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Transcription factors in immunological disease and haematological malignancies

Edited by
Tomokatsu Ikawa, Li Wu, Ashley Ng and Stephen Nutt

Coordinated by
Jacob T. Jackson

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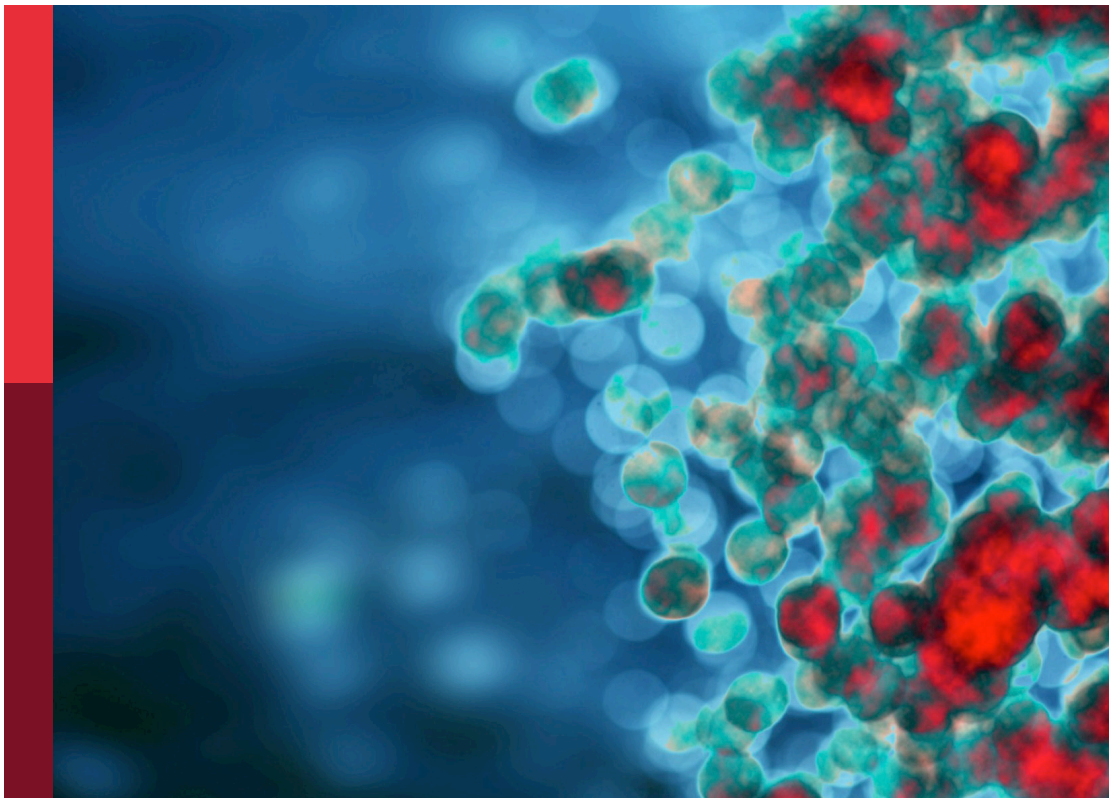

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Transcription factors in immunological disease and haematological malignancies

Topic editors
Tomokatsu Ikawa – Tokyo University of Science, Japan
Li Wu – Tsinghua University, China
Ashley Ng – The University of Melbourne, Australia
Stephen Nutt – The University of Melbourne, Australia

Topic coordinator
Jacob T. Jackson – The University of Melbourne, Australia

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Luis Graca,
University of Lisbon, Portugal

*CORRESPONDENCE
Jacob T. Jackson
jackson@wehi.edu.au

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Editorial: Transcription factors in immunological disease and haematological malignancies

Jacob T. Jackson[1]*, Ashley P. Ng[1], Stephen L. Nutt[1], Tomokatsu Ikawa[2] and Li Wu[3]

[1]Immunology Division, Walter and Eliza Hall Institute of Medical Research, The University of Melbourne, Melbourne, VIC, Australia, [2]Division of Immunology and Allergy, Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan, [3]School of Medicine, Tsinghua University, Beijing, China

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Editorial on the Research Topic
Transcription factors in immunological disease and haematological malignancies

Our Research Topic, delves into key transcription factors involved in immunological diseases and haematological malignancies, and brings to the fore cutting-edge research and thorough and targeted literature reviews. These timely works highlight the future potential of targeting transcription factors for clinical intervention in the treatment of a range of diseases in which they may be critically involved.

With regards to the original research articles, Butcher et al. employed elegant mouse models to demonstrate the T-bet+ Th17 cells, responsible for experimental autoimmune encephalitis through induction of GM-CSF, are governed by expression of GATA3 which drives expression of Egr2, Bhlhe40, and Csf2. Liang et al. reveal that RBP-J–mediated Notch signalling regulates macrophage development and activation. Their murine Parkinson's Disease (PD) model showed Notch signalling within microglia resulted in decreased tyrosine hydroxylase positive neurons that was blocked by inactivation of RBP-J that decreased infiltrating, inflammatory macrophages and activated microglia. This work showed for the first time that RBP-J–mediated Notch signalling may well play a significant role the development of PD predominantly through the regulation of the activation of microglia via NF-κB signalling. Research presented by Mah et al. reports on the important role of ING5 for normal liver cellularity in foetal development in a cell extrinsic fashion using a gene knockout mouse model. However, a third of these mice survived weaning and ING5 was not found to be required for haematopoietic stem cell self-renewal. Interestingly, the highly related ING4 transcription factor, bearing an identical homeodomain may provide some level of redundancy. Trezise et al. explored the results of a primary B cell CRISPR/Cas9-mediated screen, which illuminated key components of the pathway mediating antibody secretion. The results of these studies identifies potential candidates may be targeted for clinical treatment for antibody-mediated diseases, and potential pathogenic genes that may underlie primary antibody deficiencies. These highlight the importance of discovery research into the functional mechanisms of

transcription factors in normal and disease development, which can inform future diagnostic and therapeutic strategies.

Our Research Topic also brings together a number of in-depth scientific reviews of the literature. The first is provided by Balendran et al. with a focus on the transcription factors, NF-κB, STATs, AP-1 and IRFs with regards to their critical role in inflammatory disease. These transcription factors may serve as potential therapeutic targets in rheumatoid arthritis, through targeting with direct inhibitors, or via targeting signalling pathways that may activate these transcription factors, or exploring transcription factor interaction with a natural compound screen.

Jackson et al. delivers a comprehensive review of the role of Hhex in development, physiology and disease, where the pleiotrophic actions of Hhex have been shown to be dependent on the cellular context. Salient observations include how the function of Hhex in embryological development can be reflected in disease processes that may involve Hhex, including repression of *Cdkn2a* in the context of HSC self-renewal, emergency haematopoiesis and acute myeloid leukaemia, as well as potential roles in type 2 diabetes where both *HHEX* and *CDKN2A* variants very frequently occur together as genetic risk factors.

The literature behind the current state of knowledge for ZHX2 in normal cellular processes, including proliferation, differentiation, and metabolism homeostasis, was explored by Li et al. The involvement of ZHX2 in cancer is also reviewed with its potential role as an oncogene in hepatocellular carcinoma, clear cell renal cell carcinoma and triple-negative breast cancer increasingly recognised.

Radhakrishan et al. present an in-depth review on the role of epithelial-mesenchymal transition (EMT) transcription factors of the ZEB, TWIST and SNAIL families. These transcription factors are important in haematopoiesis, with roles in haematological malignancy increasingly recognised. Such oncogenic roles have become evident with overexpression linked to worse clinical outcomes in myeloid malignancies, with dysregulation, mutation and chromosomal aberrations involving these factors also observed in lymphoid neoplasms. Unlike Hhex, EMT transcription factor roles in haematopoiesis have been suggested to be broadly distinct from roles in embryological development.

The review by Roy et al. examines proliferation of B cells and their germinal centre development, including generation of plasmablasts and plasma cells is governed by the signalling of NF-κB. They also detail how NF-κB monomers each serve in specific roles in the differentiation/formation of plasma cells and germinal centre B cells. This body of research helps inform a number of B cell-driven diseases, including lymphoma, immunodeficiency and autoimmunity, as well as importantly allowing the authors to pose a number of outstanding questions in field.

The role of the transcription factor KLF2 in B cells, specifically in terms of development, activation, generation and maintenance of plasma cells, is reviewed by Wittner and Schuh. This report explores the function of KLF2 as both an activator and an inhibitor of various B cell functions, depending on immunological context, as well as describing KLF2's known roles in B cell malignancies such as multiple myeloma and splenic marginal zone lymphoma, and diseases such as IgA deficiencies.

Finally, Zhang et al.'s review explores the growing literature around dendritic cell (DC) differentiation. The heterogenicity of type 2 conventional DCs (cDC2), the origins of plasmacytoid DCs (pDCs), and emerging knowledge on DC3, the latter of which share features of both cDC2s and monocyte-derived DCs, is specifically explored. Insights into the transcription factors governing these cell types including IRF8, PU.1 and E2–2 and provided. The data presented in their review suggest that the development of cDCs and pDCs relies heavily on a balance between several key transcription factor pairs, notably E2.2/ZEB2 versus ID2/NFIL3 or PU.1 versus BCL11A.

Collectively, these publications shine a light on the crucial roles of a number of key transcription factors across normal development, as well as immunological and haematological malignancy. The publications highlighted in this section strongly validates the need for discovery research that can yield important new insights and novel therapeutic approaches in the treatment of diseases of unmet clinical need.

Author contributions

JJ: Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. AN: Writing – review & editing. SN: Writing – review & editing, Supervision, Funding acquisition. TI: Writing – review & editing. LW: Writing – review & editing.

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EDITED BY
Li Wu,
Tsinghua University, China

REVIEWED BY
Xiangzhi Li,
School of Life Sciences, Shandong University, China
Jacob T. Jackson,
The University of Melbourne, Australia

*CORRESPONDENCE
Chunhong Ma
machunhong@sdu.edu.cn

†These authors have contributed equally to this work

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ZHX2 in health and disease

Na Li[1†], Zhuanchang Wu[1†] and Chunhong Ma[1,2*]

[1]Key Laboratory for Experimental Teratology of Ministry of Education and Dept. Immunology, School of Basic Medical Sciences, Cheeloo Medical College, Shandong University, Jinan, Shandong, China, [2]Key Laboratory of Infection and Immunity of Shandong Province, Shandong University, Jinan, Shandong, China

As a transcriptional factor and the negative regulator of alpha fetal protein (AFP), Zinc fingers and homeoboxes 2 (ZHX2) has a well-established role in protection against hepatocellular carcinoma (HCC). However, recent studies have suggested ZHX2 as an oncogene in clear cell renal cell carcinoma (ccRCC) and triple-negative breast cancer (TNBC). Moreover, mounting evidence has illustrated a much broader role of ZHX2 in multiple cellular processes, including cell proliferation, cell differentiation, lipid metabolism, and immunoregulation. This comprehensive review emphasizes the role of ZHX2 in health and diseases which have been more recently uncovered.

KEYWORDS

ZHX2, tumor repressor, oncogene, cell differentiation, lipid metabolism, immunoregulation

Introduction

ZHX2, a member of the ZHX (Zinc fingers and homeoboxes) family, is a ubiquitous transcriptional factor that was first identified as a negative regulator of murine postnatal alpha fetal protein (AFP) (1). In 1977, Roushlatti and colleagues compared serum AFP in different mouse strains and found a gene which they called *Regulator of Alpha-fetoprotein* (*Raf*), subsequently renamed *Alpha-fetoprotein regulator 1* (*Afr1*), negatively regulated the AFP expression in adult mice (1, 2). In 2005, Perincheri et al. further refined and identified *Zhx2* as the homologous gene of *Afr1* by positional cloning (3). Human *ZHX2* was first cloned by Nagase et al. from a size-fractionated brain cDNA library in 1998 (2). In 2003, human ZHX2 was then identified as a ZHX1-interacting protein by Kawata et al. (4).

ZHX2 has been extensively studied in cancer development. ZHX2 suppresses the transcription of oncofetal genes *AFP* (1, 3, 5) and *glypican 3* (*GPC3*), and works as a tumor suppressor gene in HCC (5, 6). Subsequent studies have found that ZHX2 is widely expressed and participates in many types of cancer. Consistent with findings in HCC, low ZHX2 expression correlates with poor prognosis of thyroid cancer (7), multiple myeloma (8–10), and chronic lymphocytic leukemia (11, 12). On the contrary, ZHX2 promotes the development of ccRCC (13–15), TNBC (16), and gastric cancer (17, 18). Beyond regulating cancer development, the latest reports have shown that ZHX2 involves in several other physiological or pathological processes, including cell

differentiation and development (19–21), lipid metabolism (22–24), and viral replication (25, 26). Especially, ZHX2 is abundantly expressed in the thymus and spleen (2) and there is clear evidence supporting the involvement of ZHX2 in regulating B cell development (27), NK cell maturation (28), and macrophage polarization (29–31).

In this review, we outline these new advances in ZHX2 mediated regulation in health and diseases. We also discuss the multiple mechanisms involved in controlling ZHX2 expression and transcription.

ZHX2 protein structure and its role as a transcription factor

The human *ZHX2* gene is localized on chromosome 8q24.13 and consists of 4 exons (4). The third exon is the sole coding exon of *ZHX2* which encodes a protein of 837 amino acid residues (4). Human ZHX2 protein, like the other two family members ZHX1 and ZHX3, contains two Cys-Xaa_2-Cys-Xaa_{12}-His-Xaa_4-His-type zinc finger domains (Znf) and four homeodomains (HD) (originally thought as five HDs) (4). Besides, ZHX2 contains a proline-rich region (PRR) at position 408 to 440 between HD1 and HD2 (4). The homology of ZHX2 protein in humans and mice is as high as 87%. Kawata et al., in 2003, identified ZHX2 as a ubiquitous transcription factor. ZHX2 interacts with nuclear transcription factor Y subunit alpha (NF-YA) and forms homodimers or heterodimers with ZHX1 or ZHX3 to exert transcriptional inhibitory function (5). The amino acid sequence between residues 195 and 358 containing HD1 is required for homodimerization of ZHX2, and ZHX2 interacts with NF-YA *via* the region between 263 and 497 residues (4). Similar to full-length ZHX2, truncated ZHX2 containing residues 242-446 (ZHX2(242-446)) but not ZHX2(242-439) maintain the capability to localize in the nuclei and suppress the expression of Cyclin A/E in HCC (6). The decreased nucleic ZHX2 expression significantly correlates with poor survival of HCC patients (6). However, how ZHX2 loses its nuclear localization is completely unknown. More studies are required to define the exact nuclear localization signal (NLS) and the molecules or mechanisms regulating the nuclei translocation of ZHX2.

A growing number of genes have been identified as the ZHX2 targets, most of which are cancer-related. ZHX2 not only negatively controls the transcription of liver cancer marker genes *AFP* and *GPC3*, but also inhibits cell proliferation-related genes such as *Cdc25* (4), *Cyclin A/E* (6), and *Notch1* (32). In addition, ZHX2 represses transcription of *multidrug resistance mutation 1* (*MDR1*) (33), *lipoprotein lipase* (*LPL*) (34), *lysine demethylase 2A* (*KMD2A*) (35), and *S100 calcium binding protein A14* (*S100A14*) (7) in HCC and thyroid cancer cells. Although ZHX2 was originally reported to be a ubiquitous transcriptional repressor, recent reports uncover another face of ZHX2 as a transcriptional activator (36, 37). Jiang et al. found that Zhx2 binds *Mup* promoters and is required for high levels of Mup expression in adult mouse liver (36). ZHX2 also binds to the promoter of *phosphatase and tensin homolog (PTEN)* and subsequently promotes the transcription of *PTEN* (37). Strikingly, several non-coding RNAs have been elucidated as the ZHX2 targets, either enhanced or inhibited. ZHX2 represses transcription of *H19* (3, 38, 39), the first imprinted non-coding transcript to be identified. In glioma cells, ZHX2 binds to the promoter region of *linc00707* and negatively regulates its expression, leading to glioma cells proliferation, migration and invasion, and vasculogenic mimicry (VM) formation (40). On the contrary, ZHX2 increases transcription of *miR-24-3p* and *linc01431*, which targets *SREBP1c* (24) and PRMT1 (26) in hepatocytes respectively.

The mechanism by which ZHX2 controls target gene transcription is not fully understood. ZHX2 was originally known as an NF-YA interacting protein (4) and therefore represses transcription of *MDR1*, *Cdc25*, and *Notch1* by interacting with NF-YA (4, 6, 32, 33). However, there is no evidence for the presence of NF-YA binding sites in promoter of some other ZHX2-targeted genes, such as *Cyclin E*, or *AFP* (5, 6). A global analysis of Zhx2 targets using ChIP-seq in a murine macrophage cell line shows a significant overlap with two known apoptosis regulators Jun (41) and Bcl6 (42), which suggest a strong involvement of Zhx2 in cell apoptosis (30). In ccRCC, ChIP-seq data indicate that the genome-wide chromatin occupancy of ZHX2 overlaps with 75% of p65-binding motifs (13). ZHX2 and RelA/p65 overlapping sites also display a strong enrichment for H3K4me3 and H3K27ac, indicating that ZHX2 colocalizes with NF-κB to active gene promoters (13). In TNBC, the integrated ChIP-seq and gene expression profiling show that ZHX2 and HIF1α co-occupy transcriptional active promoters to promote gene expression (16). These studies suggest that ZHX2 may mainly serve as a transcriptional cofactor, interacting with different coactivators/repressors in different physiological circumstance to control its localization in the genome and downstream transcriptional activity. In addition, the Znf domains of ZHX2 process potential DNA-binding activity, however, whether ZHX2 can bind DNA directly and its consensus binding motif still need to be investigated.

Control of ZHX2 expression

ZHX2 expression is tightly regulated under different circumstances. A computational network study indicates ZHX2 as one of the most regulated transcription factors in myeloid cells to avoid an avalanche of transcriptional events (31). In Hodgkin lymphoma (HL), a chromosomal rearrangement far upstream region of *ZHX2* gene results in the transcriptional silence of *ZHX2*, and two transcription factors, homeodomain protein MSX1 and bZIP protein XBP1,

are identified to directly regulate ZHX2 expression (11). Furthermore, human ZHX2 is lower expressed in fetal liver, increased after birth, and silenced in HCC (43–45). Consequently, multiple mechanisms are revealed to control ZHX2 expression at different levels (Figure 1):

At the ZHX2 gene transcription level- Lv et al. found that *ZHX2* promoter region is hypermethylated in HCC, suggesting that the hypermethylation-mediated silencing of *ZHX2* is an epigenetic event involved in HCC (45). In addition, copy number analysis showed that *ZHX2* gene is amplified in various cancers, including ovarian cancer (~40%) and breast cancer (~15%). The *ZHX2* copy number significantly correlates with enhanced ZHX2 expression (16). Wu et al. (34) and Zhao et al. (37)found that Zhx2 expression can be repressed by free fatty acid in hepatocytes. Constantly, hepatic Zhx2 is reduced in mice with fatty liver, indicating that ZHX2 could be regulated by the metabolic microenvironment. This is consistent with a previous computational network study indicating ZHX2 as one of the most regulated transcription factors in myeloid cells (31). The detailed mechanisms regulating ZHX2 expression in different circumstances need to be further studied.

At the post-transcription level- microRNAs (miRNAs) are short non-coding RNAs that regulate gene expression post-transcriptionally. They generally bind to the 3'-UTR (untranslated region) of their target mRNAs and reduce protein production by destabilizing mRNA or translational silencing (46, 47). HBV-encoded proteins, particularly a well-known oncogenic protein HBx, drive the high expression of miR-155, which binds to seed sites in the 3'-UTR of the *ZHX2* mRNA and inhibit its translation (48). Similarly, HBx promotes CREB-mediated activation of miR-3188 to repress ZHX2 expression, leading to activated Notch signaling in HCC (32).

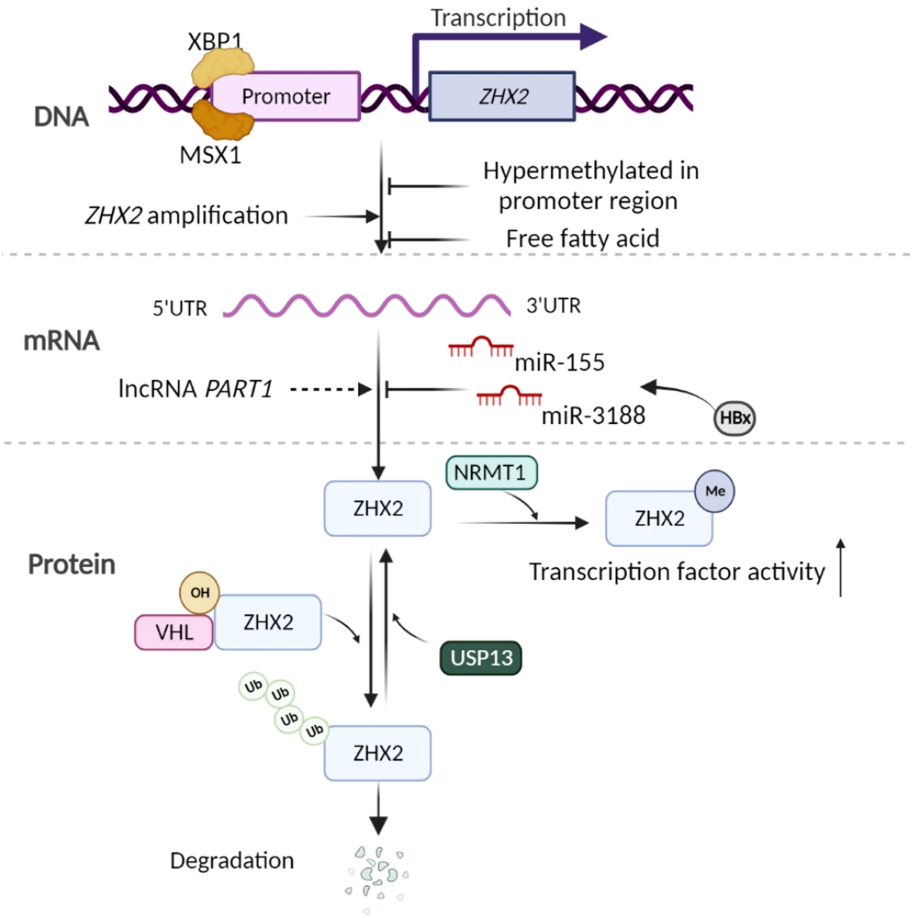

FIGURE 1
Control of ZHX2 expression. At the gene and transcription level, some transcription factors, hypermethylation of *ZHX2* promotor, and cellular stimuli such as free fatty acid are known to regulate *ZHX2* transcription. Concurrently, *ZHX2* gene amplification contributes to its enhanced expression in cancer. At the post-transcription level, miR-155 and miR-3188 upregulated by HBx inhibit *ZHX2* mRNA translation, but lncRNA PART1 promotes *ZHX2* mRNA level by altering the miRNA landscape. At the PTMs level, hydroxylated ZHX2 protein is recognized and degraded by E3 ubiquitin ligase VHL, which is inhibited by USP13-induced deubiquitination, while NRMT1-mediated Nα-methylation of ZHX2 promotes its transcription factor activity. Created using Biorender.com.

While in TNBC, lncRNA *PART1* promotes *ZHX2* transcription (49).

At the posttranslational modifications (PTMs) level- Zhang et al. report that inactivation of the von Hippel-Lindau (VHL) E3 ubiquitin ligase in ccRCC leads to the accumulation of ZHX2 protein and its nuclear localization. ZHX2 protein hydroxylation at proline 427, 440, and 464 allows VHL to bind and promote its protein degradation (13). However, a deubiquitinase USP13 inhibits the ubiquitination of ZHX2 and enhances its stability (15). A recent study found that the N-terminal methylation (Nα-methylation) of ZHX2 by the methyltransferase NRMT1 regulates its transcription factor activity and its occupancy on targeted promoters (50). Up to now, whether there are other PTMs and their roles in ZHX2 trafficking, stability, and transcriptional activity are less clear.

ZHX2 in cancer-a context-dependent tumor repressor or driver?

ZHX2 is initially identified as an AFP repressor and a tumor repressor in HCC (3, 5). Whereafter, abnormal expression of ZHX2 is reported in multiple types of tumor (6, 8, 11). Furthermore, ZHX2 expression is closely related to the malignancy and poor prognosis of B-cell lymphoma (11, 12), myeloma (8–10), lung cancer (51), and thyroid cancer (7), suggesting that ZHX2 plays an important role in tumorigenesis and cancer development. Interestingly, latest studies reported that ZHX2 functions as an oncogene in ccRCC (13, 14) and TNBC (16). Likewise, Jiang et al. reported that the whole-body knockout of *Zhx2* results in reduced liver tumors in diethylnitrosamine (DEN)-induced HCC mice (52). Therefore, ZHX2 is abnormally expressed in multiple tumors and plays different roles, either acting as a tumor suppressor or oncogene in a context-dependent manner (Figure 2). Here, we outline the role of ZHX2 in multiple tumors.

ZHX2 as a tumor suppressor in HCC and other cancers

ZHX2 regulates the posttranscriptional silencing of oncofetal genes *AFP*, and *GPC3*, both of which are expressed in fetal liver, silenced after birth, and reactivated in HCC (43–45). These suggest that ZHX2 contributes to hepatocarcinogenesis as a tumor suppressor. Consistently, our previous study showed that the nuclear ZHX2 is reduced in human HCC tissues compared with adjacent nontumor tissues and nuclear ZHX2 represses HCC cell growth by inhibition of cell cycle genes (*Cyclin A* and *Cyclin E*), demonstrating for the first time the tumor suppressor activity of ZHX2 in HCC (6). In accordance, another study detected the hypermethylation of

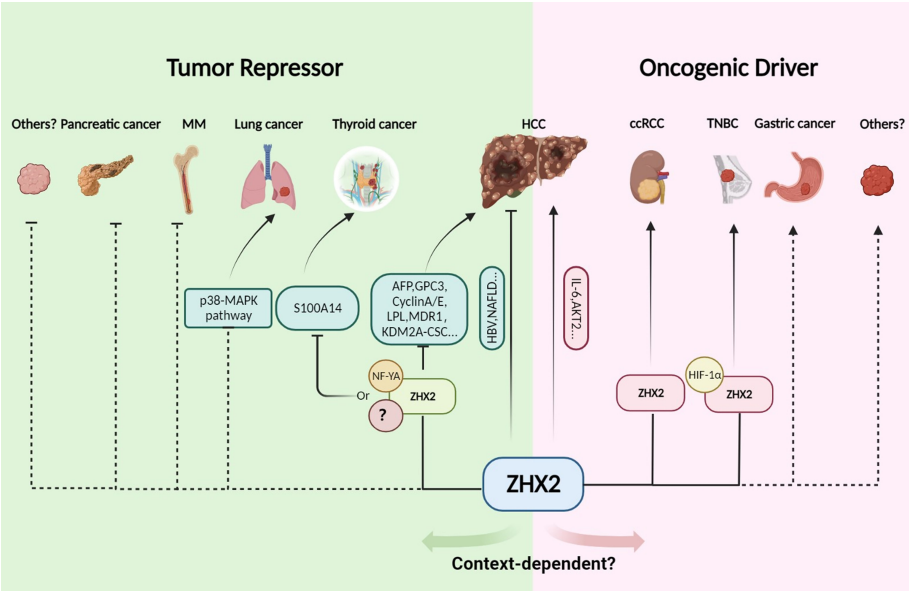

FIGURE 2
The tumor repressor or driver role of ZHX2 in cancer. In HCC, ZHX2 has a context-dependent role. ZHX2 inhibits HCC *via* multiple mechanisms, but whole body knockout of *Zhx2* reduces DEN-induced liver tumors indicating its complex roles. In HCC, lung cancer, multiple myeloma, HL, and thyroid cancer, ZHX2 acts as a tumor suppressor and transcriptionally represses AFP, GPC3, Cyclin A/E, LPL, KDM2A, and S100A14 expression *via* interacting with NF-YA or other unknown partners to restrict cancer progress. However, in ccRCC and TNBC, ZHX2 plays an oncogenic driver role by interacting with p65 and HIF1α to activate oncogenic signaling. Created using Biorender.com.

ZHX2 promoter and the silencing of ZHX2 expression in HCC tissues (45). Subsequent studies further illustrated the critical role of ZHX2 as a tumor suppressor in HCC with a variety of etiologies, including NASH-related HCC (34, 37) and HBV-related HCC (25, 32). However, there is conflicting data on the role of ZHX2 in HCC. Hu et al. reported increased ZHX2 staining in HCC tissues and higher ZHX2 expression in poorly differentiated and metastasis samples, indicating that ZHX2 might promote HCC progression (53). Jiang et al. recently showed that whole body *Zhx2* knockout ($Zhx2^{KO}$) leads to dramatically reduced liver cancer in DEN-induced HCC mouse model, indicating the oncogenic role of ZHX2 in DEN-induced liver tumor model (52). Interestingly, compared with $Zhx2^{KO}$ mice, DEN induces more tumors in liver-specific *Zhx2* knock-out mice ($Zhx2^{\Delta liv}$) (52). These data suggest that ZHX2 expression in non-parenchymal cells plays a critical role in liver carcinogenesis. Therefore, although most studies support the conclusion that ZHX2 works as a tumor suppressor in HCC, the exact role of ZHX2 in HCC needs to be further defined and ZHX2 expression in non-parenchymal cells should be deeply investigated.

The tumor suppressor role of ZHX2 has also been demonstrated in many other types of tumors including hematological tumors and solid tumors. Spectral karyotyping identified chromosomal rearrangement far upstream region of *ZHX2* gene in Hodgkin lymphoma and this aberration results in ZHX2 silencing (11, 12). Low ZHX2 is associated with poor prognosis in chronic lymphocytic leukemia and multiple myeloma (MM) (8, 54), while higher *ZHX2* mRNA correlates with better overall survival in patients with breast cancer (55) and thyroid cancer (7). ZHX2 inhibits proliferation and promotes apoptosis of lung cancer cells by inhibiting the p38-MAPK signaling pathway (51). Integrative bioinformatics analyses reveal that a miRNA-related SNP (rs3802266-G), which creates a stronger binding site for miR-181-a-2-3p in 3'UTR of *ZHX2* mRNA and consequently reduces ZHX2 expression, was significantly associated with increased risk of pancreatic cancer (56).

ZHX2 not only inhibits tumor growth but also suppresses tumorigenesis and tumor development through multiple mechanisms. Cancer stem cells (CSCs) are critical determinants of tumor relapse and therapeutic resistance (57). ZHX2 counteracts liver cancer stem cell traits by inhibiting KDM2A-mediated demethylation of H3K36 at the promoter region of stemness-associated transcription factors, such as NANOG, SOX2, and OCT4 (35). Furthermore, ZHX2 inhibits thyroid cancer metastasis (7) and is responsible for reduced chemotherapy resistance in HCC (33). ZHX2 enhances the cytotoxicity of anti-cancer drugs in HCC *via* transcriptional repression of MDR1 leading to decreased drug efflux (33). Consistently, a clinical study shows a positive correlation between high ZHX2 expression and longer survival in MM patients (8). However, a recent *in vitro* study shows that treatment of proteasome inhibitor bortezomib (BTZ) leads to enhanced ZHX2 expression which in turn promotes BTZ resistance in cultured MM cells (58). All these data reveal a widespread restriction role of ZHX2 in tumor development at multiple dimensions, including tumor cell proliferation, metastasis, stemness, and chemotherapeutic resistance.

Oncogenic role of ZHX2 in ccRCC, TNBC, and other tumors

Despite the apparent tumor repression role of ZHX2 in HCC and other cancer types, a number of studies have illustrated that ZHX2 can function as an oncogene. Recently, Zhang et al. reported in Science that the loss of tumor suppressor gene VHL in ccRCC leads to the accumulation of ZHX2 protein in the nuclear, which is correlated with poor survival in patients (13, 59). Mechanistically, ZHX2 interacts with RelA/p65 and promotes oncogenic signaling at least partially *via* activating NF-κB signaling (13). ChIP-seq and gene expression profiling show that 75% of p65 binding sites overlap with those of ZHX2 and their overlapping sites display a strong enrichment of H3K4me3 and H3K27ac (13). In addition, Zhu et al. reported that ZHX2 promotes cell growth and migration through activating MEK/ERK pathway and mediates Sunitinib resistance by regulating the autophagy in ccRCC (14). A similar phenomenon is found in studies of multiple osteosarcoma and gastric cancer (17, 18), where high expression of ZHX2 shows a significant correlation with poor survival. Further, a recent study clarified that ZHX2 functions as a cofactor of the HIF1α to promote HIF1α oncogenic signaling in TNBC (16).

Together, accumulated data demonstrate the critical role of ZHX2 in cancer, either as a tumor suppressor or as an oncogene. However, the detailed mechanism underlying the context-dependent role of ZHX2 in tumors remains largely unknown. Further investigation is required to define the genetic and environmental contexts that influence ZHX2 interaction networks and put genetic interaction networks into different tumors context.

Beyond cancer — other biological roles for ZHX2

Besides the complicated roles in tumors, recent studies suggest the involvement of ZHX2 in the regulation of cell differentiation, HBV replication, lipid homeostasis, and immune responses (Figure 3). In agreement, ZHX2 has been reported in the occurrence of chronic hepatitis B (CHB) (32, 48), metabolism-related diseases (30, 37), nerve-related diseases (19, 60, 61), and immune-related diseases (29) (Figure 3). We will discuss the role of ZHX2 in different physiological and pathological processes here.

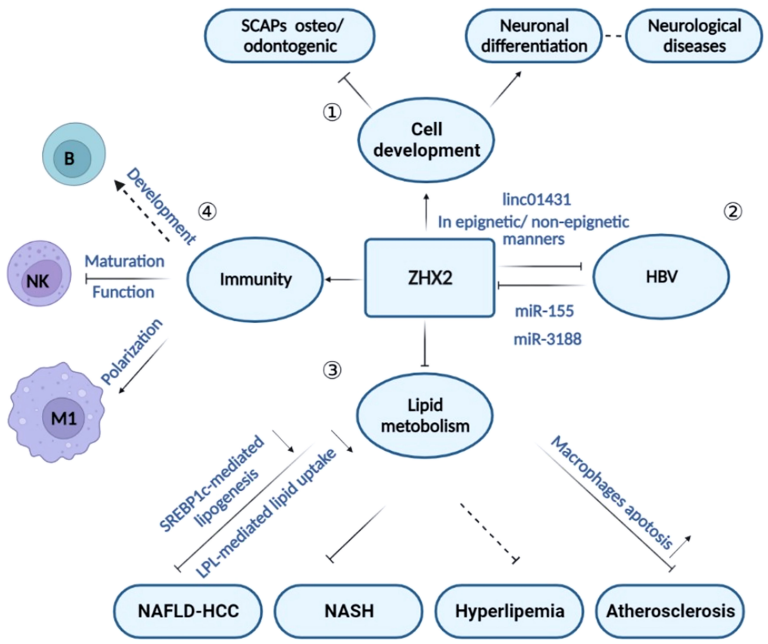

FIGURE 3
The role of ZHX2 in different physiological and pathological processes. ① Through regulating cell development, ZHX2 is implicated in inhibiting neuronal differentiation and promoting osteo/odontogenic differentiation of stem cells from SCAPs. ② ZHX2 restricts HBV replication *via* CBP/p300 and linc01431-mediated epigenetic repression or *via* inhibiting viral promoter activity in non-epigenetic manners. However, HBx protein reduces ZHX2 expression by upregulating miR-155 and miR-3188 expression. ③ ZHX2 is a critical regulator in lipid hemostasis and plays roles in atherosclerosis, NASH, and NAFLD-HCC progress. ④ ZHX2 is involved in immune regulation by influencing the development of multiple immune cell subsets.

ZHX2 in development

The first evidence indicating the involvement of ZHX2 in development comes from the critical role of Zhx2 in the postnatal repression of *Afp* and *Gpc3* in mice (3). In agreement, the dynamic expression of hepatic Zhx2 has been found during liver development and after hepatectomy (21). Zhx2 is low in fetal liver and increases after birth, while Zhx2 expression is significantly declined 24 hours after hepatectomy and then reverses to normal level (21). Therefore, ZHX2 might be a potential therapeutic target in different liver diseases which cause liver injury.

Several studies have illustrated the participation of ZHX2 in regulation of cell development of different origins, such as neurons, blood cells, and bipolar cells. Altered ZHX2 expression has been detected during erythroid differentiation (62) and B cell development (27). Concurrently, ZHX2 is responsible for macrophage polarization (29) and NK cell's terminal maturation (28). In the nervous system, ZHX2 interacts with Ephrin-B and regulates neural progenitor maintenance (19). Genome-wide analyses identified inherited CNVs (copy number variations) that affect non-genic intervals upstream *ZHX2* in autism spectrum disorder (ASD) patients (61). Exome sequencing in subjects with familial corticobasal degeneration (CBD) shows that mutations in *ZHX2* gene may cause its structural changes, indicating the possible involvement of ZHX2 in corticobasal degeneration (63). In the process of tooth root development, ZHX2 knockdown reduces the mineralization of stem cells from the apical papilla (SCAPs) and promotes SCAPs proliferation (20). Also, Zhx2 participates in the regulation of bipolar cell subset fate determination during retinal development (64). Collectively, accumulating evidence demonstrated that ZHX2 is strongly involved in the developmental processes of different cells, which is consistent with the acknowledged ZHX2-mediated transcription of stemness genes. However, much work is required to better understand the exact roles and mechanisms of ZHX2 in organogenesis and tissue repair.

ZHX2 and HBV infection

HBV is one of the well-known risk factors for HCC. According to the WHO (World Health Organization), almost one-third of the world's population has been infected with HBV at some point in their lives (65, 66). HBV infects more than 250 million individuals worldwide, and almost 1 million die annually from complications of persistent infection, cirrhosis, and HCC (66).

As a liver cancer suppressor, ZHX2 expression is significantly decreased in tumor tissue from HBV-positive

HCC patients and liver from HBV transgenic mice (48). Further studies show that HBV infection, especially the viral protein HBx reduces ZHX2 expression *via* upregulation of an oncomiR miR-155 (48) or CREB-mediated activation of miR-3188 (32), leading to liver cancer progression. In turn, ZHX2 serves as a novel restriction factor against HBV replication *via* regulating HBV promoter activities and cccDNA modifications. *In vitro* and *in vivo* experiments confirm that ZHX2 significantly inhibits HBc, HBsAg, and HBeAg expression (25), while overexpression of ZHX2 eliminates HBx-mediated proliferation of HCC cells (48). Mechanistically, ZHX2 binds to cccDNA and reduces the expression of histone regulator genes p300/CBP, leading to epigenetic repression of cccDNA (25). Alternatively, ZHX2 increases the expression of linc01431, a novel noncoding RNA for HBV restriction, which competitively binds with PRMT1 to block HBx-mediated degradation and enhances the occupancy of PRMT1 on cccDNA, thereby repressing cccDNA transcription (26). All in all, ZHX2 and HBV are mutually regulated by each other during HBV infection.

ZHX2 and lipid metabolism

Interestingly, a study in mice using the QTL (quantitative trait locus) mapping strategy identified Zhx2 as a novel regulator of plasma levels of lipids, including triglyceride (TG) (23), indicating a potential role of Zhx2 in lipid metabolism. Compared with other mouse strains, BALB/cJ mice that harbor *Zhx2* defect exhibit decreased serum lipid levels and resistance to atherosclerosis when fed a high-fat diet (30). Constantly, altered hepatic transcript levels of several genes affecting lipid homeostasis, including *Lpl*, are detected in BALB/cJ mice (23). Notably, further research shows that ZHX2 inhibits the uptake of exogenous lipids in hepatocytes by transcriptional repression of LPL expression, which leads to cell growth retardation, and suppresses the progression of NAFLD to HCC (34). Concurrently, it has been found that ZHX2 increases transcription of miR-24-3p which binds to *SREBP1c* mRNA to promote its degradation, thereby inhibiting SREBP1c-mediated lipid *de novo* synthesis (24). The involvement of ZHX2 in fatty liver disease is further confirmed by a recent study showing that Zhx2 deficiency in hepatocytes exacerbates NASH progression by transcriptional activation of *Pten* (37). Collectively, ZHX2 is a critical regulator of lipid metabolism, while we still need more studies to fully delineate the downstream network contributing to ZHX2-mediated lipid regulation.

ZHX2 and immune regulation

ZHX2 is abundantly expressed in thymus and spleen (2), and increasing studies have shown that ZHX2 affects the development and function of different immune cells and participates in the progression of a variety of immune-related diseases.

ZHX2 is involved in the process of B-cell differentiation

A study using gene expression profiling describes an interesting expression pattern of *ZHX2* in B lymphoid cells. Similar to essential transcription factors *PAX5* and *E2A*, *ZHX2* is turned on during the transition from hematopoietic stem cells (HSCs) into early-B and shows a further increase in pro-B and later stages (27). Recently, Nagel et al. confirmed that ZHX2 is significantly upregulated in B cells while ZHX1 is downregulated. The reduced expression of ZHX2 together with the activation of ZHX1 may contribute to the deregulated B-cell differentiation phenotype in HL (67). However, to date, there were no reports about the role of ZHX2 in B cell development and functions. Interestingly, a genome-wide association study reveals rs10108684, the intronic SNP of *ZHX2*, as one of the eight top-scoring associations between SNPs and vaccinia antibody levels in African-Americans, strongly suggesting the critical involvement of ZHX2 in B cell-mediated antibody production (68). In summary, ZHX2 shows a dynamic expression pattern during B cell development but its function in B cell maturation is completely unknown and requires further studies.

ZHX2 inhibits NK cell maturation and function

Natural killer (NK) cells are primarily involved in innate immunity and possess important functional properties in anti-viral and anti-tumor responses (69–71). NK cells are derived from hematopoietic stem cells (HSC) *via* a series of developmental stages, including common lymphoid progenitor (CLP), NK cell precursors (NKP), immature NK cells and mature NK cells (72, 73) Multiple internal pathways and external factors contribute to the development of NK cells from HSCs (73). Tan et al. recently showed that ZHX2 significantly restricts the terminal maturation and effector functions of NK cells both *in vivo* and *in vitro* (28). Mechanistically, ZHX2 controls NK cell maturation and function *via* two related pathways. ZHX2 down-regulates the responsiveness of NK cells to IL-15, the cytokine crucial for NK cell development and survival (74). On the other hand, ZHX2 controls the transcription of *Zeb2*, a transcription factor identified as a major driver of $CD27^{low}$ NK cell maturation (75, 76). It has been reported that Zeb2 directly or indirectly modulates IL-15-mediated survival and development of NK cells (77, 78). Zeb2 might be associated with ZHX2-mediated regulation of IL-15 signaling (77, 78). Accumulation of immature NK cells has been reported in different tumors (79). The contribution of ZHX2 in the dysregulation of tumor-infiltrating NK cells strengthens ZHX2 as an immune

checkpoint regulating NK cells. Targeting ZHX2 has great potential in NK cell-based cancer immunotherapy.

ZHX2 is a critical regulator of macrophages

Macrophages are a key subset of phagocytic cells that readily engulf and degrade dying/dead cells as well as invading bacteria and viruses (80). Macrophages are distributed widely in the body tissues and play a vital role in development, tissue homeostasis and repair, and immunity (81). Macrophages are highly plastic cells that usually present different polarization states in response to local milieu stimuli (82, 83). Recently, a computational network study indicates ZHX2 as one of the most regulated transcription factors in myeloid cells to avoid an avalanche transcription event (31) Our previous study showed that Zhx2 is an important transcription factor that regulates macrophage polarization *via* reprogramming macrophage glucose metabolism (29). *Zhx2* deletion in macrophages significantly attenuates systemic inflammation in mice, prolongs mice survival, attenuates pulmonary injury and reduces proinflammatory cytokines in septic mice (29). Specifically, loss of Zhx2 confers macrophage tolerance to LPS-induced sepsis, accompanied by reduced levels of pro-inflammatory cytokines and lactate release (29). Mechanistically, Zhx2 enhances the production of proinflammatory cytokines in macrophages by promoting glycolysis in a Pfkfb3-dependent manner (29). Accordingly, BALB/cJ strain mice are less likely to develop atherosclerosis, and this resistance to atherosclerosis can be repeated in BALB/c mice by the transfer of bone marrow-derived macrophages from BALB/cJ mice (30). That is, ZHX2 promotes macrophage survival and proinflammatory functions in atherosclerotic lesions (30). In addition, tumor-associated macrophages (TAMs) are critical modulators of the tumor microenvironment (84). The important role of ZHX2-mediated pro-inflammatory polarization of macrophages suggests that targeting ZHX2 to modulate TAM may be a promising strategy for anti-tumor immunotherapy.

