novel cell types, and further analyze and assign function to these cells and tissues to perhaps reveal unchartered but promising therapeutic avenues (reviewed in 7).

## SOLVING LONGSTANDING IMMUNOLOGICAL PROBLEMS via SINGLE CELL ANALYSIS

Memory B cells of the adaptive immune system are generated following pathogenic infection or vaccination so that they can respond against future infections (9). However, select pathogens find ways to either totally evade or, at a minimum, suppress the adaptive immune response. These conditions often lead to induction of memory B cells that are ineffective in differentiating into antibody secreting plasma cells (9). Given that memory B cells play a critical role in vaccination, understanding the basis for such heterogeneity and identifying pathogen-specific memory B cell repertoire could important clues to improve vaccine development. Single cell transcriptomic (scRNA-seq) analysis and specific gene expression programs associated with such diverse population of memory B cells are providing important information for improving future vaccine development and antibody designing for therapeutic usage (10). Another long-standing problem in human immunity is the precise role of Immunoglobulin E (IgE) antibodies. Although secreted IgE protects against infection, it might also cause major health problems, particularly during allergic reactions (11). Despite the fundamental importance of IgE in health and disease, molecular and structural insights into IgE antibodies remain incomplete. Using single cell RNA-seq, Croote et al. determined the gene expression profiles and alternative splicing patterns of IgE secreting B cells from patients with food allergies (11). Remarkably, these specific transcriptomic signatures and splicing profiles specifically associated with IgE producing B cells exhibited identical patterns in individuals who are unrelated (11). These results suggest that these antibodies or derivatives could be employed as therapeutic agents (11). Furthermore, these results might also lead to further understanding of biochemical roles of IgE antibodies in allergic reactions (11). A recent fascinating study identified heterogeneity in uncommitted hematopoietic progenitors with mixed lymphoid and myeloid potentials by single cell RNA-seq (12). Although such heterogeneity has been known for some time, this study concludes that the decision of lymphoid and myeloid lineage choice surprisingly occurs before the hematopoietic progenitor stages with combined lymphoid-myeloid potential called the early progenitor with lymphoid myeloid potential (EPLM) (12). Furthermore, the apparent multipotency of uncommitted progenitors is due to the presence of four subpopulations within these cells, each with their own developmental potentials that are not necessarily restricted to bipotency for lymphoid and myeloid lineages only (12). These results further underscore the power of single cell transcriptomics in resolving both cellular heterogeneity of immune cells as well as establishing molecular relationships amongst distinct hematopoietic precursors via identifying specific transcriptional signatures associated with them (12).

## FUNCTION OF IMMUNE CELLS IN DISTINCT ORGANS

As noted in the introduction, immune cells are found at multiple organs/tissues within the human body, raising the question of whether they behave in the same fashion at these discrete locations. Single cell analysis is making it possible to interrogate the identity and infer the function of immune cells found in disparate locations. For instance, although it has been known that immune cells are found in liver, the immunobiology of liver remains poorly understood. A new study mapped the cellular landscape of human liver using tissue dissociation techniques combined with functional assays and scRNA-seq (13). They identified 20 discrete types of hepatocytes and other cell populations including B, T, monocyte/macrophage and NK cell types (13). Combining scRNA-seq with image-based approaches provided a detailed spatial map of immune microenvironment of the human liver (13). However, there are several notes of caution raised by the authors. First, these studies show that transcriptional profiling of hepatic cell populations significantly depends on how the liver tissues are prepared as well as the viability of bulk liver homogenate (13). Second, cells dissociated from tissues might behave differently than bulk tissues—in particular, hepatocyte populations are susceptible to dissociation because of the significant heterogeneity of the liver cells (13). Thus, one should take into consideration that not all cell types will be captured with equal efficiency during scRNA-seq analysis (13). Although these single cell mapping efforts identify distinct populations of cells, they do not necessarily identify the "actual frequency of their existence" within the liver tissue of experimental origin (13). Finally, a fact that the community has grappled with in analyzing dissociated cells from tissues is how to define a "normal" tissue. For example, despite the fact that these human liver samples were obtained from "clinically acceptable, healthy liver grafts," they exhibited mildly inflamed conditions (13). Regardless of these cautionary notes, it is clear that scRNA-seq, combined with other imaging and functional (multi-modal) studies, has immense potential to create detailed cellular maps of human tissues.