Conclusions and perspectives

As a transcription factor, ZHX2 transcriptionally regulates the expression of a series of genes that participate in cell proliferation, differentiation, and metabolism homeostasis. Accordingly, ZHX2 has a broader role in regulating multiple physiological and pathological processes, including cell development, immune regulation, cancer development, and metabolism-related diseases. Significantly, ZHX2 exerts its roles in a context-dependent manner. The exact mechanisms controlling the switch of ZHX2 function in health and diseases are still not clear. Nevertheless, it remains uncertain whether ZHX2 interacts with DNA directly or indirectly *via* other transcription factors to exert its transcriptional regulation role. Future research needs to be focused on ZHX2 structure, protein interactome, and high throughput screening to clarify its transcriptional regulation and identify new targeted genes. Equally important, the mechanisms that regulate ZHX2 expression are still uncertain. Accumulated studies have suggested that different stimuli regulate ZHX2 expression at different levels including transcription, post-transcription, and posttranslational modification levels. However, the mechanisms are not yet precisely understood. Moreover, in addition to hydroxylation, ubiquitination, and Nα-methylation, other PTMs that determine the biological function and nucleocytoplasmic shuttling of ZHX2 under different circumstances need to be further explored.

Author contributions

NL, ZW, and CM designed and prepared the manuscript and the figures. CM gave guidance on the outline and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Anne Fletcher,
Monash University, Australia

REVIEWED BY
Taras Kreslavsky
Karolinska Institute, Sweden
Miriam Wöhner,
University of Erlangen Nuremberg, Germany

*CORRESPONDENCE
Stephen L. Nutt
nutt@wehi.edu.au

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An arrayed CRISPR screen of primary B cells reveals the essential elements of the antibody secretion pathway

Stephanie Trezise[1,2,3], Isabella Y. Kong[1,2,4], Edwin D. Hawkins[1,2], Marco J. Herold[1,2], Simon N. Willis[1,2] and Stephen L. Nutt[1,2]*

[1]Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia, [2]Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia, [3]Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, Harvard University, Boston, MA, United States, [4]Department of Pediatrics, Division of Pediatric Hematology/Oncology, Weill Cornell Medicine, New York, NY, United States

Background: Humoral immunity depends on the differentiation of B cells into antibody secreting cells (ASCs). Excess or inappropriate ASC differentiation can lead to antibody-mediated autoimmune diseases, while impaired differentiation results in immunodeficiency.

Methods: We have used CRISPR/Cas9 technology in primary B cells to screen for regulators of terminal differentiation and antibody production.

Results: We identified several new positive (*Sec61a1*, *Hspa5*) and negative (*Arhgef18*, *Pold1*, *Pax5*, *Ets1*) regulators that impacted on the differentiation process. Other genes limited the proliferative capacity of activated B cells (*Sumo2*, *Vcp*, *Selk*). The largest number of genes identified in this screen (35) were required for antibody secretion. These included genes involved in endoplasmic reticulum-associated degradation and the unfolded protein response, as well as post-translational protein modifications.

Discussion: The genes identified in this study represent weak links in the antibody-secretion pathway that are potential drug targets for antibody-mediated diseases, as well as candidates for genes whose mutation results in primary immune deficiency.

KEYWORDS

plasma cell, immunodeficiency, humoral immunity, *in vitro* differentiation, endoplasmic reticulum, unfolded protein response, ER associated degradation (ERAD)

Abbreviations: ASC, antibody secreting cell; CRISPR, clustered regularly interspaced short palindromic repeats; CTY, cell trace yellow; ER, endoplasmic reticulum; ERAD, ER associated degradation; FoB, Follicular B cell; UPR, unfolded protein response.

Highlights

- Study revealed key dependencies in B-cell terminal differentiation and antibody secretion.
- These genes are potential therapeutic targets for treating antibody-mediated diseases and candidate causative genes for primary antibody deficiencies.

Introduction

The differentiation of mature B cells into antibody secreting cells (ASCs) is an essential component of the adaptive immune response. The ASC compartment is comprised of short-lived proliferating plasmablasts and long-lived, generally post-mitotic, plasma cells. The antibodies produced by these cells are important for the elimination of pathogens and the persistent secretion of these antibodies after pathogen clearance provides long-term protection against re-infection. Conversely, the inability to efficiently produce antibodies results in immune deficiency. Despite the crucial roles that ASCs play in immune health, we still lack a complete understanding of the factors that regulate their differentiation and antibody secretion.

While many factors have been implicated in driving this terminal differentiation process, most of the focus to date has been on the transcription factors Irf4, Blimp-1 (encoded by *Prdm1*) and Xbp1 (1). Irf4 is essential for the initial stages of the ASC differentiation process, in part due to its role in driving expression of *Prdm1*/Blimp-1 (2–5). Blimp-1, while not required for the initiation of the differentiation process, is essential for the formation of ASCs, as it silences the expression of the genes responsible for maintaining B cell identity including *Pax5* (6–9). In ASCs, Blimp-1 maintains the expression of genes involved in antibody secretion, either through direct activation or through the recruitment of chromatin modifying complexes (9, 10).

ASCs are a highly specialized cell type, devoting approximately 70% of their transcriptome to the synthesis of the *Igh* and *Igl* chains (11). This unique transcriptional program is accompanied by a reorganization of the cellular cytoplasm to allow for the formation of parallel arrays of rough endoplasmic reticulum (ER) that is necessary to facilitate massive antibody secretion. The high rates of antibody synthesis make ASCs extremely sensitive to ER stress and, consequently, they are particularly dependent on ER stress responses such as the ER-associated degradation (ERAD) pathway and the unfolded protein response (UPR) (12). Xbp1 is a key regulator of the UPR that drives increases in cell size and ER content and promotes expression of genes involved in ER homeostasis and secretory protein production (7, 9). Xbp1 is not required for the differentiation or survival of ASCs, however, the UPR activity and secretory capacity of *Xbp1*-deficient ASCs is greatly diminished (9, 13, 14). It is highly likely that there are additional, as yet unknown, genes which are also essential for the generation and function of ASCs.

We have previously performed a comprehensive transcriptional analysis of the terminal differentiation process from naïve B cell through to long-lived bone marrow plasma cells (11). This study revealed that despite differences in anatomical location, lifespan and proliferation status, ASCs share a core transcriptional signature. In addition to known regulators of ASC biology, *Prdm1*, *Irf4* and *Xbp1*, this ASC gene signature contained many genes whose functions have not been previously characterized or have not been examined in the context of ASCs. To interrogate the function of these genes, we have developed a CRISPR-Cas9 mediated arrayed targeted screen in primary mouse B cells, with the ability to measure multiple parameters in parallel, including antibody secretion. We have used this system to identify genes positively and negatively influencing the differentiation, proliferation, survival and secretion capacity of ASCs. Several of the genes identified in these screens as being required for ASC differentiation or antibody secretion have been implicated in primary antibody deficiencies. In most primary antibody deficiency patients, the genetic cause remains undetermined, therefore, the additional hits from these screens represent excellent candidates for the genes that underpin these diseases. Conversely, the genes identified as negative regulators of differentiation may play roles in preventing antibody-mediated autoimmune diseases or allergy.

Results

An arrayed targeted CRISPR screen for primary murine B cells

We sought to establish a CRISPR-Cas9 based screening system, which would allow the identification of genes that are essential for the generation, survival and/or antibody-secreting capacity of ASCs. While conventional pooled CRISPR-Cas9 screens can detect genes required for differentiation and survival, they are not able to assess defects in antibody secretion (15, 16). We optimized a 96-well transfection and primary B cell transduction protocol that consistently results in transduction rates above 80% (Supplementary Figures 1A, B). To test this system, cells were transduced with sgRNAs targeting *Sdc1*, which encodes CD138, a surface marker that serves as a proxy for ASC differentiation. Naïve splenic B cells were isolated from Cas9 transgenic mice and stimulated for 24 hours with LPS before lentiviral transduction with sgRNAs. Following transduction, the cells were returned to culture under LPS stimulation for a further three days before analysis (Figure 1A). At three days post-transduction, there were few detectable $CD138^+$ cells within the sgRNA transduced populations (Supplementary Figure 1C). To ensure that this system could block the differentiation of B cells, we measured the effect of targeting *Prdm1*, an essential driver of the differentiation process (8), and *Plpp5*, an ASC signature gene that does not influence differentiation (17). At three days post-transduction, cells transduced with sgRNAs targeting *Prdm1* showed an 80-90% decrease in the proportion of differentiated cells (Supplementary Figure 1D). In contrast, cells transduced with sgRNAs targeting *Plpp5* did not display any difference in the proportion of $CD138^+$ cells compared to controls (Supplementary Figure 1D). To examine the antibody-secreting capacity of the transduced cells, the concentration of IgM in the culture supernatant was measured by ELISA. Cells transduced with sgRNAs targeting *Prdm1* showed a 95% reduction in IgM secretion relative to the control, while *Plpp5* targeting sgRNAs did not impact on antibody secretion rates (Supplementary Figure 1E). From these

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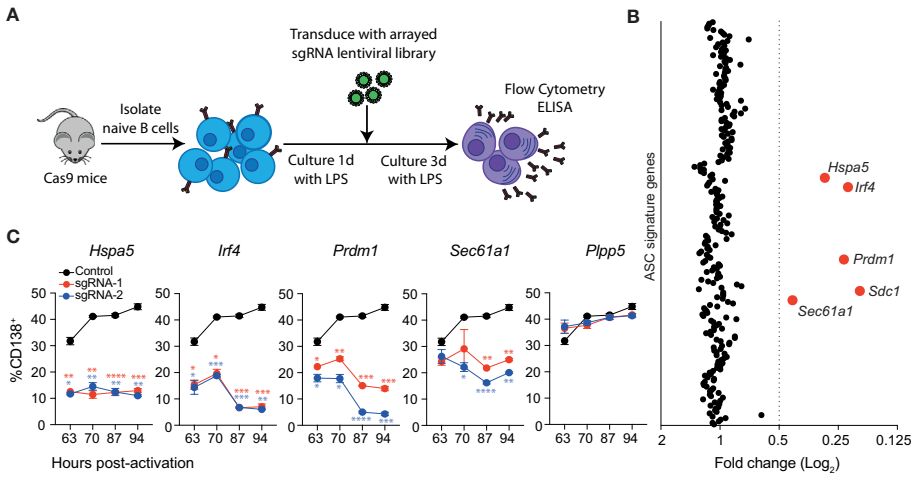

FIGURE 1
Identification of genes essential for LPS driven B cell differentiation *in vitro*. **(A)** Workflow of targeted CRISPR screen. Naïve splenic B cells were isolated from Cas9 expressing transgenic mice, activated with LPS and transduced with an arrayed lentiviral library that co-expressed specific sgRNAs and BFP. Three days after transduction, cells were analyzed by flow cytometry and culture supernatant by ELISA. **(B)** Average fold changes in the proportion of transduced cells (BFP$^+$) that express CD138 for each targeted gene relative to the untransduced control. Genes with a fold change ≤0.5 are labelled and highlighted in red. Data points represent the mean of 2 independent sgRNAs from 2 replicate experiments. **(C)** Proportion of CD138$^+$ cells among cells transduced with sgRNAs (BFP$^+$) targeting *Hspa5*, *Irf4*, *Prdm1*, *Sec61a1* or the *Plpp5* control at the indicated time post-activation with LPS. Data points represent the mean of triplicate wells and error bars indicate the S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data in **(C)** is representative of 3 independent experiments.

data, we conclude that, despite the short timeframe of the assay, this system is suitable for identifying genes that are essential for B cell differentiation and antibody secretion.

Identification of positive regulators of ASC differentiation

We used this system to interrogate the ASC gene signature to identify novel regulators of ASC differentiation *in vitro*. Of the 301 originally defined ASC signature genes, we screened sgRNAs corresponding to 258 protein-coding genes (Table S1). Naïve splenic B cells were transduced with an arrayed lentiviral library containing two sgRNAs against each gene such that each well received a single sgRNA and were cultured as above (Figure 1A). The impact of each sgRNA on differentiation was determined by examining the proportion of transduced (BFP$^+$) cells that expressed CD138. The cut-off for genes of interest was arbitrarily set to sgRNAs that reduced the proportion of CD138$^+$ cells by 50% relative to the untransduced controls for each plate (Figure 1B). In agreement with Supplementary Figures 1C, D, sgRNAs targeting *Prdm1* or *Sdc1* resulted in a decrease in CD138$^+$ cells. We also observed a reduction in differentiated cells following transduction with sgRNAs targeting *Irf4, Hspa5*, and *Sec61a1*. There was strong agreement between the effect of sgRNA pairs directed against the same gene, and a consistent effect of targeting the same gene across replicate screens (Supplementary Figure 2A). These results demonstrate that most genes within the ASC signature are not required for differentiation to CD138$^+$ ASCs, at least in the context of this *in vitro* assay.

The single timepoint examined in the screen assay does not provide any information as to how these genes are influencing the kinetics of the differentiation process. To investigate this, we repeated the assay, focusing on the genes of interest and including multiple timepoints (Figure 1C). As expected, targeting *Plpp5* did not have any effect on the frequency of differentiated cells at any examined timepoint, while targeting *Irf4* or *Prdm1* resulted in a significant reduction in differentiated cells at all examined timepoints. Similarly, *Hspa5* targeting resulted in a significantly decreased frequency of CD138$^+$ cells at all timepoints. *Hspa5*, encodes Grp78 or BiP, a major regulator of the UPR, which binds to ER stress sensors, keeping them in an inactive state (18). It is likely that the *Hspa5* targeted cells have unrestrained activation of their UPR, resulting in cell death, and that the cells undergoing differentiation and upregulating antibody production would be the most sensitive to this stress. *Sec61a1* encodes the largest subunit of the Sec61 complex, which controls the co-translational or post-translational transport of polypeptides into the ER lumen and peptide insertion into the ER membrane (19, 20). *Sec61a1* targeted cells initially showed similar rates of differentiation to untransduced cells, however, at later timepoints there was a significant decrease in the frequency of differentiated cells. It is of interest that the ASC signature examined in this screen contains 36 other genes that are considered components of the UPR that did not impact on ASC differentiation rates.

Negative regulators of ASC differentiation

We hypothesized that this screening assay, with minor modifications, would also be suitable to identify negative regulators of the B-cell differentiation process (Figure 2A). In contrast to the screen for positive regulators, the cells were cultured in LPS + IL-4 as this condition induces a relatively weak differentiation response and, therefore, enhanced differentiation rates should be more apparent. We also introduced the sgRNAs into unstimulated B cells, to allow the

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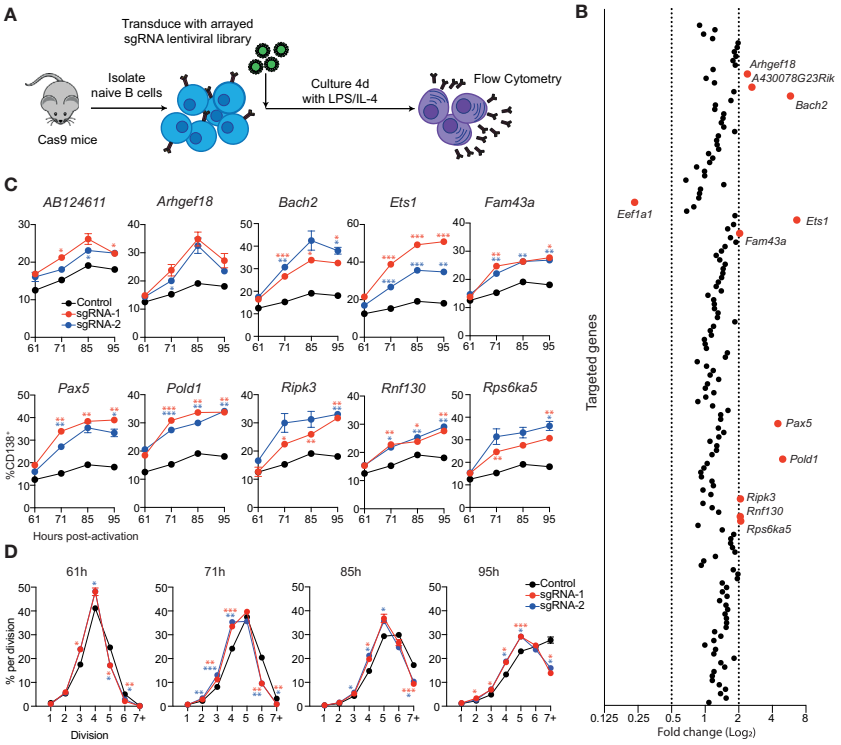

FIGURE 2
Identification of genes that repress ASC differentiation *in vitro*. **(A)** Overview of experimental workflow for targeted arrayed CRISPR/Cas9 screen. Naïve splenic B cells were isolated from Cas9 expressing transgenic mice and transduced with an arrayed lentiviral sgRNA library. Following transduction, cells were cultured in LPS + IL-4 for 4 days before analysis by flow cytometry. **(B)** Each data point represents the average fold change in the proportion of transduced cells (BFP$^+$) that are CD138$^+$ for both sgRNAs targeting a particular gene relative to the untransduced controls on the same plate. Genes with a fold change of ≤0.5 or ≥2 are labelled and highlighted in red. Data points represent the mean of 2 independent sgRNAs from 2 replicate experiments. **(C)** Naïve B cells from Cas9 transgenic mice were transduced with sgRNAs targeting the indicated genes and cultured in LPS. At the indicated timepoints, the proportion of CD138$^+$ cells within the transduced (BFP$^+$) population was assessed by flow cytometry. **(D)** Naïve B cells from Cas9 transgenic mice were labelled with the division tracking dye CTY, transduced with sgRNAs targeting *Pold1* and cultured in LPS. At the indicated timepoints, the dilution of CTY within the transduced (BFP$^+$) population was assessed by flow cytometry. Data points represent the mean of triplicate wells and error bars indicate the S.E.M. **(C, D)** are representative of 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

targeting of these genes early in the differentiation process. To validate this approach, B cells were transduced with sgRNAs directed against *Bach2*, as *Bach2*$^{-/-}$ B cells display enhanced differentiation (21) and *BACH2* variants are associated with many autoimmune and allergic diseases (22). The transduction rate of unstimulated B cells, although reduced compared to that of activated B cells, was sufficient for the development of a robust assay (Supplementary Figure 3A). At 4 days post-transduction, *Bach2* sgRNA transduced cells displayed a 4-fold increase in the rate of differentiation compared to the untransduced controls (Supplementary Figure 3B).

To identify potential negative regulators of ASC differentiation, we reanalyzed the RNAseq data that was used to generate the ASC gene signature and focused on genes that had a 3-fold higher expression in follicular B cell (FoB) samples compared to all ASC subsets (≤0.05 false discovery rate (FDR), ≥32 fragment per kilobase million reads (FPKM) in FoB samples) (11). This strategy generated a candidate list of 155 genes that are downregulated during differentiation that included many canonical B cell genes including, *Cd19*, *Cd22*, *Ms4a1* (*Cd20*), *Bcl6*, *Pax5* and *Ebf1* (Supplementary Figure 3C and Table S2). An arrayed lentiviral library was generated that contained two sgRNAs targeting each gene within this gene list. In agreement with the validation experiments, targeting *Bach2* resulted in an increased proportion of CD138$^+$ cells (Figure 2B). The other sgRNAs that resulted in a large increase in differentiation were directed against *Ets1*, *Pax5* and *Pold1*. We also observed a more modest effect in targeting *AB124611*, *Arhgef18* (or *A430078G23Rik* which is the same gene as *Arhgef18)*, *Fam43a*, *Ripk3*, *Rnf130* and *Rsp6ka5*. There was an additional gene, *Eef1a1*, which encodes a translation elongation factor, that resulted in a decrease in the proportion of CD138$^+$ cells.

To analyze the kinetics of differentiation in the targeted cells we performed a time course in cultures supplemented with LPS (Figure 2C) or LPS + IL-4 (Supplementary Figure 3D). Cells transduced with sgRNAs targeting *AB124611* or *Arhgef18* displayed a slight increase in differentiation in both conditions at all timepoints. All other genes resulted in a significant increase in differentiation at multiple timepoints, with the transcription factors *Bach2*, *Ets1*, *Pax5* and the DNA polymerase *Pold1* targeted cultures having the most pronounced impact. *Pold1* is involved in lagging strand synthesis during DNA replication and G1 to S-phase transition (23, 24) Indeed, we observed that *Pold1* targeted cells display delayed proliferation kinetics (Figure 2D) that may be indirectly driving increased rates of differentiation by slowing cell cycle progression (25, 26). The pathways through which *AB124611* (unknown function), *Arhgef18/ A430078G23Rik* (guanine nucleotide exchange factor), *Fam43a* (unknown function), *Ripk3* (necroptosis pathway), *Rnf130* (E3

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ubiquitin ligase) and *Rps6ka5* (S6 kinase family) limit ASC differentiation is unclear and requires further investigation.

Identification of B cell proliferation and survival regulators

To investigate potential regulators of B cell proliferation or survival, we reanalyzed the data from the positive regulator screen, this time examining the total live cell number (Figure 3A). 10 genes which influenced B cell survival and/or proliferation were identified (*Cdv3, Hspa5, Irf4, Rpl10, Rpl15, Rpl23a, Rps6, Sec61a1, Sumo2, Vcp*). Comparison with the differentiation results demonstrated that some of these genes (*Irf4*, *Hspa5* and *Sec61a1*) affected both cell number and differentiation, while the other genes identified only influenced cell number (Supplementary Figure 2B). *Irf4* has been linked to cell division in activated B cells as it directly induces the expression of genes involved in proliferation, including *Myc* (27, 28). *Sumo2* has previously been implicated in proliferation and cell survival as Sumo2-deficient mouse embryonic fibroblasts have decreased cell cycling and an increased cell death compared to WT cells (29). All the genes within the ASC gene signature that encode ribosomal proteins (*Rpl10, Rpl15, Rpl23a*, *Rps6*) were identified as having a strong effect on cell number. As efficient protein translation is essential for cell division and survival, it is unsurprising that targeting these genes would have a dramatic effect on cell numbers. As discussed previously, *Hspa5*/Grp78 is a major regulator of ER homeostasis and a reduction in Grp78 concentration can result in cell death (18). *Vcp* and *Selk*, a gene that was just above our fold change cut-off, both encode components of the ERAD pathway, which is responsible for detecting misfolded proteins and targeting them for proteasomal degradation before they can accumulate and trigger the terminal UPR (30–33). Curiously, there are additional genes within the ASC gene signature (*Derl1, Derl3, Edem3, Herpud1, Hsp90b1, Os9, Sel1l*) that encode components of the ERAD pathway which, when inactivated, did not have a clear impact on the total live cell number. This may reflect a redundant role between family members within this pathway.

By measuring cell number, we could not dissect the effects of genes that affected cell survival and genes that affected proliferation. Therefore, to interrogate these processes, Cas9 expressing B cells were labelled with the division tracking dye Cell Trace Yellow (CTY), activated for 24 hours with LPS, transduced with sgRNAs targeting the genes of interest and CTY dilution was assessed at multiple

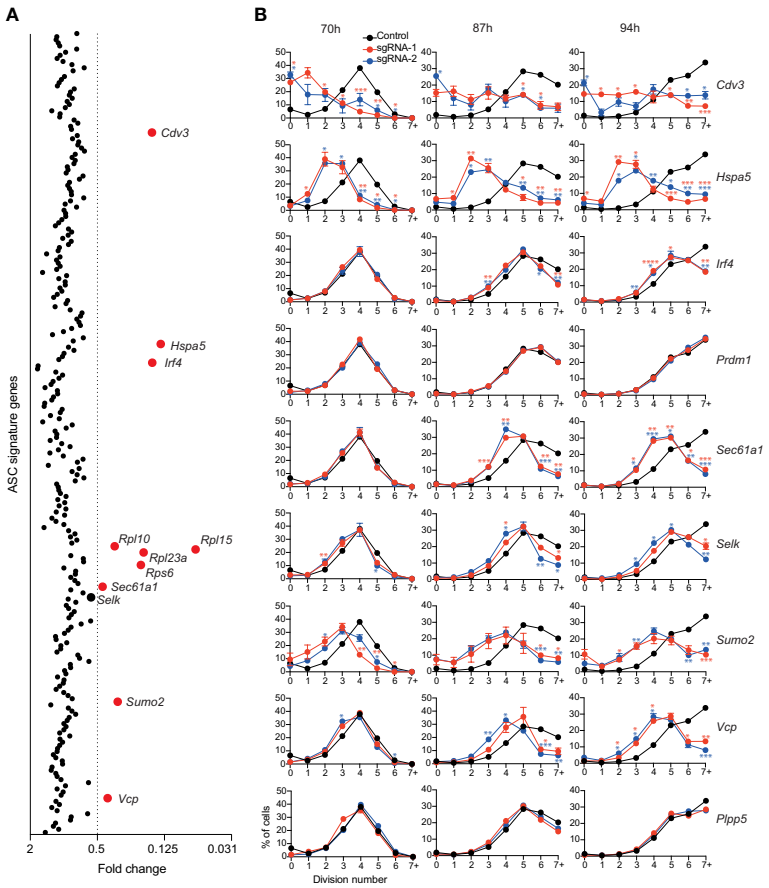

FIGURE 3
Genes affecting total live cell number. **(A)** Experimental workflow is described in Figure 1A. Average fold changes in the total number of live cells for each targeted gene relative to the untransduced control. Genes with a fold change of ≤0.5 are labelled and highlighted in red. Data points represent the mean of 2 independent sgRNAs from 2 replicate experiments. **(B)** Naïve B cells from Cas9 transgenic mice were labelled with the division tracking dye CTY, activated with LPS and transduced with sgRNAs targeting *Irf4*, *Prdm1*, *Cdv3*, *Sumo2*, *Sec61a1*, *Hspa5*, *Selk*, *Vcp* or the *Plpp5* control. At the indicated timepoints, the dilution of CTY was assessed by flow cytometry. Data points represent the mean proportion of cells in each division from triplicate wells. Error bars indicate the S.E.M. Representative of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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timepoints post-transduction. The genes encoding ribosomal proteins are essential for many basic cellular processes and were therefore excluded from further investigation. B cell division and differentiation are linked processes, with the probability of differentiation increasing with each division, therefore genes that were identified as regulators of differentiation were included even if they did not meet the reduction in cell number threshold (34, 35). We also included *Plpp5*, which does not influence B cell number, as an additional control. The CTY dilution profiles of cells transduced with sgRNAs targeting *Prdm1* and *Plpp5* overlapped with the untransduced controls, indicating that these genes do not influence B cell proliferation (Figure 3B). It has previously been demonstrated that *Irf4*-deficient B cells have a reduced proliferative capacity in response to LPS compared to WT B cells (27, 36). This proliferative defect was confirmed by our data and was most notable at later timepoints where cells transduced with *Irf4* targeting sgRNAs had stalled in their proliferation (Figure 3B). Targeting *Cdv3, Hspa5*, *Sumo2* or *Vcp* caused a dramatic reduction in proliferation capacity while targeting *Selk* or *Sec61a1* resulted in a less severe alteration in cell division. Clearly, there are many direct and indirect approaches to target B cell proliferation and thus impact on ASC differentiation and function.

Identification of antibody secretion regulators

An advantage of performing these screens in an arrayed format is that it allows for the identification of genes that regulate antibody secretion, the predominant function of ASCs. We assayed antibody production by measuring the concentrated of secreted IgM in the culture supernatants using ELISAs. To account for variation in cell numbers between cultures, results were normalized to IgM secretion per cell. Within the ASC gene signature, there were 35 genes that influenced antibody secretion (Figure 4A). The reduction in IgM

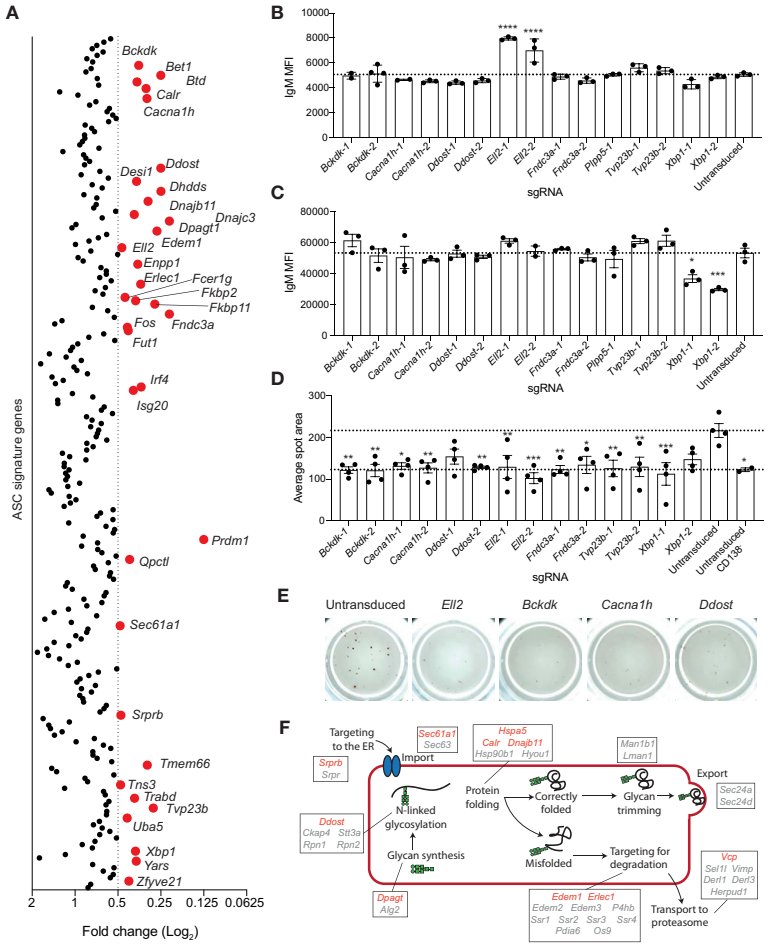

FIGURE 4
Genes essential for antibody secretion. **(A)** Experimental workflow is described in Figure 1A. The concentration of IgM in the culture supernatant was measure by ELISA and normalized to the live cell number. Data are presented as average fold change in IgM per cell for each targeted gene relative to the untransduced control. Genes with a fold change of ≤0.5 are labelled and highlighted in red. Data points represent the mean of 2 independent sgRNAs from 2 replicate experiments. **(B, C)** Naïve B cells from Cas9 transgenic mice were activated with LPS, transduced with sgRNAs targeting *Xbp1*, *Ell2*, *Bckdk*, *Cacna1h*, *Ddost*, *Fndc3a*, *Tvp23b*, or the *Plpp5* control, and cultured for a further 3 days before analysis. Mean fluorescence intensity (MFI) of IgM on the **(B)** plasma membrane or **(C)** total cellular IgM. **(D, E)** At 2 days post-transduction, transduced and untransduced cells were sorted and re-cultured overnight before transfer to ELISpot plates. **(D)** Average spot size formed and **(E)** representative wells are shown. Error bars indicate S.E.M. and dotted lines indicate the mean of the untransduced samples. Data in **(B–E)** are representative of 2-3 independent experiments. **(F)** Overview of the ER protein folding pathway (KEGG pathway: mmu04141) with genes within the ASC gene signature labelled. Red indicates secretion screen hits and grey indicates genes that did not reach the fold change cut-off. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

secretion after targeting *Irf4*, *Prdm1* and *Sec61a1* is reflective of the block in differentiation, whereas the remaining 32 genes are potential specific regulators of the antibody-secretion process (Supplementary Figures 2C, D). Genes whose disruption specifically impaired antibody secretion can be segregated into several groups; genes known to be required for antibody secretion (*Xbp1*, *Ell2*), genes involved in protein folding and ERAD (*Calr*, *Dnajb11*, *Edem1*, *Erlec1*, *Srprb*), genes involved in post-translational modifications (*Ddost*, *Dhdds*, *Dpagt1*, *Fut1, Uba5*), and genes with an unknown function or whose function is not obviously linked with antibody secretion (*Bckdk*, *Bet1*, *Cacna1h*, *Dnajc3*, *Enpp1*, *Fcer1g*, *Fkbp2*, *Fkbp11*, *Fndc3a*, *Fos*, *Isg20*, *Qpctl*, *Tmem66*, *Tns3*, *Trabd*, *Tvp23b*, *Yars*, *Zfyve21*).

There are multiple stages in the secretion process that these genes may be either directly or indirectly regulating and several of these genes (*Bckdk, Cacna1h, Ddost, Ell2, Fndc3a*, *Tvp23b*) were selected for validation and a more in-depth investigation of their role in antibody secretion. *Xbp1* and *Plpp5* were included as additional controls. To determine whether these genes were influencing the transcriptional switch from producing the membrane bound form of immunoglobulin produced by B cells to the secretory form expressed in ASCs, we examined the amount of membrane bound IgM present on the plasma membrane of transduced CD138$^+$ cells (Figure 4B). Cells transduced with sgRNAs targeting *Ell2* displayed an increase in membrane bound IgM, which is in line with the known role of Ell2 in promoting the usage of the distal *Igh* polyadenylation site to drive the production of secretory transcripts (37). No other sgRNAs affected the levels of membrane bound IgM, suggesting that these genes regulate processes further along the antibody-secretion pathway. Decreased antibody secretion may also be due to a reduction in the production of IgM protein, therefore, the total IgM production capacity of the CD138$^+$ cells was determined by sequential membrane bound and intracellular labelling of IgM with the same antibody (Figure 4C). As expected, *Xbp1* targeted cells had decreased levels of IgM as *Xbp1*-deficient ASCs are known to have a reduced capacity to upregulate immunoglobulin production (13, 14). No other targeted genes resulted in decreased total IgM levels, suggesting that they do not regulate the protein production capacity of ASCs per se. The proportion of IgM$^+$ cells was consistently greater than 90% for all sgRNAs, demonstrating that the reduction in IgM secretion was not due to increased frequencies of isotype switched cells (data not shown). To examine the rate of secretion per cell, naive should have special character for i B cells were stimulated with LPS for 24 hours then transduced with sgRNAs. At 2 days post-transduction, BFP$^+$ cells were sorted and returned to culture to recover for 24 hours before transfer to ELIspot plates. The average spot size was reduced for all genes and was comparable to that observed for undifferentiated (CD138$^-$) B cells (Figure 4D, E). Thus, all sgRNAs examined appear to reduce antibody secretion downstream of protein synthesis.

Discussion

The generation, survival and function of ASCs is critical for an effective adaptive immune response and underpins the protective immunity elicited by all current vaccinations. Previous work has identified a core group of expressed genes that are shared between all ASC subsets (11) and interrogating this signature provided us with the opportunity to identify novel regulators of ASC biology. We developed an arrayed CRISPR/Cas9-mediated screening system which allowed for the identification of factors essential for the differentiation, survival, proliferation and antibody secretion capacity of primary B cells at a very high resolution.

The ASC gene signature consists of genes encoding proteins of diverse functional categories including gene expression and translation, UPR, protein transport, post-translational modifications, metabolism, receptors and signaling pathways (11, 12). In light of this it was surprising that only five genes within the ASC gene signature were essential for the differentiation to CD138$^+$ ASCs to occur *in vitro*, including *Sdc1*, the gene that encodes CD138, *Irf4*, *Prdm1*, *Sec61a1* and *Hspa5*. *Irf4* and *Prdm1* are well known regulators of ASC formation and function (8, 9, 38). *Sec61a1* and *Hspa5*, encode proteins important in protein translocation into the ER and ensuring correct protein folding. *Sec61a1* has been implicated in the differentiation and survival of human ASCs as *SEC61A1* haploinsufficiency causes decreased rates of differentiation *in vitro* and decreased plasmablast populations *in vivo* (39). This study identified two families with *SEC61A1* mutations, one with a nonsense mutation resulting in haploinsufficiency (p.E381*) and the other with a point mutation (V85D). Interestingly, neither of these mutations resulted in changes in peripheral B cell populations, however, plasmablast populations were reduced. Furthermore, the mutation of *SEC61A1* in multiple myeloma cell lines results in UPR activation and cell death (40, 41). This suggests that the absence of differentiation observed in *Sec61a1* targeted mouse B cells is likely due to increased cell death during the differentiation process as cells try to increase their rates of antibody synthesis. *Hspa5* encoded Grp78 is a key regulator of the UPR due to its function as an ER chaperone protein (18). Grp78 binds to unfolded or misfolded proteins in the ER lumen to facilitate correct protein binding, however, it also binds to the ER stress sensors, IRE1a, PERK and ATF6, keeping them in an inactive state. In the absence of Grp78, the ER stress sensors activate downstream processes including the UPR, and if left unrestrained will induce cell death. It is likely that this terminal UPR activation is occurring in the *Hspa5* targeted cells resulting in the decrease in differentiation, survival and proliferation observed in this study.

By altering the parameters of our genetic screen, we were also able to identify 10 genes that act as negative regulators of ASC differentiation. This list included four regulators of gene expression, *Bach2*, *Pax5*, *Ets1* and *Pold1*. Bach2 is known to represses the expression of *Prdm1* (21, 42) and *Bach2*$^{-/-}$ B cells display increased rates of differentiation, as was also evident in our screen results. Pax5 is a master regulator of B cell identity, and its inactivation in mature B cells results in cells reverting to an earlier progenitor stage (43). Although downregulation of *Pax5* expression is one of the earliest stages of the ASC differentiation process (6), and Pax5 represses many ASC genes (44, 45), this process is not essential as differentiation proceeds if *Pax5* cannot be downregulated (46). Furthermore, others have reported that RNAi knockdown of *Pax5* expression in activated B cells did not alter the rate of differentiation (47). In contrast, we observed increased rates of differentiation in the targeted cells, suggesting that Pax5 downregulation, while not essential, may still be a limiting step in normal ASC differentiation. This discrepancy is potentially due to a more complete loss of *Pax5* following CRISPR

editing while the residual levels of *Pax5* following RNAi may be sufficient for the differentiation process to occur normally. Ets1 has been shown to negatively regulate ASC differentiation specifically induced by the TLR9 ligand CpG (48, 49), however our data suggests a broader function for Ets1 in controlling the rate of ASC differentiation. Interestingly, Ets1 is proposed to act by maintaining *Pax5* expression and post-translationally inhibiting Blimp-1 (49). In keeping with this gatekeeper function, variants in *ETS1* has been linked to several autoimmune conditions including systemic lupus erythematosus (50) and multiple sclerosis (51). Interestingly, targeting *Pold1* caused an increase in differentiation to a similar extent as these key transcription factors. *Pold1* encodes the catalytic subunit of the DNA polymerase delta (PolD) complex, which is involved in the synthesis of the lagging strand during DNA replication and in several DNA damage repair pathways (23), and its mutation in humans results in immunodeficiency (52). We found that *Pold1* loss resulted in a slowed cell cycle in activated B cells. This coupled with prior reports showing B cells that spend a prolonged time in G1 display dramatically increased rates of differentiation (25, 26) suggest that *Pold* loss indirectly increased to rate of ASC differentiation by slowing the cell cycle.

Many of the genes identified in this screen as being essential for antibody secretion encode components of the ER protein processing pathway. This pathway involves a multitude of processes (targeting to the ER, polypeptide import, folding, *N*-linked glycosylation, recognition of misfolded proteins, and targeting of misfolded proteins for degradation) (18) and hits from this screen have been implicated in almost every stage of this pathway (Figure 4F) *Srprb* encodes a component of the signal recognition complex, which is controls the co-translational targeting of polypeptides to the ER (53). *Calr* and *Dnajb11* are involved in maintaining ER homeostasis through their roles as chaperones to promote correct protein folding (54, 55). *Edem1* and *Erlec1* are components of the ERAD pathway (56, 57). *Ddost*, *Dhdds*, *Dpagt1* and *Fut1* are all involved in post-translational modification, with *Ddost*, *Dhdds* and *Dpagt1* being required for the synthesis and attachment of *N*-linked glycosylations and *Fut1* being a factor regulating protein fucosylation (58–61). Correct protein glycosylation is essential for facilitating correct protein folding, preventing protein degradation by the ERAD pathway, trafficking from the ER to the golgi, movement through the golgi and transport to the plasma membrane (62). All of these processes are required for antibody secretion, therefore, targeting genes regulating the addition of glycans is likely affecting at least one of these processes.

Several recent studies have also used a CRISPR-Cas9 screening approach to identify regulators of ASC differentiation (15, 63, 64). Although each group used independently curated gene lists for their boutique sgRNA library, making a direct comparison of the results difficult, a relatively small number of common genes essential for ASC differentiation were identified in each study (*Prdm1*, *Irf4* and *Hspa5*). It is also noteworthy that the prior studies identified glycosylation machinery and components of the ERAD and UPR pathways as being essential for ASC differentiation and/or survival (63, 64), while, with the exception of *Hspa5* and *Sec61a1*, we observed that targeting these pathways specifically disrupted antibody secretion. A potential explanation for this discrepancy is that these previous screens all used the induced germinal center culture system, where the B cells were kept alive for longer (65). The shorter timeframe of our screen may allow for this block in antibody secretion to be detected before the accumulation of protein becomes high enough to trigger the terminal UPR, leading to a selective loss of ASCs. These caveats aside, these studies collectively provide a wealth of new information on genes required for ASCs differentiation and function.

The genes required for antibody secretion represent excellent candidates for the development of new small molecules to treat antibody-mediated diseases including autoimmune conditions, allergy, transplant rejection and the plasma cell malignancy multiple myeloma. It is interesting to note, however, that there are 27 additional genes within the ASC gene signature that are implicated in the UPR that did not have a measurable effect on antibody secretion in this assay (Figure 4F). There may be redundancy in this pathway so targeting only one gene at a time may not have any effect on secretion rates. The genes identified by this screen may also highlight potential weak links in the antibody secretion process that may underlie immunodeficiency syndromes. As highlighted above, human *SEC61A1* haploinsufficiency has recently been demonstrated to cause a primary antibody deficiency through impaired ASC differentiation (39), whereas *IRF4* haploinsufficiency has been linked to Whipple's disease caused by the inability to control infection with the bacteria *Tropheryma whipplei* (66). Mutations in the *BTD* gene cause Biotinidase deficiency, a treatable deficiency in biotin that may have an immunodeficiency component (67, 68). Mutations in several other regulators identified in our screen may also result in antibody immunodeficiency, including *CACNA1H*, a calcium channel linked to epilepsy. Two patients with *CACNA1H* mutations have been reported to show selective antibody deficiency (69), whereas patients harboring mutations in the genes encoding ribosomal proteins have been documented to develop common variable immune deficiency (70). The remaining genes from our screen that are involved in ASC differentiation, proliferation or antibody production represent strong additional candidates for primary antibody deficiency genes.

Materials and methods

Mice

Experimental mice were bred and maintained on a C57BL/6 genetic background and housed in the Walter and Eliza Hall Institute (WEHI) animal facility in a specific pathogen free environment. Animal experiments were conducted in accordance with protocols approved by the WEHI animal ethics committee. Rosa26-lox-STOP-lox-Cas9-IRES-GFP mice (71) were bred with B6-Cre-deleter mice to generate the constitutive Cas9 transgenic strain.

B cell isolation and culture

Naïve splenic B cells were isolated using a B cell isolation kit (Miltenyi Biotech) and cultured in B cell medium (RPMI 1640, 10% FCS, 2 mM L-Glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 1% non-essential amino acids) supplemented with 10 μg/mL LPS (Sigma-Aldrich) ±10 ng/mL mouse IL-4 (R&D Systems). For proliferation analysis, non-proliferating lymphocytes were separated using a Percoll (GE Healthcare) density gradient prior to B cell

isolation and cells were labelled with the division tracking dye, CTY (Invitrogen).

Flow cytometry

Cells were stained with monoclonal antibodies specific for CD138 (281-2; BioLegend) or IgM (331.12; eBioScience). Intracellular staining was performed using BD Cytofix/Cytoperm (BD Biosciences). Cell viability was determined by the addition of 1 µg/mL Propidium Iodide (PI; Sigma-Aldrich), 1 µg/mL FluoroGold (Sigma-Aldrich) or 1 µL/mL eFluoro-780 (eBioscience).

Enzyme-linked immunosorbent assay (ELISA)

Plates were coated with anti-IgM (1 µg/mL; Southern Biotech) overnight. Plates were washed with PBS/0.04% Tween-20, PBS, then water before the addition of cell culture supernatant or IgM standard (TEPC183; Sigma-Aldrich) to the appropriate wells. After 4 hours, plates were washed and incubated with anti-IgM-HRP (1 µg/mL; Southern Biotech) for a further 4 hours. Plates were washed and bound IgM was visualized by the addition of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich) substrate solution (0.54 mg/mL ABTS, 10.5 mg/mL citric acid, 15 mg/mL trisodium citrate dihydrate, 0.03% hydrogen peroxide).

Enzyme-linked immunospot (ELISpot)

Multiscreen HA plates (Millipore) were coated with anti-IgM diluted in 0.2 M carbonate buffer for 4 hours. Plates were washed with PBS before cells in B cell medium were added. Plates were then incubated at 37°C 10% CO_2 for 14-18 hours. Plates were washed as in the ELISA method before the addition of anti-IgM-HRP. IgM secreting cells were visualized by the addition of 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich) solution (0.05 M sodium acetate, 0.25 mg/mL AEC, 2% *N,N,*-Dimethyl Formamide, 0.03% hydrogen peroxide).

Production of lentiviral vectors

Individual sgRNA plasmids were obtained from the Sanger Arrayed Mouse Whole Genome Lentiviral CRISPR Library (Sigma-Aldrich, #MSANGERG) that co-expressed BFP. HEK293T cells were maintained in DMEM/10% FCS and plated 16 hours prior to transfection at a density of $2x10^4$ or $1.5x10^6$ cells for 96-well and 10 cm^2 plates respectively. pMDL1-gag-pol, pCAG-Eco, pRSV-REV and sgRNA plasmids were combined at a ratio of 3:2:2:3. Fugene6 Transfection Reagent (Promega) was added to the plasmid mix at a ratio of 3 µl FuGENE6 to 1 µg DNA and incubated for 30 minutes before the FuGENE6-DNA mixture was added to HEK293T cultures. Transfected HEK293T cells were BFP^+ (Supplementary Figure 1A). Lentivirus containing supernatant was collected 48 hours post-transfection, and either used fresh or stored at -80 °C.

Transduction of primary B cells

Non-tissue culture treated 96-well plates were coated with Retronectin (32 µg/mL; produced in house) for 4 hours and plates were blocked with PBS/2% BSA prior to the addition of cells and lentiviral containing supernatant. Each well received only a single sgRNA expressing lentiviral supernatant. Plates were then centrifuged at 1200 rpm for 90 minutes. Following centrifugation, supernatant was removed, and cells were resuspended in B cell medium containing LPS ± IL-4. The rate of transduction (proportion of BFP^+ cells (Supplementary Figure 1B) and impact of CRISPR sgRNA on ASC differentiation (proportion of total BFP^+ cells that are $CD138^+$) and cell number was determined by flow cytometry, and the impact on antibody secretion was quantified by ELISA. The proportion of BFP^+ $CD138^+$ ASCs and the antibody secretion rate were compared to untransduced controls for each plate.

Analysis of publicly available RNAseq data

To generate a list of FoB specific genes, we reanalyzed RNA-sequencing data published by Shi et al. (GSE60927) (11). The count table was downloaded and gene with at least 1 count per million (CPM) in at least three samples were included downstream analysis (72, 73). Count data were normalized using the trimmed mean of M-values (TMM) method, and differential gene expression analysis was performed using the limma-voom pipeline (limma version 3.40.6) (72, 74, 75). FoB specific genes had a 3-fold higher expression in FoB samples compared to all ASC subsets (≤0.05 false discovery rate). Heatmaps of logCPM were generated using pheatmap.

Statistical analysis

Statistical significance was determined by two-way ANOVA with multiple comparisons.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Walter and Eliza Hall Institute Animal Ethics Committee.

Author contributions

Conceptualization, ST and SN. Methodology, ST, IK, and MH. Investigation, ST and IK. Writing – original draft, ST and SN. Writing – review and editing, ST, IK, EH, MH, SW, and SN.

Resources, EH, MH, SW, and SN. Supervision, EH, SW and SN. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1089243/full#supplementary-material

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Krüppel-like factor 2: a central regulator of B cell differentiation and plasma cell homing

Jens Wittner and Wolfgang Schuh*

Division of Molecular Immunology, Department of Internal Medicine 3, Nikolaus-Fiebiger-Center, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

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EDITED BY
Stephen Nutt,
The University of Melbourne, Australia

REVIEWED BY
Zhoujie Ding,
Monash University, Australia
Jasper Cornish,
The University of Melbourne, Australia

*CORRESPONDENCE
Wolfgang Schuh
wolfgang.schuh@uk-erlangen.de

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The development of B cells, their activation and terminal differentiation into antibody-producing plasma cells are characterized by alternating phases of proliferation and quiescence that are controlled by complex transcriptional networks. The spatial and anatomical organization of B cells and plasma cells inside lymphoid organs as well as their migration within lymphoid structures and between organs are prerequisites for the generation and the maintenance of humoral immune responses. Transcription factors of the Krüppel-like family are critical regulators of immune cell differentiation, activation, and migration. Here, we discuss the functional relevance of Krüppel-like factor 2 (KLF2) for B cell development, B cell activation, plasma cell formation and maintenance. We elaborate on KLF2-mediated regulation of B cell and plasmablast migration in the context of immune responses. Moreover, we describe the importance of KLF2 for the onset and the progression of B cell-related diseases and malignancies.

KEYWORDS

KLF2, plasma cells, quiescence, B cells, integrins, IgA, mucosal immunity, multiple myeloma

Introduction

Krüppel-like factor 2 (KLF2) is a transcription factor of the Krüppel-like factor (KLF) family whose members are characterized by a C-terminal zinc finger DNA-binding domain. The family name originated from the phenotype of a Drosophila loss-of-function mutant with abnormal segmentation of the abdominal region of the Drosophila larva ("Krüppel" mutant, Krüppel: German word for cripple). In Drosophila, the *krüppel* gene is one of the so-called *gap* genes, a group of genes responsible for the development of the Drosophila larvae and their segmentation (1, 2). The KLF family consists of 17 members in vertebrates, all of which are involved in the control of differentiation, proliferation, cell adhesion, and migration processes in a variety of cell types (3, 4). KLF2 was first described by Anderson and colleagues in 1995 and originally named lung Krüppel-like factor (LKLF) due to its high expression in the lung (5). The importance of KLF2 during embryonic development was revealed in 1997 by Kuo and colleagues using a genomic knockout mouse model for the *Klf2* gene. Their study demonstrated that KLF2-deficient embryos died between days E12.5 and E14.5 due to hemorrhage, defective blood

vessels, and an abnormal tunica media *in utero* (6). Thus, KLF2 has an essential function in embryonic development and in endothelial cell biology. From the time point of its discovery in the late 1990s, numerous studies have revealed a crucial role for KLF2 during proliferation, differentiation, activation, and positioning of B and T cells, and other immune cells (4, 7). The loss of function of KLF2 is associated with diseases, such as arteriosclerosis, adipogenesis, thrombosis, and lymphoma (3, 4, 7–12). The role of KLF2 has been intensively studied in T-lymphoid cells and it becomes increasingly evident that KLF2 also acts as an important regulator of different aspects of B cell biology. Therefore, in this review article, we discuss the relevance of KLF2 during B cell differentiation and activation as well as its function of KLF2 as a regulator of B cell and plasma cell homing. Finally, we elaborate on how KLF2 contributes to B cell-related diseases and malignancies.

Expression of KLF2 in B-lymphoid cells

Expression of KLF2 in early B cell progenitors in the bone marrow (BM) was discovered in a mouse model with tetracycline-controllable expression of the pre-B cell receptor (pre-BCR) (13). The pre-BCR is part of a critical checkpoint in early B cell development, which tests the ability of newly formed immunoglobulin (Ig) μ-heavy chains (μHC) to functionally pair with the surrogate light chain components VpreB and λ5. Pre-BCR-mediated signals result in clonal expansion of pre-B cells, suppression of apoptosis, targeting of the VDJ-recombination machinery to the *Ig light chain* (*IgL*) loci, and allelic exclusion (14, 15). Analyses of changes in the transcriptome upon tetracycline-controlled pre-BCR induction, uncovered KLF2 as a pre-BCR-induced gene (13). KLF2 expression in pre-B cells was confirmed in KLF2:GFP reporter mice (16). Pre-BCR signals result in Erk5 phosphorylation, which in turn activates the transcription factors Mef2c and Mef2d by phosphorylation. Phosphorylated Mef2c and Mef2d, in turn, activate transcription of the *Klf2* gene and, in parallel, of immediate-early genes, encoding for the transcription factors Jun and Fos, as well as the early growth response proteins Egr1 and 2 that induce pre-B cell expansion (17). In addition, Mef2c/d transcription factors induce IRF-4, a transcription factor important for the termination of pre-B cell expansion and the initiation of immature B cell differentiation (18). Over time, KLF2 accumulates in proliferating pre-B cells and inhibits the Mef2c/d-mediated transcription of the immediate-early genes Jun and Fos and Egr1/2, thus, contributing to the termination of pre-B cell expansion (17). Along this line, ectopic expression of KLF2 resulted in a block of pre-B cell proliferation concurrent with decreased *c-myc* and increased *p21* and *p27* mRNA abundances (19) (Figure 1). However, KLF2-deficient mice displayed normal pre-B and immature B cell compartments (16, 20), suggesting that in the absence of KLF2, termination of pre-B cell expansion still occurs and is presumably mediated through *Irf-4* upregulation. As aforementioned, activation of Mef2c/2d by pre-BCR signals results in the upregulation of *Irf-4* expression. Subsequently, IRF-4/IRF-8-mediated upregulation of the transcription factors Aiolos and Ikaros was shown to downregulate pre-BCR expression and to impair cell cycle progression and thereby pre-B cell expansion (21).

As aforementioned, KLF2 expression is induced by the pre-BCR in early B cell development and is maintained in immature B and follicular (Fo) B cells (13, 16, 20, 22). Marginal zone (MZ) B cells

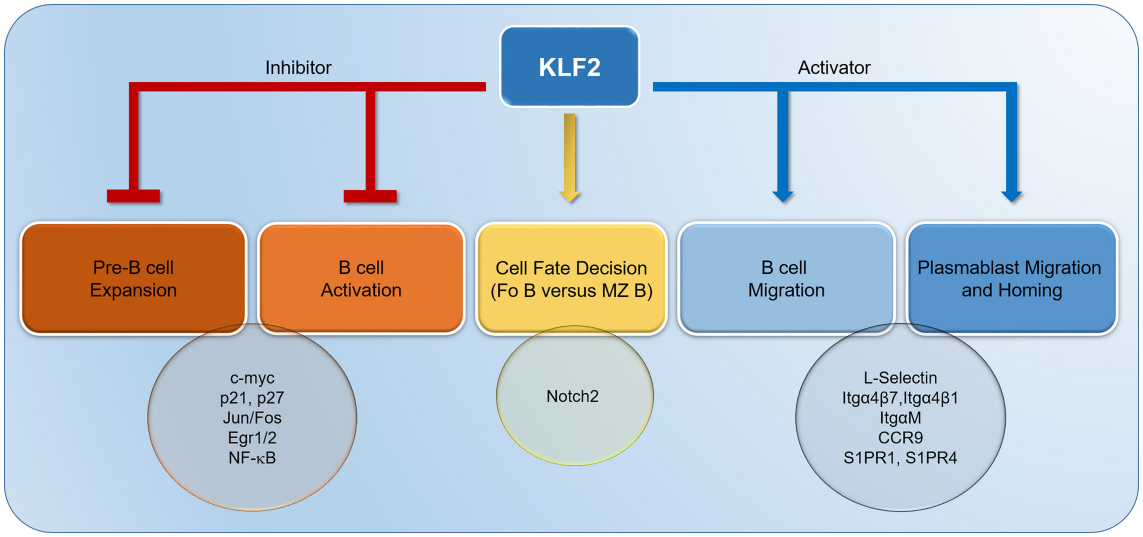

FIGURE 1
Krüppel-like factor-2 (KLF2) contributes to the termination of pre-B cell expansion through inhibition of Jun/Fos and Egr1/2. Moreover, KLF2 inhibits the proliferation of pre-B cells and the activation of naïve, mature B cells by downregulating c-myc and upregulating p21 and p27. In B cells, KLF2 suppresses NF-κB activation. Furthermore, KLF2 represses Notch2 signaling in naïve B cells, thereby driving B cell differentiation to follicular B cells. KLF2 controls the migration of B cells and plasmablasts by positively regulating L-Selectin, Itgα4β7, Itgα4β1, ItgαM, Chemokine receptor 9 (CCR9), Sphingosine-1-Phosphate-Receptor (S1PR) 1 and S1PR4. IgA plasmablast homing to gut-associated lymphoid tissues (GALT) is mediated by KLF2-regulated factors Itgα4β7 and CCR9. Itg, integrin.

show low abundances of KLF2 mRNA and protein whereas B1 cells in the peritoneum display the highest abundance of KLF2 (16, 20, 22). Activation of splenic B cells *in vitro* with LPS, anti-CD40/IL-4 or anti-IgM (anti-BCR) led to decreased KLF2 mRNA and protein abundances (16, 20, 22, 23). In this context, ectopic expression of KLF2 in LPS-activated, proliferating B cells led to an inhibition of B cell activation, expansion, and plasmablast differentiation (19). Therefore, KLF2 acts as a quiescence factor that keeps mature B cells in a resting state. The function of KLF2 as an important quiescence regulator was already postulated in 2000, when KLF2 was found in comparative transcriptome analyses to be highly abundant in resting, naïve B, and anergic B cells, but downregulated in activated B cells (24). In mature B cells, *Klf2* gene expression might be driven by the transcription factor Foxo1 (similar to the Foxo-1-mediated regulation of the *Klf4* gene) as Foxo1-binding sites were found in the *Klf2* promoter and Foxo1-binding to the *Klf2* promoter was described (25). In support, *Klf2* mRNA was reduced in Foxo1-deficient B cells (26). B cell activation results in PI3K-Akt-mediated phosphorylation of Foxo1. Phosphorylated Foxo1 is transported out of the nucleus and becomes transcriptionally inactive (27). Consequently, *Klf2* expression is terminated, which in turn, might enable B cell proliferation and differentiation.

B cells in secondary lymphoid organs can be activated by antigen in either a T cell-dependent (TD) or a T cell-independent (TI) manner. TD activation leads to the formation of a germinal center (GC) reaction in which the BCR of the activated B cell undergoes affinity maturation and Ig class switch recombination occurs. As a result of the GC reaction, B cells with a high affinity BCR either differentiate to memory B cells (Bmem) or to plasma cells (28–31). One study unraveled increased *Klf2* RNA abundances in $CD80^+$/$PD\text{-}L2^+$ Bmem that were shown to quickly differentiate into antibody-secreting cells but did not form new germinal centers (32). Furthermore, single-cell RNAseq of isotype-switched Bmem uncovered a cluster of *Klf2*-expressing Bmem. The cells in this cluster were characterized by low abundances of *Cr2* (CD21), intermediate abundances of *Fcer2a* (CD23), and expressed *Klf2*, *Vimentin-1* and *Prostate androgen-regulated mucin-like protein 1* (*Parm-1*). Based on these characteristics, the authors of this study defined cluster I cells as transitional Bmem (33). Although KLF2 has been detected in Bmem subsets, its functional relevance for Bmem is so far mostly unknown. We speculate that KLF2 in Bmem might functionally contribute to their tissue distribution and retention. In this context, KLF2 expression in Bmem correlated with expression of factors critical for homing and migration, such as Integrin (Itg)β7, Sphingosine-1-phosphate-receptor 1 (S1PR1) and C-C chemokine receptor CCR6 expression (34). Additionally, it is tempting to speculate that KLF2 might keep Bmem in the resting state until they encounter their specific antigen.

GC B cells that differentiate to plasma cells undergo a dramatic morphological change characterized by an increase in cell size and an enlargement of the endoplasmatic reticulum (ER) (31). This process is controlled by a complex regulatory network of transcription factors. Blimp-1 (encoded by the *Prdm-1* gene) is the key transcription factor that drives plasma cell differentiation by promoting Ig production and secretion, and by repressing B ell activation-signature transcription factors Pax5, Bcl-6, Bach2 and the enzyme Activation-induced cytidine deaminase (AID, encoded by the *Aicda* gene) (31). Activated B cells first differentiate into proliferating plasmablasts that are migratory and then into mature, resting plasma cells (35). In plasmablasts in the blood, expression of KLF2 and its target gene *S1pr1* was detected (36). Migration along the sphingosine-1-phosphate (S1P) gradient guides plasmablasts from lymph nodes and spleen to lymph and blood (36). Analysis of KLF2:GFP reporter mice revealed KLF2 expression in IgM and IgA plasmablasts in the blood. In lymphoid organs, the highest frequency of KLF2-positive cells was found within the IgA plasmablast population in mesenteric lymph nodes (mLN), suggesting a pivotal role of KLF2 for IgA plasmablasts and IgA plasma cells (37).

Functional role of KLF2 in peripheral B cell subsets

The regulatory role of KLF2 in B cell proliferation and activation was primarily analyzed *in vitro* by overexpression approaches and by studying loss-of-function mutants of *KLF2* and their ability to activate NF-κB signaling. Regarding the regulation of quiescence, ectopic expression of KLF2 in pre-B cell cultures and in LPS-activated B cells led to the downregulation of *c-myc* and upregulation of the cell cycle inhibitors *p27* and *p21* (19). Moreover, as shown in monocytes, KLF2 interferes with NF-κB activation (4, 38), a mechanism that might also apply for B cells and B lymphoma cells. Accordingly, *KLF2* loss-of-function mutations as found in human lymphoma cells impaired KLF2-mediated NF-κB suppression in a B lymphoma cell line (11), a topic that will be discussed later in the review article.

To study the functional relevance of KLF2 during B cell development and activation *in vivo*, mouse models with a conditional B cell-specific deletion of a floxed *KLF2* gene were generated. To achieve B cell-specific deletion, either *mb1cre* or *CD19cre* deleter mouse strains were used (16, 20, 22). The B cell-specific deletion of KLF2 resulted in enlarged spleens with an expansion of Fo B cells and MZ B cells (16, 20, 22). KLF2-deficient Fo B cells showed enhanced CD21 surface expression and altered BCR-mediated calcium signals, and thus, as concluded from these parameters and changes in the global gene expression profile partially resembled MZ B cells (16, 20, 22). Fo B and MZ B cells are functionally distinct B cell subsets. Fo B cells migrate between lymphoid organs and give rise to GC upon activation. MZ B cells are a specialized B cell subset located in the splenic marginal zone and their mobility, in contrast to Fo B cells, is limited to shuttling between the marginal zone and the B cell follicle to facilitate antigen transport (39). MZ B cells can develop either from transitional B cells or from follicular B cells (40). Their differentiation is driven by Notch2 signaling. Deletion of *Notch2* or its ligand *Dll-1* resulted in a loss of MZ B cells (41, 42). In an elegant study, induction of Notch2IC (intracellular domain of Notch2 that interacts with DNA-binding protein RBPJ and regulates transcription) resulted in the conversion of Fo B cells to MZ B cells. Upon induction of Notch2IC signaling, *Klf2* (besides

Irf-8 and *Foxo1*) was downregulated (43). These findings are supported by the expansion of MZ B cells observed in KLF2-deficient mice and suggest a role of KLF2 in the cell fate decision and the imprinting of the cellular identity of Fo B versus MZ B cells (Figure 1). As described later, loss-of-function mutations of human KLF2 are frequently found in splenic marginal cell lymphoma (SMZL) and play a role in disease onset and/or progression. Immunization experiments showed an increased immune response to TI antigen type 2 (TNP-Ficoll) antigens in B cell-specific *KLF2*-deficient animals compared to controls, which might be due to the observed expansion of MZ B cells and the altered phenotype of KLF2-deficient Fo B cells (22). Immunization with the TD antigen TNP-KLH, however, resulted in reduced antigen-specific IgG titers upon boost immunization. Antigen-specific IgG plasma cells as determined by ELISpot analyses were unaffected in the spleen but were virtually absent in the BM, indicating that loss of KLF2 affects plasmablast homing and/or plasma cell survival in the BM (20).

Importantly, KLF2 deletion profoundly affected mucosal immune responses. KLF2-deficiency resulted in reduction and phenotypic alterations of peritoneal B1 cells (16, 20, 44). Mice with a B cell-specific KLF2 deletion develop fewer and smaller Peyer's patches (PP) and natural IgA in the serum was reduced (16, 20, 22). Furthermore, B cell-specific deletion of KLF2 resulted in drastically reduced secretory IgA (SIgA) in the gut lumen concomitant with reduced IgA plasma cells in the intestinal lamina propria (LP). IgA plasmablasts and plasma cells, however, accumulated in the mLN and PP, although PP were smaller in size and numbers. Immune responses to immunization with soluble recombinant Flagellin, an immunodominant protein of Salmonella typhimurium, were blunted. In summary, B cell-specific deletion of KLF2 in B cells in mice led to a phenotype similar to that observed in human IgA deficiencies (37).

KLF2-regulated genes in B cells and plasma cells

KLF2 acts a major regulator of thymic exit and T cell migration by regulating S1PR1 (45–47). In peripheral murine B cell subsets, one study also described direct binding of KLF2 to the *edg1* promoter (the *edg1* gene encodes for S1PR1) in murine MZ B cells by chromatin immunoprecipitation (ChIP) (22). In this study, *S1pr1* mRNA was shown to be downregulated in KLF2-deficient MZ B cells and upregulated in Fo-deficient B cells despite the lack of KLF2 binding to the *edg1* promoter in Fo B cells (22). Two other independent studies demonstrated that S1PR1 mRNA and protein were not significantly altered in KLF2-deficient Fo B cells (16, 20). Therefore, the involvement of KLF2 in the regulation of *S1pr1* expression in MZ B cells and Fo B cells remains unresolved. In IgA plasmablasts, however, RNASeq data confirmed the KLF2-dependent regulation of *S1pr1* and *S1pr4* mRNAs, which were both significantly reduced in KLF2-deficient IgA plasmablasts in the mLN (37). Therefore, KLF2-mediated regulation of S1PRs might contribute to plasmablast migration and homing to the bone marrow as well as mucosal effector sides (Figures 1, 2).

The chemokine receptor CXCR5 recognizes the chemokine CXCL13 and is important for the positioning of B and T cells inside the B cell follicles in lymph nodes and the spleen (48) and for the shuttling of MZ B cells between the follicle and the marginal zone of the spleen (39). In T follicular helper (TFH) cells, KLF2 binds directly to the *Cxcr5* promoter (as shown by ChIP) and represses *Cxcr5* expression. Downregulation of KLF2 caused by ICOS signals *via* Foxo1 resulted in *Cxcr5* upregulation that is critical for TFH-positioning in the B cell follicle (49). In contrast to the well described regulation in TFH cells, KLF2-mediated regulation of *Cxcr5* in B cells remains controversial: one study described downregulation of CXCR5 mRNA and protein in KLF2-deficient MZ B cells and an upregulation in KLF2-deficient Fo B cells (22). However, two other studies were not able to confirm this regulation (16, 20). Therefore, it remains unclear whether KLF2 might be involved in the regulation of MZ B cell-shuttling between the marginal zone and the follicle, or in Fo B cell-positioning within the follicle as shown for TFH cells. Hence, resolving the role of KLF2 in MZ B-shuttling and Fo B cell migration within the follicle will require more sophisticated spatial and temporal analyses.

Genome-wide microarray RNA expression analyses in Fo B cells in two different mouse strains with a B cell-specific *Klf2* deletion (either *CD19Cre*- or *mb1Cre*-mediated) identified the surface receptors L-Selectin (CD62L) and Integrin (Itg) β7, which are important for migration and homing, as KLF2-regulated factors (16, 20) (Figure 1). While L-Selectin as a major factor of leucocyte extravasation, plays an important role in B cell migration from blood to lymph nodes, Itgβ7 is known for its specific role in mucosal lymphocyte migration. It was demonstrated by chromatin immunoprecipitation (ChIP) that KLF2 directly binds to the *Itgβ7* promoter in B cell lines (50). On protein level, loss of surface L-Selectin and surface Itgα4β7 was demonstrated in KLF2-deficient splenic Fo B cells and B cells in the blood (16, 20). Moreover, in KLF2-deficient $TACI^+/CD138^+$ IgA plasmablasts, Itgα4β7 was downregulated (37). As the Itgα4 chain was virtually absent on the surface of KLF2-deficient IgA plasmablasts, not only Itgα4β7 but also surface expression of Itgα4β1, which is critical for BM homing, is impaired (37). Besides downregulation of L-Selectin and Itgβ7, a significant reduction of *S1pr4* and an increase of *S1pr3* transcripts in KLF2-deficient Fo B cells was detected (16). While S1PR3 plays a role for MZ B cell positioning but is dispensable for lymph node motility, the function of S1PR4 in B cells is unclear (39, 51).

KLF2 directly induces Blimp1 during Th1 cell differentiation by binding to the *Prdm1* promoter (52) but it remains unclear whether Blimp1 is also controlled by KLF2 during plasma cell differentiation. Based on the findings that KLF2-deficent mice had reduced numbers of antigen-specific IgG-secreting plasma cells in the BM and that natural IgA was reduced in their serum, the effect of KLF2 deletion on plasmablast and plasma cells subsets was thoroughly assessed by our group (20, 37). Plasmablasts were defined as $CD19^+/B220^+/TACI^+/CD138^+$ cells with a high frequency of proliferating $Ki67^+$ cells, whereas plasma cells were identified as $CD19^{lo/neg}/B220^-/TACI^+/CD138^+$ which are non-proliferating (35, 37). Analysis of plasma cell compartments in B

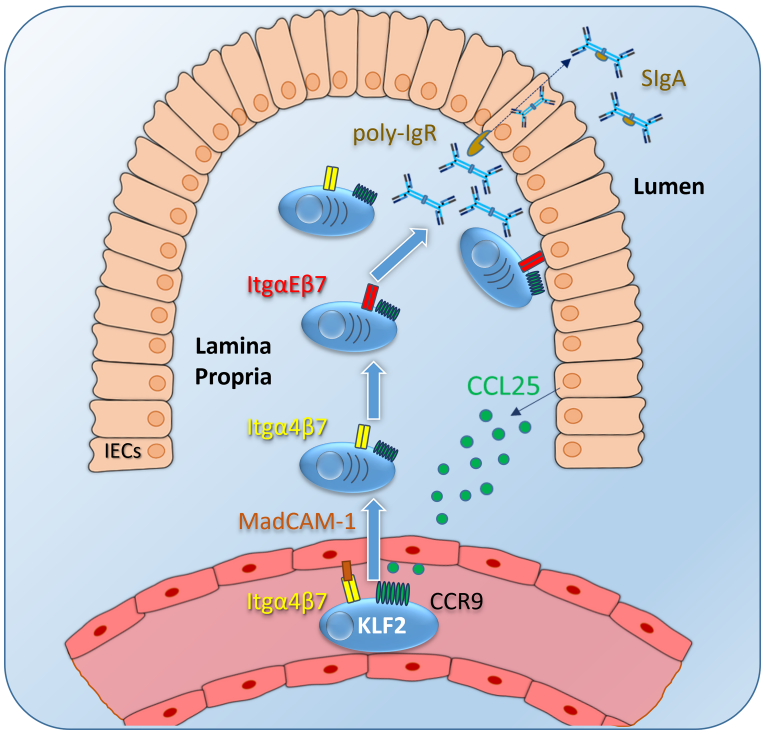

FIGURE 2
IgA plasmablast homing to the intestinal lamina propria (LP): Intestinal epithelial cells (IECs) express CCL25, which is presented on glucosamine-glycans on endothelial cells of venules as a ligand for the CCR9 receptor on IgA-expressing plasmablasts (and other immune cells). Integrin α4β7 is activated upon CCR9 signaling and binds to its ligand MadCAM-1, followed by plasmablast migration to the intestinal LP. Itgα4β7 and CCR9 expression is induced by KLF2 in IgA plasmablasts. Inside the LP, IgA plasmablasts differentiate into IgA-secreting plasma cells, a subset of those express ItgαEβ7 to localize close to the IECs. This mechanism might facilitate the binding of dimeric IgA to the poly-Ig receptor and might subsequently promote the transcytosis of dimeric IgA through the epithelial layer to the gut lumen. Itg, integrin; SIgA, secretory IgA.

cell-specific KLF2-deficient mice revealed a severe dysregulation of the compartmentalization of IgA plasmablasts and IgA plasma cells. In these mice, IgA plasmablasts and IgA plasma cells were virtually absent in the BM, reduced in the blood, the spleen and importantly, the intestinal LP. However, IgA plasmablasts and IgA plasma cells accumulated in mLN of KLF2-deficient mice (37). RNAseq as well as flow cytometric analyses of KLF2-deficient IgA plasmablasts compared to controls identified L-Selectin, Itgβ7, ItgαM, and chemokine receptor CCR9 as KLF2-regulated factors (37). Surface CCR9 on IgA plasmablasts was significantly reduced concomitant with an impaired migration towards a CCL25 gradient *in vitro* (37). Together, reductions of Itgβ7 and CCR9 expression in KLF2-deficient IgA plasmablasts led to compromised IgA responses caused by impaired migration from mLN to the LP of the small intestine and colon (37). Hence, KLF2 regulates the expression of the important gut-associated lymphoid tissue (GALT)-homing factors Itgβ7 and CCR9 (Figures 1, 2). Upon KLF2-regulated expression of CCR9 and Itgβ7, IgA plasmablasts are attracted to the LP by gradients of CCL25, the ligand of CCR9. CCL25 is secreted by e.g., intestinal epithelial cells (IEC) (53). CCL25-binding to CCR9 activates Itgα4β7 that binds to MadCAM-1 on endothelial cells and leads to the extravasation of plasmablasts to the mucosal LP (54, 55). Inside the intestinal LP, IgA plasmablasts differentiate to mature IgA plasma cells. A subset of those express ItgαEβ7 which enables them to bind to E-Cadherin on IECs, a mechanism that might promote dimeric IgA binding to the poly-IgR and facilitate transcytosis of dimeric IgA to the gut lumen [(56), Figure 2].

In addition to the regulation of Itgα4β7, the expression of the ItgαM chain was also affected in KLF2-deficient IgA plasmablasts. ItgαM is a binding partner of Itgβ2, which is important for lymph node egress of B cells (57). Moreover, ItgαM was absent on KLF2-deficient IgA plasmablasts compared to their wildtype counterparts (37). The dysregulation of ItgαM together with the aforementioned reduction of S1PR1 might be the cause for the observed accumulation of IgA plasmablasts/plasma cells in the mLN and in the remaining PP of KLF2-deficient mice (37). Hence, KLF2 might be involved in the process of lymph node exit of IgA plasmablasts presumably by regulating ItgαMβ2 and S1PR1.

In summary, KLF2 contributes to the control of the quiescent, resting state of mature B cells and pre-B cells by controlling cell cycle regulators (c-myc, p21, and p27) and immediate-early transcription factors (such as Jun, Fos, and Egr1/2), respectively. Moreover, KLF2-regulated genes are crucial for migration and homing of naïve B cells, activated B cells, and plasmablasts. KLF2-regulated gene products include integrins (Itgα4β7, Itgα4β1, and ItgαM), selectins (L-Selectin), and chemokine receptors (CCR9) as well as Sphingosin-1-phosphat-receptors (S1PR1, S1PR3, and S1PR4) in IgA plasmablasts. By regulating the expression of these factors, KLF2 controls the exit of IgA plasmablasts from the lymph node as well as their homing to the intestinal LP.

KLF2 in B cell-related diseases and malignancies

Splenic marginal zone lymphoma

In humans, splenic marginal zone lymphoma (SMZL) is a low-grade B cell lymphoma, with variable clinical course. Clinical diagnosis is rather difficult as specific phenotypic and genetic markers are lacking. In approximately one third of SMZL cases, the IgHV1-2 heavy chain that harbors few somatic mutations and a long CDR3 region is expressed (58, 59) and approximately one third of SMZL cases harbor a hemizygous deletion of chromosome 7q with a so far unsolved role in the pathogenesis of SMZL (60–62). Transcriptome and mutational analyses have revealed candidate genes that may contribute to disease onset and/or progression. Mutations were predominantly detected in the *KLF2* and the *NOTCH2* genes. KLF2 was inactivated by mutations in 42% of SMZL patients/cases (11). This is in line with findings that KLF2-deficient mice display a strong expansion of MZ B cells (16, 20, 22). Based on the mutations found in SMZL patients, expression constructs with genes encoding for different KLF2 mutant forms were generated. The effect of these KLF2 mutants on NF-κB activation was assessed in *in vitro* reporter assays in HEK293T cells and OCI-LY19 B-lymphoma cells. KLF2 mutants failed to suppress NF-κB activation in contrast to non-mutated KLF2 (11). Constitutive activation of the NF-κB signaling pathway contributes to SMZL pathogenesis by promoting MZ B cell survival and expansion (63, 64).

Multiple myeloma

The hallmark of Multiple Myeloma (MM), a malignant disease, is the expansion of plasma cells. Clinical signs include hypercalcemia, renal failure, anemia, and bone lesions. Moreover, MM is characterized by plasma cell expansion in the BM and the presence of free IgL chains, the so-called Bence Jones proteins that can be found in the serum and the urine of MM patients (65). Genetic predispositions such as mutations in the *N-RAS*, *K-RAS* or *EGR1* genes as well as translocations are primary events in the onset of MM (65, 66). Deregulation of histone methylation can also contribute to MM. In this context, the chromosomal translocation t (4,14) (p16;q32) can be found in up to 20% of MM patients. This translocation results in the overexpression of WHSC1, a histone H3 lysine 36 (H3K36) methyltransferase (67). Furthermore, the KDM3a histone demethylase that catalyzes the removal of H3K9 mono- and di-methylations, is expressed in MM lines and was shown to be essential for MM cell proliferation and survival. *KLF2* was identified as a target gene of KDM3a. KLF2 is highly expressed in MM cell lines (68). Downregulation of KLF2 resulted in an impairment of MM cell proliferation and in the induction of apoptosis. *IRF-4* was identified a KLF2-regulated gene in MM cell lines. Together, KDM3a, KLF2, and IRF-4 regulate the expression of *ITGβ7*, an essential integrin for MM homing to and adhesion in the BM (69). As aforementioned, ITGβ7 is a crucially important KLF2-regulated target gene in healthy B cells and plasma cells. Therefore, KLF2 is involved in MM cell adhesion and BM homing. Moreover, KLF2 is involved in the regulation of the angiogenic factors EGFL7 and ITGβ3 in MM cells. KLF2 expression was increased by ITGβ3 signaling which in turn led to upregulation of EGFL7, thereby enhancing MM cell expansion (70). In contrast to naïve B cells, MM cells proliferate in the presence of KLF2. As aforementioned, KLF2 in MM cells promotes their proliferation and survival. Therefore, the complex interplay of the various signaling pathways implicated in the pathogenesis of MM (i.e., the RAS/RAF/MEK/ERK, the PI3K/AKT, the JAK/STAT, and the NF-κB pathways (71) with the KLF2 signaling network in MM cells needs to be further investigated.

IgA deficiencies

As aforementioned, B cell-specific deletion of *Klf2* in the mouse resulted in a profound disturbance of the localization of IgA plasma cells concurrent with the absence of SIgA in the gut lumen and feces (37). These phenotypes are strikingly similar to those found in human IgA deficiencies (72). Loss of Itgβ7, a central player of IgA plasmablast/plasma cell homeostasis, is implicated in the human Kabuki syndrome. In a corresponding mouse model, deletion of the gene encoding for the Kmt2d histone methyltransferase led to a decrease of Itgβ7 expression, which consequently resulted in a defective homing of IgA plasmablasts to the gut (73). As *Itgβ7* is also a direct target gene of KLF2, it will be of great interest to study the effect of KLF2 loss-of-function mutations on the onset and progression of gut-related diseases, such as Ulcerative colitis and Crohn's disease.

B cell abnormalities

Recently, a novel mutation in the human *KLF2* gene was discovered that leads to the disruption of the highly conserved zinc finger domain required for the nuclear transport and DNA-binding. The patients showed lymphopenia with decreased B cell numbers, lower numbers of switched memory B cells, and reduced serum IgG1. Moreover, L-Selectin on blood B cells was downregulated. In addition, this mutation also resulted in an imbalance of various T cell subsets (74).

Future perspectives

KLF2 is a central regulator of not only B cell and plasma cell differentiation, activation, and migration, but is equivalently important in other immune cells. KLF2 alterations have been associated with a multitude of diseases, such as adipogenesis, atherosclerosis, thrombosis, asthma, arthritis (3, 4, 7–9, 12). Thus, the challenge for further studies will be the identification and characterization of the KLF2-regulated signalosome, transcriptome, and proteome in various cell types in immune responses and diseases.

Author contributions

JW and WS conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Li Wu,
Tsinghua University, China

REVIEWED BY
Yu-Jun Dai,
Sun Yat-sen University Cancer Center (SYSUCC), China
Marcus O. Muench,
Vitalant Research Institute, United States

*CORRESPONDENCE
Tim Thomas
tthomas@wehi.edu.au
Anne K. Voss
avoss@wehi.edu.au

†These authors have contributed equally to this work and share senior authorship

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The chromatin reader protein ING5 is required for normal hematopoietic cell numbers in the fetal liver

Sophia Y.Y. Mah[1,2], Hannah K. Vanyai[1,2], Yuqing Yang[1,2], Anne K. Voss[1,2*†] and Tim Thomas[1,2*†]

[1]Epigenetics and Development Division, Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia, [2]Department of Medical Biology, University of Melbourne, Melbourne, VIC, Australia

ING5 is a component of KAT6A and KAT7 histone lysine acetylation protein complexes. ING5 contains a PHD domain that binds to histone H3 lysine 4 when it is trimethylated, and so functions as a 'reader' and adaptor protein. KAT6A and KAT7 function are critical for normal hematopoiesis. To examine the function of ING5 in hematopoiesis, we generated a null allele of *Ing5*. Mice lacking ING5 during development had decreased foetal liver cellularity, decreased numbers of hematopoietic stem cells and perturbed erythropoiesis compared to wild-type control mice. *Ing5*$^{-/-}$ pups had hypoplastic spleens. Competitive transplantation experiments using foetal liver hematopoietic cells showed that there was no defect in long-term repopulating capacity of stem cells lacking ING5, suggesting that the defects during the foetal stage were not cell intrinsic. Together, these results suggest that ING5 function is dispensable for normal hematopoiesis but may be required for timely foetal hematopoiesis in a cell-extrinsic manner.

KEYWORDS

ING5, chromatin, KAT6A, KAT7, fetal, hematopoiesis

Introduction

The mammalian inhibitor of growth (ING) family consists of five proteins, ING1 to ING5, defined through sequence homology (1, 2). These proteins are chromatin 'reader' proteins as they all contain a plant homeodomain (PHD) finger (3), which is prominent in chromatin adaptor proteins (4). ING proteins are involved in the regulation of a wide range of critical cellular processes including DNA repair, apoptosis, cell cycle and epigenetic regulation of gene expression *via* association with histone acetyltransferases (HATs) or histone deacetylases (HDACs) (5, 6).

The PHD fingers of all mammalian ING proteins and yeast orthologues bind to mono-, di- or tri-methylated lysine 4 of histone H3 (H3K4me1/2/3) (7). The strongest binding occurs with H3K4me3. The affinity decreases 10-fold with the removal of successive methyl groups (8–17). This binding is specific to H3K4 methylation, as histone H3 methylated at

other lysine residues (lysine 9) or histone H4 mono-, di- or trimethylated at lysine 20 (H4K20me1/2/3) is not bound (3, 8). H3K4me3 is enriched in the promoter regions and immediately downstream of transcription start sites of active genes and is associated with gene activation (7, 18). Dysregulation of ING proteins is associated with aberrant gene expression in cancers (19–22).

ING5 has been detected in distinct protein complexes, which contain a MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) protein family enzyme subunit. The MYST proteins are a family of histone acetyltransferases with diverse roles in chromatin regulation (23–25). ING5 was found in KAT6A (formerly known as MOZ, monocytic leukaemia zinc finger gene), KAT6B (formerly MORF or Querkopf) and KAT7 (formerly HBO1) complexes (26). Both KAT6A and KAT7 have essential roles in regulating the hematopoietic system (27–30). KAT6A is essential for the formation of definitive hematopoietic stem cells (HSCs) during embryonic development (27), and the maintenance of HSC in adult bone marrow (28). The role of KAT6A in hematopoiesis is dependent on its acetyltransferase activity (31). These findings suggest that how the acetyltransferase activity is directed to chromatin is important for the function of these proteins. KAT6A is required for pre-B cell proliferation in germinal centres and for the maturation of CD8+ T cells (32–34). KAT7 also has essential roles in the hematopoietic system, and like KAT6A, KAT7 is critically important for maintenance of HSCs (29), as well as in lineage commitment, in particular erythropoiesis (35) and T cell development (36). KAT7 function is also critical in thymic epithelial cells for clonal selection of T cells in the thymus (37).

Chromosomal translocations involving the *KAT6A* gene (24, 38, 39) or the *KAT6B* gene (40–42) cause aggressive forms of acute myeloid leukaemia. KAT7 dysregulation is associated with a variety of cancers (43) and, similar to *KAT6A*, *KAT7* chromosomal translocations cause leukaemia (44). Both KAT6A and KAT7 are promising targets for anti-cancer therapy (45–47), in particular leukaemia, and a drug inhibiting the enzymatic activity of KAT6A is in clinical trials.

Since the complexes containing the MYST family proteins KAT6A and KAT7 have essential roles in hematopoiesis, and both contain the adaptor protein ING5 we undertook a study to examine the role of ING5 in hematopoiesis.

Materials and methods

Animal strains and alleles

Mice with *loxP* sites flanking exons 3 to 5 of the *Ing5* gene (*Ing5*^{fl}) were generated by OZgene Pty Ltd, Bently, Western Australia, using Bruce 4 ES cells, which are C57BL/6 derived. Removal of exons 3 to 5 was achieved by crossing the *Ing5*^{fl} mice to a *Cre*-deleter mouse strain (48), which produced a frame shift and a premature stop codon and generated the germline deleted *Ing5*$^{-}$ allele used in this study. The region of deletion (Supplementary Figure 1; Figure 1A) encodes part of the N-terminal ING domain and the nuclear localisation signal. Mice carrying the *Ing5*$^{-}$ allele were backcrossed to wild-type C57BL/6J mice for more than 8 generations. Mice were genotyped by PCR using a common forward oligonucleotide 1 (TGCTGGG ACTGTTTACAAATTAGA) together with a reverse oligonucleotide 2 (AAAGGAGTGAACAATACAGCATGA) detecting the wild-type (322 bp product) allele or a reverse oligonucleotide (ATGTACCGAATGTGGGAACTAAAT) detecting the null allele (528 bp product) in the same reaction. Quantitative reverse transcriptase PCR using a pair of oligonucleotides amplifying cDNA 3 prime of the deleted exons (forward CCAGAAGCCTGAGTGTCTCC and reverse TGCCAGTCTGTTGATGAAGC) was performed on RNA isolated from E10.5 embryos using Qiagen RNeasy Mini Kit (Qiagen 217004) followed by cDNA synthesis using Super Script III Reverse Transcriptase (Invitrogen 18080085) and RT-qPCR amplification using SYBR Hi-ROX (Bioline QT605-05). Values for RT-qPCR are displayed as arbitrary units relative to the housekeeping gene *Pgk1*.