Another recent study maps tissue resident macrophages in murine lung and identifies two subpopulations of interstitial macrophages via single cell transcriptomics (14). This study showed that two populations of macrophages in the murine lung were phenotypically distinct and exhibited differences in their intratissue localization (14). While one population of these cells lies close to tissue nerve bundles, the other population is more closely associated with blood vessels and thus, presumed to play a critical role in maintaining blood vessel integrity and antifibrotic activity (14). Although known for some time, these observations show that the immune cells of the same lineage but residing in different locations can function differently depending on the local tissue microenvironment (14). Thus, combining single cell transcriptomics with functional studies and spatial information could lead to identifying novel immune cell populations with characteristic molecular profiles and distinct tissue localizations, presumably performing distinct functions at these locations.

## IDENTIFICATION OF NOVEL CELL TYPES AND STATES

It is well-known that the vertebrate immune system consists of the innate and adaptive arms, responding to immediate challenge and responding to threats via acquired antigen receptors, respectively. While the innate immune arm in mammals are formed from cells of the myeloid lineage (granulocytes, mast cells, monocytes/macrophages, and dendritic cells), the cells constituting the adaptive immune system are primarily composed of B and T lymphocytes (15). However, the recent discovery of innate lymphoid cells (ILCs) that constitute a rare sub-population of lymphocytes, has challenged this binary notion. Unlike T and B cells, ILCs do not express specific cell surface antigen receptors or undergo clonal expansion when stimulated *ex vivo* (16, 17). Instead, ILCs express cytokine receptors likely to sense environmental threats and rapidly produce a distinct set of cytokines in response to these signals (16–18). Discovery of ILCs has accelerated the need for unbiased methodologies to profile immune cell types solely on the basis of cellular and/or molecular signatures rather than on cell surface markers, because so far immune cells have been traditionally profiled based on surface receptor expression (18). In trying to characterize the immune repertoire in zebrafish, a recent study generated a comprehensive atlas of cellular signatures of lymphocytes defined by their unique transcriptomic profiles in steady state and after challenging the immune system to induce short term inflammation (18). This scRNA-seq analysis led to the surprising finding that zebrafish possesses cytokine producing ILC-like cells much like mammals, potentially involved in responding to environmental threats (18). Thus, scRNA-seq allows to identify heterogeneous cell population and different cellular states in an unbiased fashion based on specific transcriptomic signatures, rather than their surface receptor expression profile (18).

## SINGLE CELL ANALYSIS AND HETEROGENEITY

Heterogeneity in gene expression is important to elucidate because it might indicate the existence of new and yet unidentified subpopulations in such milieu. But, heterogeneity in gene expression profile could also provide novel insights into the function of a given gene or sets of genes (8, 19). For instance, even in an apparent homogeneous population, variation in gene expression will likely arise from stochastic gene expression in addition to various dynamic cellular states like the cell cycle or circadian rhythm (19–21). The steady state level of RNA expression could indicate a static cellular state, but it does not directly reveal status of dynamic processes such as cellular differentiation, cell cycle or circadian rhythm (8, 19–21). In dealing with heterogeneity, one should also be careful about dealing with variability in biological samples, which could be confounding and problematic for further downstream analysis (7, 22, 23). There are two broad types of variability or noise in these experiments: technical variability and biological

variability. Technical variability/noise is usually due to changes in sample preparation or processing that might vary depending on the protocols used and experimental conditions (7, 22). In contrast, biological variability might arise due to differences in environmental perturbation or inherent genetic variances of derived biological samples (7, 22).