Tissue collection

Foetal livers were isolated from E14.5 embryos, and cells were dissociated by passing through a 40 µm cell strainer (Corning 352340). Bone marrow, thymus and spleen cells were isolated from transplant recipients using standard techniques. Peripheral blood and tissue cell counts were obtained using an automated hematology analyser (ADVIA 120; Siemens).

Hematopoietic transplant experiments

All irradiated recipients were CD45.1^{+} C57BL/6 female mice aged 6-8 weeks. Mice received irradiation (2 x 5.5 Gy 3 h apart) and were given drinking water supplemented with 10 mg/ml of neomycin (Sigma N6386) for 3 weeks post-irradiation. Competitive transplantation experiments were conducted using 1x10^{6} bone marrow cells from femurs of CD45.1^{+} mice (competitor cells) combined with 0.5 x 10^{6} cells of CD45.2^{+} E14.5 foetal livers (donor). Cells were injected into the tail vein of irradiated CD45.1^{+} host mice. Three hosts were used per donor. Peripheral blood was isolated at 4 weeks and 16 weeks post-transplantation for analyses. Haematopoietic organs were recovered at 16 weeks post-transplantation.

Flow cytometry

For hematopoietic organs, equal numbers of cells were stained with primary antibodies (Supplementary Table 1) using standard procedures. Cell types were identified using cell surface markers as shown in Supplementary Table 2. Expression of CD34 and CD135 were used to identify a stem cell population, designated LT-HSCs, and progenitor cell populations, as previously published (49–51) or, alternatively, SLAM markers CD150 and CD48 (52, 53) were used to identify a stem cell population designated HSCs and progenitor

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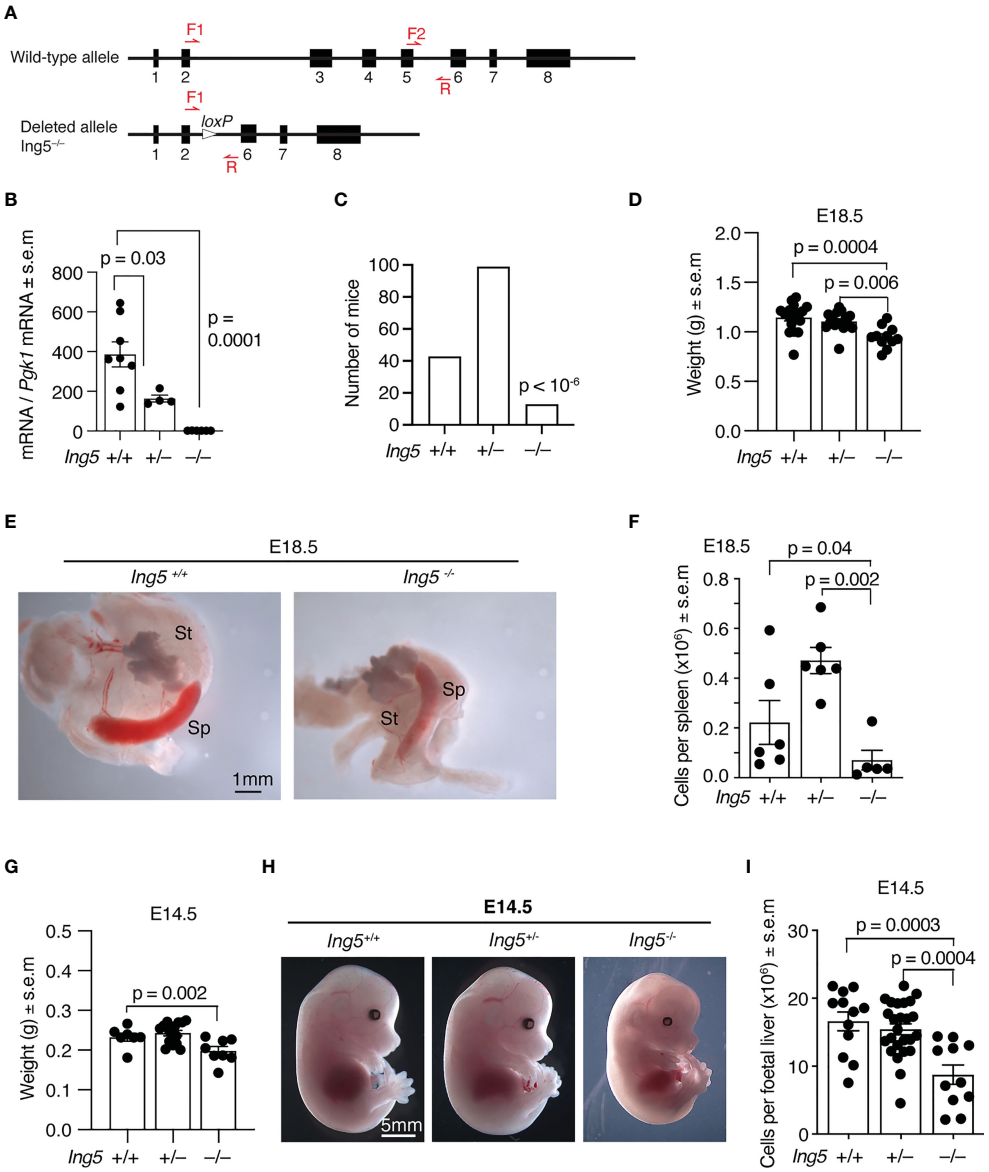

FIGURE 1
Gross phenotypic effects of loss of ING5. **(A)** The wild-type and germline null allele of the *Ing5* gene used in this study. Exons are numbered. Red arrows indicate position of oligonucleotide primers used for genotyping. *LoxP*, Cre-recombinase target sequence. Details of the targeting strategy and the intermediate conditional allele are displayed in Supplementary Figure 1. **(B)** Levels of *Ing5* mRNA quantified by RT-qPCR in whole E10.5 $Ing5^{-/-}$, $Ing5^{+/-}$ and $Ing5^{+/+}$ embryos. N = 6 $Ing5^{-/-}$, 4 $Ing5^{+/-}$ and 8 $Ing5^{+/+}$ embryos. **(C)** Numbers of $Ing5^{-/-}$, $Ing5^{+/-}$ and $Ing5^{+/+}$ mice generated by mating $Ing5^{+/-}$ x $Ing5^{+/-}$ mice at 3 weeks of age (weaning). A total of 155 mice were genotyped. $Ing5^{-/-}$ were underrepresented at weaning ($p < 10^{-6}$). **(D)** Body weight of E18.5 foetuses. N= 11 $Ing5^{-/-}$, 15 $Ing5^{+/-}$ and 17 $Ing5^{+/+}$ foetuses. **(E)** Representative images of $Ing5^{-/-}$ and $Ing5^{+/+}$ E18.5 spleens. St, stomach; Sp, spleen. **(F)** Quantification of the number of cells in the spleen at E18.5 using an automated hematology analyser. **(G)** Body weights of E14.5 foetuses. N = 8 $Ing5^{-/-}$, 16 $Ing5^{+/-}$ and 7 $Ing5^{+/+}$ foetuses. **(H)** Representative images of $Ing5^{-/-}$, $Ing5^{+/-}$and $Ing5^{+/+}$ foetuses at E14.5. **(I)** Foetal liver cellularity at E14.5. The number of nucleated hematopoietic cells was quantified using an automated hematology analyser. N = 11 $Ing5^{-/-}$, 25 $Ing5^{+/-}$ and 12 $Ing5^{+/+}$ foetuses. Each dot represents one foetal liver. Data are displayed as mean ± s.e.m. and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test **(B, D, F,G, I)**, or are displayed as absolute numbers and were analysed by cumulative binomial probability analysis **(C)**. Each dot represents one animal **(B, D, F, G, I)**.

cell populations. After excess secondary antibodies were removed by washing with FACS buffer, all pelleted cells were resuspended in Fluoro-Gold viability dye (8 µg/ml; Sigma 39286). Samples were then analysed on flow cytometry analysers, LSRIIW, LSRIIC, or Fortessa1 (BD Biosciences). Data were analysed using FlowJo v10.4 (Treestar). Representative gating strategies are displayed in Supplementary Figures 2-7.

Statistical analysis

Data are presented as means ± s.e.m. and were analysed using a graphing and statistics software (Prism 9 version 9.4.1, GraphPad; or R version 4.2.2, The R Foundation for Statistical Computing Platform). The specific statistical tests used, and number of observations are stated in the figure legends.

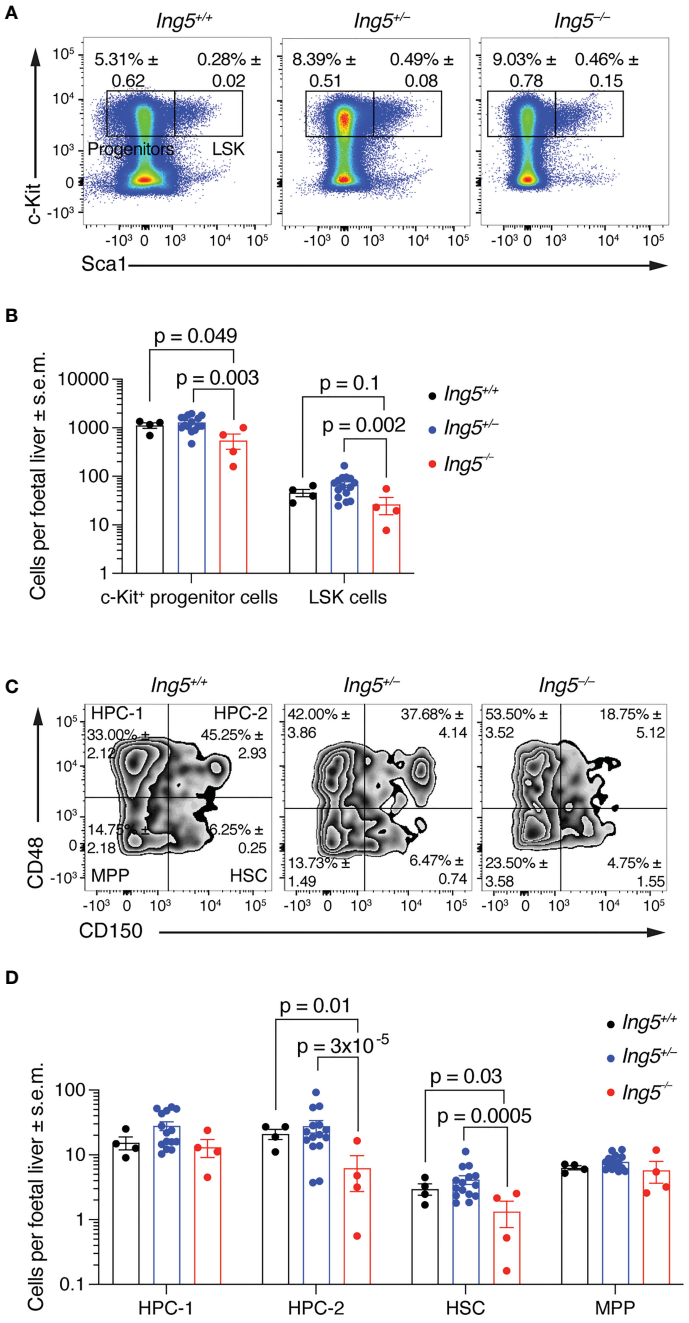

FIGURE 2
Loss of ING5 causes a reduction in HSC and progenitor numbers. E14.5 foetal liver hematopoietic cell suspensions were analysed for HSC and progenitors within the LSK compartment using SLAM markers CD48 and CD150. Gating strategy as shown in Supplementary Figure 2.
(A) Representative flow cytometry plots for each genotype gated on live cells. Numbers within plots are average percentage ± s.e.m. of total live cells.
(B) Bar graph showing the number of LSK and c-Kit$^+$ progenitor cells per foetal liver. **(C)** Representative flow cytometry plots for identification of stem and progenitor cells using CD150 and CD48 gated on the LSK compartment. Numbers within plots are average percentage ± s.e.m. of total LSK cells.
(D) Bar graph showing the number of stem and progenitor populations per foetal liver. N = 4 *Ing5*$^{-/-}$, 15 *Ing5*$^{+/-}$ and 4 *Ing5*$^{+/+}$ foetuses. Data are displayed as mean ± s.e.m. and were analysed after log transformation by two-way ANOVA with genotype and cell type as the independent factors followed by Šídák's multiple comparisons test. Each dot represents data from one animal. HPC-1, hematopoietic progenitor cells 1; HPC-2, hematopoietic progenitor cells 2; HSC, hematopoietic stem cells; MPP, multipotent progenitor cells. Detailed gating strategy in Supplementary Figure 2. Supplementary Table 1 displays antibodies used, and Supplementary Table 2 cell surface markers used for various hematopoietic cell populations.

Results

To examine the role of ING5 in hematopoiesis we generated a mouse strain in which exons 3, 4 and 5 were deleted in the germline (Supplementary Figure 1; Figure 1A). Exons 3 to 5 encode part of the conserved N-terminal ING domain and the nuclear localisation signal. Splicing around exons 3 to 5 results in a frameshift obliterating the PHD finger. Therefore, no functional protein is produced from the mutated locus. Quantitative reverse transcriptase PCR was used to assess *Ing5* mRNA levels in whole

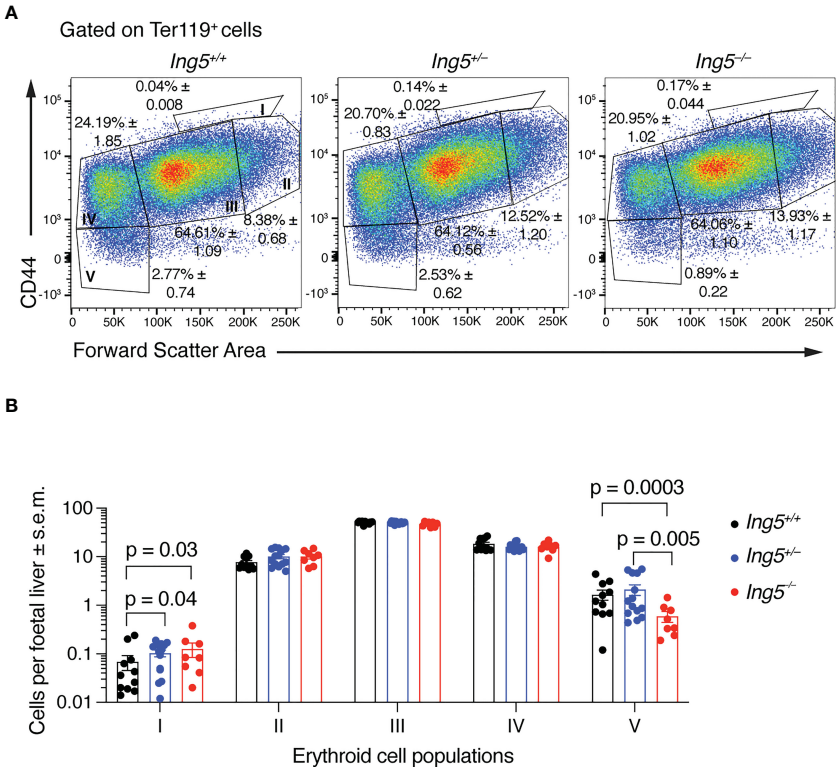

FIGURE 3
Loss of ING5 results in an increase in proerythroblasts and a decrease in the mature erythrocyte population. **(A)** Representative flow cytometry plots for each genotype, gated on Ter-119+ cells. Population I, proerythroblasts; II, basophilic erythroblasts; III, polychromatic erythroblasts; IV, orthochromatic erythroblasts; IV, orthochromatic erythroblasts and reticulocytes; V, mature red blood cells. Numbers within plots are average percentage ± s.e.m. of the total Mac-1^{neg} Gr-1^{neg} CD45.2^{neg} Ter-119^{+} cells. **(B)** Bar graphs of the number cells in each sub-population of erythroblasts per foetal liver. N = 8 *Ing5*$^{-/-}$, 14 *Ing5*$^{+/-}$ and 11 *Ing5*$^{+/+}$ foetuses. Data are displayed as mean ± s.e.m. and were analysed after log transformation by two-way ANOVA with genotype and cell type as the independent factors followed by Šídák's multiple comparisons test. Each dot represents number of cells of one animal. Gating strategy in Supplementary Figure 3.

E10.5 embryos from heterozygous intercross matings. No *Ing5* RNA was detected in *Ing5*$^{-/-}$ embryos using primers that amplify mRNA encoded 3' of the genomic deletion, showing that the mutation targets the *Ing5* locus and that any splicing out of frame results in degradation of the out-of-frame mRNA (Figure 1B).

Using this allele on an inbred C57BL/6 background, we studied the effect of loss of ING5 by generating homozygous embryos from heterozygous intercross matings. Only a third of the number of *Ing5*$^{-/-}$ mice expected were present at weaning ($p < 10^{-6}$, Figure 1C) with some mice dying soon after birth. At E18.5 before birth, *Ing5*$^{-/-}$ foetuses were externally morphologically normal, although smaller than wild-type and *Ing5*$^{+/-}$ foetuses (p = 0.0004 and 0.006; Figure 1D). Noteworthy was a reduction in spleen cellularity in E18.5 *Ing5*$^{-/-}$ foetuses compared to wild-type and *Ing5*$^{+/-}$ littermate controls (p = 0.04 and 0.002; Figures 1E, F), which resembled, but was not as significant as the loss of spleen cellularity in *Kat6a*$^{-/-}$ (*Moz*$^{-/-}$) foetuses (27). A moderate growth retardation was visible at E14.5 in *Ing5*$^{-/-}$ foetuses compared to wild-type littermate controls (p = 0.002; Figures 1G, H). A reduction in foetal liver cellularity to 53% of wild-type was apparent in E14.5 *Ing5*$^{-/-}$ foetuses (p = 0.0003; Figure 1I). This reduction in foetal liver cells was still apparent when the moderate growth retardation was taken into consideration (62% of wild-type; p = 0.005; data not shown).

To determine the role of ING5 in foetal liver hematopoiesis, we examined the hematopoietic stem cell compartment at E14.5 using flow cytometry (Supplementary Figure 2). The number of lineage negative (Lin^{neg}) c-Kit positive (c-Kit^{+}) progenitor cells per foetal liver was significantly reduced in *Ing5*$^{-/-}$ foetuses compared to wild-type and *Ing5*$^{+/-}$ littermate controls (p = 0.049 and 0.003; Figures 2A, B). *Ing5*$^{-/-}$ foetuses also showed a tendency of a reduction in Lin^{neg}, c-Kit^{+}, Sca-1^{+} (LSK) cells compared to wild-type and *Ing5*$^{+/-}$ littermate controls (p = 0.1 and 0.002; Figures 2A, B). Examining the stem cell compartment further by subdividing the LSK population based on the expression of CD48 and CD150 (52, 53), we found a significant decrease in the hematopoietic progenitor cell 2 (HPC-2) population (p = 0.01 and $3x10^{-5}$; HPC-2; Figures 2C, D) and the HSC populations in *Ing5*$^{-/-}$ foetuses compared to wild-type and *Ing5*$^{+/-}$ littermate controls (p = 0.03 and 0.005; Figures 2C, D).

Together, the results presented in the previous section suggested that the principal effect of loss of ING5 was on the stem and progenitor cell populations. In E14.5 foetal livers, the majority of progenitors are required to generate definitive erythroid cells in large numbers to support the growth of the embryo. Therefore, we examined erythropoiesis using flow cytometry (54) (Supplementary Figure 3). This analysis showed that there was an accumulation of proerythroblasts (population I) in *Ing5*$^{-/-}$ and *Ing5*$^{+/-}$ foetuses compared to wild-type littermate controls ($p = 0.03$ and 0.04; Figures 3A, B) but not in basophilic (II), polychromatic (III) or orthochromatic erythroblasts and reticulocytes (IV; Figures 3A, B). Interestingly, *Ing5*$^{-/-}$ foetuses displayed a significant reduction in mature erythrocytes compared to wild-type and *Ing5*$^{+/-}$ littermate controls ($p = 0.0003$ and 0.005; Figures 3A, B). Therefore, loss of ING5 resulted in a reduction in progression through the proerythroblasts stage of erythropoiesis and a reduction in mature erythrocytes at E14.5, which were mild enough to allow development of the *Ing5*$^{-/-}$ foetuses to term.

To further examine the function of ING5 in hematopoiesis we performed competitive foetal liver hematopoietic cell transplants. We transplanted 500,000 E14.5 foetal liver test cells mixed with 1,000,000 adult bone marrow competitor cells. A minimum of three individual foetal livers (biological replicates) for each genotype were transplanted, each into three recipients (technical replicates) making a total of at least nine transplants for each genotype studied. The technical replicates for each biological replicate were averaged. The foetal liver cells had a CD45.2 cell surface phenotype and both the recipient and competitor cells had a CD45.1 cell surface phenotype (Figure 4A). Examination of the peripheral blood (Supplementary Figure 4) at 4 weeks post-transplantation (Figures 4B, C) or at 16 weeks post-transplantation (Figures 4D, E) showed no significant differences in the number of white blood cells or the proportion of leukocyte populations, apart from a slight increase in CD4+ T cells in the *Ing*$^{+/-}$ sample compared to the *Ing5*$^{-/-}$ (Figure 4E).

After 16 weeks, we examined the stem cell compartment in the bone marrow of the transplanted mice studied in Figure 4, using the flow cytometric gating strategy shown in Supplementary Figure 5. Bone marrow cellularity was not significantly different between genotypes (Figure 5A). No significant differences between genotypes were seen in stem or early progenitor cell populations generated by the transplanted cells (Figures 5B-D) identified using two methods, namely by subdividing LSK cells based on CD135 and CD34 expression (Figures 5B, C; detailed gating strategy in

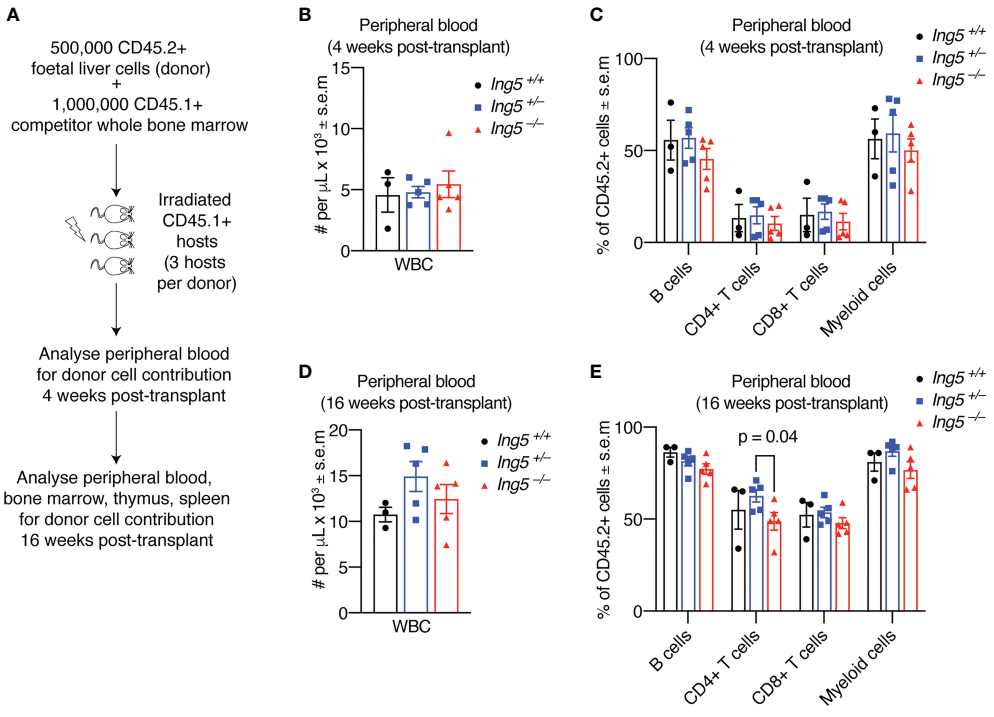

FIGURE 4
Loss of ING5 does not have a major effect on the contribution of donor cells to mature cell types in the peripheral blood after short-term or long-term reconstitution. Analysis of peripheral blood by flow cytometry 4 weeks and 16 weeks after competitive foetal liver cell transplantation. **(A)** Protocol for competitive transplantation of foetal liver cells into irradiated recipient mice and subsequent analyses. **(B)** Quantification of nucleated white blood cells in the peripheral blood using an automated hematology analyser 4 weeks after transplantation. **(C)** Proportion of CD45.2$^+$ donor cells among mature cell types in the peripheral blood 4 weeks post-transplantation. **(D)** Quantification of nucleated white blood cells in the peripheral blood using an automated hematology 16 weeks after transplantation. **(E)** Proportion of CD45.2$^+$ donor cells among mature cell types in the peripheral blood at 16 weeks post-transplantation. N = 5 *Ing5*$^{-/-}$, 5 *Ing5*$^{+/-}$ and 3 *Ing5*$^{+/+}$ foetal liver samples, transplanted into 3 recipients each. Each dot represents the average of three recipient mice that received cells from the same donor. Data are displayed as mean ± s.e.m. and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test. Gating strategy in Supplementary Figure 4.

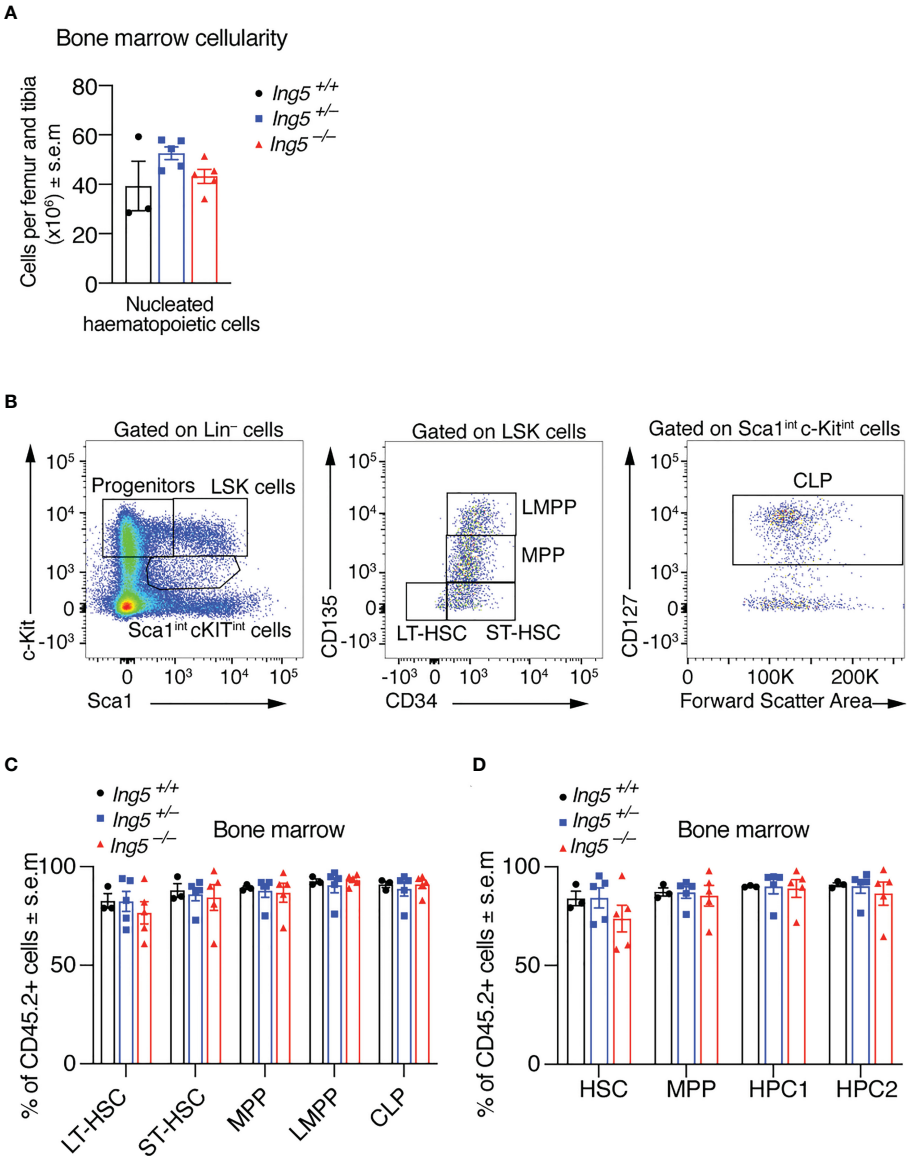

FIGURE 5
Loss of ING5 does affect donor cell contribution to the production of HSCs and progenitor cells in the bone marrow after competitive foetal liver cell transplantation. Analysis of the bone marrow 16 weeks after competitive foetal liver cell transplantation. **(A)** Quantification of nucleated hematopoietic cells in the bone marrow of recipients using an automated hematology analyser. **(B)** Gating strategy to identify stem and progenitor subsets using CD135, CD34 and CD127. **(C)** Proportion of CD45.2$^+$ donor cell contribution to each major subtype of HSC and progenitor cells identified using CD34, CD135 and CD127. **(D)** Proportion of CD45.2$^+$ donor cell contribution to each major subtype of HSC and progenitor cells identified using CD48 and CD150. N = 5 *Ing5*$^{-/-}$, 5 *Ing5*$^{+/-}$ and 3 *Ing5*$^{+/+}$ foetal liver samples in **(A, C, D)**. Data are displayed as mean ± s.e.m. and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test. Each dot represents the average of three recipients that received cells from the same donor. Detailed gating strategy in Supplementary Figures 2; 5.

Supplementary Figure 5) or CD150 and CD48 expression (Figure 5D; gating strategy in Supplementary Figure 5). Similarly, no significant differences in the contribution of donor cells of different genotypes to common lymphoid progenitors (CLPs) were detected (Figures 5B, C). The transplanted cells were distinguished from host or competitor cells by the CD45.2 cell surface phenotype. No major significant differences in the B cell linage were observed between recipients receiving *Ing5*$^{-/-}$ or *Ing5*$^{+/+}$ littermate control foetal liver cells when bone marrow and spleen were examined (Figures 6A-E; Supplementary Figure 6). A slight difference was seen between the proportion of T2 and marginal zone B cells in the spleen between *Ing5*$^{-/-}$ and *Ing5*$^{+/-}$, but *Ing5*$^{-/-}$ and *Ing5*$^{+/+}$ donor cells were not significantly different (Figure 6E). Similarly, no significant differences in the T cell linage were observed between recipients receiving *Ing5*$^{-/-}$ or *Ing5*$^{+/+}$ littermate control foetal liver cells when the thymus was examined (Figures 6F-H; Supplementary Figure 7).

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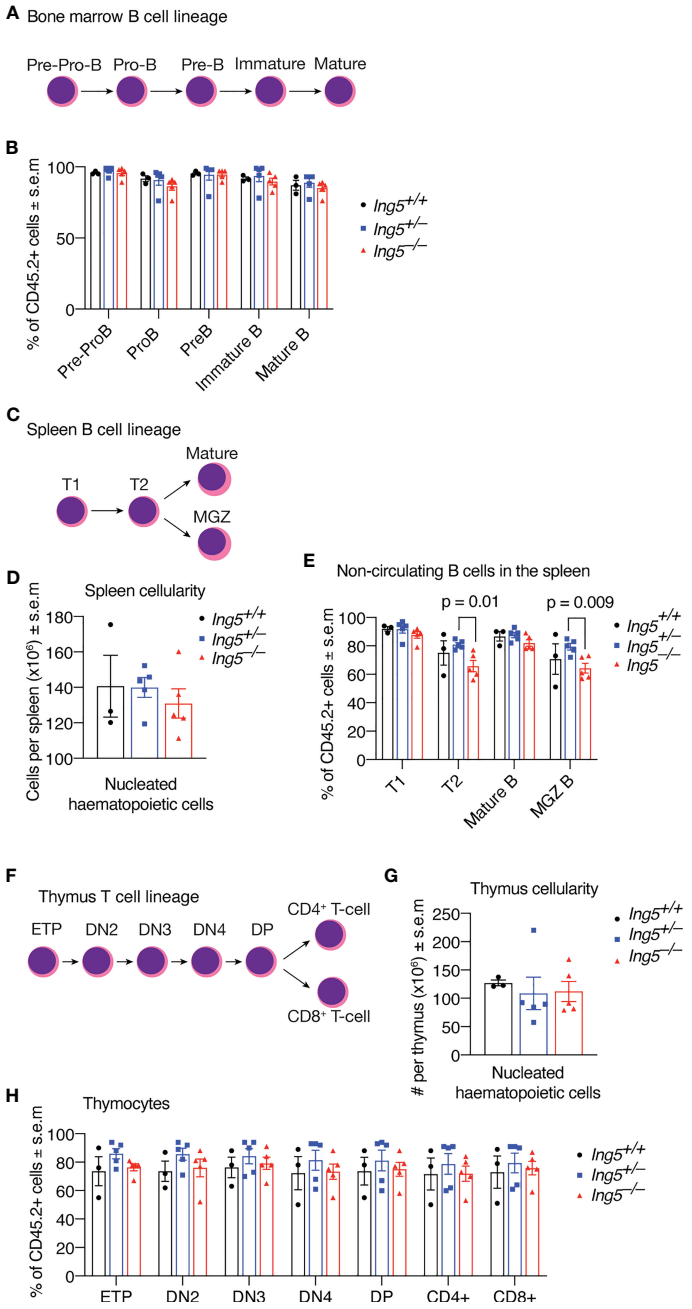

FIGURE 6
Loss of ING5 does not have a major effect on donor cell contribution to lymphoid development in bone marrow, spleen and thymus after competitive foetal liver cell transplantation. Analysis of bone marrow, spleen and thymus cells by flow cytometry 16 weeks after competitive foetal liver cell transplantation. **(A)** Major subtypes of B cell progenitors in the bone marrow. **(B)** Proportion of CD45.2$^+$ donor cell contribution to each major subtype of B cell progenitors and mature B cells in the bone marrow. **(C)** Major subtypes of B cells in the spleen. **(D)** Quantification of nucleated hematopoietic cells in the spleen. **(E)** Proportion of CD45.2$^+$ donor cell contribution to each major subtype of B cell progenitors and mature B cells in the spleen. **(F)** Major subtypes of T cells in the thymus. **(G)** Quantification of nucleated hematopoietic cells in the thymus. **(H)** Proportion of CD45.2$^+$ donor cells contribution to each major subtype of T cells progenitors and mature cell types. N = 5 *Ing5*$^{-/-}$, 5 *Ing5*$^{+/-}$ and 3 *Ing5*$^{+/+}$ foetal liver samples transplanted into 3 recipients each. Each dot represents the average of the three recipients that received cells from the same donor. Data are displayed as mean ± s.e.m. and were analysed by one-way ANOVA followed by Tukey's multiple comparisons test. MGZ B cells: marginal zone B cells; T1, transitional zone 1 B cells; T2, transitional zone 2 B cells. ETP, early thymic progenitors; DN2-4, double negative (CD4^{neg} CD8^{neg}) cell stage 2-4; DP, double positive for CD4 and CD8.

Overall these results show that while there are significant differences in the cellularity of the foetal liver and foetal stem and progenitor cells, these differences are not cell-intrinsic to the long-term repopulating stem cells.

Discussion

In this study we observed that about two thirds of the *Ing5*$^{-/-}$ mice died between birth and weaning, presumably accounting for

the deaths observed soon after birth. The surviving *Ing5*$^{-/-}$ mice were normal and fertile. We observed some abnormalities in hematopoiesis in *Ing5*$^{-/-}$ foetuses. Since ING5 is a member of the KAT6A and KAT7 chromatin regulatory complexes, this suggested that, like KAT6A and KAT7, ING5 may have a function in the hematopoietic stem cell compartment. At E18.5 the spleens of *Ing5*$^{-/-}$ foetuses were severely cytopenic. The spleen, a site of erythropoiesis during foetal development, is populated by HSCs coming from the foetal liver (55). This phenotype is similar to, although not as severe as the phenotype of foetuses lacking the histone acetyltransferase KAT6A (MOZ) (27). Similar to *Kat6a* heterozygous foetuses (27), foetuses lacking ING5 showed a reduction in the numbers of CD48^{+} CD150^{+} HSCs.

During development, the rapidly growing foetus requires the production of a large number of red blood cells. Examining erythropoiesis, we found an increase in the most immature erythroid progenitor cells and a reduction in the number of mature red blood cells. These findings are similar to the effect of a conditional deletion of *Kat7* or *Kat6a* on erythropoiesis in midgestation embryos (27, 29). Loss of either KAT7 or KAT6A causes a delay in erythropoiesis maturation resulting in the accumulation of early erythroid progenitors that does not prevent differentiation of mature definitive red blood cells (27, 29). In addition, disrupted erythropoiesis was also observed after shRNA knockdown of *Kat7* (*Hbo1* (35);.

In contrast to loss of KAT6A or KAT7 function, loss of ING5 function does not affect the ability of ING5 deficient cells to repopulate the hematopoietic system of a lethally irradiated recipient mouse. This suggests that, unlike the effects of loss of KAT6A or KAT7, the effects of loss of ING5 are not cell autonomous but affect the interaction of hematopoietic cells with surrounding cells, particularly in the foetal spleen. Alternatively, other aspects of foetal development affecting hematopoietic cells may affect foetal liver hematopoiesis, which might include a developmental delay.

The results described in this paper suggest that the ING5 adaptor protein is not required for many of the essential, cell-intrinsic functions of either KAT6A or KAT7 in the hematopoietic system. KAT6A and KAT7 are the enzyme subunits of their respective complexes, which, in addition to ING5, contain BRPF1-3 (KAT6A and KAT7) (26, 35, 56, 57) or JADE1-3 (KAT7) (26, 58). The BRPF and JADE family proteins are also adaptor proteins containing bromo and PHD domains and so direct their respective complexes to chromatin (59). Indeed, alternative splice variants of JADE may lack the ING5 binding domain, resulting in a KAT7 complex which does not contain ING5 and has a changed histone lysine specificity (11, 58). This suggests that ING5 has a specific role in modulating the activity of chromatin regulatory complexes in which it is found. KAT7 has a global function in regulating H3K14ac (46, 60–62), but under different conditions has been shown to acetylate H4 (26). KAT6A has a function in regulating H3K23ac (63) and H3K9ac at specific loci (64–67). H3K14ac and H3K23ac are the two most abundant histone acetylation modifications (68), suggesting that the complexes generating the modifications are present throughout the genome (62). However, there is an enrichment of at least H3K14ac and KAT7 at transcription start sites (62, 69). Transcription start sites are also enriched for H3K4me3 (7, 18), the modification bound by ING5 (3, 8). It is possible that the function of ING5 is related to the enrichment of these complexes at transcription start sites rather than at all genomic locations where KAT6A and KAT7 are found.

ING5 has an identical domain structure to ING4 and these domains are highly conserved (2). ING4 is an alternative subunit for the KAT7 complex (26) and has also been pulled-down in the KAT6A complex (70), suggesting that there might be a degree of redundancy between ING4 and ING5 in the KAT6A complex as well as in the KAT7 complex.

In conclusion we have shown that ING5 has a function in the hematopoietic system during foetal development. However, the loss of ING5 does not cause severe defects of the same magnitude as the complete loss of the function of the protein complexes in which it is found.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Walter and Eliza Hall Institute Ethics Committee.

Author contributions

Contribution: SM, YY, HV conducted experiments and analysed data. TT and AV wrote the manuscript. TT and AV conceived and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1119750/full#supplementary-material

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Stephen Robert Daley,
Queensland University of Technology,
Australia

REVIEWED BY
Arístides López-Márquez,
Sant Joan de Déu Research Institute
(IRSJD), Spain
Brian Laidlaw,
Washington University in St. Louis,
United States

*CORRESPONDENCE
Jacob T. Jackson
jackson@wehi.edu.au

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The Haematopoietically-expressed homeobox transcription factor: roles in development, physiology and disease

Jacob T. Jackson[1]*, Stephen L. Nutt[1,2] and Matthew P. McCormack[3,4]

[1]Immunology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia, [2]Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia, [3]The Australian Centre for Blood Diseases, Monash University, Melbourne, VIC, Australia, [4]iCamuno Biotherapeutics, Melbourne, VIC, Australia

The Haematopoietically expressed homeobox transcription factor (Hhex) is a transcriptional repressor that is of fundamental importance across species, as evident by its evolutionary conservation spanning fish, amphibians, birds, mice and humans. Indeed, Hhex maintains its vital functions throughout the lifespan of the organism, beginning in the oocyte, through fundamental stages of embryogenesis in the foregut endoderm. The endodermal development driven by Hhex gives rise to endocrine organs such as the pancreas in a process which is likely linked to its role as a risk factor in diabetes and pancreatic disorders. Hhex is also required for the normal development of the bile duct and liver, the latter also importantly being the initial site of haematopoiesis. These haematopoietic origins are governed by Hhex, leading to its crucial later roles in definitive haematopoietic stem cell (HSC) self-renewal, lymphopoiesis and haematological malignancy. Hhex is also necessary for the developing forebrain and thyroid gland, with this reliance on Hhex evident in its role in endocrine disorders later in life including a potential role in Alzheimer's disease. Thus, the roles of Hhex in embryological development throughout evolution appear to be linked to its later roles in a variety of disease processes.

KEYWORDS

transcription factor, Hhex, diabetes, haematopoiesis, leakamia, AML, T-ALL

1 Background

The Haematopoietically expressed homeobox gene (Hhex), also known as Hex, Xhex in *Xenopus* and Prh (proline rich homeodomain), was first identified in chicken haematopoietic cells, as well as cells of the liver and lungs, with homologues noted in chickens, Xenopus, mice and humans (1–5). Hhex is a non-clustered/divergent/orphan

homeobox gene, members of which are distinct from the clustered (Hox) homeobox genes, in that they are spread throughout the genome. The genomic structure of human Hhex was shown to comprise 4 exons (Figure 1) located on chromosome 10 whilst in mice Hhex is located on chromosome 19 (2, 5). The Jayaraman laboratory first showed a role for Hhex in haematopoiesis using chicken cells at a similar developmental state to that of megakaryocytic-erythroid progenitors (MEPs). Transformation of these cells by Hhex, specifically the myeloblasts, induced them to proliferate *in vitro* (6). Early analysis of haematopoietic cell lines at various stages of differentiation quickly revealed Hhex was weakly expressed in T cells and plasma cells, but abundant in developing B cells (7). Hhex is also found in myeloid and osteoclastic progenitors along with MEPs, but downregulated during differentiation (7).

The first study of Hhex noted the DNA binding capacity of the homeodomain and thus its potential for transcriptional regulation (1). Depending on the context and cofactor interactions, Hhex can act as a transcriptional repressor or activator (8, 9). The activation domain of Hhex, regardless of cell type, was determined to be at the carboxy-terminus (10), while the N-terminus of Hhex may be responsible for inhibiting DNA binding by the homeodomain and that may also enable Hhex to form oligomers within the nucleus to mediate its function as discussed further below (Figure 1) (11, 12).

As a key regulator of development and haematopoiesis, expression of Hhex must be tightly controlled, and its role as a repressor is key to its utility in haematopoietic stem and progenitor cells. In a study employing murine haematopoietic cell lines it was shown that Hhex was regulated by an element in its first intron (13). This haematopoietic-specific enhancer is bound by GATA-1, GATA-2 and c-Myb (13, 14). Hhex was also identified as a GATA-binding partner in human endothelial cells where its expression is induced by transforming growth factor (TGF)-β1 with Hhex then driving Flk-1 expression and downregulating vascular endothelial growth factor (VEGF) signalling (15).

Following translation, Hhex protein is regulated by and interacts with a number of proteins in undertaking its functions. In humans, oligomers of Hhex, in the form of octamers, have been shown to bind with high affinity to numerous locations within the promoter of *Goosecoid* and the DNA is wrapped by Hhex binding to promote transcriptional repression (16). These oligomeric forms of Hhex are highly stable, resisting both chemical and thermal denaturing (17, 18). Hhex also regulates the retention of Groucho/Transducin-like enhancer protein (TLE) proteins in the nucleus *via* direct binding, and this Hhex/TLE interaction is important for transcriptional repression (Figure 1) (19).

It also was reported that Hhex bound Jun *via* helix III of the Hhex homeodomain implying a role of Hhex in cytokine/growth factor signalling (20). In a haematopoietic cell line, K562, the N-terminal proline-rich domain of Hhex was observed to interact with the proteasome, specifically the HC8 subunit within the 20S and 26S proteasomes (21). Whilst Hhex was cleaved slowly by the proteasome, this process was not required for the transcriptional repression mediated by Hhex (21). Truncated forms of Hhex, formed subsequent to the proteolysis process, were still able to bind DNA (21). Hhex can be phosphorylated by the β subunit of CK2 at residues S163 and S177, an event that inhibits DNA binding by Hhex, which in turn is reversible by dephosphorylation (22). In human U937 cells, Hhex was reported as a potential negative regulator of eukaryotic translation initiation factor 4E (eIF4E) in myeloid cells (23). In this context, Hhex was thought to regulate cellular translation by inhibiting eIF4E-dependent Cyclin D1 mRNA transport (23). HOXA9 was required for eIF4E function, which in turn competes with Hhex as a functional repressor of eIF4E, and if dysregulated can lead to leukemogenesis (24). Moreover, eIF4E-dependent nuclear export of Cyclin D1 and ornithine decarboxylase mRNAs is stimulated by HOXA9 (24).

Together, these results clearly demonstrate Hhex regulates and is regulated by diverse intracellular processes depending on the

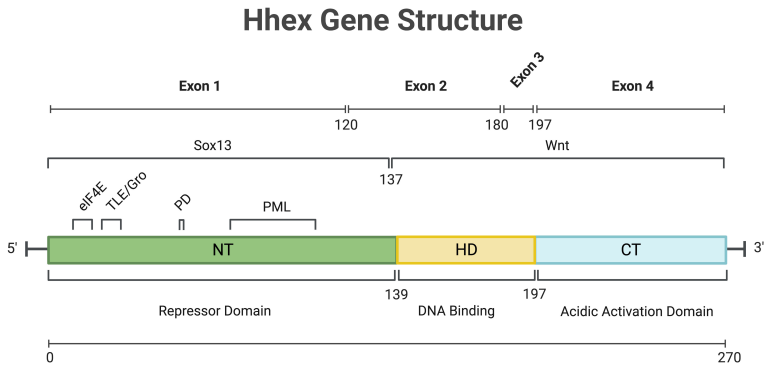

FIGURE 1
Overview of Hhex gene structure in humans. Numbers indicate amino acid position. C-Terminal (CT), eukaryotic Initiation Factor-4A (eIF4a), Homeo-Domain (HD), N-Terminal (NT), Phosphorylation Domain (PD), Promyelocytic Leukaemia protein (PML), SRY-Box Transcription Factor 13 (Sox13), Transducin-Like Enchancer/Groucho (TLE/Gro), Wingless/Integrated Signalling domain (Wnt). Gene and protein sequence information was obtained from NCBI (NM_002729.5 and NP_002720.1 respectively). Created with BioRender.com.

cellular context and warrant further research to fully understand the post-translational roles of Hhex in diverse cell types.

2 Role of Hhex in embryogenesis

Hhex plays a fundamental role in embryogenesis in many organisms throughout evolution including in fish, amphibians, birds, mice and humans, demonstrating its highly evolutionarily conserved role in vertebrate development, which is also strikingly revealed by amino acid sequence alignment, particularly with regards to the homeodomain (Figure 2).

2.1 Humans

Hhex expression was detected in a human cDNA library of oocytes and embryos (up to 10 weeks old) along with other Hox family genes including HOXD8, HOXD1 and OCT1, as well as HOXA7 exclusively in oocytes (25). In human ESCs and inducible pluripotent stem cells (iPSCs), Hhex overexpression was able to induce hepatoblasts (26). The same group also showed that Hhex was driving hepatogenesis through repression of eomesdermin (EOMES) expression (Figure 3) (27). Moreover, in human iPSCs, Hhex, and its closely related orphan homeobox gene Hlx, enhance early-phase reprogramming, whilst blocking pluripotency in somatic cells (28). Hhex expression was also found to be restricted by Sonic Hedgehog (Shh) activity in a human ES model of pancreatic development where Hhex was one of several epithelium markers, along with HNFα, Pax6 and PTF1α to be downregulated by Shh (Figure 3) (29). In a recent study, Hhex was demonstrated to be a "gatekeeper" of pancreatic development in human IPSCs, with its deletion resulting in liver and duodenum development (30). This commitment to pancreatic development driven by Hhex was observed in combination with other transcription factors including FOXA1, FOXA2 and GATA4 (30). Additionally, inhibition of all-trans retinoic acid was also noted to downregulate HHEX in a pancreatic endoderm model using hESCs (31). Whilst there is a clear importance of Hhex demonstrated in human embryological development, much of what we understand regarding its key developmental roles has nevertheless been extensively gleaned from murine studies as detailed below.

2.2 Mice

Hhex was initially observed as exhibiting endodermal expression, marking developing liver and foregut, as well as mesodermal expression with nascent blood islands in the visceral yolk sac of murine embryos (32). Further characterisation of the murine embryo by *in situ* hybridisation revealed Hhex was expressed in the chorion of the ectoplacental cavity and weakly in the visceral endoderm of the future yolk sac at E7.5, in liver and thyroid tissues only at E9.5 and in the foetal liver, lung and thyroid at E12.5-15.5 (33). Hhex was further shown to be essential for liver formation in the murine embryo at day E9.5 (33). As well as being important for thyroid and lung, Hhex Knock Out (KO) mice revealed that Hhex was involved in the hepatic ectoderm, as well as a role in monopoiesis, with embryonic lethality ultimately resulting by E10.5 (33). Bogue, et al. showed Hhex plays a role in murine foregut organogenesis including the thymus, where Hhex is downregulated by E18.5 (34). Whilst the specification of thyroid

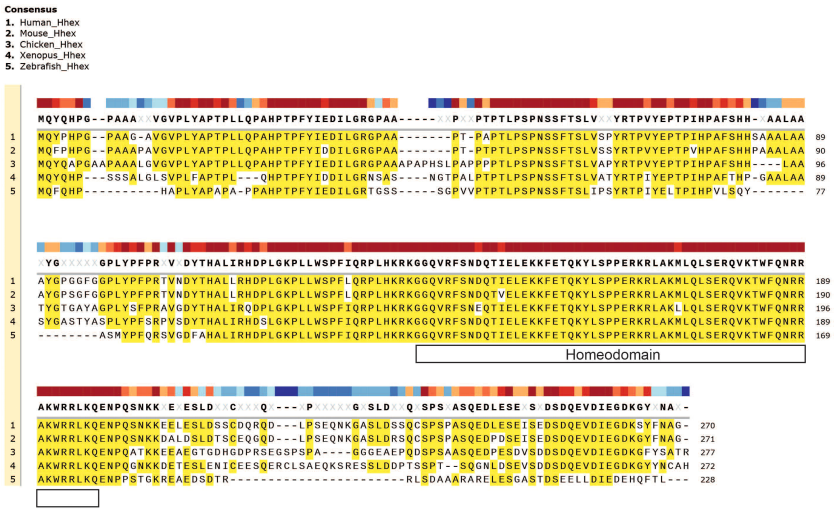

FIGURE 2
Evolutionary conservation of Hhex between divergent species. Gene amino acid sequence alignment of Hhex between species including Human, Mouse, Chicken, Xenopus and Zebrafish. Amino acid alignment highlighted in yellow. The topmost, colour-coded bar indicates the degree of conservation between species. Gene sequences were obtained from UniProt and aligned with MUSCLE alignment using SnapGene software (Version 5.2.4).

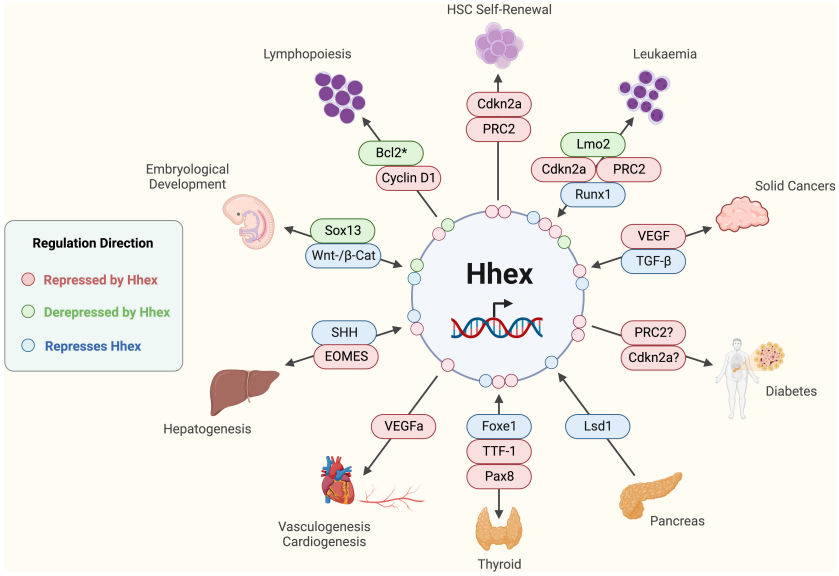

FIGURE 3
Established functions and interactions of Hhex in mammalian cells in various tissue and disease contexts. *Potentially other anti-apoptotic molecules are involved depending on the immunological cell type in question. Acute Myeloid Leukaemia (AML), B-cell lymphoma 2 (Bcl2), Cyclin-dependent kinase 2a (Cdkn2a), Eomesodermin (EOMES), Forkhead box e1 (Foxe1), Haematopoietic Stem Cell (HSC), (Pax8), Lim domain only 2 (Lmo2), Lysine-specific demethylase 1 (Lsd1), Polycomb Repressive Complex 2 (PRC2), Runt-related transcription factor 1 (Runx1), Sonic Hedgehog (SHH), SRY-box transcription factor 13 (Sox13), T cell Acute Lymphoblastic Leukaemia (T-ALL), Tumour Growth Factor-beta (TGF-β), Thyroid Transcription Factor-1 (TTF-1), Vascular Endothelial Growth Factor (VEGF), Wingless-Integrated/β-Catenin (Wnt/β-Cat). Created with BioRender.com.

cells does not require Hhex (35), it is nevertheless required for normal thyroid development, where by E10 it is the organ with the highest Hhex expression, with its expression remaining high until E18.5 (34). Additionally, the absence of Pax8 failed to affect the expression of Hhex in the developing thyroid at E9, but Hhex was undetectable at E10, suggesting that Pax8 is required for maintaining Hhex expression, but not the induction of its expression (35). These studies collectively point to later roles for Hhex in endocrinology. It was also shown that Hhex is vital for developing lung, bile duct, gall bladder and pancreas. High, and essential, expression of Hhex in the developing liver endoderm was also reported, potentially linking to Hhex's vital importance in additionally providing the necessary organ environment to facilitate haematopoiesis, detailed further in subsequent sections of this review (34, 36).

Further characterisation of Hhex's role in endodermal development within the embryo showed that in the absence of Hhex-anterior visceral endoderm (AVE) repression, Bmp2 is not present in the proximal visceral endoderm and Wingless/Integrated 3 (Wnt3) and Nodal are not properly limited to the posterior epiblast (37). Hhex-AVE null embryos then exhibited later initiation of the primitive streak and impaired patterning within the anterior primitive streak (37). Other studies noted that Hhex expression lacked asymmetry in the anterior visceral endoderm of murine embryos and that TLE4 expression could also induce endodermal expression of Hhex (38, 39). Using a fluorescent marker to track Hhex expression in the early endoderm, the importance of Hhex for self-renewal was demonstrated with the absence of Hhex allowing cellular proliferation and differentiation (40). The requirement of Hhex in the endothelial tissues during murine embryological development of the forebrain was also reported, in addition to liver and thyroid, which may be related to Hhex polymorphisms as risk factors in neurological diseases such as Alzheimer's disease discussed later in this review (41).

A link to β-catenin/Wnt signalling was first suggested when Hhex expression was ablated in the developing mouse embryo by conditional deletion of β-catenin at E7.5 in the prospective definitive endoderm of the neural plate stage embryos (Figure 3) (42). In addition, within the ventral foregut endoderm of the developing mouse embryo, SRY-Box Transcription Factor 13 (Sox13), a known Wnt/TCF signalling repressor, was shown to directly interact with Hhex, where Hhex blocks Sox13 repression of Wnt/TCF, whilst Wnt/TCF could in turn de-repress Hhex (Figures 1, 3) (43). This implies the presence of a positive feedback loop in which Hhex can amplify Wnt/TCF signalling to drive development of the murine embryo.

The first reported target of Hhex in development was regulation of the sodium-bile acid cotransporter protein *via* a Hhex response element (HRE) in the promoter (44). It was also found that Hhex directly binds and represses endothelial cell-specific molecule-1 (ESM-1), *via* the evolutionarily conserved HRE-1, revealing the essential role of Hhex in the formation of the vascular endothelium

in the developing embryo (45). Vasculogenesis and cardiac development was also found to require Hhex, with VEGFa levels repressed by Hhex in murine embryos (Figure 3) (46). It was also revealed in mice that urokinase-type plasminogen activator (uPA) induces angiogenesis *via* reducing the transcription activity of Hhex which causes de-repression of the VEGF receptor expression (47).

Liver and pancreatic development in the anterior definitive endoderm were also observed to be driven by $Hhex^{+}Cxcr4^{+}$ cells upon isolating and culturing the cells *in vitro* (48). Hhex was also observed to induce liver development in an *in vivo* system, independently of Cxcr4 expression, with lack of Hhex also inhibiting pancreatic development (49). This study showed that Hhex controls the proliferation rate of the endodermal cells in the leading edge which allows it to grow beyond the cardiogenic mesoderm when the gut tube is closing and the positioning of these cells is essential for pancreatic specification (49).

Organogenesis in the murine embryo endoderm was demonstrated to be induced by Hhex by promoting hepatoblast development in the stromal environment by allowing continued differentiation (49, 50). Moreover, recent studies have shown that pluripotent stem cells expressing wildtype Hhex can facilitate normal liver development in both mice and pigs otherwise lacking Hhex, which in the absence of Hhex results in embryonic lethality (51).

Mechanistically, it was observed that HNF3β and GATA-4 motifs in the Hhex promoter transactivate Hhex in the liver allowing tissue-specific expression of Hhex (52). Using embryoid bodies, Hhex was shown to synergise with BMP-4, inducing upregulation of Albumin, Afp carbamoyl phosphate synthetase, transcription factor 1 and CCAAT/enhancer binding protein alpha, leading to secretion of Albumin and transferrin, and inducing a pro-hepatic gene signature which included fibrinogens, apolipoproteins and cytochromes (53). Hhex was also observed to repress Shh cell signalling in hepatocyte proliferation of the developing mouse embryo, by translocating to the nucleus and mediating transcriptional repression (54). This process was facilitated by GPC3 binding CD81, which would otherwise bind to Hhex, keeping it from entering the nucleus (54). The transcriptional repression by Hhex was also blocked *via* Shh, which itself is bound by GPC3, that in turn downregulates its function as well as it binding to CD81 (54). It was also reported using Hhex KO mice that Hhex is necessary for hepatic differentiation in the endoderm *via* VEGF signalling, independently of endothelial cells (55). An important function of Hhex in bile duct formation was also suggested using Notch2 KO mice, where Hhex expression declined perinatally, normalised post-weaning, and remained elevated in icteric 6-month-old mice, thereby suggesting a role in promoting secondary bile duct formation (56).

In a genome-wide computational analysis study, aimed at identifying cis-regulatory transcription factors, Hhex was reported to be controlling embryonic blood and endothelial development at E11.5 mouse embryos, shown *via* β-galactosidase reporters (57). The use of embryonic stem cell (ESC)-derived Hhex KO embryoid bodies revealed that they lacked macrophage potential whilst endothelial cells expanded (58). In contrast, Hhex overexpression in embryoid bodies reduces cell numbers by upregulating Flk-1 and increases the number of blast colony forming cells (BL-CFCs) formed with haemangioblast characteristics (58).

A potential role of Hhex within the bone of murine embryos was shown at E15.5, with changes to intracellular localisation of Hhex during development (59). The same group also observed Hhex expression in the chondrocyte cell line, ATDC5, which increased with differentiation, and when Hhex was overexpressed, it induced a necroptotic-like cell death (60). A role of Hhex in central nervous system (CNS) neurons during murine embryonic development was discovered by the observation of inhibition of axonal growth when Hhex is prematurely expressed (61). This may be linked to Hhex's potential role in neurological diseases (see below).

2.3 Zebrafish

Zebrafish have been used to study the role of Hhex in the embryological development of vertebrates. This first study in zebrafish specifically observed Hhex expression with the yolk syncytial layer, equivalent to the murine visceral endoderm, acting as a transcriptional repressor (62). Initially, Hhex is regulated by the maternal Wnt pathway, and later by the Bmp-mediated pathway, with overexpression of Hhex downregulating both pathways, whilst concordantly upregulating chordin (62). Bmp and Fgf are required in liver development of zebrafish by specifically blocking Hhex and Prox1 expression within that tissue, but not in the neighbouring endoderm and mesoderm (63). In addition, within the dorsal yolk syncytial layer of zebrafish, Hhex is activated by Wnt/β-catenin, along with Vega1 and 2 *via* the action of Boz, which in turn allow the repression of Hhex (64).

Hhex is also vital for zebrafish haemangioblast development where it functions downstream of *Cloche*, a gene that plays an important role in haemangioblast differentiation (65). Scl and Hhex induce each other's expression suggesting that they may also compensate for each other's functions (65). A role for Hhex was also revealed as a transcriptional regulator of the VEGFC/FLT4/PRX1 signalling pathway that is necessary for development of the vascular system in zebrafish (66).

Normal hepatopancreatic duct (HPD) formation in the zebrafish embryo also requires Hhex, as shown using Hhex KO zebrafish (67). The need for Hhex in HPD formation was also verified in a subsequent study, where Hhex was shown to be necessary in both the endoderm and the yolk syncytial layer for HPD fate (68). The mutation of Telomere Maintenance 2 (Tel2) was observed to repress Hhex, whilst unmutated Tel2 engaged with Hhex's promoter to facilitate Hhex expression necessary for normal liver regeneration in zebrafish (69).

Late thyroid development in zebrafish embryos was also observed to require Hhex, along with the homeobox transcription factor Nk2.1a, also known as Thyroid Transcription Factor-1 (TTF-1) in mammals (70). Indeed, another study also noted Hhex, along with Pax2a and Nk2.1, tightly regulated Bcl2l in the developing

thyroid of zebrafish, implying a potential role of Hhex in regulation of pro-survival molecules (71), a finding that will be covered more extensively later in the context of lymphopoiesis.

2.4 Xenopus

Hhex was first identified in *Xenopus* by Newman et al, and denoted *Xhex*, where in the gastrula stage embryo, it was found to be expressed in the dorsal endomesoderm, which in turn gives rise to the liver (3). Hhex also plays an important role in anterior development originating from the endoderm (72) and was also observed to be important in vasculature development where it may have a role in the VEGF/Flk-1 signalling pathway in vascular endothelial cells (3). Overexpression of maternal Wnt/β-catenin and TGF-β signals induced ectopic Hhex and Cerberus, both early gene markers of the anterior endomesoderm, whereas blocking these pathways, downregulated expression of Hhex and Cerberus (73). Expression of the BMP antagonists Noggin and Chordin was found to allow normal Hhex and Cerberus expression, and conversely Hhex mRNA, injected ventrally, upregulated ectopic Cerberus (73). This research goes some way to describe the initial gene expression events associated with Hhex expression within the anterior endoderm required for normal development of the *Xenopus* foregut and liver (73). Another study suggested that Hhex promotes anterior identity in the *Xenopus* embryos by directly repressing Goosecoid, as well as being required for endodermal anterior patterning (9, 74, 75). These studies underscore the complex roles of Hhex in regulating the expression of multiple genes during the development of the *Xenopus* embryo.

Promoter analysis within the *Xenopus* embryos revealed that β-catenin represses Hhex expression indirectly *via* the homeodomain repressor Vent2, but conversely, subsequently drives liver organogenesis (76). Moreover, it was shown in both murine and *Xenopus* embryos that a 4.2kb upstream region of the Hhex gene was important for Hhex expression in endothelial precursor cells, liver and thyroid where an intronic component was required and adequate for normal anterior visceral endoderm and anterior definitive endoderm development (77).

Hhex is also required for normal cardiogenesis in *Xenopus* embryos, where its expression is induced in the endoderm *via* the Wnt/β-catenin signalling antagonist Dkk-1 and Hhex goes on to then regulate diffusible heart-inducing factor (78). Hhex, along with Cer1, was necessary for the Sox17 signalling pathway required for cardiac mesoderm formation in murine Embryonic Stem Cells (mESCs) (79).

Hhex plays a vital role in embryological development from organisms such as fish and amphibians, up to mice and humans. Universally, across all organisms analysed, Hhex is crucial for endodermal and mesodermal development within the embryo, giving rise to the foregut, thyroid, pancreas (in mice and humans), liver, haematopoietic and vascular systems. Hhex operates as a transcriptional repressor, in combination and in conflict with various other transcription factors and developmental proteins depending on the organism, but consistently in relation to Wnt/β-catenin signalling which appears to govern Hhex expression. Bmp is also consistently repressed by Hhex expression in mice, fish and amphibians, with the observation yet to be made in humans. Whilst in humans and mice, Shh is able to repress Hhex expression. These fundamental roles of Hhex in the formation of key organs and tissues in the developing embryo, foreshadow a continuing importance of Hhex in haematological and endocrinological diseases as discussed below.

3 Role of Hhex in HSC development and maintenance

As its name suggests, Hhex plays a central role in haematopoiesis in vertebrates with its haematopoietic expression detected across fish, amphibians, birds and mammals (1, 3, 62). The first haematopoietic progenitors found within the developing embryo are within the initial site of primitive haematopoiesis within the liver, the haemangioblast, and Hhex is essential for its development (80). Early studies revealed a high level of Hhex expression across many branches of haematopoiesis including within myeloid and erythro-megakaryocytic progenitor cells, with both lineages downregulating Hhex with differentiation, as well as within B cells, Natural Killer (NK) cells, dendritic cells (DCs) and immature T cell progenitors in the thymus (6, 7, 81). Whereas another study showed that, conversely, Hhex overexpression in haematopoietic progenitor cells results in a failure to contribute to mature blood lineages (82). An important role for Hhex was also demonstrated in erythropoiesis, specifically with regard to the globin genes, where Hhex is upregulated, along with Id2, in high-foetal haemoglobin conditions in human erythroblasts (83). Creation of murine Hhex KO ESC cocultures, where haematopoietic progenitor cells (HPCs) developed *in vitro*, showed that loss of Hhex delayed haemangioblast formation and caused an accumulation of CD41+ and $CD41^{+}$/c-Kit^{+} cells, thought to be the earliest HPCs, as well as impairing further haematopoietic development by impeding their proliferation (84).

Our own studies revealed that Hhex was essential for murine HSC self-renewal and emergency haematopoiesis following myeloablation (85). In these settings, Hhex directly represses Cdkn2a *via* PRC2 complex-mediated repression, in a similar mechanism as observed in acute myeloid leukaemia (AML) (85, 86). Cdkn2a is highly upregulated when Hhex is deleted in both HSCs and AML, and the absence of Cdkn2a rescues the defective HSC self-renewal and emergency haematopoiesis observed in Hhex KO mice (Figure 3) (85, 86). The repression of Hhex, and resultant expression of Cdkn2a and Cdkn1b, was also noted to be necessary for osteoclastogenesis in mice, indicating similar relationship between Hhex and cyclin dependent kinase inhibitors in the context of osteoclasts (87).

Within both the embryo and adult, an evolutionarily conserved non-coding region in the Hhex locus was found to bind the important HSC transcription factors Gata2, Scl, Fli1, Pu.1 and Ets1/2 and to be essential for HSC development, haematopoiesis

and homeostasis (88). The methyltransferase SETD8 was shown to be an erythroid specific repressor of Hhex, along with Gata2 and Hlx, with Hhex being upregulated when SETD8 was deleted (89).

These studies collectively illustrate the key role of Hhex in adult HSCs and haematopoiesis, continuing from Hhex's necessity in the haemangioblast of the developing embryo. The repression of Cdkn2a *via* PRC2 by Hhex is central to its function and Hhex appears to utilise this mechanism in the context of AML. Being able to inhibit Hhex may therefore be a clinical strategy in the context of some haematological malignancies.

4 Role of Hhex in lymphopoiesis

Several laboratories have now shown that Hhex plays a pivotal role in lymphopoiesis. The impaired B cell development exhibited in Hhex null mice was first reported in the context of a RAG1-deficient blastocyst complementation system which circumvented the embryonic lethality of Hhex KO mice (90). This study noted deficiencies in mature B cells, pre-B cells and $CD5^+$ B cells as well as the presence of a $CD19^+B220^-$ aberrant B cell population within the bone marrow of Hhex KO mice (90). Moreover, studies using Lck-Hhex transgenic mice showed that overexpression of Hhex in T cells impacted their development, demonstrating that downregulation of Hhex is necessary for normal T cell development (91).

A critical role for Hhex in early murine lymphopoiesis was most clearly demonstrated using haematopoietically inducible KO mice and competitive bone marrow transplantation assays, where the absence of Hhex blocked lymphoid cell development beyond the common lymphoid progenitor (CLP) cell stage (81). This defect in lymphopoiesis was characterised by the formation of a Pro-B-like aberrant ($CD19^+B220^-$) B cell population which was defective in IL-7/Stat5 signalling capacity with an increased level of apoptosis in the few remaining B cell progenitors (81, 92). However, expression of constitutively active Stat5 transgene failed to rescue the defective lymphopoiesis observed in the absence of Hhex, indicating that defective IL-7 signalling in this context was not the primary cause of the lymphopoietic defect observed (93). In contrast, transgenic expression of the potent anti-apoptotic molecule, Bcl2 was able to restore normal lymphopoiesis in Hhex null mice, both *in vitro* and *in vivo*, thus showing that Hhex plays a key role in inhibiting apoptosis during lymphopoiesis (Figure 3) (93).

Hhex-null CLPs exhibited downregulation of the cell cycle gene, Cyclin D1, which was shown to play a key role in the lymphoid developmental block, as retroviral overexpression of Cyclin D1 rescued lymphopoiesis *in vitro* (Figure 3) (81). Interestingly, in the human myeloid cell line (U937), Hhex was a reported as a negative regulator during translation of eIF4E protein, which in turn inhibited eIF4E-dependent transport of Cyclin D1 mRNA within the cell (23, 94). It was also demonstrated by the same laboratory that loss of Hhex resulted in aberrant nuclear function of eIF4E, where eIF4E is normally required for nuclear transport of Cyclin D1 mRNA into the cytoplasm (23, 94), a process stimulated by HOXA9 (24). Additionally, whilst the crucial cell cycle inhibitor Cdkn2a was observed to be upregulated in the absence of Hhex, its absence did not restore the impaired lymphopoiesis observed in the Hhex null mice, thus collectively suggesting that regulation of cell cycle is not the primary role of Hhex in the context of lymphoid development (93).

Beyond lymphoid development, Hhex plays specific downstream roles in both T regulatory cells (Tregs) and NK cells. The expression and function of Foxp3, the critical transcription factor required by Tregs, is negatively regulated by Hhex, which binds directly to the Foxp3 locus, with Hhex overexpression resulting in a failure to suppress the immune response in murine models of Treg function (95). In contrast, TGF-β/Smad3 signalling, which promotes Treg activity, was found to downregulate normal Hhex expression in Tregs (95). In the context of NK cells, repression of Hhex expression is required for normal development (96). Conversely, Hhex was shown to directly repress the expression of the pro-apoptotic protein BIM to allow normal NK cell survival (97).

Recently, Hhex, in concert with transcriptional corepressor TLE3, was also revealed to be a key regulator of germinal centre B cells developing into memory B cells through induction of the transcription factor Ski (98). The absence of Hhex in memory B cells resulted in an upregulation of Bcl-6, which was also shown to directly repress Hhex in germinal centre B cells (98). Similar to its function in CLPs, the Bcl-6 target gene, Bcl2 was able to rescue the Hhex KO phenotype in memory B cells (Figure 3) (98). This suggests that the significant role of Hhex in maintenance of cell survival remains important throughout B cell development.

5 Role of Hhex in leukaemia

Given the vitally important role of Hhex in haematopoiesis, it is no surprise that Hhex has increasingly revealed itself as playing a fundamental role in both the development and maintenance of various haematological malignancies, most notably in the context of T-ALL and AML. Upregulation of Hhex expression was first seen in the peripheral blood of B cell leukaemia patients (99) and dysregulation of Hhex was then subsequently suggested to be a contributing factor to B cell leukaemogenesis (7). Hhex was found to induce murine B cell leukaemia in the AKXD model as a consequence of retroviral insertion upstream of Hhex and mEg5 (100). In this system, both Hhex and mEg5 were upregulated following retroviral insertion but only Hhex was expressed highly in these samples (100). Subsequent studies using bone marrow transplants in lethally irradiated recipient mice of retrovirally overexpressed Hhex in HPCs showed Hhex induced T cell lymphomas (82).

Whilst these findings indicate the potential involvement of Hhex in B and T-cell leukaemogenesis, in the case of lymphoma one group noted a clear reduction in Hhex expression in all human B cell lymphoma classes they tested, with the exception of oncogenic activation (101). Indeed, in some primary cutaneous T cell lymphoma (CTCL) patient samples HHEX was shown to be deleted (102). Specifically, the deletion of HHEX *via* a 10q23.33-10q24.1 chromosomal deletion resulted in a loss of heterozygosity

in about half of the patient samples, thereby being suggestive of a role for HHEX in the aetiology of CTCL (102). Although, in the context of anaplastic large cell lymphoma (ALCL), HHEX was not observed to drive the pathogenesis of disease, as its overexpression induced apoptosis and differentiation and its expression was repressed by TGFβ/SMAD-pathway in ALCL cell lines (103).

The utility of Hhex has also been strongly established in the development of T-ALL in both mice and humans. The clearest evidence for this was shown in a murine model Lmo2-induced leukaemia in mice and T-ALL patient samples, where it was revealed upregulation of Hhex as an integral part of a broader induction of an HSC transcriptional programme and where Hhex could additionally phenocopy the action of Lmo2 in early leukaemogenesis (Figure 3) (104). In a study using Rag-deficient NOD mice, T-ALL initiation was potentially caused by the loss of T cell progenitor checkpoint regulation, with induction of a HSC gene programme including Hhex, as well as Lmo2, Lyl and Kit (105). Indeed, the +1 enhancer element of HHEX was shown to be directly bound by LMO2/FLI1/ERG in human T-ALL (14). HHEX was also observed as a direct transcriptional target of LMO2 in human early T-cell Precursor (ETP)-ALL (106). The same group also found CD2-Lmo2 transgenic mice required Hhex to be expressed for development of T-ALL, implying Hhex as a crucial mediator of the oncogenic functions of Lmo2 (106). We also observed that Hhex is required for the radio-resistance of Leukemic Stem Cells (LSCs) in a similar mouse model of human ETP-ALL (107).

Deacetylation treatment was also observed to downregulate Lmo2 expression and its target Hhex in T-ALL (108). Ldb1 and Lmo2 were also reported to bind the promoters of Hhex, Lyl1 and Nfe2, resulting in their upregulation in HSPCs and human ETP-ALL cell lines, as well as pre-leukaemic Lmo2 transgenic thymocytes in the murine Lmo2-induced T-ALL model (109). Induced deletion of Ldb1 conversely downregulates Hhex expression in murine T-ALL (109). Hhex was observed to be repressed by NKK-3 in human T-ALL samples. The same group noted that HHEX activated AUTS2, part of the chromatin modulating PRC1 complex, which in turn mediated MSX1 expression (110). Collectively, these studies underscore the interplay between other transcription factors, especially Lmo2 and its binding partners Ldb1 and Lyl1, in regulating the expression of Hhex leading to the development of T-ALL.

Dysregulation of Hhex is also well-documented in terms of its involvement in AML where nuclear Hhex was downregulated, whilst eIF4E was upregulated (23). Use of CD11c-Hhex transgenic mice revealed that high levels of Hhex during myeloid development may induce myeloid leukaemia, with higher cell cycle rates observed, although leukemogenesis was slow (18 months of age), implying that further mutation(s) were required in addition to Hhex overexpression (91).

Another group discovered an AML patient with a NUP98/HHEX chromosomal translocation as the only cytogenetic aberration and made a murine version of this genetic lesion (111). With a 9-month latency, the bone marrow bearing this lesion gave rise to a transplantable acute leukaemia, bearing similar gene dysregulation found in the more clinically common homeobox gene fusion NUP98/HOXA9 translocation (111). AMLs driven by NUP98-Hhex fusion, along with other NUP98-oncoprotien fusions, exhibit an induced aneuploidy *via* a weakening in the mitotic spindle checkpoint (112). Indeed, in the most commonly observed form of numeric aneuploidy in AML, trisomy 8, the HHEX gene body is repressed by hypermethylation and may serve as a potential diagnostic feature of the disease (113). Hhex overexpression was also detected in AML patients with the t (8, 21)(q22;q22) translocation and studies in Kasumi-1 cells, a leukaemic cell line which bears the 8:21 chromosomal translocation, showed that Hhex was required for their survival (114). These observations point to Hhex, and other Hox genes, as being both gene fusion partners and drivers for the promotion of AML development.

In the K562 myelogenous cell line, Hhex was shown to influence leukemogenesis through repression of VEGF *via* its promoter region, but required TLE co-repression to mediate its function (115). Dasatinib, a BCR-ABL/Src kinase inhibitor, reduced phosphorylation of Hhex, which in turn allowed Hhex-mediated repression of VEGF and VEGFR-1 leading to a reduction in cell survival (116). In the context of Acute Pro-myelocytic Leukaemia (APL) analysis of 18 patients showed PML-RAR-α reduced HHEX expression by targeting its promoter, which then downregulated VEGF-A, and thus the pro-angiogenic response in APL (117).

We have shown using a murine model of AML, specifically MLL-ENL, that Hhex was required for both the initiation and propagation of AML, with loss of Hhex resulting in the upregulation of $p16^{INK4a}$ and $p19^{Arf}$, leading to myeloid differentiation and growth arrest (86). Mechanistically, we demonstrated that Hhex represses PRC2-mediated epigenetic repression of Cdkn2a by binding to the Cdkn2a locus and directly interacting with the PRC2 to enable H3K27me3-mediated epigenetic repression (86). Hhex was also observed to be a direct target of Runx1, a transcription factor with known tumour-suppressor function, where Hhex combined with Flt3-ITD to induce AML in mice (Figure 3) (118). Hhex expression, in combination with a mutant form of additional sex combs-like 1 (Asxl1) an epigenetic modulator often mutated in myeloid leukaemia, was also found to enhance Runx1-ETO and Flt3-ITD-driven myeloid leukaemia *via* upregulation of Myb and Etv5 in mice (119). We also observed that Hhex overexpression induced self-renewal of murine IL-3 dependent promyelocytes *in vitro* (120). Moreover, this function of Hhex required nuclear localisation and structure function analysis demonstrated a requirement of the DNA-binding and N-terminal–repressive domains of Hhex for promyelocytic transformation (120). Despite Hhex containing a PML-interaction domain (Figure 1), it did not require PML for transformation, nor did it require $p16^{INK4a}$ and $p19^{Arf}$ indicating Hhex did not require PRC2-mediated epigenetic repression for this particular process unlike what we observed for the induction of AML (120). Nevertheless, Hhex could still cooperate with growth factor (IL-3) independence to cause pro-myelocytic leukaemia in mice (120). It is increasingly clear that Hhex plays a vital, but context dependent, role in the pathology of AML, but typically requires cooperative mutations in growth factor signalling pathways.

In summary, across multiple types of haematological malignancy, Hhex appears to be a key player in the disease development. Whilst technically challenging, greater focus should be placed on developing effective ways to target Hhex and its interacting partners in leukaemia patients. There would be clinical benefit in being able to effectively drug Hhex, and related transcription factors, particularly in the context of AML, where in many countries an aging population is resulting in a dramatically increasing disease burden of AML, and where existing therapies are currently limited and suboptimal.

6 Role of Hhex in solid cancers

In addition to Hhex's well-established roles in leukaemogenesis, it has been reported to contribute to the development of a range of solid tumours including those with endocrine functions such as in the breast, prostate and thyroid, as well as the liver, cervical and bile duct cancers.

Several studies point to Hhex playing an important part in the development of breast cancer. Hhex is expressed in breast epithelial cells, with its intracellular localisation regulated and altered by malignancy of these cells (121). Hhex was also noted to upregulate the NIS (sodium iodine symporter) promoter which is specifically upregulated in breast tissue with lactation (121). Work using a breast cancer cell line (MCF-7) showed that Hhex transcriptionally controlled endoglin and inhibited cell migration (122). Subsequent work from the same laboratory reported that siRNA Knock Down (KD) of Hhex in breast cancer cells enhanced their proliferation in part due to VEGF signalling (Figure 3) (19, 123). Moreover, Hhex overexpression impaired breast tumour growth in mice, which may help explain the poor prognosis which is associated with breast cancer patients exhibiting low Hhex expression (123). HHEX expression was also confirmed to be lower in human breast cancer compared to pre-cancerous tissue, potentially contributing to the worse clinical outcomes observed in breast cancer patients bearing low levels of HHEX expression (124, 125). In addition, type 2 diabetes (T2D) single nucleotide polymorphisms (SNPs) in Hhex (rs11187146) and Cdkn2a/b (rs1333049) were linked as being as additive risk factors in likelihood of developing and dying from breast cancer in an American patient cohort (126). Overall, these studies suggest that lower Hhex expression is a poor prognostic indicator in breast cancer and further study is needed to better understand its function in this disease.

In prostate cancer, the protein kinase CK2 was shown to impede Hhex by phosphorylation-induced inhibition of Hhex's DNA binding, allowing increased proliferation and migration of prostate cancer cell lines (127). In addition, inhibition of CK2 blocked Hhex phosphorylation resulting in reduced cell proliferation (127). The same laboratory previously suggested that Hhex controlled the expression of endoglin in the inhibition of prostate cancer cell line migration (123). In the prostate cancer cell line PNT2-C2, TGF-β signalling downregulated Hhex expression, whilst also increasing Hhex phosphorylation (Figure 3) (128). Additionally, when looking at another endocrine organ, the thyroid, and given the vital role Hhex plays in its development, it was perhaps unsurprising to find Hhex reported as highly expressed in thyroid patient tumour samples with nuclear localisation (129).

It may be expected, given its important role in development of the liver, that Hhex may play a role in cancer development and progression within this organ. Indeed, the absence of Hhex appears to be necessary for the progression of hepatocellular carcinoma (HCC) with Hhex overexpression increasing known tumour suppressor genes p53 and Rb, whilst downregulating c-Jun and Bcl2, well known proto-oncogenes (130). These observations also correlated with reduced tumorigenicity in mice, with Hhex expression denoting poorly differentiated HCC, suggesting that absence of Hhex expression may serve as a biomarker of HCC progression (130). Studies of HCC have also revealed that Hhex interacts with the potent oncogenic transcription factor, c-Myc (131). KD of Hhex using siRNA showed increased proliferation in HCC (131). Whilst c-myc drives metabolism and proliferation, Hhex appears do the opposite, causing decreased c-Myc activity and reduced tumour growth in a murine xenograft model of HCC (131). However, another study found that Hhex was nevertheless expressed in the majority of HCC cell lines (132).

In cholangiocarcinoma (CCA), more commonly known as bile duct cancer, Hhex was also found to be highly expressed and to operate in a positive feedback loop with Notch3, which itself is important in CCA, as well as inducing Wnt signalling (133). CCA tumour growth was reduced with siRNA KD of Hhex in a xenograft model, and Hhex overexpression in cholangiocytes increased their proliferation (133). Interestingly, whilst Hhex is suggested to be a positive regulator in the context of bile duct cancer, in contrast it appears to operate as a negative regulator in the context of liver carcinoma, which may hint at the underlying of role of Hhex in embryological development of these two organs.

The importance of the methylation status of the Hhex gene was noted in melanoma patients, where those with hypermethylated Hhex exhibited significantly worse levels of overall disease-free survival, as well as disease specific survival and lymph node metastasis, compared to patients with hypomethylated Hhex gene (134). The methylation status of Hhex was also shown to be relevant in cervical squamous cell carcinoma (CSCC), where hypomethylated HHEX was also observed as a positive prognostic indicator in patients (135). Moreover, another study uncovered HHEX as a potential biomarker in CSCC, speaking to its importance in the pathology of the disease (136).

In summary, in addition to Hhex's well-established role in haematological malignancy, the absence of Hhex, and in some settings its overexpression, serves as important drivers of solid tumour development, potentially stemming from its role in the embryological development of the organs from which the cancer is derived. These observations suggest that a better understanding of how Hhex mediates its normal developmental as well as its aberrant tumour-promoting functions may aid the development of more targeted therapeutics for cancer patients.

7 Role of Hhex in pancreas and diabetes

As previously discussed, Hhex plays a vital role in the embryological development of the organs of the vertebrate foregut including the pancreas. Moreover, Hhex also remains functionally relevant in the pancreas in the adult organism. Specifically, it was revealed within adult pancreas that Hhex is expressed in somatostatin-secreting delta cells (137). Use of two mouse models of pancreatic deletion of Hhex showed it is needed for pancreatic development (137). Moreover, decreased somatostatin in Hhex KO pancreatic islets caused impaired paracrine inhibition of insulin released from beta cells (137). In beta cells the Hhex locus is targeted by Lsd1 which facilitates H3K3me1/2 methylation-mediated repression of Hhex preventing beta to delta cell transition (Figure 3) (138). This suggested that compromised paracrine control may be partly responsible for T2D through the acceleration of beta cell exhaustion and failure (137). Hhex RNA and protein was also revealed in humans as being highly expressed in the pancreas, specifically the islets, exocrine acini and ductal epithelium, but not detected at significant levels in liver parenchyma and colonic epithelium (139). The overexpression or KD of Hhex in *Xenopus* showed that it is essential for the ventral pancreas formation, *via* Vpp1 expression in ventral pancreatic progenitor cells, as well as liver development (140). This finding was also verified in *Drosophila* in that Hhex is equally important in glucose metabolism, as revealed in tissue specific KD studies (141).

Increasingly, and perhaps unsurprisingly, Hhex has been observed as a notable risk factor in a number of endocrinological and metabolic diseases that involve the pancreas. A number of allelic SNPs (rs1111875, rs5015480 and rs7923837) within the Hhex gene have been implicated to varying degrees as T2D risk factors, with a Genome-Wide Association Study (GWAS) linking rs5015480 with gestational diabetes mellitus with these studies and meta-analyses showing that the ethnic background of the patient population is the most important factor as to whether a Hhex SNP risk factor allele applies and to what extent (Supplementary Table 1) (142). Indeed, a study of T2D patients using ATAC-seq also detected Hhex in open chromatin peaks, amongst other candidate genes associated with T2D and islet dysfunction (143). In murine studies, Hhex may potentially play a broader role in metabolism beyond the pancreas, such as in the liver which was shown to have high Hhex expression, but which decreased in response to high fat feeding (144). This study however also conflicts with that of Costapas et al, who reported that pancreatic islets exhibited lower Hhex expression (139, 144). Nevertheless, the modulation of Hhex expression within the liver in response to dietary metabolism suggests that Hhex SNPs may play a relevant role as a risk factor in T2D susceptibility (144).