Additionally, the heterogeneity exhibited in scRNA-seq data could also be due to variability in the expression of a given gene in various cells. This in turn could depend on relative expression state of a given gene compared to other genes in the same signaling pathway (8, 19–21). Variability in level of expression of a given gene across different cells might also reflect how tightly the transcription of this gene is regulated (19). It is now generally believed that nuclear transcription occurs due to the result of RNA polymerase II activity in short bursts giving rise to a set of transcripts, which are processed and transported from the nucleus to the cell cytoplasm for functional usage (24). It then stands to reason that genes resulting from higher transcriptional bursts but lower frequency of expression produce more noise than genes that are expressed due to less frequent transcriptional bursts (19, 24–26). Additionally, it is also shown by Padovan-Merhar et al. that increasing cellular volume or content can result in enhanced transcription because both transcriptional burst size and frequency changes with cellular content/volume and with cell cycle (25). In this regard, it is worth considering an early study of scRNA-seq analysis of bone marrow-derived dendritic cells treated with LPS, which demonstrated extensive bimodal pattern of gene expression and splicing with two distinct patterns of cellular states (27). While the variation is likely due to a number of factors, including developmental stages of the cells, cytokine signaling of a subset of fast-responding bone marrow derived dendritic cells could affect the whole population in part due to changes in transcriptional bursting and alternate splicing (19, 27). In a more recent study, Wu et al. addressed how immunoglobulin (Ig) class switching is triggered in activated B cells (28). Although class switch recombination (CSR) is an important process to generate antibody diversity, the mechanism for transcriptional requirement from upstream promoter region of the Ig constant region (I) for targeting of activation-induced deaminase (AID) enzyme for class switching to IgE and IgG1 remained unclear until recently (28). This study, via single cell analysis in a murine model system, identified an early population of B cells that express Iε but not Iγ1 transcripts in response to IL4 signaling (28). This is likely a result in promoter switching to IgE and occurs at lower levels than Iγ1 (28). Hence, heterogeneity in transcriptional activation of Ig promoters is a likely mechanism responsible for targeting of AID to switch to IgE, which could typify transcriptional activation for many gene networks even in identical and apparently homogenously activated B cells (28).

## HETEROGENEITY AND ALTERNATIVE SPLICING

To understand true transcriptional diversity in cells requires not only to determine total transcript levels but isoform levels as well (5). A computational approach called RNA velocity measures the time derivative of RNA abundance and is capable of distinguishing spliced vs. un-spliced mRNAs in scRNA sequencing analysis. The beauty of RNA velocity is that it can predict the future state of an individual cell on a timescale of hours (29). This appears to be an exciting computational development that could significantly help in analyzing and identifying lineage development and cellular dynamics, which is of particular value when dealing with limited biological samples like human tissues (7, 29). But there are also experimental approaches to identifying splicing variants at single cell level. Using a novel nanopore long-read RNS-seq at single cell level, Byrne et al. experimentally identified thousands of unannotated transcription units, consisting of start and end sites, and hundreds of alternative spliced transcripts in murine B1a cells, suggesting existence of extensive splicing isoforms in these cells (30). Peritoneal cavity derived B-1a cells are distinct from the conventional B2 cells due to their differences in origin of development, their cell surface marker expression and their functions in immune response (31). For example, patients with autoimmune disorders like Systemic Lupus Erythematosus (SLE), Sjogren's syndrome and rheumatoid arthritis exhibit higher levels of B-1 cells when compared to normal subjects (30). Interestingly, hundreds of genes that are specifically expressed in B1a cells exhibit multiple spicing variants, including B cell specific surface receptors, raising the possibility that distinct populations of B1 cells express alternatively spliced protein isoforms, including cell surface receptors, and thus they might respond to different stimuli both quantitatively and/or qualitatively (30). Recognition of such heterogeneity across B1a cell population based on alternative splicing signatures could have important ramifications in better understanding and possible therapeutic potential in treating autoimmune disorders.

## HETEROGENEITY DURING LIGAND DEPENDENT DIFFERENTIATION

Immune cells are characterized by surface expression of specific receptors, those that generally respond to signaling via engagement of cognate ligands for differentiation along particular lineages. However, due to high degree of heterogeneity in immune cell lineages, it remains to be determined whether there are sub-populations within a specific lineage that respond differentially (both in a qualitative and quantitative sense) to ligands. To illustrate differential response of immune cells to specific ligands, Chea et al. used single cell analysis, which revealed that there is significant heterogeneity in response of fetal liver-derived ILC progenitors to Notch signaling (32). It is well-known that Notch signaling is required for T cell development, although it is not required for development of fetal liver-derived ILCs (32). Using scRNA-seq, this current work identified two distinct subpopulation of fetal liver-derived ILCs—one that is sensitive to Notch signaling for their proliferation while the other is independent of Notch (32). Hence, the heterogeneity exhibited during ILC development is defined by distinct transcriptional signatures and their differential requirement for Notch signaling (32). Another example of