Interestingly, Hhex may function in the pancreas *via* a similar fashion to that which was observed in HSCs and leukaemia, by directly repressing Cdkn2a, as a consistent SNP in the Cdkn2a gene (rs10811661) is often concurrent with SNPs within the Hhex gene as well-established risk factors in the development of T2D (Supplementary Table 1). Indeed, in a study of T2D patients Hhex and Cdkn2a polymorphisms were detected in about half of patients, where it was shown a CpG site was introduced or removed associated with the differential methylation the SNP-CpG site of Hhex in pancreatic islets (Figure 3) (145). Moreover, Cdkn2a, along with several other genes, was also associated with both differential methylation of DNA of the CpG-SNP site within islets and the DNA methylation of surrounding CpG sites, suggesting that this may be a molecular means by which Hhex SNPs associated with T2D mediate their effect in patients (145).

There were also conflicting separate studies regarding the role of T2D SNPs affecting low birth weight when inherited by the offspring (Supplementary Table 1). Given the role of Hhex SNPs in T2D and glucose metabolism, its influence on the risk of T1D development, polycystic ovary syndrome (which shares an insulin resistance link with T2D) and metabolic syndrome was also explored in humans with studies revealing no such link from several GWAS studies (Supplementary Table 1). Interesting though, Hhex's association with T2D, which extends to high body weight index, may also have further a role in adipocyte development *in vitro* where lack of Hhex impairs expression of PPAR-gamma protein and impedes adipogenesis (146). Based on the evidence produced thus far, Hhex does not appear to play a role in birth weight, PCOS, metabolic syndrome or T1D development despite a clear relationship with glucose metabolism in T2D, but it may be involved in adipocyte development.

Several studies have hinted at how Hhex may be regulated and which functions it performs within the pancreas. Hhex is upregulated in human islets by gastrin hormone treatment (50) and Aldh1a2 KD reduced Hhex expression, along with Prox1, in the pancreas and liver (147). Using hESCs it was shown that Hhex, along with Pax6, may be repressed by Aristaless related homeobox (ARX) in that ARX KO pancreatic progenitor cells exhibited an upregulation of Hhex and conversely when ARX was re-expressed, Hhex was then downregulated (148). Whilst Hhex is not required in ductal cell function of adults, KD of Hhex in pancreatic progenitor cells can cause pancreatitis (149). However, Hhex is vital in early life for maintenance of ductal homeostasis and allowing ductal hypersecretion as a cause of chronic pancreatitis in children (149). Ferreira et al. went on to show that the G-protein coupled receptor Npr3 is repressed by Hhex and thereby the potential secretion by ductal cells (149). Within islets, delta cell specific-Hhex was shown to control cAMP and concentration of intracellular calcium *via* histone post-translation changes, which in turn modulates Cav1.2 calcium channel and adenylyl cyclase 6 (AC6) and secretion of somatostatin (150). These histone modifications that epigenetically control secretion of somatostatin within islets were mediated by a super complex composed of the Cullin 4B-RING E3 ligase (CRL4B) and interestingly, the PRC2 methyltransferase complex (Figure 3) (150).

The strong association of SNPs risk factors in T2D for both Hhex and Cdkn2a across a broad spectrum of human ethnicities, combined with observations that epigenetic modifications made *via* Hhex within pancreatic islets involve PRC2, are tantalising. This strongly suggests that Hhex's well-documented function in HSCs and leukaemia *via* PRC2-mediated repression of Cdkn2a may also be one of its primary roles in the adult pancreas. However, further research is still required to resolve this hypothesis more conclusively.

8 Role of Hhex in endocrinology

Perhaps unsurprisingly given its important role in the developing thyroid gland and pancreas in the embryo, Hhex continues to play an important part in the endocrine system and in endocrinological diseases. The expression of Hhex was observed in both early undifferentiated thyroid cells and in the adult thyroid gland of both rats and humans, as well as in differentiated follicular thyroid cell lines (151, 152). Cells of the thyroid line FRTL-5 decreased their levels of Hhex expression in response to thyroid stimulating hormone (TSH) (152), with another study in differentiated human thyroid cells reporting that Hhex was not required for thyroid-specific gene expression induced by TSH (153). The thyroglobulin promoter was shown to be repressed by Hhex, which in turn blocked the activation of thyroid transcription factor-1 (TTF-1, also known as NKX2-1) and Paired box 8 (Pax8) (Figure 3) (152). In a subsequent publication, the same laboratory noted that TTF-1 enhanced the promoter activity of Hhex in rat FRTL-5 cells, and that the mRNA of both TTF-1 and Hhex was co-expressed in human thyroid tissues (154). In another report following on from that work, Puppin, et al, identified a relationship between Pax8 and Hhex, where Pax8 induced Hhex protein expression in a thyroid cell line and induced Hhex promoter activity in non-thyroidal cell lines (155). Hhex, along with Pax8, Foxe1 (Forkhead Box E1, also known as TTF-2) and E-Cadherin, were also observed to be downregulated in response to the functional inactivation of TTF-1 in PCCI3 thyroid cells (156). Whilst Hhex has minimal impact on thyroid specific gene expression (153), Foxe1 is required for NIS expression as shown in FRTL-5 cells (157). And it is also worth noting that Hhex upregulates the NIS promoter within breast tissue (121), suggesting a potentially similar mechanism of function for Hhex within both the breast and thyroid. Indeed, in the precursor cells of developing thyroid Hhex, along with Pax8, TTF-1 and Foxe1, operate in a highly inter-related network governing normal thyroid development (Figure 3) (35, 158, 159). Collectively, this research clearly shows the close relationship between the transcription factors Hhex and Pax8 in regulating TFF-1 expression to govern thyroid function.

Potentially conflicting reports exist showing that Hhex was absent in oncogene-transformed thyroid cell lines (Pellizzari, 2000), however another study from the same laboratory showed that Hhex was actually highly expressed in thyroid tumour samples from patients and concentrated within the nucleus (129, 152). Hhex, along with notably Pax8 and NIS amongst other thyroid specific genes, was also observed to be significantly decreased in patients in both benign thyroid tissues and carcinomas suggesting a potential involvement in a de-differentiation process (160).

Given the well-established role of Hhex in thyroid development within the embryo, the effect of mutations within Hhex was examined. Although Hhex mutations were found to not be a driver of thyroid dysgenesis (TD), PAX8 R52P mutation was implicated (161). Whilst another group subsequently examined the thyroid tissue of Chinese children suffering from TD for Hhex mutations (162), they also failed to show any link between Hhex mutations and TD, along with FOXE1, TTF-1 and PAX8, but still observed a correlation with the intronic mutation rs2275729, although owing largely to the small study size, further work is required to determine its potential importance (162). In addition, heterozygous Hhex mutations were detected in a small fraction (8/110) of congenital hypothyroidism patients which ultimately went on to develop TD (163).

The adrenal gland may also have a requirement for Hhex, with a meta-analysis of patients detecting a Hhex SNP (rs2497306) associated with levels of serum dehydroepiandrosterone sulphate (DHEAS), which is produced by the adrenal gland and associated with aging (164). Moreover, the rs2497306 SNP was also observed to be negatively associated with serum DHEAS levels of female RA patients (165). Additionally, the mild endocrine disruptor DDT (Dichloro-diphenyl-trichloroethane) was found to disrupt the Hhex-mediated regulation of cellular proliferation within rat adrenal cortex (166). These observations collectively suggest a role for Hhex in regulating processes within the thyroid and adrenal glands that warrant further investigation.

9 Miscellaneous roles of Hhex in neurological and other diseases

A number of studies have examined the potential role of Hhex in Alzheimer's Disease (AD) in terms of SNPs that are known to be risk factors in T2D. However, several GWASs ultimately concluded that Hhex SNP rs1544210 was not specifically associated with late-onset AD (167–169). However, another meta-analysis found whilst Hhex SNP rs1544210 was not statistically significant in analysis of their 3 included studies (p=0.04, 0.09 and 0.29), there was a trend towards association with late-onset AD susceptibility (170). In a European patient cohort study of 110 candidate polymorphisms, Hhex SNP rs1111875, a major risk factor in T2D, was found to be a highly significant risk factor ($p<0.00001$) for AD, but only with the accompanying GSTM3 (rs7483) SNP (171). Whereas in a Korean population it was shown that Hhex polymorphisms observed in T2D (rs1111875 and rs5015480) were not associated with AD or Parkinson's Disease (PD) (172), Hhex T2D SNP rs1544210 was associated with greater dementia and AD risk in a Swedish population (173).

Simpson et al. investigated how Hhex may influence neuronal biology, noting that Hhex had broad expression in CNS neurons in adults, including neurons of the corticospinal tract following spinal damage, and was amongst the most potent inhibitors of neurite growth (61). However, in adults Hhex expression was substantially reduced in immature cortical and peripheral neurons (61). In early immature cortical neurons, Hhex overexpression impaired both the initial axonogenesis including the axonal elongation growth rate with domain deletion analysis suggesting Hhex acted in this context as a transcriptional repressor (61). In the context of multiple sclerosis (MS), the HHEX SNP rs7923837, is a known risk factor of the disease (174). This observation may be related to more metabolically active lymphocytes in the blood of MS patients, which also express significantly less HHEX, but also bear far greater

nuclear rs7923837 SNP Hhex, when compared to healthy controls (174). Recently, the microglia of mice were shown to decrease their Hhex expression when socially stressed or administered with agonists to TLR-2 and TLR-4 (175). Conversely, Hhex overexpression dampened the expression of inflammatory genes associated with TLR-4 induction, collectively suggesting that Hhex may be repressed by inflammatory signals (TLR-2/4) which can then contribute to neuro-inflammation in microglia (175). These findings suggest the potential of therapeutic intervention targeting Hhex in the treatment of neuro-inflammation in certain disease settings.

There are also a number of reports of Hhex function in various aspects of physiology including angiogenesis, milk production, muscle and lung function, as well as various diseases including psoriasis, hepatic and gallstone disease. In a study of milk production in dairy cows Hhex was reported to be targeted by miR-148 and regulate VEGFA, NRP1 and MYH10 with these genes in turn targeted by miRNAs miR-186, miR-148 and miR-141/200a respectively (176). A potential role for Hhex in terms of lactation, specifically protein localisation, was noted *in vitro* and Hhex may also play a role in mammary cell differentiation and tumorigenesis (121). In lung fibroblasts, Hhex expression was induced in response to TGF-β1, as was miR-21-3p which targets Hhex (177).

In muscle, Hhex was observed to upregulate gene expression of SMemb/Non-muscle Myosin Heavy Chain-B *via* the cAMP-Responsive element (178). In vascular smooth muscle cells, Hhex overexpression promoted G0/G1 to S-phase cell cycle transition, inducing cell cycle genes including CDK2, CDK6, CyclinB2 and CyclinD2, and inhibiting apoptosis, with the authors linking this to a potential role in vascular proliferative disease (179). Indeed, another study noted Hhex promoted vasculogenesis *via* VEGF as it was associated with increased vascular density in a rat model of stroke (180). In the skin lesions of psoriasis patients, Hhex mRNA and protein was found to be significantly lower in mesenchymal stem cells which suggested a role for Hhex in angiogenesis *via* its known influence on the VEGF signalling pathway (181, 182).

Hhex was also shown to be a novel bile acid-induced FXR/Fxr target gene following chronic bile acid exposure in hepatocytes with the FXR/Fxr binding to a conserved intronic enhancer in both human and mouse Hhex (183). The prevalent Hhex T2D risk factor SNP rs1111875 was found to be significantly associated with development of gallstone disease and is suggested as a potential biomarker (184). Additionally, Hhex was shown to be necessary in the formation of hepatic cysts of the bile duct in a liver conditional KO model in mice, resulting in increased expression of PC1/2 in the absence of Hhex (185). These data suggest Hhex may play an important role in various liver diseases.

These seemingly disparate involvements of Hhex in various organs and tissues all likely hint at a continuation of the utility of Hhex beyond embryonic development (Figure 4). For example, Hhex was noted as playing an important role in the development of liver, vascular endothelium and forebrain which may link to the reported observations above. Hhex may well also have undocumented functions in breast, muscle and myelination

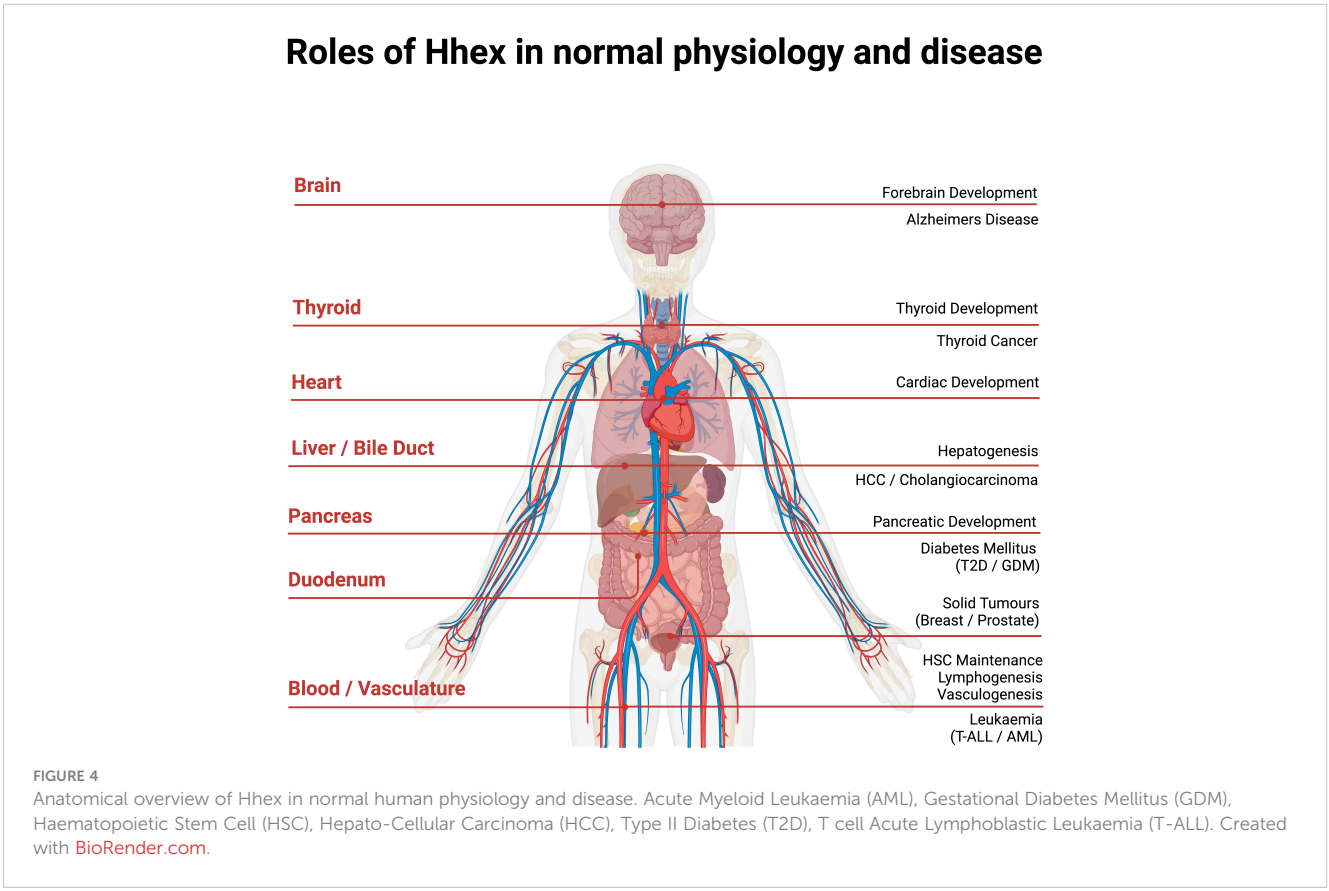

FIGURE 4
Anatomical overview of Hhex in normal human physiology and disease. Acute Myeloid Leukaemia (AML), Gestational Diabetes Mellitus (GDM), Haematopoietic Stem Cell (HSC), Hepato-Cellular Carcinoma (HCC), Type II Diabetes (T2D), T cell Acute Lymphoblastic Leukaemia (T-ALL). Created with BioRender.com.

during embryology. Further research into the Hhex's functions in both development of the embryo and adult will elucidate more clearly if its functions are maintained in the adult or whether it is redeployed in additional roles.

10 Conclusions/perspectives

This review reveals that Hhex is a crucial transcription factor throughout vertebrate evolution and the lifespan of the organism from embryo to adult. Hhex acts as a powerful transcriptional repressor, notably of PRC2 target genes such as Cdkn2a in HSCs, leukaemia and potentially in diabetes, given that SNPs in Hhex are typically noted as a risk factor alongside Cdkn2a. Hhex also plays a distinct role in maintaining pro-survival genes during lymphopoiesis. Additionally, Hhex itself appears to be regulated during embryological development by the Wnt/β-catenin signalling pathway in which it operates in a positive feedback loop. Moreover, Hhex is reported to repress genes in many other contexts including Eomes in hepatogenesis, Sox13 in the foregut endoderm, ESM-1 in the vascular endothelium, VEGF in vasculogenesis and cardiogeneisis, goosecoid in *Xenopus* anterior identity and the thyroglobulin promoter governing TTF-1 and Pax8 in the thyroid gland (Figure 3). Many of the diseases where Hhex manifests as a driving or contributing factor echo Hhex's embryonic functions within the affected organ, where Hhex continues to play an important role. Thus, more extensive research into the exact role of Hhex in haematological malignancies, solid tumours, diabetes and thyroid diseases, may offer the greatest immediate benefits for diseases where Hhex is already heavily implicated and greater therapeutic intervention is still required. As such more broadly, further study into Hhex's precise mechanisms of action and direct binding partners may contribute to tackling disruptions to embryonic development, diseases of the adult endocrine system and malignancies.

Author contributions

JJ wrote the manuscript and created the figures and table. MM helped plan the manuscript. MM and SN provided invaluable feedback in drafting the manuscript, figures and table. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author MM is an employee of the company iCamuno Biotherapeutics.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1197490/full#supplementary-material

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Ashley Ng,
The University of Melbourne, Australia

REVIEWED BY
Leonid A. Pobezinsky,
University of Massachusetts Amherst, United States
Lifei Hou,
Harvard Medical School, United States
Lisa M. Minter,
University of Massachusetts Amherst, United States
Koichi Yuki,
Harvard Medical School, United States

*CORRESPONDENCE
Jinfang Zhu
jfzhu@niaid.nih.gov

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GATA3 induces the pathogenicity of Th17 cells via regulating GM-CSF expression

Matthew J. Butcher[1], Rama Krishna Gurram[1], Xiaoliang Zhu[1], Xi Chen[1], Gangqing Hu[2,3], Vanja Lazarevic[4], Keji Zhao[2] and Jinfang Zhu[1]*

[1]Molecular and Cellular Immunoregulation Section, Laboratory of Immune System Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States, [2]Laboratory of Epigenome Biology, Systems Biology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, United States, [3]Department of Microbiology, Immunology, and Cell Biology, School of Medicine, West Virginia University, Morgantown, WV, United States, [4]Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States

T-bet-expressing Th17 (T-bet$^+$RORγt$^+$) cells are associated with the induction of pathology during experimental autoimmune encephalomyelitis (EAE) and the encephalitic nature of these Th17 cells can be explained by their ability to produce GM-CSF. However, the upstream regulatory mechanisms that control *Csf2* (gene encoding GM-CSF) expression are still unclear. In this study, we found that Th17 cells dynamically expressed GATA3, the master transcription factor for Th2 cell differentiation, during their differentiation both *in vitro* and *in vivo*. Early deletion of *Gata3* in three complimentary conditional knockout models by Cre-ERT2, *hCd2*^{Cre} and *Tbx21*^{Cre}, respectively, limited the pathogenicity of Th17 cells during EAE, which was correlated with a defect in generating pathogenic T-bet-expressing Th17 cells. These results indicate that early GATA3-dependent gene regulation is critically required to generate a *de novo* encephalitogenic Th17 response. Furthermore, a late deletion of *Gata3* via Cre-ERT2 in the adoptive transfer EAE model resulted in a cell intrinsic failure to induce EAE symptoms which was correlated with a substantial reduction in GM-CSF production without affecting the generation and/or maintenance of T-bet-expressing Th17 cells. RNA-Seq analysis of *Gata3*-sufficient and *Gata3*-deficient CNS-infiltrating CD4$^+$ effector T cells from mixed congenic co-transfer recipient mice revealed an important, cell-intrinsic, function of GATA3 in regulating the expression of *Egr2*, *Bhlhe40*, and *Csf2*. Thus, our data highlights a novel role for GATA3 in promoting and maintaining the pathogenicity of T-bet-expressing Th17 cells in EAE, via putative regulation of Egr2, Bhlhe40, and GM-CSF expression.

KEYWORDS

GATA3, experimental autoimmune encephalomyelitis, Th17, pathogenicity, GM-CSF, Bhlhe40

Introduction

As an important part of the adaptive immune system, CD4 T helper (Th) cells play central roles in orchestrating immune responses to a variety of infections as well as during allergic and/or autoimmune reactions via the production of unique sets of cytokines (1). In response to foreign or self-antigen-laden antigen presenting cells, naïve T cells differentiate into distinct Th effector lineages through a combination of T cell receptor (TCR) activation and differentiating cytokine cues. As a result, lineage-specific transcription factors are induced and initiate the differentiation of specific Th effector cell lineages. The master lineage transcription factors for each lineage are T-bet (Th1), GATA3 (Th2), RORγt (Th17), and Foxp3 (Treg), respectively (1, 2). For Th17 cell differentiation, IL-6, IL-21, and/or IL-23 signaling induces the Th17-lineage transcription factor RORγt via Stat3 activation, and RORγt then works in conjunction with the pioneering transcription factors BATF/IRF4 and Stat3 to regulate the expression of effector cytokines IL-17A and IL-17F (1, 3, 4). During Th2 cell differentiation, a combination of TCR stimulation and IL-4-Stat6 signaling is sufficient to drive GATA3 expression and the production of Th2-related cytokines, including IL-4, IL-5, and IL-13 (1). In the case of Th1 cell differentiation, TCR activation together with IL-12- and/or IFNγ-mediated signaling induces T-bet expression and endow T-bet$^+$ cells with the capacity to produce IFNγ (1).

However, the one-transcription factor-one fate model is over simplified and there are many *in vivo* experimental contexts in which multiple master lineage transcription factors can be co-expressed (1, 2). In fact, GATA3 is expressed by all T cells at various expression levels *in vivo* and its expression is tightly regulated to an appropriate level for optimal T cell survival and proliferation (1, 5). Additionally, there are multiple instances in which GATA3 is co-expressed at intermediate or high levels with other master-lineage transcription factors. For example, a subset of colonic Foxp3$^+$ Tregs can co-express either GATA3 or RORγt, and GATA3/RORγt co-expressing cells have been observed in asthmatic patients and models of allergic inflammation (6, 7). Additionally, in a model of enforced expression of GATA3, *de novo* Th17 cell differentiation was still able to occur, suggesting that GATA3 may not intrinsically block RORγt$^+$ Th17 cell differentiation (8). Similarly, T-bet and GATA3 can be co-induced or co-expressed during Th1 differentiation *in vitro* (9–11) and both can be expressed dynamically in Tregs (12). Lastly, there are situations in which T-bet and RORγt can be co-expressed. For example, T-bet$^+$RORγt$^+$ Th17 cells have been found in the gut and in the central nervous system (CNS), where they are able to co-produce IL-17A and IFNγ (13–17). Thus, while the expression of primary lineage defining transcription factors is critically required for the lineage commitment of Th subsets, dynamic expression of the master regulators of other lineages may endow the established cell lineages with additional functions.

One experimental model in which T-bet$^+$RORγt$^+$ Th17 cells have garnered significant attention is experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. In EAE, a peripheral immunization with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) results in the generation of several autoimmune demyelinating Th subsets, including T-bet$^-$ RORγt$^+$ (Th17), T-bet$^+$RORγt$^+$ (T-bet-expressing Th17), T-bet$^+$RORγt$^-$ (Th1) cells, which are all found in the CNS at the peak of the disease. Several experimental lines of evidence have shown that 2D2-transgenic *in vitro* polarized Th17 cells are sufficient to induce EAE symptoms in transfer models. However, neither Th1-related IFNγ, nor Th17-related IL-17A, IL-17F, IL-21, IL-22 cytokines are required to provoke EAE symptoms (18–21). Instead, granulocyte-macrophage colony stimulating factor (GM-CSF) has emerged as a key pro-encephalomyelitic cytokine that is both required for EAE and is secreted by encephalitic Th17 and Th1 cells in the CNS (22–24). However, the exact mechanisms through which GM-CSF-production is regulated within T cells are less clear. IL-1β and IL-23 cytokine signaling are required for *in vivo* GM-CSF production during EAE and the transcription factors c-Rel, NF-kB1, RUNX1, RORγt, Bhlhe40 have been proposed to affect T cell *Csf2* expression (22–30). Of the aforementioned transcription factors, *Bhlhe40* is particularly noteworthy as it is induced within T cells upon TCR stimulation and *Bhlhe40*$^{-/-}$ mice have been demonstrated to be deficient in GM-CSF production in T cells *in vivo* (31, 32). Thus, Bhlhe40-dependent GM-CSF production within encephalitic T cells has emerged as a key pro-inflammatory pathway in EAE, although the precise mechanisms through which Bhlhe40 expression is regulated are currently unclear.

Innate lymphoid cells (ILCs), the innate counterparts of Th cells, also express T-bet, GATA3 and RORγt, for the development and functions of group 1 ILCs (ILC1s), ILC2s and ILC3s, respectively (33). Like T-bet$^+$RORγt$^+$ Th17 cells, NKp46$^+$ ILC3s in the intestinal lamina propria also express both T-bet and RORγt (34). Strikingly, GATA3 plays important role in the development of NKp46$^+$ ILC3s and regulates optimal production of IL-22 (35). Since ILC and Th subsets often utilize similar transcriptional machinery for their development and functions, we hypothesized that GATA3 may also have an important function in regulating the generation and functions of T-bet$^+$RORγt$^+$ Th17 cells.

Here we report that *de novo* differentiating Th17 cells dynamically express GATA3 ranging from an early intermediate level to a late low level. Complimentary experimental models designed to probe the functions of GATA3 in EAE revealed that while the early intermediate expression of GATA3 is dispensable for the initial differentiation of Th17 cells, it is required to generate encephalitogenic T-bet-expressing Th17 cells and to provoke EAE symptoms. Interestingly, when *Gata3* was deleted at a later stage, following the generation of Th17 cells in the draining lymph node, the re-transfer of these effector T cells in an adoptive transfer EAE model revealed that the production of GM-CSF was drastically reduced without affecting the overall proportion of IFNγ/IL-17A-producing T cells or relative T-bet-expressing Th17 cell percentages. Further co-adoptive transfer experiments revealed that the GATA3-mediated GM-CSF regulation effect was cell intrinsic. Transcriptomic analyses through RNA-Seq revealed that GATA3 regulated the expression of *Bhlhe40* and *Egr2* in a cell-intrinsic manner. Together, these results suggest a novel regulatory pathway involving GATA3, Egr2, Bhlhe40, and GM-CSF in EAE.

Materials and methods

Mice

Gata3$^{fl/fl}$ [Taconic line 355 (36)], Cre-ERT2-*Gata3*$^{fl/fl}$ mice [Taconic line 8445 (37)], *Cd45.1/Cd45.2* C57BL/6 (Taconic line 8422), *Cd45.1* C57BL/6 (Taconic line 7), *Tcra*$^{-/-}$ (Taconic line 98) and C57BL/6 mice were ordered from the NIAID-Taconic repository or the Taconic. h*Cd2*^{Cre}*Gata3*$^{fl/fl}$ mice has been reported recently (38). *Tbx21*^{Cre} mice [Jax line 024507 (39)] were crossed with *Gata3*$^{fl/fl}$ mice to generate *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ mice. *Rorc*$^{E2\text{-}Crimson}$ mice (35) were crossed with *Gata3*^{ZsGreen} (40) and *Foxp3*^{RFP} [Jax line 008374 (41)] reporter mice to generate *Rorc*^{E2Crimson}*Gata3*^{ZsGreen}*Foxp3*^{RFP} triple reporter mice. 2D2 mice were purchased from the Jackson Laboratory (JAX line 006912). All mice were imported, bred, and housed within the National Institute of Allergy and Infectious Diseases (NIAID) specific pathogen-free animal facilities. Unless otherwise specified, all experimental mice were used between 6-16 weeks of age under an animal study protocol approved by the NIAID Animal Care and Use Committee.

In vitro CD4 T cell cultures

Naïve T cells ($CD3^+CD4^+CD45RB^{hi}CD25^-$ from C57BL/6 mice or $CD3^+CD4^+CD45RB^{hi}CD25^-Foxp3^-ROR\gamma t^-GATA3^-$ from *Gata3*^{ZsGreen}*Rorc*$^{E2\text{-}Crimson}$*Foxp3*^{RFP} mice) were isolated from peripheral lymph nodes via cell sorting (FACSAria, BD Biosciences). The isolated naïve T cells were subsequently cultured under Th17 conditions (1 µg/ml anti-CD3; 2 µg/ml anti-CD28; 10 µg/ml anti-IL-4, 10 µg/ml anti-IFNγ; 0.5 ng/ml TGFβ1, 10 ng/ml IL-1β, 20 ng/ml IL-6, 10 ng/ml IL-23) in complete RPMI1640 media (Invitrogen, 10% FBS (Hyclone), 200 mM Glutamine, 100 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco)) for 0-72 hr as indicated.

Experimental autoimmune encephalomyelitis

For EAE experiments, MOG_{35-55}/Complete Freund's Adjuvant (CFA) and MOG_{35-55}/Incomplete Freund's Adjuvant (IFA) emulsions were prepared. For MOG_{35-55}/CFA preparations, 0.4 mg/ml of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, NIAID peptide core facility) was emulsified 1:1 in CFA (BD) supplemented with *Mycobacterium tuberculosis* extract H37Ra (Difco, 4 mg/ml). For MOG_{35-55}/IFA preparations, 0.4 mg/ml of MOG35-55 peptide was emulsified 1:1 with IFA (BD).

To induce active EAE, 8-12-week-old sex matched mice were injected subcutaneously with MOG_{35-55}/CFA (50µl/flank) and 200 ng of Pertussis Toxin ('Ptx', Calbiochem) intraperitoneally on days 0 and 2 of the experiment. Immunized mice were subsequently harvested at the indicated time points or at the peak of EAE symptoms. EAE clinical scores and body weights were collected daily and scored as follows: 0 – asymptomatic, 1 – tail paralysis, 2 – hindlimb paresis, 3 – hindlimb paralysis, 4 – unilateral forelimb paralysis and hindlimb paralysis, 5 – moribund or death. To isolate draining lymph node effector T cells for adoptive cell transfer EAE experiments, the indicated donor mice were immunized with MOG_{35-55}/CFA and Pertussis Toxin, and the draining lymph nodes were subsequently collected six days post-immunization. $CD3^+CD4^+CD44^{hi}$ T cells were collected from the draining lymph nodes by cell sorting for the cell transfer procedure. For some experiments involving 2D2 cells, naïve 2D2 cells were isolated by cell sorting ($CD3^+CD4^+CD45RB^{hi}CD25^-V\beta11^+$) and transferred intravenously ($2x10^6$ cells/mouse) to *Cd45.1/Cd45.2* hosts before immunization. To induce EAE in *Tcra*$^{-/-}$ recipients in adoptive cell transfer EAE experiments, $4x10^6$ donor cells were transferred intravenously and the recipient mice were injected with MOG_{35-55}/IFA and Pertussis Toxin (i.p. d0, d2). The *Tcra*$^{-/-}$ recipient mice were monitored daily for EAE clinical symptoms as described above. In some experiments involving Cre-ERT2-*Gata3*$^{fl/fl}$ mice or CD4 T cells, the mice were also injected with 100 µl of tamoxifen (T5648; Sigma-Aldrich, 4mg/ml) or a vehicle control (corn oil) on immunization d0 or cell transfer d0.

Tissue preparation

For the isolation of CNS-infiltrating cells for flow cytometry experiments, mice were perfused via cardiac puncture with cold PBS immediately following euthanasia. The brain and spinal cord were subsequently dissected, minced finely, and digested with 1 U/ml Liberase TM (05401119001; Roche) and 0.3 U/ml DNase I (10104159001; Roche) in incomplete RPMI1640 media for 30 minutes at 37°C. The tissues were mechanically disrupted via repetitive pipetting and filtered through a 70 µm cell strainer (Fisher Scientific). The resulting cell suspension was centrifuged through a percoll density gradient (38% - 70%) and mononuclear cells were collected from the interphase. The mononuclear cell suspension was washed and resuspended in culture medium for flow cytometry. For the preparation of lymph node or splenic cell suspensions, lymph nodes (inguinal, axillary, brachial) and spleens were isolated sterilely and mechanically disrupted using a 70 µm cell strainer. Erythrocytes were lysed from the resulting splenic cell suspension using ACK lysis buffer (Fisher Scientific). The final cell suspensions were washed and resuspended in culture medium (re-stimulated samples) or FACS buffer (non-stimulated samples) for flow cytometry.

Flow cytometry and cell sorting

To detect intracellular cytokine production, cells were re-stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 500 nM ionomycin (Sigma Aldrich) in complete RPMI 1640 media for 5 hours in the presence of 1X Brefeldin A (Biolegend) for the last hour of the incubation. Following re-stimulation, single cell suspensions were

first incubatedwith anti-CD16/CD32 (2.4G2) antibodies (15 minutes, 4°C) and subsequently stained for extracellular antigens (20 minutes, 4°C). Cytokines and transcription factors were stained using the Foxp3 staining buffer set (00-5523-00, eBioscience) according to the manufacturer's instructions. The samples were acquired using an LSR-II, Fortessa, or FACS Symphony cytometer (BD Biosciences), and the results were analyzed using FlowJo software (Tree Star, v10). The following antibodies were used in the study: CD3 (17A2), CD4 (RM4-5), CD44 (IM7), CD25 (PC61.5), CD45RB (C363-16A), T-bet (04-46), RORγt (Q31-378), GATA3 (TWAJ), Foxp3, IFNγ (XMG1.2), IL-17A (eBio17B7), GM-CSF (MP1-22E9), Bhlhe40 (Dec1, NB100-1800), Egr2 (erongr2), TNFα (MP6-XT22), CD45.1 (A20), CD45.2 (104), Vb11 (RR3-15), CD11b (M1/70), CD45 (30-F11), F4/80 (BM8), Gr1 (RB6-8C5), Tmem119 (106-6), and Goat anti-Rabbit secondary antibody (Thermofisher, A-11008). For FACS sorting experiments, single cell suspensions were prepared sterilely and stained as described above. Specified live cell populations were sorted using a FACS Aria (BD Biosciences) and collected into complete RPMI 1640 media. The isolated populations were washed twice with PBS and subsequently used for downstream applications.

RNA-Seq analysis

CNS-infiltrating $CD3^+CD4^+CD44^{hi}CD45.1^+CD45.2^+$ and $CD3^+CD4^+CD44^{hi}CD45.1^-CD45.2^+$ T cells were sorted directly into 300 µl of Qiazol (Qiagen) from vehicle or tamoxifen treated *Cd45.1/Cd45.2* C57BL/6 and *Cd45.2* Cre-ERT2-$Gata3^{fl/fl}$ mixed co-transfer EAE $Tcra^{-/-}$ hosts by cell sorting. Total RNA was extracted and cDNA libraries were prepared using the Smart-Seq2 method (42) as previously described (43). Multiplex sequencing reads of 50 bp were generated by the NHLBI DNA Sequencing and Computational Biology Core and sequence reads were mapped to the mouse genome (mm9) using bowtie 2 with the default settings (44). Gene expression was measured by RPKM (45) and differentially expressed genes were identified using Partek Flow (Partek). Differentially expressed genes were imported into Ingenuity Pathway Analysis and analyzed using the Core Analysis settings. Th17-related genes and pathways that connect *Gata3*, *Egr2*, *Bhlhe40*, and *Csf2* were built using the differential expression data, and the build and connect features of Ingenuity Pathway Analysis. The RNA-Seq datasets have been deposited at the Gene Expression Omnibus database under the accession no. GSE227394.

Statistics

Statistical differences between experimental groups were determined by a two-tailed Student's *t* test, Bonferroni-holm multiple comparison-corrected Student's *t* tests, or one way ANOVA with Tukey post-hoc comparison tests as appropriate with Prism 7 software. For all statistical comparisons, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All summary data are reported as mean ± standard error of the mean.

Results

GATA3 is dynamically expressed during *de novo* Th17 cell differentiation both *in vitro* and *in vivo*

GATA3 is important for the development of $NKp46^+$ ILC3s that express both T-bet and RORγt (35). To test whether GATA3 also plays a role in T-bet-expressing Th17 cells, we first examined the kinetics of GATA3 expression by flow cytometry during *de novo* Th17 polarization *in vitro* (Figures 1A, B). As expected, naïve T cells expressed a low baseline level of GATA3. However, the expression of both GATA3 and RORγt was induced within 24 hours of culture. GATA3 expression was then gradually reduced to lower levels over the next 48 hours of culture. The dynamic expression of GATA3 in RORγt-expressing cells was further assessed using naïve T cells from the $Gata3^{ZsGreen}Rorc^{E2\text{-}Crimson}Foxp3^{RFP}$ triple reporter mice (Figures 1C, D). Again, GATA3-ZsGreen and RORγt-E2-Crimson were co-induced within 24 hours of Th17-polarizing culture conditions, and GATA3-ZsGreen expression was subsequently reduced back to a low level over the next 48 hours. We next examined the kinetics of GATA3 expression in differentiating Th17 cells *in vivo* using $MOG_{35\text{-}55}$/CFA immunized C57BL/6 mice (Figures 1E, F). In the unimmunized naïve C57BL/6 mice, few $ROR\gamma t^+$ Th17 cells were present within the lymph nodes and all of them were $GATA3^{low}$. On the fourth day post immunization, CD4 T cells in the draining lymph nodes began to co-express GATA3 and RORγt, however, by the sixth day post immunization, GATA3 expression within the $ROR\gamma t^+$ Th17 cells returned to a low state akin to naïve T cells. We also assessed MOG-antigen specific CD45.2 + 2D2 cells that were adoptively transferred to $CD45.1^+CD45.2^+$ host mice which were subsequently immunized with $MOG_{35\text{-}55}$/CFA (Figures 1G, H). As expected, in the unimmunized state, $CD45.2^+$ naïve 2D2 T cells retained a naïve phenotype and did not express RORγt or GATA3 within the naïve $CD45.1^+CD45.2^+$ hosts. However, the donor 2D2 T cells co-expressed GATA3 and RORγt on the fourth day post-immunization and the $ROR\gamma t^+$ Th17 cells downregulated GATA3 to a low state on the sixth day post-immunization. Taken together, these data demonstrate that GATA3 is dynamically regulated during a *de novo* Th17 cell differentiation both *in vitro* and *in vivo*.

Early expression of GATA3 is essential to generate pathogenic T-bet$^+$ Th17 cells and to induce EAE

To determine what effects early GATA3 expression might have on the development of a Th17 cell response *in vivo*, we utilized three complimentary *Gata3* conditional knockout mouse strains in the EAE model. First, we immunized the Cre-ERT2-$Gata3^{fl/fl}$ mice with MOG/CFA with or without tamoxifen pretreatment on day 0. CD4 T cells from the draining lymph nodes (dLNs) of these immunized mice were isolated on day 6 post immunization and then transferred into the $Tcra^{-/-}$ recipient mice. In this adoptive transfer EAE experiments, CD4 T effector cells from tamoxifen-

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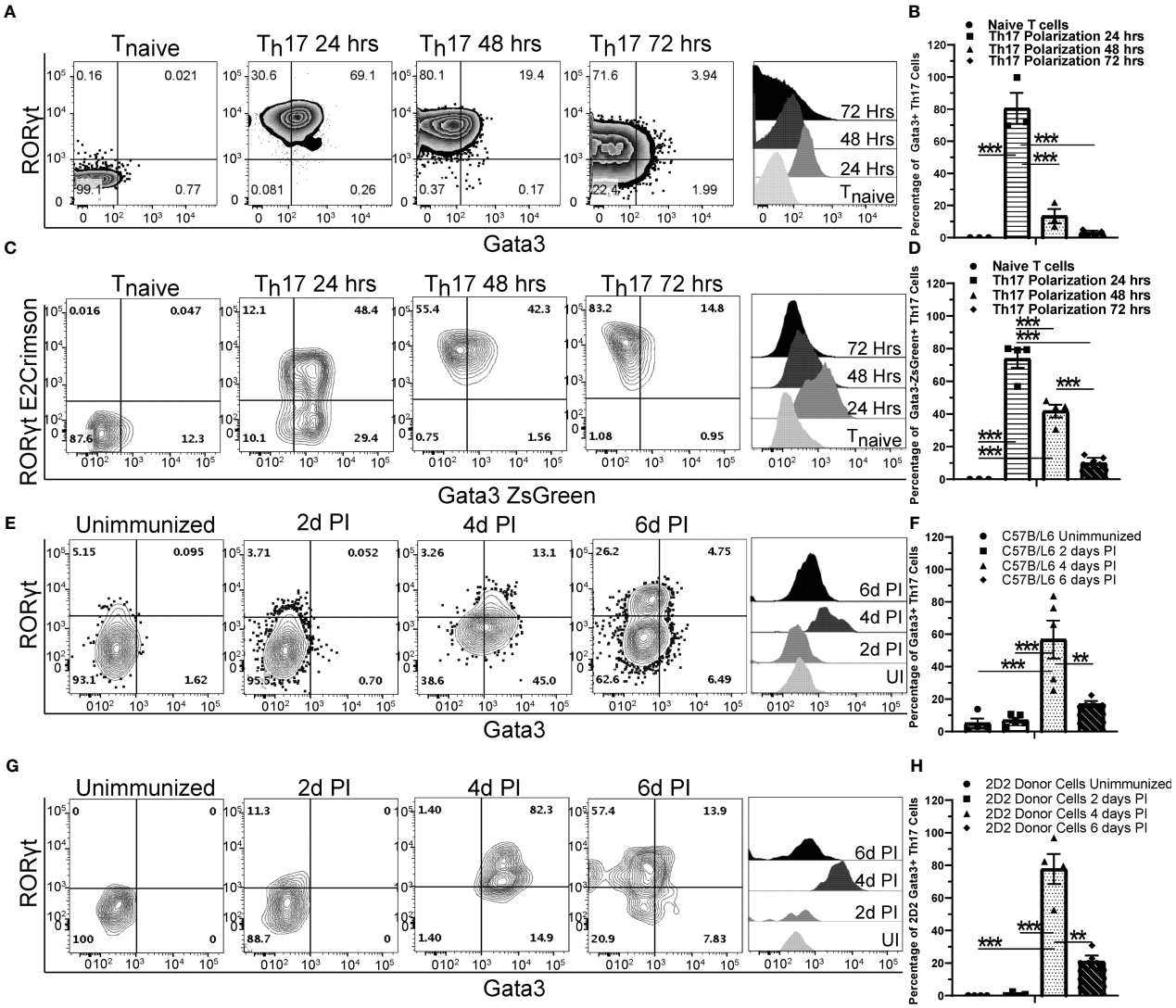

FIGURE 1
GATA3 is transiently induced during Th17 cell differentiation. **(A, B)** Naïve C57BL/6 CD4 T cells cultured under Th17 polarization conditions and monitored for GATA3 expression at 0, 24, 48, and 72 hr. **(A)** Representative FACS plots at the indicated timepoints depicting GATA3 and RORγt staining amongst $CD4^+CD44^+Foxp3^-$ T cells and representative GATA3 histograms within $CD4^+CD44^+Foxp3^-ROR\gamma t^+$ T cells. **(B)** Mean % of GATA3-expressing (i.e., $GATA3^+ROR\gamma t^+$ Th17) cells among $CD4^+CD44^+Foxp3^-ROR\gamma t^+$ populations in **(A)** from three independent experiments. **(C, D)** Representative RORγt and GATA3 reporter expression by $Rorc^{E2\text{-}Crimson}Gata3^{ZsGreen}Foxp3^{RFP}$ CD4 T cells cultured under Th17 polarization conditions as in **(A)**. **(D)** Mean % of GATA3-expressing (i.e., $GATA3^+ROR\gamma t^+$ Th17) cells among $CD4^+CD44^+Foxp3^-ROR\gamma t^+$ populations in **(C)** from two independent experiments. **(E–H)** The *in vivo* kinetics of GATA3 expression within draining lymph node (dLN) Th17 cells in response to $MOG_{35\text{-}55}$/CFA immunization. **(E, F)** Representative GATA3 and RORγt staining **(E)** within $CD4^+CD44^+Foxp3^-$ dLN T cells and the mean % of GATA3-expressing cells among $CD4^+CD44^+Foxp3^-ROR\gamma t^+$ T cells **(F)** from immunized C57BL/6 mice (n=5-8 mice/timepoint from three independent experiments). **(G, H)** Representative GATA3 and RORγt staining **(G)** within donor 2D2 $CD4^+CD44^+Foxp3^-$ dLN T cells and the mean % of GATA3-expressing cells among 2D2 $CD4^+CD44^+Foxp3^-ROR\gamma t^+$ T cells **(H)** from immunized 2D2 naïve T cell recipient *Cd45.1/Cd45.2* mice (n=4 mice/timepoint from two independent experiments). UI – unimmunized, PI – post immunization. For statistical comparisons, a one-way ANOVA was conducted with Tukey *Post-Hoc* testing for group comparisons. Significance levels are denoted as follows: **p <0.01; ***p <0.001.

pretreated Cre-ERT2-$Gata3^{fl/fl}$ dLNs were unable to elicit EAE symptoms in new $Tcra^{-/-}$ hosts, in comparison to CD4 effector cells from vehicle-treated Cre-ERT2-$Gata3^{fl/fl}$ dLNs (Figure 2A). To assess the effects of deleting *Gata3* on the development of 'non-pathogenic' or 'pathogenic' Th17 cells in MOG-immunized mice, we quantified the frequency of Th subsets that either expressed T-bet and RORγt (Figures 2B, C) or IFNγ and IL-17A (Figures 2D, E) in dLNs six days post immunization. Interestingly, tamoxifen pre-treated Cre-ERT2-$Gata3^{fl/fl}$ mice failed to generate a 'pathogenic' T-$bet^+ROR\gamma t^+$ Th17 cell response in comparison to vehicle-treated controls (Figures 2B, C); which corresponded with a failure to generate $IFN\gamma^+IL\text{-}17A^+$ Th17 cells (Figures 2D, E). However, since many other cell types, including ILCs, NK cells, NKT, and CD8 T cells, rely on GATA3 for their development and functionality, and recent publications have suggested that meningeal $NKp46^+$ ILCs help to regulate Th17 cell-mediated neuroinflammation in the CNS (46, 47), we were concerned that the failure to mount a T-$bet^+ROR\gamma t^+$ Th17 response might be reflective of the functions of

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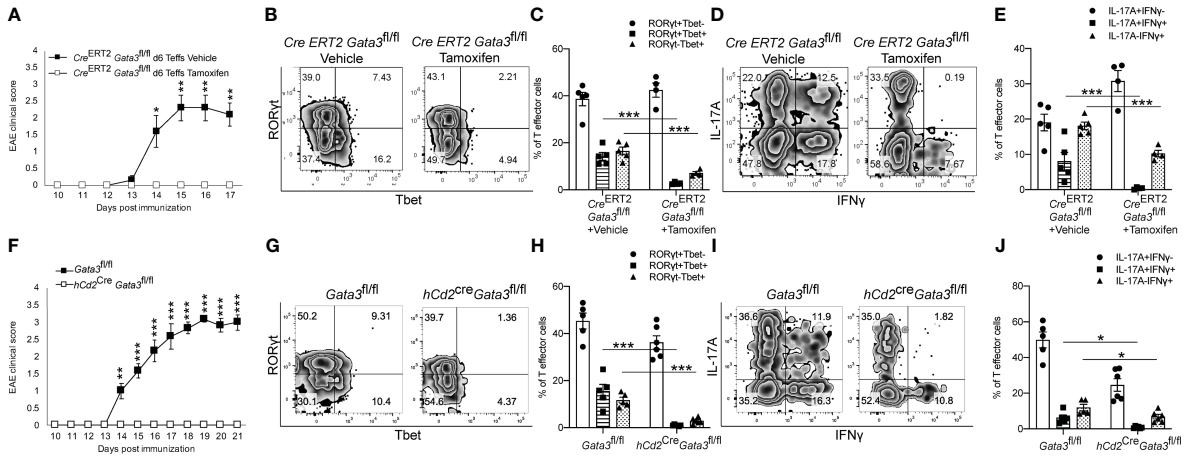

FIGURE 2
Early GATA3 expression is required to generate a *de novo* T-bet$^+$ Th17 cell response and EAE symptoms. **(A)** Mean EAE clinical scores from *Tcra*$^{-/-}$ hosts that received 4x10^6 CD4$^+$CD44$^+$ T cells harvested from the draining lymph nodes (dLNs) of Cre-ERT2-*Gata3*$^{fl/fl}$ mice 6 days after MOG/CFA immunization with vehicle or tamoxifen treatment (n=12 mice/group from three independent experiments). On the day of the cell transfer procedure, the *Tcra*$^{-/-}$ host mice were boosted with MOG$_{35-55}$/IFA and Pertussis Toxin as described in the methods. **(B, C)** Representative T-bet and RORγt staining amongst d0 Vehicle or Tamoxifen-treated Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD3$^+$CD4$^+$CD44$^+$Foxp3$^-$ T cells **(B)**, and the mean percentages of T-bet and RORγt expressing T cell subsets **(C)**. **(D, E)** Representative IFNγ and IL-17A staining amongst Vehicle or Tamoxifen treated Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD3$^+$CD4$^+$CD44$^+$Foxp3$^-$ T cells **(D)**, and the mean percentages of IFNγ and IL-17A expressing T cell subsets (**E**, n =5 mice/group from two independent experiments). **(F)** Mean EAE clinical scores from MOG$_{35-55}$/CFA immunized *Gata3*$^{fl/fl}$ and *hCd2*^{Cre}*Gata3*$^{fl/fl}$ mice. n=12 mice/group from three independent experiments. **(G, H)** Representative T-bet and RORγt staining amongst *Gata3*$^{fl/fl}$ and *hCd2*^{Cre}*Gata3*$^{fl/fl}$ d6 post-immunization dLN CD3$^+$CD4$^+$CD44$^+$Foxp3$^-$ T cells **(G)**, and the mean percentages of T-bet and RORγt expressing T cell subsets **(H)**. **(I, J)** Representative IFNγ and IL-17A staining amongst *Gata3*$^{fl/fl}$ and *hCd2*^{Cre}*Gata3*$^{fl/fl}$ d6 dLN CD3$^+$CD4$^+$CD44$^+$Foxp3$^-$ T cells **(I)**, and the mean percentages of IFNγ and IL-17A expressing T cell subsets **(J)**. n=6 mice/group from two independent experiments. For statistical comparisons, unpaired student's T tests were used. Significance levels are denoted as follows: *p <0.05; **p <0.01; ***p <0.001.

GATA3 in ILCs rather than a T cell intrinsic defect. To rule out the functions of GATA3 in non-T cells, we crossed *hCd2*^{Cre} mice with *Gata3*$^{fl/fl}$ mice to assess the effects of a complimentary mature T cell-restricted *Gata3* knockout (*hCd2*^{Cre}*Gata3*$^{fl/fl}$) on EAE induction (Figures 2F, J). Interestingly, *hCd2*^{Cre}*Gata3*$^{fl/fl}$ mice similarly failed to develop EAE in comparison to *Gata3*$^{fl/fl}$ controls (Figure 2F). In addition, *hCD2*^{Cre}Gata3$^{fl/fl}$ mice failed to generate a T-bet$^+$RORγt$^+$ Th17 cell response within the draining lymph nodes in comparison to *Gata3*$^{fl/fl}$ mice (Figures 2G, H); which also corresponded with a failure to generate IFNγ$^+$IL-17A$^+$ Th17 cells (Figures 2I, J). These data demonstrate that early GATA3 expression during *de novo* Th17 cell differentiation is required to generate a pathogenic T-bet$^+$RORγt$^+$ Th17 cell response.

Next, to determine whether GATA3 is required in pathogenic T-bet$^+$RORγt$^+$ Th17 cells, we crossed *Tbx21*^{Cre} mice with *Gata3*$^{fl/fl}$ mice to generate a *Gata3* conditional knockout mouse model with GATA3 deficiency only in T-bet-expressing/expressed cells (*Tbx21*^{Cre}*Gata3*$^{fl/fl}$). Again, *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ mice were resistant to developing EAE symptoms in comparison to *Gata3*$^{fl/fl}$ controls (Figure 3A). However, as T-bet-expressing NKp46$^+$ meningeal ILCs (46, 47) have been reported to play a critical role in regulating Th17-mediated neuroinflammation and GATA3 regulates the development and functionality of ILC1 and NK cells, we sought to test the role of GATA3 in T-bet$^+$RORγt$^+$ Th17 cells through the adoptive transfer model of EAE (Figure 3B). In transfer EAE experiments, CD4 effector cells harvested from dLNs of the *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ 6 days post immunization were only able to elicit mild EAE symptoms in comparison to *Gata3*$^{fl/fl}$ CD4 effector cells (Figure 3B), suggesting that the resistance to EAE conferred by the *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ conditional knockout is mediated by T-bet$^+$ T cells. *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ mice were unable to generate and/or maintain T-bet$^+$RORγt$^+$ Th17 cells within the draining lymph nodes of immunized mice (Figures 3C, D). In addition, as we observed before, *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ CD4 effector cells were also unable to generate IFNγ$^+$IL-17A$^+$ Th17 cells (Figures 3E, F). Together, all our results demonstrate that T-bet$^+$ Th17 cells require early GATA3 expression during *de novo* Th17 cell differentiation for their development and encephalitic functions in EAE.

GATA3 is essential for GM-CSF expression by pathogenic T-bet$^+$RORγt$^+$ Th17 cells

As we found that GATA3 is induced and subsequently downregulated to a low expression state during Th17 cell differentiation *in vivo*, and that early GATA3 expression is essential for the generation of T-bet$^+$RORγt$^+$ Th17 cells, we were curious as to what effects late post-differentiation maintenance levels of GATA3 might have on the pathogenicity of established Th17 cells in EAE. To examine how late maintenance levels of GATA3 might affect the pathogenicity of Th17 cells in EAE, we assessed the effects of a late *Gata3* deletion on the pathogenicity of Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 effector cells in transfer EAE experiments. We first compared the frequency of IFNγ and IL-17A positive CD4 effector cells within the d6 draining lymph nodes of *Cd45.1/Cd45.2*

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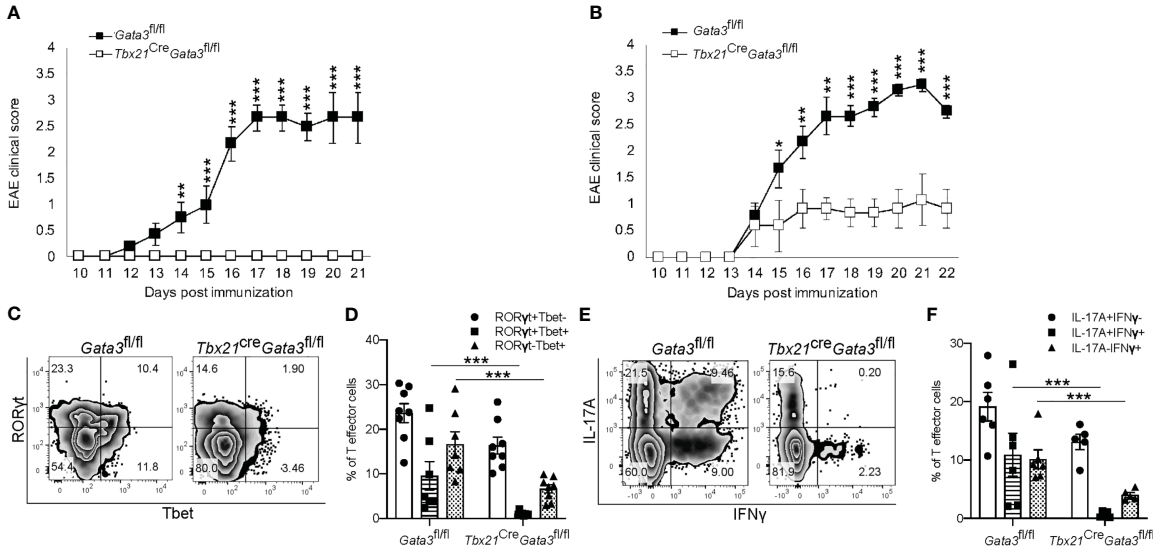

FIGURE 3
GATA3 expression is required for the development and/or maintenance of early T-bet$^+$ Th17 cells. **(A)** Mean EAE clinical scores from MOG_{35-55}/CFA immunized *Gata3*$^{fl/fl}$ and *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ mice. n=10 mice/group from three independent experiments. **(B)** Mean cell transfer EAE clinical scores from *Tcra*$^{-/-}$ mice that received d6 dLN *Gata3*$^{fl/fl}$ or *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ donor CD4$^+$CD44^{hi} T cells and immunized with MOG_{35-55}/IFA. n=10 mice/group from three independent experiments. **(C, D)** Representative T-bet and RORγt staining amongst *Gata3*$^{fl/fl}$ and *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ d6 post-immunization dLN CD3$^+$CD4$^+$CD44^{hi}Foxp3$^-$ T cells **(C)**. The mean percentages of T-bet- and RORγt-expressing CD4 T cell subsets within the d6 dLN **(D)**. **(E, F)** Representative IFNγ and IL-17A staining amongst *Gata3*$^{fl/fl}$ and *Tbx21*^{Cre}*Gata3*$^{fl/fl}$ d6 post-immunization dLN CD3$^+$CD4$^+$CD44^{hi}Foxp3$^-$ T cells **(E)**. The mean percentages of IFNγ and IL-17A positive dLN CD3$^+$CD4$^+$CD44^{hi}Foxp3$^-$ T cell subsets. n=6 mice/group from two independent experiments. For statistical comparisons, unpaired student's T tests were used. Significance levels are denoted as follows: *p <0.05; **p <0.01; ***p <0.001.

and Cre-ERT2-*Gata3*$^{fl/fl}$ mice (Figures 4A, B). As expected, the frequencies of IFNγ and IL-17A positive CD4 effector cells were similar amongst *Gata3*-sufficient *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ mice, confirming that IFNγ$^+$IL-17A$^+$ Th17 cells were efficiently generated before deletion of *Gata3*. To assess the effects of a late *Gata3* deletion on the pathogenicity of Cre-ERT2-*Gata3*$^{fl/fl}$ effector cells, d6 dLN CD4 effector cells were transferred to *Tcra*$^{-/-}$ recipients, which were subsequently treated with corn oil (vehicle) or Tamoxifen on post transfer day 0 (Figure 4C). Interestingly, Tamoxifen-treated Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 effector cells *Tcra*$^{-/-}$ recipients were resistant to transfer EAE in comparison to vehicle treated controls (Figure 4C), indicating that late maintenance levels of GATA3 are also required for Th17-mediated encephalomyelitis. To determine how a late *Gata3* deletion might affect the frequency of IFNγ- and IL-17A-producing CD4 effector cells, we phenotyped donor *Gata3*-sufficient and deficient effector cells from transfer EAE recipient mice. In contrast to the early *Gata3* deletion model in which *Gata3*-deficient cells were unable to generate T-bet$^+$RORγt$^+$ Th17 cells, deleting *Gata3* at the post-differentiation stage did not affect the frequency of IFNγ or IL-17A positive CD4 effector cells in the CNS (Figures 4D, E) nor in the spleen (Figures 4F, G). Instead, a late *Gata3*-deletion resulted in a substantial reduction in GM-CSF-producing Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 effector cells in the CNS (Figures 4H, I) and the spleen (Figures 4J, K). As prior work has demonstrated that GM-CSF is an effector cytokine critically required for the recruitment and activation of CNS mononuclear cells and for EAE induction (22, 23, 48), the inability of late tamoxifen-treated Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 effector cells to produce GM-CSF likely explains why these transferred CD4 effector cells were unable to induce EAE. However, as the cytokines IL-23 and IL-1β are required for GM-CSF induction (48), we were concerned that the effects of late GATA3^{low} expression on GM-CSF production might be reflective of a less inflammatory environment rather than a cell intrinsic effect. Thus, to determine if a late deletion of *Gata3* affects GM-CSF production in a cell intrinsic manner, we conducted mixed congenic co-transfer EAE experiments. In brief, *Cd45.1/Cd45.2* and *Cd45.2* Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD4$^+$ T effector cells were collected from MOG_{35-55}/CFA immunized donor mice, mixed at a 1:1 ratio, and then transferred to vehicle- or tamoxifen-treated *Tcra*$^{-/-}$ recipients. We first assessed the relative percentages of *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ donor cells pre-transfer (Figures 5A, B) and post-transfer in the vehicle- or tamoxifen-treated *Tcra*$^{-/-}$ recipients' CNS (Figures 5C, D) and spleen (Figure 5E). *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 effector cells were equally present in the starting population (Figures 5A, B) and in the vehicle- and tamoxifen-treated *Tcra*$^{-/-}$ recipient spleens following the transfer (Figure 5E). However, we detected a slight reduction in the frequency of CNS-infiltrating Cre-ERT2-*Gata3*$^{fl/fl}$ effector cells within the tamoxifen-treated *Tcra*$^{-/-}$ mice (Figures 5C, D) *vs* vehicle control recipients, suggesting that a late *Gata3* deletion confers a slight cell-intrinsic disadvantage to *Gata3*-deficient Cre-ERT2-*Gata3*$^{fl/fl}$ effector cells in comparison to *Gata3*-sufficient effector cells (Figures 5C, D). We next assessed the phenotypes of the donor cells in the CNS (Figures 5F–I). As in our single population transfer EAE experiments (Figure 4), we observed a similar distribution of IFNγ and IL-17A positive *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 effector cells in the vehicle-treated

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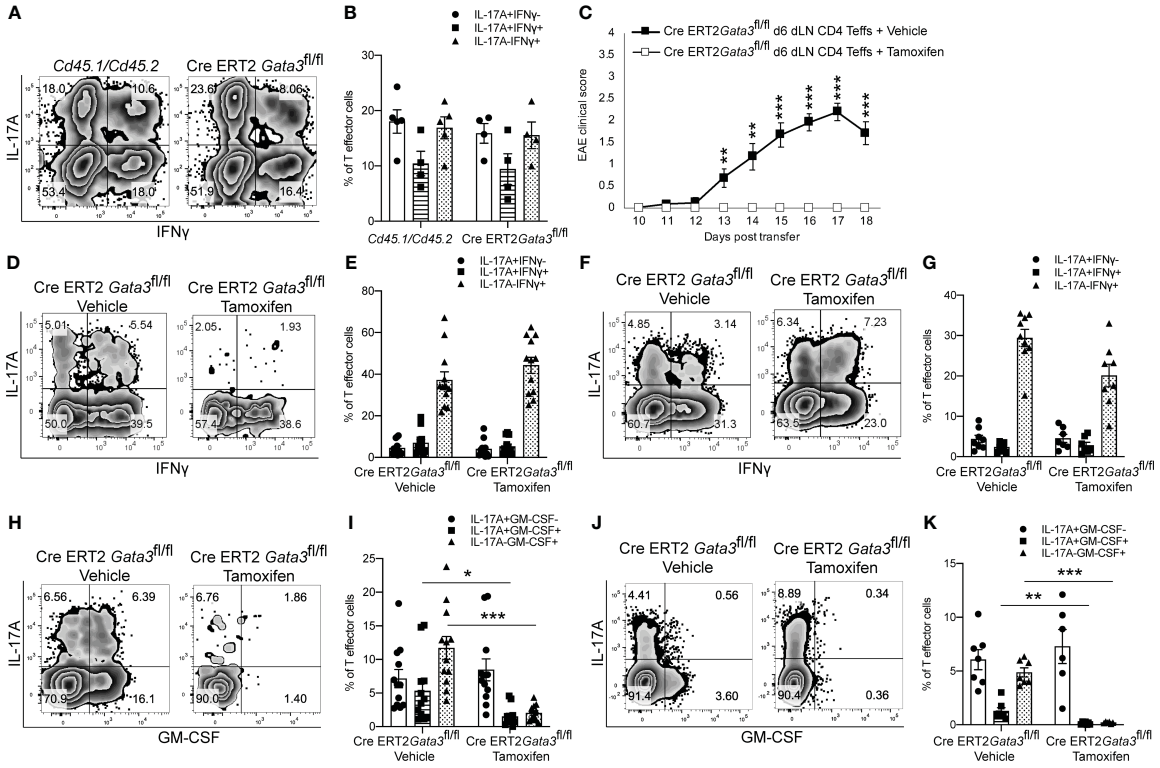

FIGURE 4
GATA3 is required for GM-CSF production and CD4 T cell-mediated encephalomyelitis. **(A, B)** Characterization of the pre-transfer d6 dLN CD3$^+$CD4$^+$CD44^{hi} *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ populations. **(A)** Representative IFNγ vs IL-17A staining and **(B)** the mean percentages of IFNγ and IL-17A CD4$^+$CD44^{hi}Foxp3$^-$ T cell subpopulations in the pre-transfer isolates. **(C)** Mean transfer EAE clinical scores from *Tcra*$^{-/-}$ hosts that received 4x10^6 Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD4$^+$CD44^{hi} T cells **(A, B)** and a vehicle or tamoxifen treatment. n=15 mice/group from four independent experiments. (D-G; H-K) Characterizations of the Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 T cells within vehicle or tamoxifen treated *Tcra*$^{-/-}$ hosts post-transfer. Representative IFNγ and IL-17A staining of vehicle or tamoxifen treated Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 T cells in the *Tcra*$^{-/-}$ host CNS **(D)** and the spleen **(F)**. The mean percentages of IFNγ and IL-17A subpopulations within the CNS **(E)** and spleen **(G)**. Representative GM-CSF and IL-17A staining of vehicle or tamoxifen treated Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 T cells within the *Tcra*$^{-/-}$ host CNS **(H)** and the spleen **(J)**. The mean percentages of IL-17A and GM-CSF positive donor CD4 T effector cells within the CNS **(I)** and the spleen **(K)**. n=12 recipient mice/group from four independent experiments. For statistical comparisons, unpaired student's T tests were used. Significance levels are denoted as follows: *p <0.05; **p <0.01; ***p <0.001.

and tamoxifen-treated *Tcra*$^{-/-}$ recipients. However, when we assessed *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 T effector cells for their ability to produce GM-CSF, we noticed that GM-CSF staining was dramatically reduced in a cell-intrinsic manner in the tamoxifen-induced *Gata3*-knockout Cre-ERT2-*Gata3*$^{fl/fl}$ effector cells (Figures 5H, I). Thus, these data together suggest that a late *Gata3* deletion does not affect the maintenance, stability, or ability of Th17 cells to generate T-bet$^+$ Th17 cells; instead, encephalitic CD4 T cells intrinsically require low levels of GATA3 to efficiently produce GM-CSF.

GATA3 is required for normal expression of *Bhlhe40*, *Egr2* and *Csf2*

To gain insight as to how late expression of GATA3 might regulate GM-CSF within established CD4 effector cells, we compared the transcriptomes of CNS-infiltrating *Gata3*-sufficient and *Gata3*-deficient CD4 effector cells in the mixed *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ co-transfer EAE model (Figure 6) at the peak of EAE. Comparison of gene expression between CNS-infiltrating *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ CD4$^+$CD44^{hi} T effector cells that were isolated from the same vehicle-treated *Tcra*$^{-/-}$ recipients did not reveal much differentially regulated genes. On the other hand, by comparing the transcriptomes of CNS-infiltrating *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ CD4$^+$CD44^{hi} T effector cells isolated from tamoxifen-treated *Tcra*$^{-/-}$ recipients at the peak of EAE, we identified 97 differentially expressed genes, of which 72 were significantly down-regulated in *Gata3*-deficient effector cells. Genes associated with Th1-related responses including *Ifng, Il2, Penk, Ccl1*, and *Il18r1*, genes associated with T cell-B cell signaling including *Cd40lg*, *Tnfsf11*, and *Tnfsf14*, and *Csf2* expression, consistent with our results above (Figures 4, 5), were downregulated (Figure 6). Interestingly, we also detected altered expression of several transcription regulators, including down-regulated expression of *Bhlhe40* and *Egr2*, and up-regulated expression of *Vhl* in *Gata3*-deficient Cre-ERT2-*Gata3*$^{fl/fl}$ vs *Gata3*-sufficient *Cd45.1/Cd45.2* T effector cells. *Vhl*, *Egr2*, and *Bhlhe40* are of note as *Vhl* is an important regulator of the HIF1a hypoxic-response pathway in T cells (49) and *Vhl* has been implicated as a potential upstream regulator of *Bhlhe40* (*Stra13*) in human RCC4 cells (50). In addition, Miao and colleagues have

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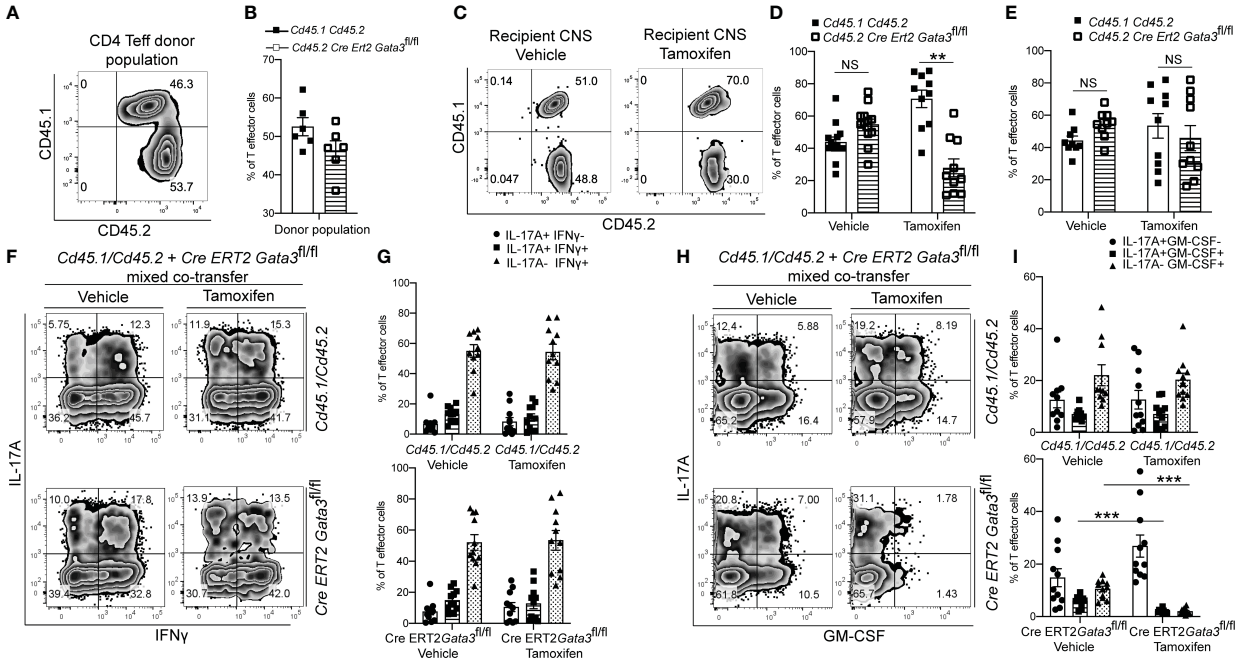

FIGURE 5
The post-differentiation effects of GATA3 on GM-CSF production is cell intrinsic. Mixed co-transfers of CD45.1$^+$CD45.2$^+$ C57BL/6 and CD45.2$^+$ Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD4$^+$ T effector cells were conducted. **(A, B)** The starting ratios of FACS-sorted donor *Cd45.1/Cd45.2* C57BL/6 and *Cd45.2* Cre-ERT2-*Gata3*$^{fl/fl}$ d6 dLN CD4 T effector cells shown as a representative FACS plot **(A)** and population means **(B)**. **(C-E)** The post-transfer ratios of donor CD45.1$^+$CD45.2$^+$ C57BL/6 and CD45.2$^+$ Cre-ERT2-*Gata3*$^{fl/fl}$ cells within host *Tcra*$^{-/-}$ mice treated with corn oil (vehicle) or tamoxifen. **(C)** Representative CD45.2 and CD45.1 staining within the CNS. The mean percentages of corn oil or tamoxifen treated CD45.1$^+$CD45.2$^+$ C57BL/6 and CD45.2$^+$ Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 T cells within the CNS **(D)** and spleen **(E)**. **(F, G)** Representative post-transfer corn oil or tamoxifen treated CD45.1/CD45.2 and Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 T effector IFNγ and IL-17A staining **(F)** and subpopulation means **(G)** within the CNS of *Tcra*$^{-/-}$ recipient mice. **(H, I)** Representative post-transfer donor *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ donor CD4 T effector GM-CSF and IL-17A staining **(H)** and subpopulation means **(I)** within the CNS of *Tcra*$^{-/-}$ recipient mice. n=12 mice/group from three independent experiments. For statistical comparisons, unpaired student's T tests were used. Significance levels are denoted as follows: *p <0.05; **p <0.01; ***p <0.001.

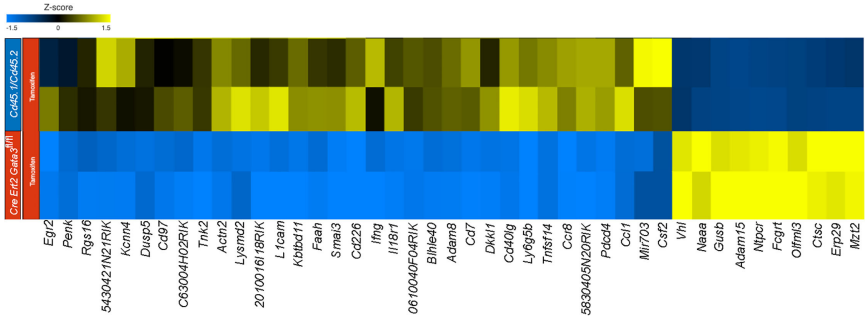

FIGURE 6
GATA3 regulates pro-inflammatory gene expression in EAE. RNA-Seq analysis was performed using CNS-infiltrating CD4$^+$CD44^{hi}CD25$^-$ CD45.1$^+$CD45.2$^+$ C57BL/6 and CD45.1$^-$CD45.2$^+$ Cre-ERT2-*Gata3*$^{fl/fl}$ donor T effector cells from tamoxifen-treated *Tcra*$^{-/-}$ co-transfer EAE mice at the peak. Differentially expressed genes were identified using Partek Flow Genomic Suite (Partek) and curated based on an FDR threshold of <0.05. Differentially expressed genes were clustered and displayed as a heatmap. The results are representative of biological duplicates.

shown that Egr2 can bind to the *Bhlhe40* locus within CD4 T cells in ChIP experiments (51), and *Bhlhe40* has been shown to directly regulate *Csf2* expression in knockout and ChIP experiments (31, 52).

We further confirmed the regulation of *Egr2 and Bhlhe40* expression by GATA3 at the protein level by flow cytometry. In agreement with the RNA-Seq results, CNS-infiltrating *Gata3*-sufficient *Cd45.1/Cd45.2* CD4$^+$ T effector cells expressed Egr2, while the late *Gata3*-knockout Cre-ERT2-*Gata3*$^{fl/fl}$ CD4$^+$ effector cells expressed less Egr2 (Figures 7A, B). Bhlhe40 expression followed a similar pattern, with CNS-infiltrating *Cd45.1/Cd45.2* CD4 effector cells expressing higher levels of Bhlhe40 than the late *Gata3*-knockout CD4 effector cells (Figures 7C, D). Interestingly, in agreement with our prior observations that

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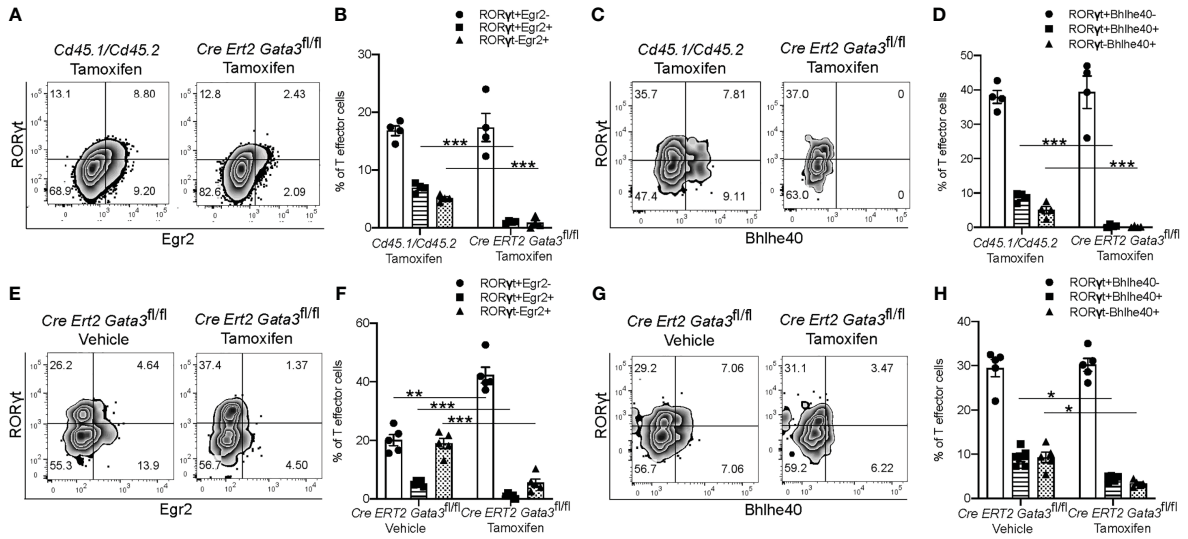

FIGURE 7
GATA3 is required for normal Bhlhe40 and Egr2 expression at all stages. **(A–D)** The expression of Egr2 and Bhlhe40 within *Gata3*-sufficient (*Cd45.1/Cd45.2*) and *Gata3*-deficient (Cre-ERT2-*Gata3*$^{fl/fl}$) CNS-infiltrating CD3^{+}CD4^{+}CD44^{hi}Foxp3^{-} T effector cells from co-transfer EAE *Tcra*$^{-/-}$ recipient mice treated with tamoxifen (cell transfer d0). **(A)** Representative Egr2 and RORγt staining and summary statistics **(B)** amongst tamoxifen treated CNS-infiltrating CD4^{+}CD44^{hi}Foxp3^{-} *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ cells. **(C)** Representative Bhlhe40 and RORγt staining and summary statistics **(D)** amongst tamoxifen treated CD4^{+}CD44^{hi}Foxp3^{-} *Cd45.1/Cd45.2* and Cre-ERT2-*Gata3*$^{fl/fl}$ cells. n=4 mice/condition from two independent experiments. **(E–H)** The expression of Egr2 and Bhlhe40 within *Gata3*-sufficient (Vehicle, d0) or *Gata3*-deficient (Tamoxifen, d0) day 6 dLN CD3^{+}CD4^{+}CD44^{hi}Foxp3^{-} T effector cells from MOG$_{35-55}$/CFA-immunized Cre-ERT2-*Gata3*$^{fl/fl}$ mice. **(E)** Representative Egr2 and RORγt staining and the corresponding summary statistics **(F)** from vehicle control or tamoxifen treated d6 dLN Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 T effector cells. **(G)** Representative d6 dLN CD4 T effector Bhlhe40 and RORγt staining and the corresponding summary statistics **(H)** from immunized and vehicle or tamoxifen treated Cre-ERT2-*Gata3*$^{fl/fl}$ mice. n=4 mice/condition from two independent experiments. For statistical comparisons, unpaired student's T tests were used. Significance levels are denoted as follows: *p <0.05; **p <0.01; ***p <0.001.

GATA3 expression is not restricted to RORγt^{+}T-bet^{+} 'pathogenic' Th17 cells, and that the percentage of IFNγ^{+}IL-17A^{+} Th17 and IFNγ^{-}IL-17A^{+} Th17 cells were unaffected in late *Gata3*-knockout effector cells, the expression of Egr2 and Bhlhe40 were not restricted to RORγt^{+} cells. Instead, both RORγt^{+} Th17 and RORγt^{-} CD4 effector cells were able to express Egr2, Bhlhe40, and GM-CSF, suggesting that the GATA3-dependent expression of Egr2 and Bhlhe40 is not Th17 cell specific. To determine if early GATA3 might also affect the expression of Egr2 and Bhlhe40 in differentiating T cells, we revisited our early tamoxifen-inducible Cre-ERT2-*Gata3*$^{fl/fl}$ d6 draining lymph node *Gata3* deletion model. In the d6 draining lymph node, *Gata3*-sufficient vehicle control Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 effector cells, including both RORγt^{+} Th17 and RORγt^{-} T cells, expressed Egr2 (Figures 7E, F) and Bhlhe40 (Figures 7G, H). On the other hand, tamoxifen-treated *Gata3*-knockout Cre-ERT2-*Gata3*$^{fl/fl}$ CD4 effector cells were largely Egr2 and Bhlhe40 negative (Figures 7E–H).

To gain insight as to how GATA3 might regulate *Bhlhe40* and thereby *Csf2* expression, we analyzed our RNA-Seq results in an Ingenuity Pathway Analysis (Figure 8). Based on this analysis, there are several ways in which TCR signaling-dependent GATA3 expression via Crebbp, Fos, Myc, Cebpa, and/or STAT6 might help induce *Egr2*. Egr2, or possibly GATA3 itself, may help to directly induce *Bhlhe40*, and Bhlhe40 in turn regulates *Csf2* expression resulting in GM-CSF-dependent encephalomyelitis in EAE.

Discussion

GATA3 is the master transcription factor for Th2 cell differentiation and ILC2 development (53). It also plays an important role during T cell and ILC development at multiple stages (37, 54). In fact, GATA3 is expressed by all T cell and ILC subsets albeit at different levels (54, 55). We have previously reported that GATA3 regulates the development of NKp46^{+} ILC3s that express both RORγt and T-bet (35). Furthermore, it regulates the expression of IL-22 in ILC3s. In the present study, we found surprising new regulatory roles for GATA3 in regulating Th17 responses in autoimmune neuroinflammation. GATA3 expression is induced during *de novo* Th17 differentiation both *in vitro* and *in vivo*. Consistent with the previous finding that GATA3 regulates the development of T-bet/RORγt co-expressing ILC3s, it also regulates the differentiation of T-bet/RORγt co-expressing Th17 cells. Furthermore, continuous expression of GATA3 is required for GM-CSF expression in EAE.

In terms of the regulation of GATA3 expression, it is known that T cell receptor activation induces initial GATA3 expression *in vitro* under Th2 polarizing conditions, and GATA3 can help to enforce the Th2-program via a positive reinforcement loop involving autocrine IL-4 production (56, 57). Co-expression of RORγt and GATA3 may be explained by the induction of GATA3 within developing Th17 cells by IL-4 from a secondary cellular source. However, as our *in vitro* Th17 polarization

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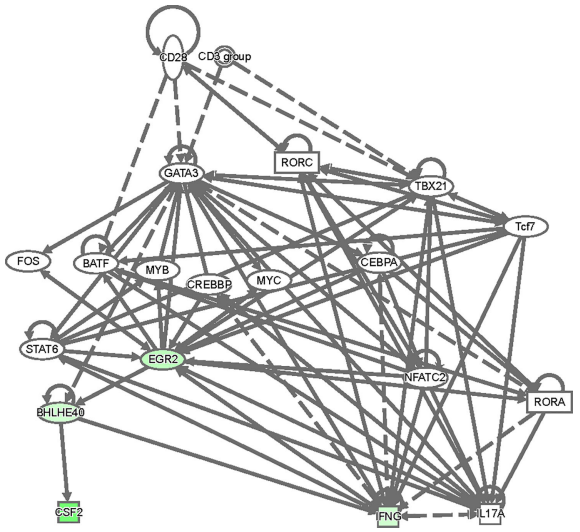

FIGURE 8
A model of regulatory network involving GATA3, Egr2, Bhlhe40 and Csf2. The top differentially expressed genes between $CD4^+CD44^{hi}CD25^-CD45.1^+CD45.2^+$ C57BL/6 and $CD45.1^-CD45.2^+$ Cre-ERT2-*Gata3*$^{fl/fl}$ donor T effector cells in Figure 6 were used in an ingenuity pathway analysis to visualize regulatory connections between GATA3, Egr2, Bhlhe40 and Csf2. Downregulated genes from the dataset were overlaid (green).

conditions included an anti-IL-4 neutralizing antibody, TCR-driven expression of GATA3 in the absence of IL-4 would be the most likely explanation for the transient induction of GATA3 within developing Th17 cells.

We used three complimentary *Gata3* deletion models (Cre-ERT2-*Gata3*$^{fl/fl}$, *hCd2*^{Cre}*Gata3*$^{fl/fl}$ and *Tbx21*^{Cre}*Gata3*$^{fl/fl}$) to study the functions of an early GATA3 induction within nascent Th17 cells in EAE. These different models essentially yielded similar results: all three mouse strains were unable to develop notable EAE symptoms correlated with an inability to generate encephalitogenic T-bet$^+$ Th17 cells within the draining lymph node or CNS following an immunization with MOG_{35-55}. These results are noteworthy for several reasons. Firstly, despite the observation that RORγt and GATA3 are co-induced during Th17 differentiation, T-bet$^-$RORγt$^+$ Th17 cells were still able to develop with or without functional GATA3 protein; suggesting that Th17 cells do not intrinsically require GATA3 for their development, an observation that we have recently reported (38). Secondly, per these data, GATA3 is necessary for the development of encephalitogenic T-bet$^+$ Th17 cells, presumably from T-bet$^-$ Th17 cells. Thirdly, the deletion of *Gata3* did not result in an increase in IFNγ-production or T-bet$^+$RORγt$^-$ Th1 cells within the draining lymph nodes or CNS. These results were surprising as prior work *in vitro* has demonstrated that GATA3 actively represses Runx3 protein-regulated production of IFNγ within *in vitro* polarized Th2 cells (58) and T-bet and Runx protein are required for the development of pathogenic IFNγ-producing Th17 cells (59). Since *de novo* T-bet$^+$ Th17 cells failed to develop in our *Gata3*-conditional knockout models, determining which genes are responsible for the observed phenotypes proved to be technically challenging and remains an open question. It is likely that GATA3 regulates the balance between RORγt and T-bet during the differentiation of T-bet$^+$ Th17 cells as it does during the development of NKp46$^+$ ILC3s.