ligand dependent lineage commitment in lymphoid cells was provided by an elegant study by Berthault et al. (33). Given there are multiple distinct stages of differentiation associated with commitment of hematopoietic precursors to lymphoid lineage, the identification of molecular steps involved in this process has been difficult to precisely elucidate (33). Beginning with fetal liver derived precursor cells, this study employed scRNA-seq to elucidate how these cells commit to particular lineage choices by identifying transcriptomic signatures characteristic of B and T cell subsets (33). In particular, identifying the "loss of B cell potential," which indicate a "T cell bias signature" or a "loss of T cell potential," indicating a "B cell bias signature" was helpful in characterizing sequential events in this process (33). Surprisingly, majority of precursor cells express both signatures albeit at low levels and such co-expressed signatures persisted through multiple stages of differentiation (33). However, interleukin 7 (IL-7) signaling resolved these lineage choice pathways by quantitatively regulating the lymphoid progenitors via stabilizing the B cell specific transcripts, suggesting a crucial role for cytokine signaling in lymphoid cell fate decisions (33).

## SINGLE CELL ANALYSIS IN AIDING DISEASE HETEROGENEITY

We now know that many patients do not respond to treatments because recent data shows that roughly 90% of drugs are only effective for $<$ 50% of patients (34). The ineffectiveness could be due to the fact that there is substantial cellular heterogeneity (both across intra- and inter-sample variations) in patient population, which can significantly impact therapy response across multiple cell types and thousands of specific genes (34). Moreover, in contrast to ensemble analysis, single cell analysis could lead to identification of individual clones and associated biomarkers, thereby leading to more precise targeting of each clone (34, 35). For instance, scRNA-seq profiling led to the identification of particular B-cell receptor signaling pathways and gene expression patterns in non-Hodgkin lymphomas (36). Such distinct molecular profiles could possibly explain the differences in therapy response to BCR-pathway inhibitors (36). Likewise, profiling of circulating tumor cells in multiple myeloma via single cell analysis led to further classification of this disease and identification of relevant genes and quantitative assessment of their expression patterns that could be important for future treatment and prognosis (37). It is known that most adult B cell lymphomas exhibit a germinal center B cell phenotype (38). But it remains unclear whether these lymphoma derived B cells retain the functional characteristics of true germinal center B cells or they are halted at certain stages of the germinal center maturation reaction, a notion proposed based on ensemble analysis, which shows a co-expression pattern of follicular- and germinal center B cell-specific genes (38, 39). However, by combining scRNA-seq, phenotypic and genetic analyses of follicular and germinal center-derived B cells with modeling, these studies revealed that although bulk patient samples exhibited mixed profiles of gene expression, germinal center-derived and follicular lymphoma-derived B cells showed distinct transcriptional signatures at higher resolution (39). Hence, they conclude that the B cell lymphoma arises not due to a blockade in a specific stage of germinal center B cell maturation process, but rather these cells have undergone germinal center maturation and acquired novel and dynamic gene expression profiles to increase lymphoma heterogeneity (39).

## MECHANISTIC INSIGHTS FROM SINGLE CELL ANALYSIS

While single cell assays have been primarily used for identification of heterogeneous or rare cell types, it has not been widely used to determine transcriptional mechanisms. However, to move beyond these important but often descriptive features, single cell analysis must be able to provide significant insights into mechanistic pathways. Indeed, there are some examples of elegantly using single cell analysis to address transcriptional mechanisms. Mostly by combining various *in vitro* assays with transcriptomics and functional assays, these studies demonstrate that the field of single cell analysis could move beyond descriptive analysis to providing mechanistic insights. Rothenberg and colleagues used a combination of scRNA-seq, *in vitro* differentiation assays along with flow cytometry and time-lapse live cell imaging to address lineage commitment mechanisms during T cell development (40). The transcription factor Bcl11b is expressed in all T cell lineages and necessary for commitment to such lineages from precursor cells but the mechanism of how this factor is turned on and maintain expression throughout T cell lineages remained unclear (40). This study identified three distinct steps to turn on *Bcl11b* expression: (i) an early commitment step, where the locus becomes "poised" for expression, which is dependent on two T cell lineage-restricted transcription factors, TCF-1 and GATA-3, (ii) a more "permissive" step that is dependent on Notch signaling, and (iii) a third "amplitude-control" step to modulate *Bcl11b* gene expression, that requires another transcription factor, Runx1, already present from early precursor cells (40). These stepwise and stage-specific mechanisms act in an orchestrated fashion, thereby tightly regulating transcriptional activation of *Bcl11b* that is necessary for developmental commitment of T cell lineage (40). Another study comprehensively characterized transcriptional and differentiation regulation of myeloid progenitor populations *de novo* (41). They show that simply analyzing cell populations by their cell surface receptor expression does not accurately reflect sub-populations of progenitor cells (41). However, by adopting a multi-modal approach, including scRNA-seq, fluorescent activated cell sorting (FACS), functional assays, chromatin profiling (using H3K4me2 as a mark), genetic perturbation, and computational modeling, the authors could profile myeloid cell precursor sub-populations and further suggest that transcriptional priming in myeloid cells is coupled with *in vivo* developmental commitment (41). Their model also proposes a circuitry of potential transcription factor

activity within and between myeloid sub-populations (41). Therefore, a combination of genetic perturbation with scRNA-seq and computational modeling enables to further identify the critical players of transcriptional programs during the myeloid differentiation process (41). The same group also used a novel technique called, CRISP-Seq, that combines CRISPR-pooled techniques with scRNA-seq to study the transcriptional pathways regulating bifurcation of monocyte/macrophage and dendritic cell lineages (42). This study identified two critical transcription factor, Cebpb and Irf8 that are critical for such lineage choices and further illustrated the potential of such a highly multiplexed screening strategy to identify "transcriptional rewiring" often associated with inflammatory and antiviral pathways (42).

To elucidate the regulatory check points of B cell development from early hematopoietic precursors through to naïve B cells, Pe'er et al. combined single-cell mass-cytometry together with a computational algorithm to construct developmental trajectories to monitor this progression (43). This comprehensive analysis of human B lymphoid developmental stages allowed them to uncover previously unidentified subsets of B cells that undergo immunoglobulin gene rearrangement by aligning protein co-expression profiles (43). Phenotypically ordering these various stages, they could also identify the role of IL7 mediated phosphorylation of STAT5 in defining these developmental sub-populations of B cells (43). Hence, by combining computational algorithms with scRNA-seq, they identified cellular checkpoints during B cell development that were coordinated with other cellular events like cell cycle status, apoptosis and IgH gene rearrangement, thereby establishing a more complete "ordered" model of B cell development (43). In a more recent study, Miyai et al. used scRNA-seq analysis to unravel mechanism of transcriptional priming of multipotent hematopoietic progenitors to B cell lineage (44). While it is known that stem cell fate is primarily dictated by a set of core transcription factors and associated epigenetic changes, the transcriptional regulatory mechanisms and the cross-talk amongst these transcription factors involved during cell fate decisions remain incomplete (44). A multi-modal approach, which included single cell analysis, demonstrated an unexpected multi-step, sequential transcriptional priming process occurring in three waves, before the regulatory cross-talk begins for B cell commitment (44). The early-wave include activation of transcription factor genes like *Fos* and *Jun*, a mid-wave exhibited upregulation of factors like *Cebpb* and *Tead2* and finally a late-wave that included factors like *SpiB* and *Irf4* as well as genes encoding chromatin regulators like *Ezh2* (44). It is known that scRNA-seq usually suffers from under-representation of lowly expressed mRNAs, which is generally termed "dropout" that hides important relationships amongst various genes and transcriptional pathways in a given cell, thereby limiting accurate mechanistic predictions (45). An algorithm to predict gene interaction pathways and transcription factor targets has been recently developed, which is expected to greatly aid in analyzing scRNA-seq data and accurately deduce even lowly expressed mRNAs in such datasets (45). Taken together, these multi-modal approaches clearly show that in the near future more studies combining scRNA-seq with functional and perturbation experiments as well as computation will be undertaken to move beyond phenomenology and identify transcriptional regulatory mechanisms in the immune system (5).

## FUTURE PERSPECTIVES

The bewildering complexity of the mammalian immune system for proper function and appropriate responses to foreign pathogens at the right time is regulated by an elaborate network of cellular and tissue interactions. Hence, to achieve a systems level understanding and mechanistic elucidation of the immune system necessitates identification and characterization of its resident cells with their substantial heterogeneity. Clearly, single cell analysis, though mostly in the realm of transcriptomics/RNA-seq, is providing us with tools to achieve such a feat (46). In the near future, when the single cell data sets are compared and combined with ensemble level profiles from various patient population (e.g., The Cancer Genome Atlas, TCGA; http://cancergenome.nih.gov/), they are likely to identify molecular targets and novel avenues appropriate for therapeutic interventions (47). However, a limitation of over relying on transcriptomic studies is that it is naturally assumed that the message levels accurately correspond to protein levels (48). This is often not true, and the field of single cell analysis needs to advance beyond transcriptomics to technologically develop and subsequently incorporate single cell proteomics, metabolomics, lipid profiles and imaging at high throughput scale comparable to and compatible with RNA-seq (47, 48). Moreover, the studies of isolated, dissociated cells must be combined with *in situ* single cell studies in tissues and organs to provide more meaningful spatial and cellular residency data.

It should be noted though that single cell analysis has come a long way and recent developments in this space raise considerable hope that these studies will move beyond their current, predominantly discovery-driven realm to a mechanistic and hypothesis-driven realm and the mysteries of the immune system will ultimately be resolved. For example, high throughput single cell chromatin contact analysis (Hi-C) is enabling us to decipher the genome architecture in distinct cell types at single cell resolution, which when combined with transcriptomic and proteomic data should provide mechanistic insights into cellular heterogeneity and differentiation (49). New and exciting development in this field now also provides a pathway to carry out cellular profiling via genome topology as the only variable (50). Because the 3D genome structure is usually of high information content with many molecular features, it could be employed in cluster analysis to profile distinct cellular types (50). For example, the promoter-enhancer looping is known to regulate differential gene expression in a cell type dependent fashion. Employing the feature of differentially formed but established cell type–specific promoter-enhancer loops (based on cell type–purified bulk Hi-C), this study could unambiguously separate the single cells into specific clusters of immune cell types (50). Finally, an exciting new advancement called super resolution chromatin tracing that uses super resolution

microscopy is revealing at single cell level how the genome is folded into topologically associated domains (TADs) and cooperative interactions at this level even in the absence of cohesion (a co-factor necessary for binding of CCCTC-binding factor CTCF), thus, TADs are likely to be units of chromatin folding (51). This is a breakthrough in studying structure and function of the genome and expected to significantly advance this field (51).

It is worthwhile to ponder why no two cells in an animal could be identical. Raj and colleagues argue that in general, there are two reasons why any two cells might differ from each other and these might not be mutually exclusive (48). First, the fate of the cell could be a deterministic outcome—cells receiving distinctly different instructions, leading to different outcomes. Second, a stochastic or probabilistic outcome– cells behave functionally differently with distinct outcomes, although they receive the same set of instructions. In case of the immune cells, we could perhaps imagine an additional scenario that depending on whether the immune cells of the same lineage landing in distinct anatomical locations "acquire" new functions depending on the "new" tissue niche (changing from deterministic to stochastic fate) or "inherit" distinct functions (remain deterministic) even before they arrive at their final destination. Certainly, we are into really exciting times!

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

## ACKNOWLEDGMENTS

Work in the author's laboratory is generously supported by the Division of Program Coordination, Planning, and Strategic Initiatives, Office of the Director and the Intramural Research Program of the National Institutes of Health, National Institute on Aging. The author thanks Dr. Ranjan Sen and Dr. Richard Conroy for critical reading of the manuscript and many stimulating discussions. AR assumes sole responsibility for the views expressed herein.

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