Once GATA3 has been induced and subsequently downregulated, mature Th17 cells express low levels of GATA3. However, low levels of GATA3 expression are still required for eliciting EAE symptoms. In contrast to the effects of an early *Gata3* deletion on T cell priming, a late post-developmental deletion of *Gata3* did not affect the relative proportions of IFNγ$^+$IL-17A$^+$ or T-bet$^+$RORγt$^+$ 'pathogenic' Th17 cells in the CNS or periphery, but still prevented the development of encephalomyelitis symptoms. The presence of T-bet$^+$RORγt$^+$ 'pathogenic' Th17 cells allowed us to study gene regulation mediated by GATA3. Strikingly, this late *Gata3* deletion resulted in a defect in the production of GM-CSF, which has regarded as a pro-encephalomyelitic cytokine that is secreted by encephalitic Th17 and Th1 cells in the CNS (22–24). This effect is cell intrinsic as demonstrated by mixed congenic transfer EAE experiments. Transcriptomic analyses of *Gata3*-sufficient and *Gata3*-deficient (late *Gata3* deletion by tamoxifen) CNS-infiltrating CD4 effector cells from our mixed congenic transfer EAE model revealed stark reductions in the expression of *Bhlhe40* and *Egr2*, and enhanced expression of *Vhl* within late-*Gata3*-deficient CD4 effector cells. These results are noteworthy as in EAE experiments, CNS-infiltrating *Bhlhe40*$^{-/-}$ CD4 T cells are virtually unable to produce GM-CSF; Lin and colleagues have demonstrated that Bhlhe40 can directly regulate *Csf2* expression (31, 52). Like GATA3 (56, 57), *Bhlhe40* has been reported to be induced in response to TCR stimulation (31, 32); and Bhlhe40 and GM-CSF reporter mice have demonstrated that both are strongly expressed by CNS-infiltrating T cells in comparison to CD4 effector cells in the periphery (48, 52). These results suggest that TCR-dependent maintenance of GATA3 expression may help to regulate *Bhlhe40* and *Csf2* expression within the CNS. In addition, Vhl is an important regulator of the HIF1a hypoxic-response pathway in T cells (49) and has been implicated as a potential upstream regulator of *Bhlhe40* (*Stra13*) in human RCC4 cells (50). Lastly, Miao and colleagues have demonstrated that Egr2 can bind to the *Bhlhe40* locus within CD4 T cells in ChIP experiments (51). Since both Bhlhe40 and Egr2 are also regulated by early TCR signaling, it is likely that GATA3 is required for the maintenance of Bhlhe40 and Egr2 expression within the CNS which leads to GM-CSF production by encephalitic Th17 cells. While the results presented here highlight a novel role for GATA3 in regulating GM-CSF production, the results are limited in that it is unclear if the effects of GATA3 on GM-CSF are direct, indirect via regulation of Egr2 and Bhlhe40, or a mixture thereof. Additional studies will be needed to determine the exact regulatory mechanisms though which GATA3 affects T cell intrinsic GM-CSF production.

Altogether, our data demonstrate that dynamic GATA3 expression during Th17 cell differentiation is required for Th17-mediated encephalomyelitis in EAE. An early deletion of GATA3 during Th17 cell differentiation blocked the development of 'pathogenic' T-bet$^+$RORγt$^+$ Th17 cells, however, a late deletion of GATA3 at the established T effector stage allowed the presence of T-bet$^+$RORγt$^+$ Th17 cells. Nevertheless, GATA3 is still critically required for encephalomyelitis, which is associated with a reduction in the expression of GM-CSF and its regulators. Thus,

our study highlights a novel role for GATA3 in promoting the pathogenicity of T-bet$^+$ Th17 cells in EAE, via putative regulation of Egr2, Bhlhe40, and GM-CSF expression.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GSE227394 (GEO), https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227394.

Ethics statement

The animal study was reviewed and approved by National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee.

Author contributions

JZ conceived the project. MB performed most of the experiments. RG and XZ performed some *in vitro* and *in vivo* experiments. XC contributed to the RNA-Seq experiments. GH and MB performed bioinformatic analysis. VL and KZ made intellectual contributions and edited the manuscript. MB and JZ wrote the manuscript. JZ supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Li Wu,
Tsinghua University, China

REVIEWED BY
Fengyang Chen,
Hangzhou Medical College, China
Ganesan Ramamoorthi,
Moffitt Cancer Center, United States

*CORRESPONDENCE
Adrian A. Achuthan
aaa@unimelb.edu.au

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Targeting transcription factors for therapeutic benefit in rheumatoid arthritis

Thivya Balendran[1], Keith Lim[2], John A. Hamilton[1] and Adrian A. Achuthan[1*]

[1]Department of Medicine, Royal Melbourne Hospital, The University of Melbourne, Parkville, VIC, Australia, [2]Department of Medicine, Western Health, The University of Melbourne, St Albans, VIC, Australia

Rheumatoid arthritis (RA) is a destructive inflammatory autoimmune disease that causes pain and disability. Many of the currently available drugs for treating RA patients are aimed at halting the progression of the disease and alleviating inflammation. Further, some of these treatment options have drawbacks, including disease recurrence and adverse effects due to long-term use. These inefficiencies have created a need for a different approach to treating RA. Recently, the focus has shifted to direct targeting of transcription factors (TFs), as they play a vital role in the pathogenesis of RA, activating key cytokines, chemokines, adhesion molecules, and enzymes. In light of this, synthetic drugs and natural compounds are being explored to target key TFs or their signaling pathways in RA. This review discusses the role of four key TFs in inflammation, namely NF-κB, STATs, AP-1 and IRFs, and their potential for being targeted to treat RA.

KEYWORDS

transcription factors, rheumatoid arthritis, cytokines, NF-κB, AP-1, STAT and IRF

1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that mostly affects joints. Joint inflammation is initiated and maintained by a complex interaction between many cells, including T cells, dendritic cells, B cells, macrophages, neutrophils, osteoclasts, and fibroblast-like synoviocytes (FLS) (1). These cells can release pro-inflammatory cytokines, chemokines, reactive oxidative species, matrix metalloproteinases (MMPs) and autoantibodies into synovial joints and thus contribute inflammation, cartilage damage, osteoclast activation, and bone destruction (2–4).

Many pro-inflammatory mediators have been implicated in the pathogenesis of RA (5). For example, tumor necrosis factor (TNF), interferons (IFNs), interleukin (IL) -1β, IL-2, IL-4, IL-6, IL-8, IL-17, IL-18, IL-21, IL-22, IL-23 and granulocyte macrophage-colony stimulating factor (GM-CSF) have been suggested to play a central role in RA pathogenesis (6–8). These cytokines activate key transcription factors (TFs), such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), interferon regulatory factors (IRFs), and signal

transducer and activator of transcription (STAT) proteins, which can further promote the production of pro-inflammatory mediators (9). Therefore, targeting these key TFs or the signaling pathways associated with these TFs is a feasible strategy for treating RA. While several synthetic drugs are currently being trialed aimed at targeting key TFs in RA, several natural compounds have also been explored as potential alternative treatment options with a focus on targeting TFs. In this review, we summarize the role of four families of TFs, namely NF-κB, STATs, AP-1 and IRFs, in the pathogenesis of RA, and provide an update on the latest preclinical and clinical trials targeting them.

2 NF-κB

2.1 NF-κB signaling pathway

The NF-κB signaling pathway controls many biological processes, but its dysregulation is often associated with inflammation, for example, that associated with RA. Activated NF-κB is observed in RA synovium in early and late stages of joint inflammation and initiation of inflammation is triggered by NF-κB activation in both T cells and antigen presenting cells (10). Different extracellular and/or intracellular stimuli (e.g., TNF, IL-1β, IL-6, MMPs and RANKL) can activate the NF-κB signaling pathway, either directly or indirectly (11). The NF-κB family is composed of five structurally related members that include NF-κB1/p50 (precursor p105), NF-κB2/p52 (precursor p100), RelA/p65, RelB, and c-Rel which bind to specific DNA and κB enhancer elements that mediate the transcription of target genes (12, 13). NF-κB is activated via two different pathways. Direct activation includes canonical and non-canonical pathways, mediated by inhibitor of kappa B (IκB) kinase (IKK) and NF-κB-inducing kinase (NIK), respectively. The indirect activation of NF-κB is interconnected with other cellular pathways, including mitogen-activated protein kinase (MAPK), Rho, and phosphoinositide 3-kinase (PI3-K) (11).

2.1.1 Canonical pathway

In inflammatory conditions, such as in RA, cytokines, chemokines and free radicals provide signals that lead to degradation of IκB protein resulting in the disassociation of NF-κB (12, 13) (Figure 1). Activation of the canonical pathway occurs

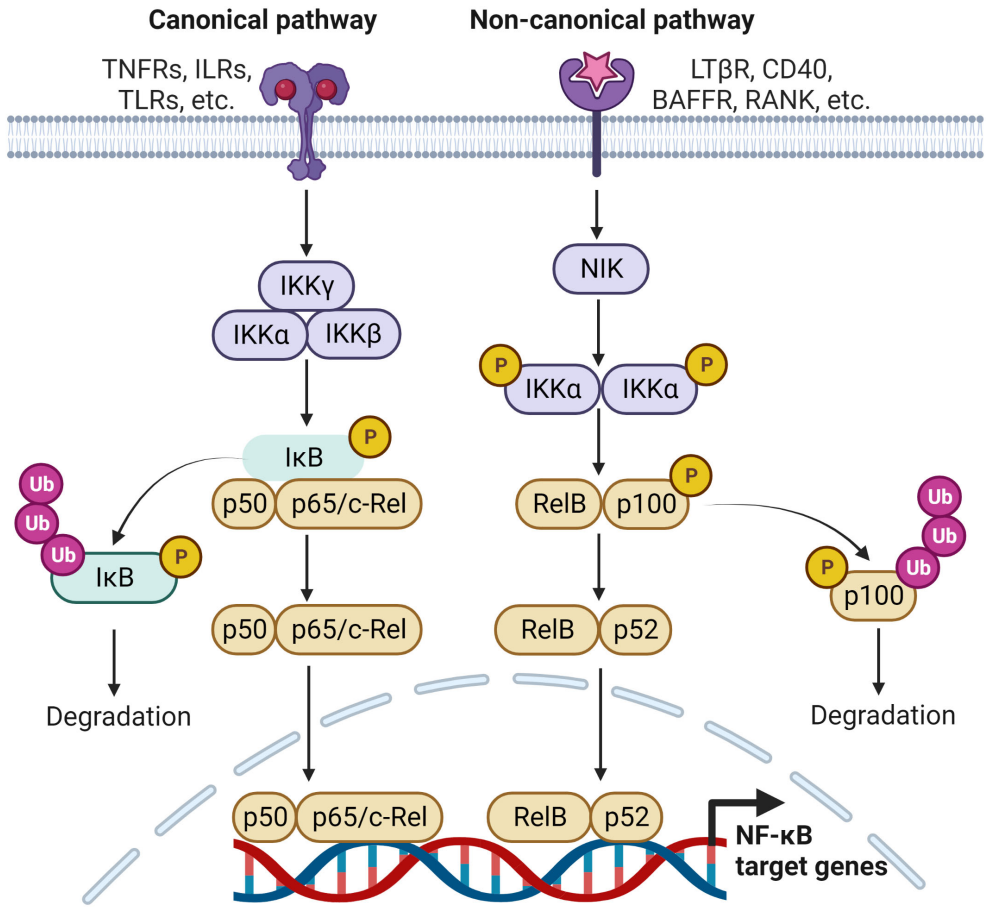

FIGURE 1
Signaling pathways leading to the regulation of NF-κB target genes. The activation of NF-κB involves two signaling pathways, the canonical and non-canonical pathways. Both are activated through engagement with distinct receptors, leading to transcriptional regulation several NF-κB target genes that are responsible for immune and inflammatory responses.

through stimulation of the TNF receptors, Toll-like receptors (TLRs), interleukin receptors, pattern recognition receptors (PRRs), T cell receptors (TCRs) and B cell receptors (BCRs) (14, 15). The canonical pathway has an IKK complex, comprising IKKα and IKKβ, the homologous catalytic subunits, and IKKγ, a regulatory subunit of the complex that activates IKKβ (14). Receptor activation stimulates numerous kinases, such as transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), receptor-interacting protein kinase 1 (RIP1), MAPK kinase ERK1 (MEKK1) and TANK-binding kinase (TBK1), which phosphorylate IKKβ and activate the IKK complex (16). Activated IKKβ then phosphorylates IκBα and activates its downstream TFs, RelA/p50 and p50/c-Rel (13). The liberated RelA/p50 and p50/c-Rel translocate to the nucleus and activate the transcription of NF-κB-dependent inflammatory genes (14, 17).

2.1.2 Non-canonical pathway

Noncanonical NF-κB pathway respond to a certain type of stimulus, such as the lymphotoxin β receptor (LTβR), CD40, the B-cell activating factor receptor (BAFFR) and receptor activator of NF-κB (RANK) (18) (Figure 1). NIK is essential for the activation of this pathway and is central to the signaling that activates IKKα and forms a functional cooperation with IKKα to phosphorylate p100. Phosphorylation of p100 stimulates the partial proteasomal processing of p52 (11). This generates NF-κB2/p52 through the degradation of the p100 C-terminal IκB-like structure, and leads to the nuclear translocation of p52/RelB occurs (19).

2.2 NF-κB-regulated genes

NF-κB regulates more than 150 genes involved in anti-apoptosis, cell proliferation, immunity, and inflammation. It plays a key role in regulating the activation, survival, and differentiation of innate and adaptive immune cells (20). In RA, a deregulated NF-κB signaling pathway contributes to the pathogenic process and activates both immune and non-immune cells (e.g., FLS) through transcriptional regulation of inflammatory mediators, including TNF, IL-1, IL-2, IL-6, IL-8, IL-9, IL-12, IL-18, IL-23, GM-CSF, VEGF, RANKL, MCP-1, MIP-2, CXCL1, CXCL10, RANTES, ICAM-1, VCAM-1, MMPs, and COX-2. (10, 13, 20, 21). These NF-κB-regulated inflammatory mediators have been reported to play a crucial role in the pathogenesis of RA by activating both immune and non-immune cells.

T cells and macrophages are key responders to the NF-κB signaling pathway. Deregulated NF-κB signaling causes aberrant activation of T cells and each member of the NF-κB family is responsible in activating different types of T cells in RA. RelA and c-Rel activate naïve T cells by inducing TCR activation. c-Rel promotes the transcription of Foxp3, a key regulator of Tregs (22). NF-κB differentiates Th1 and Th17 cells by inducing IL-12 production and promotes IL-17 synthesis in Th17 cells, and thereby recruiting neutrophils and monocytes to sites of inflammation. Th17 cells contribute to inflammation by regulating expression of TNF, IL-1β, IL17, IL-21, and IL-22 (23). Noncanonical NF-κB regulates Th17 to induce GM-CSF. On the other hand, Th2 responses are regulated by NF-κB1/p50. In macrophages, NF-κB induces a range of inflammatory mediators, including TNF, IL-1β, IL-6, IL-12, and COX-2. Activated c-Rel is essential for IL-12B expression and also for NF-κB-ATF3-CEBPδ transcriptional circuit, which enables macrophages to analyze the responses received from persistent and transient TLR4 stimulation (24). In FLS, NF-κB p50/p52 and NFATc1 respond to RANKL and exhibit an inflammatory response along with osteoclast activation and osteoclast genesis (18). NFATC1 is a major TF that regulates osteoclast differentiation (25). Together with NFATC1, RelB regulates osteoclast formation (26). Given the broad range of inflammatory roles of NF-B, its targeting might be beneficial for treating RA.

2.3 Current treatments targeting NF-κB

2.3.1 Synthetic drugs

Conventional disease-modifying antirheumatic drugs (cDMARDs) and biological DMARDs (bDMARDs) have been used to treat RA for many decades. Methotrexate (MTX) is a first-line drug widely used to treat RA, while bDMARDs, such as TNF inhibitors, have been used since 1980. Currently, five main classes of TNF-inhibiting bDMARDs are available: etanercept, adalimumab, certolizumab pegol, golimumab, and infliximab. A recent clinical trial suggests switching from TNF inhibitors to tacrolimus (TAC) after acquiring low disease activity. TAC is an immunosuppressant that can block the calcineurin pathway in T cells by inhibiting cytokine production and T cell proliferation (27). Artemisinin-type compounds inhibit several receptor-coupled signaling pathways that include IL-1, TNF, RANKL, growth factor receptors, and TLRs (4). Terfenadine and Fexofenadine have recently been identified as more cost-effective and safer TNF inhibitors (28). Regulation of RANKL levels is maintained by bDMARDs (e.g., Denosumab) (29). All the above-mentioned drugs target cytokines that can activate NF-κB, thereby indirectly suppressing its activity. The long-term use of these drugs and the need to increase the dosage for an effective result can lead to adverse effects, such as osteoporosis, hyperlipidemia, hepatitis, tuberculosis, malignancies, and adrenal insufficiency (30). Furthermore, there is an increase in resistance to these drug in 30% of cases of RA (31).

Since there is a need for a different approach to reduce side effects, recent studies focus directly on targeting NF-κB, thus potentially achieving more precision in treating RA (Table 1). Tetrandrine, a bisbenzylisoquinoline, blocks NF-κB/RelA (32). Iguratimod is a new synthetic targeted DMARD (stDMARD) that inhibits the translocation of NF-κB to the nucleus and is approved only in China and Japan for RA treatment (33). Small-interfering RNA (siRNA) targeting NF-κB, delivered in combination with MTX inside a liposome capsule, prevents its release in the circulation, avoiding possible adverse effects of MTX (17). Chen

et al., have demonstrated that low molecular weight polyethyleneimine cholesterol polyethylene glycol encapsulates siRNA as an efficient way to silence NF-κB/p65 to restore an anti-inflammatory microenvironment in RA (34). Drug delivery via nanocarriers is now being explored to deliver controlled doses of drug of interest to promote cell/tissue specific treatment, thus minimizing the potential side effects (43).

2.3.2 Natural compounds

To minimize side effects caused by synthetic drugs, many studies are now focusing on natural compounds that can alleviate RA disease (Table 1). Celastrol, triptolide, resveratrol, curcumin, myricetin, fisetin and quercetin have been identified to hopefully reduce RA severity by targeting numerous cytokines, signaling pathways and proteases (41, 44). Numerous studies have shown the effect of celastrol on actively improving RA severity through suppression of the following: ROS-NF-κB-NLRP3 signaling (37), HIF expression and ROS release (36), the PI3-K/AKT/mTOR axis (45, 46) and NF-κB by degrading IκB (44, 47). Resveratrol, a polyphenol, activates sirt1, which suppresses the transcriptional activity of NF-κB/p65 by deacetylation and inhibits the COX/MMP pathway and the production of IL-1β, IL-6, and TNF (48). Curcumin suppresses the expression of NF-κB by upregulating that of miR-124 (39). Emerging findings suggest that treating RA patients with vitamin D supplementation can lower RANKL and CXCL10 levels, and suppress activation of NF-κB (18). Glucosamine prevents the demethylation of particular CpG sites in the promotor region of IL-1β, thereby preventing NF-κB from binding to the promotor region and suppressing the expression of IL-1β (49). These studies indicate the potential of natural compounds to not only target NF-κB, but also to suppress inflammation in RA.

3 JAK/STAT

3.1 JAK/STAT signaling pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a key signaling pathway important in governing many biological processes, including cell differentiation, proliferation, and immune functions. Several studies have identified that the JAK/STAT signaling pathway is deregulated in RA (50, 51). Many of the proinflammatory cytokines, including TNF, IL-β, IL-6, IL-7, IL-8, IL-12, IL-15, IL-17, IL-23, IL-32, IFN and GM-CSF, that are highly expressed in RA are known to be regulated by JAK/STAT signaling pathway (50, 52). The JAK family has four members, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), while the STAT family of TFs consists of seven members, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Upon receptor ligation, JAKs are autophosphorylated, and recruit and phosphorylate members of the STAT family (Figure 2). Phosphorylated STATs dissociate from the receptor and form homo or heterodimers before translocating to the nucleus to activate the transcription of STAT-regulated genes (53). STATs bound to gene promoters can be dephosphorylated by nuclear protein tyrosine phosphatases (N-PTPs) and subsequently exit the nucleus to the cytoplasm for further activation cycles (54). Negative regulators of the JAK/STAT pathway, such as PTPs, protein inhibitors of activated STAT (PIAS), and suppressors of cytokine signaling proteins (SOCS), play crucial roles in controlling STAT-regulated gene expression (55–57).

JAKs and STATs are activated by stimulation with various cytokines (51, 58). JAK1 is phosphorylated by four types of cytokine receptor families: (i) cytokine receptor with γ_c (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors); (ii) receptors with gp130 subunits (IL-

TABLE 1 Synthetic drugs and natural compounds targeting NF-κB either directly or indirectly.

Target	Drugs	Effects on NF-κB-regulated inflammatory factors	Study type	Reference (s)
Synthetic drugs				
NF-κB	Tetrandrine	Inhibits IL-1β, TNF and IL-6	Clinical trial NCT05245448	(32)
NF-κB	Iguratimod	Inhibits prostaglandin E2, bradykinin, IL-1β, IL-6, IL-8, GM-CSF, TNF and COX-2	Clinical trial NCT03855007	(33)
NF-κB	siRNA	Inhibits IL-1, TNF, IFNγ and IL-6 production	In vitro	(17, 34)
Natural compounds				
NF-κB	Vitamin D	Inhibits RANK, CXCL10, and IL-17a.	Clinical trial NCT04344405	(18)
NF-κB	Celastrol	Inhibits IL-1β, TNF, substance P, β-endorphin, MMP9, COX-2, c-Myc, TGF-β, c-JUN, JAK1, JAK3, IKKβ, SYK, MMP3 and MEK1.	In vitro	(31, 35–37)
NF-κB	Curcumin	Inhibits IL-1, TNF, and IL-6. Increases IL-10	In vitro	(38, 39)
NF-κB	Resveratrol	Inhibits COX-2, iNOS, TNF, MMP3, MMP13	In vitro	(40, 41)
NF-κB	Quercetin	Inhibits IL-1β, IL-6, IL-8, IL-13, TNF and IL-17	In vitro	(42)

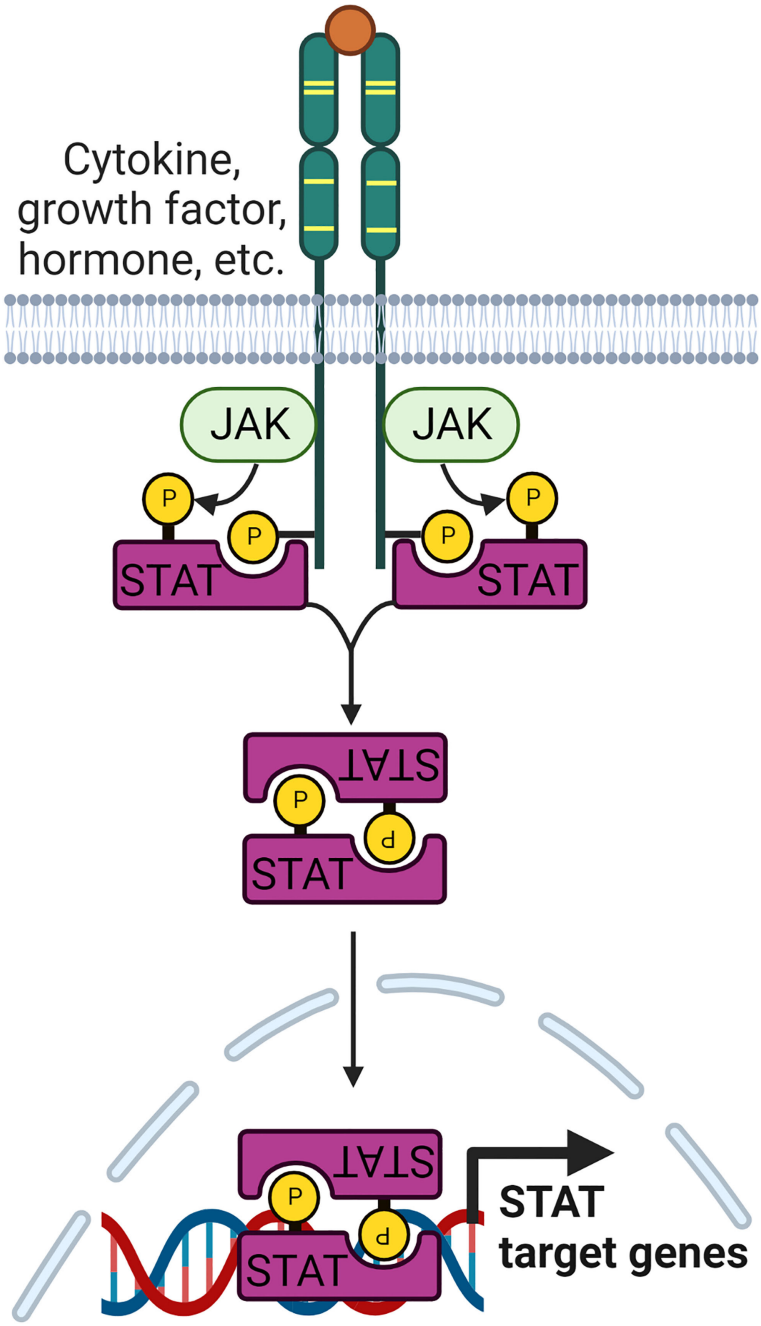

FIGURE 2
Signaling pathway leading to the regulation of JAK/STAT target genes. The family of JAK kinases are activated upon receptor ligation. Subsequently, they recruit STAT family proteins and phosphorylate them. Phosphorylated STATs form homo or heterodimers before being translocated to the nucleus to regulate transcription of STAT target genes.

6, IL-11, IL-27, oncostatin M (OSM), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), cardiotrophin-like cytokine (CLC), and ciliary neurotrophic factor (CNTF) receptors; (59) (iii) class 2 cytokine receptors (IL-10 family, type 1 and 2 IFN receptors); and (iv) IL-3, IL-5, and GM-CSF receptors. As for JAK1, JAK2 is activated by (i) the gp130 receptor family, (ii) the IL-3R family (IL-3, IL-5R and GM-CSF receptors), (iii) the class 2 cytokine receptor family, and (iv) single chain receptors, such as growth hormone, thrombopoietin, prolactin and erythropoietin receptors. JAK3 is activated by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors and TYK2 is activated by IFN type 1, IL-6 family, IL-10 family, IL-12, IL-13, and IL-23 receptors (60).

STAT1 is activated by IFNγ, IL-2, IL-6, IL-7, IL-21, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TNF, hepatocyte growth factor (HGF) and angiotensin 2. It has been found that IFNα/β are the only cytokines that can activate STAT2. STAT3 is activated by the IL-6 cytokine family (IL-6, IL-11, IL-27, IL-31, CNTF, OSM, and LIF), the IL-10 cytokine family (IL-10, IL-

19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29), GM-CSF, IL-2, IL-7, IL-21, IFNα/β, and leptin. STAT4 is activated by IL-12, IL-23 and IFNα/β whereas STAT5 is activated by the IL-3, the IL-2 cytokine family (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21), prolactin, EGF, GM-CSF, PDGF and GH. STAT6 is activated only by IL-4 and IL-13 (58, 61, 62).

3.2 STAT-regulated genes

STATs bind directly to DNA regulatory elements and regulate gene transcription. STAT1, STAT3, STAT4, STAT5, and STAT6 have been shown to be highly expressed in RA (63). STATs often interact with other TFs, which assemble in the promotor or enhancer regions of target genes. Examples of such interactions are STAT1-STAT2 with IRF9, STAT1 with NF-κB, STAT3 with Jun, STAT3 with IRF4, RORγt, and BAFT in T cells, and STAT1 with IRF1, IRF8 and PU.1 in macrophages. These complex transcription networks highlight the fact that multiple TFs can be involved in regulating gene expression in various cell types.

3.2.1 STAT1

Various STAT proteins play a different role in different cell types that lead to RA pathogenesis. In synovial macrophages, STAT1 activates CXCL9 and CXCL10, which recruit T cells, induce Th1 differentiation, and upregulate IFNγ production (64, 65). STAT1 activation is essential for activated IRF1 and TLR3 in macrophages (66). STAT1 can induce iNOS expression and produce NO, which can reduce cell migration, while suppressing STAT3 activity (67). Most importantly, STAT1 regulates MMP3 and MMP13, thereby inducing cartilage degradation in the knee joint (68). A study by Kuuliala et al. has suggested that the activation of STAT1 and STAT6 in circulating leukocytes helps predict the response to treatment in RA (69).

3.2.2 STAT3

STAT3 is another major TF involved in inflammation. STAT3 induces angiogenesis, transcription of B cell lymphoma protein 2 (BCL-2), several MMPs, including MMP1, 3, and 13, and cyclins (70, 71). STAT3 activates RORγt, which induces IL-6 production and leads to Th17 polarization and stabilization (72). It can inhibit fibroblast apoptosis (73), the function of STAT1, and the expression of IFNα (74).

3.2.3 STAT4, STAT5, and STAT6

The production of the Th1-driven cytokine, IL-12, is mediated through STAT4. STAT5 promotes the production of $CD4^+$ T cells (75). A few studies have reported the role of STAT5 in GM-CSF-induced CCL17 production (76, 77), a chemokine found to be important in inflammatory arthritis (78). IL-4 transduces signal through STAT6, which regulates the Treg cell response (55, 69).

3.3 Current treatments targeting JAK/STAT

3.3.1 Synthetic drugs

JAK/STATs are key regulators of cytokines produced in RA pathogenesis and therefore, are considered as feasible drug targets (60). Currently, JAK inhibitors (JAKi) are used as third-line therapy for RA patients with disease recurrence after using MTX and bDMARDs. JAKi are tsDMARDs, and they competitively inhibit by binding to the ATP binding site of the kinase domain present in JAKs, thereby inhibiting the JAK phosphorylation and preventing STAT activation (60). Among the currently available JAKi, baricitinib and tofacitinib are pan JAKi (Table 2). Baricitinib, tofacitinib and upadacitinib are approved by the FDA for the treatment of RA (87), while filgotinib and peficitinib are being evaluated (82). Tofacitinib is highly selective for JAK1 and JAK3, with less selectivity for JAK2 and TYK2 (64). Baricitinib inhibits

TABLE 2 Synthetic drugs and natural compounds targeting JAK/STAT either directly or indirectly.

Target	Drugs	Effects on JAK/STAT-regulated inflammatory factors	Study type	Reference(s)
Synthetic drugs				
JAK1/3	Tofacitinib	Inhibits STAT1, STAT3, STAT5, CXCL9, and CXCL10	FDA-approved	(64, 79)
JAK1/2	Baricitinib	Inhibits IL-6, IL-12, IL-23, IFNγ, CXCL9 and CXCL10	FDA-approved	(73)
JAK1	Upadacitinib	–	FDA-approved	(80)
JAK1	Fligotinib	Inhibits STAT1 and STAT5	FDA-approved	(81)
JAK3	Peficitinib	–	FDA-approved	(82)
Natural compounds				
JAK2/3	Notopterol	Inhibits STAT5	In vitro	(83)
JAK2	Genkwanin	Inhibits STAT3	In vitro	(84)
JAK1/2	Kaempferol	Inhibits STAT1 and STAT3	In vitro	(85)
STAT1/3	EGCG	Inhibits iNOS and ICAM-1	In vitro	(86)

JAK1 and JAK2, while moderately inhibits TYK2 and JAK3 (88). One study showed a similar safety profile for baricitinib and tofacitinib, but a better clinical outcome with baricitinib (89). The introduction of a nanostructure-based drug delivery system enables site-specific delivery of tofacitinib and the JNK inhibitor SP600125 (90). Upadacitinib and filgotinib selectively inhibit JAK1 and have been proven to be efficient in phase 2 and 3 studies (91). Another study demonstrated that baricitinib combined with MTX and upadacitinib with MTX can effectively inhibit the JAK/STAT signaling pathway (82).

3.3.2 Natural compounds

Although JAKi function effectively in RA patients, they are expensive for broader application and demonstrate adverse effects, including hepatotoxicity, gastrointestinal perforations, thromboembolism, herpes zoster, and tuberculosis; therefore, some studies are focusing on exploring natural compounds that can inhibit JAK/STAT signaling (87) (Table 2). Notopterol is a natural compound that effectively inhibits JAK2/JAK3 and suppresses the production of CXCL2, CXCL9, CXCL10, CXCL12, CCL5, IL-1β, IL-6, and TNF levels in bone marrow-derived macrophages. Genkwanin, a flavone, inhibits the JAK/STAT pathway by binding to JAK2 and NF-κB, reducing TNF, NO and IL-6 levels, while increasing IL-10 production (84, 92). Quercetin, epigallocatechin-3-gallate (EGCG), resveratrol, curcumin, genistein, chlorogenic acid, swertiamarin, cyanidin, ferulic acid, baicalein, falcarindiol, cinnamaldehyde and cryptotanshinone have been found to be effective in inhibiting JAK/STATs.

4 AP-1

4.1 Activation of AP-1

The activator protein-1 (AP-1) is proposed to play an important role in inflammation and pathogenesis of RA (93). Increased levels of c-Fos and c-Jun in RA synovium are correlated with disease severity (94). In the initial phase of RA, ROS activated AP-1, but in the late phase, proinflammatory cytokines can upregulate AP-1 (95). It is a leucine zipper TF composed of Fos, Jun, and ATF families of proteins (96). Fos proteins (FosB, Fra-1, Fra-2, c-Fos) heterodimerize with members of the Jun family, whereas Jun proteins (c-Jun, JunB and JunD) can heterodimerize and/or homodimerize with members of the Fos family to form transcriptionally functional complexes that bind to the promotor region of AP-1 sites (97) (Figure 3). The dimer composition of AP-1 and the active state of the Jun and Fos components determine the target of AP-1 (98). Jun : Jun and Fos : Jun dimers selectively bind to AP-1 motifs, known as the 12-O-tetra-decanoylphorbol-13-acetate (TPA) responsive element (TRE) and the cAMP-responsive element (CRE) (99).

AP-1 is activated primarily by MAPK signaling. The three main subfamilies in the MAPK signaling pathway, extracellular signal-regulated kinases (ERKs), p38, and c-Jun N-terminal kinase (JNK), are essential for activation of AP-1 (96). MAPKs are activated by a cascade of phosphorylation events, wherein activated mitogen-activated protein kinase kinase kinase (MAPKKK) phosphorylates mitogen-activated protein kinase kinase (MAPKK), which finally phosphorylates MAPK (100). IL-1β, IL-6, TNF, TGF-β, and TPA up-regulates AP-1 through the MAPK pathway (101, 102). ERK1/2 is activated through a signaling cascade via phosphorylation of Ras, Raf, and MEK 1/2 (98). Stimulation of TLR4, IL-1R, and TNFR activates MyD88 and TAK1, which activates MKK4/7 or MKK3/6, thereby activating c-Fos/c-Jun of AP-1 by JNK and p38, respectively (103, 104). CXCL1 induces c-Jun phosphorylation in RA synovial fibroblasts (RASFs), and increased activation of AP-1 is observed in CXCL1 treated cells (93). AP-1 is activated by enhanced PI3K/AKT activation through stimulation of TNF- and thrombin-induced EGFR transactivation in chondrocytes (105, 106). Another study in RASFs showed that myostatin-induced TNF expression through the PIK3-AKT-AP-1 signaling pathway by activating the c-Jun binding site found in the TNF gene promoter region (107). Activating transcription factor 2 (ATF2), a member of the AP-1 TF family, is highly expressed in RA FLS activated via ERK and MAPK. Sprouty2 can inhibit ATF2 overexpression by inhibiting the phosphorylation of ERK and MAPK (108).

4.2 AP-1-regulated genes

4.2.1 Fos

AP-1 selectively regulates a range of cytokines, chemokines, proteinases, and TFs. Each member of the AP-1 family differentially regulates genes. c-Fos/AP-1 induce the expression of MMPs (e.g., MMP1, 2, 3, 8, 9, and 13) and cytokines (e.g., IL-23) (109, 110). MMPs are mainly regulated by IL-1β-induced c-Fos/AP-1; most genes in the MMP family have an AP-1 binding site in the promoter regions near the TATA box and a mutation in the AP-1 binding site completely suppresses MMP expression (111). MMPs are essential for cartilage joint matrix breakdown and MMP13 predominantly degrades cartilage by cleaving type 2 collagen (111). IL-1β can induce osteoclast genesis directly and/or indirectly through RANKL signaling. Integration of RANKL and M-CSF signaling requires Fos/AP-1 (112).

4.2.2 Jun

c-Jun differentially regulates cyclooxygenase-2 (COX-2) and arginase-1 (ARG-1) and promotes macrophage activation, thus contributing to arthritis progression (113). JunB can control Th17 differentiation by inducing the expression of RORγt and RORα, while suppressing the expression of Foxp3 (72). JunB synergizes with c-MAF and GATA3 and induces activation of IL-4, which induces Th2 cell differentiation (114). cJun and JunB together activate AKT1 by binding directly to its promoter region (115).

4.2.3 NFAT

The nuclear factor of activated T cells (NFAT) is suggested to play a role in the pathogenesis of RA (116). AP-1 interacts with NFAT and cooperatively forms an AP-1/NFAT complex, which enhances transcriptional activity compared to Fos-Jun or NFAT

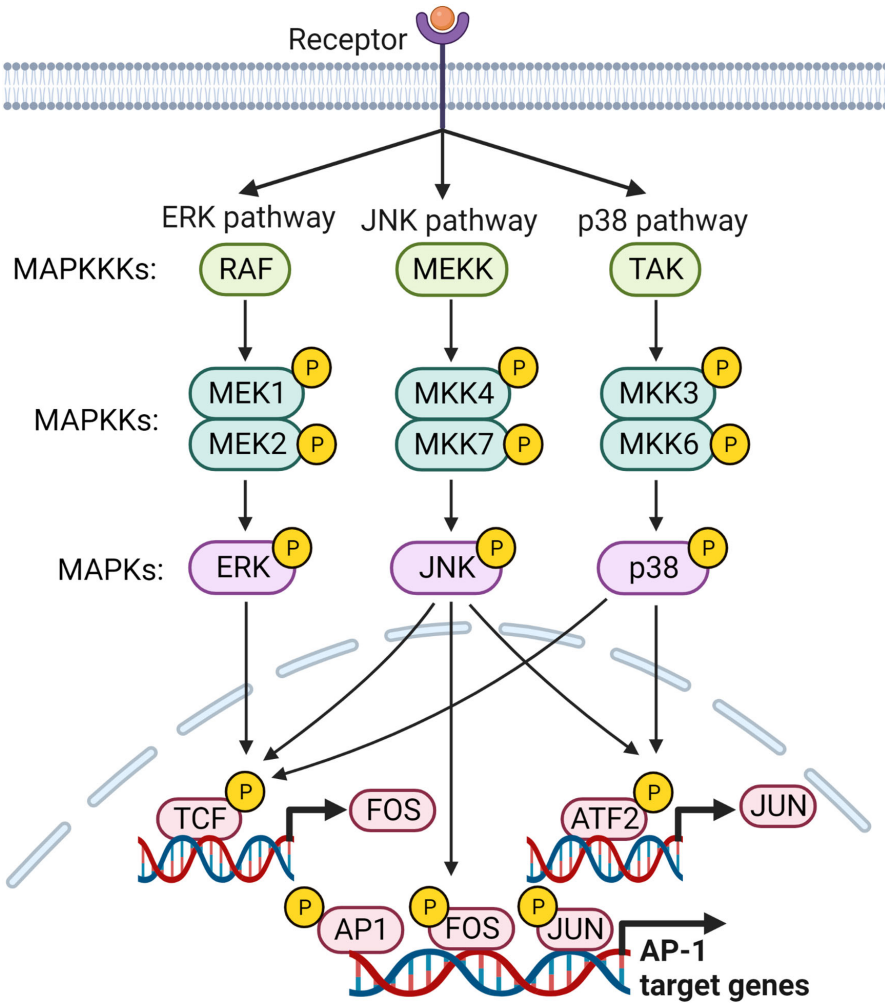

FIGURE 3
MAP kinase signaling pathways leading to the regulation of AP-1 target genes. Members of MAP kinases, ERK, JNK and p38, are responsible for activating transcription factors that regulate transcription of FOS and JUN genes and their subsequent activation. Subsequently, activated AP-1 binds to the promoter regions of its target genes and regulates their expression.

binding and regulates most cytokines. It regulates IL-2, which is required for Treg proliferation (114). As AP-1 regulates some important inflammatory mediators that promote RA, it serves as a treatment target to alleviate RA. Synthetic drugs and natural compounds targeting AP-1 are being studied at present.

4.3 Current treatments targeting AP-1

4.3.1 Synthetic drugs

Given the role of AP-1 in the regulation of key inflammatory mediators known to promote RA, targeting it is a potential treatment solution; however, there are no FDA-approved AP-1 inhibitors available in the clinic. Many *in vitro* and *in vivo* studies are currently focusing on drugs that can inhibit AP-1 (Table 3). CKD-506 is an orally administered hydroxamate that blocks the activation of AP-1 and NF-κB transcription in peripheral blood mononuclear cells isolated from RA patients (117). T-5224, a molecular inhibitor of c-Fos/AP-1, inhibits the DNA binding of c-Fos/c-Jun, thus inhibiting IL-1β, IL-6, TNF, MMP1, 3, and 13. N-(3-acetamidophenyl)-2-[5-(1H-benzimidazol-2-yl) pyridin2-yl] sulfanylacetamide can disrupt the interaction between AP-1 and NFAT and blocks the transcription of IL-2 and some cyclosporin A-sensitive cytokines (126). A cyclin-dependent kinase 4/6 (CDK) inhibitor (CDKi) blocks AP-1 transcription by decreasing Jun stability, thus blocking the production of MMP3 and attenuating cartilage destruction in the collagen-induced arthritis model (119). A novel JNK inhibitor, 11H-indeno[1,2-b] quinoxaline-11-one oxime, has been shown to not only inhibit JNK phosphorylation but also block the transcriptional activity of AP-1 and NF-κB (120). Roflumilast, a selective phosphodiester-4 inhibitor, inhibits the production of IL-1β, IL-6, TNF, CCL5, CXCL9, CXCL10, MMP3, and MMP13 by blocking the transcriptional activity of AP-1 and NF-κB (118). Many of these synthetic drugs targeting AP-1 show promise in preclinical studies, but further research and clinical trials are needed before obtaining FDA approval.

TABLE 3 Synthetic drugs and natural compounds targeting AP-1 either directly or indirectly.

Target	Drugs	Effects on AP-1-regulated inflammatory factors	Study type	Reference (s)
Synthetic drugs				
AP-1	CKD-506	Inhibits TNF, IL-6, IL-8, MMP1, and MMP3	Clinical trial NCT04204603	(117)
AP-1	Roflumilast	Inhibits CCL5, CXCL9, CXCL10, MMP3 and MMP13	*In vivo*	(118)
c-Fos	T-5224	Inhibits MMP1, 3, 13, TNF, IL-6, and IL-1β	*In vivo*	(111)
Jun	CDKI	Inhibits MMP1 and MMP3 production via AP-1 signaling pathway	*In vitro*	(119)
JNK	11H-indeno[1,2-b] quinoxalin-11-one oxime	Inhibits IL-6 production by inhibiting AP-1 and NF-κB pathway	*In vitro*	(120)
Natural compounds				
AP-1	Thymoquinone	Inhibits TNF and IL-6	*In vivo*	(121)
AP-1	Actin K	Inhibits VCAM-1	*In vitro*	(122)
AP-1	Apigenin-4´-O-α-L-rhamnoside	Inhibits MMP1, MMP3, RANKL and TNF	*In vitro*	(102)
AP-1	Thymoquinone	Inhibits ICAM-1, VCAM-1, MAPK, MMP3, MMP13, and COX-2	*In vitro*	(123, 124)
AP-1	Extract of *Sigesbeckia orientalis*	Inhibits IL-1β, IL-6, IL-8, COX-2, MMP9, MAPKs	*In vivo*	(125)
c-Jun	Melittin	Inhibits COX-2, MMP1, MMP3, MMP8 and MMP13	*In vitro*	(106)

4.3.2 Natural compounds

While there are no FDA-approved AP-1 inhibitors available, many studies have explored natural compounds that can potentially block components in the AP-1 pathway (Table 3). Antcin K is an extract taken from a medicinal mushroom, *Antrodia cinnamomea*, that inhibits vascular cell adhesion molecule 1 (VCAM-1) and monocyte adhesion to RASFs by inhibiting the phosphorylation of p38 and MEK1/2-ERK (122). Apigenin-4´-O-α-L-rhamnoside, a natural flavonoid, exhibits inhibitory mechanisms against MMP1, MMP3, TNF, and RNAKL in RA FLS by inhibiting the MAPK/JNK/p38 pathway (102). Anticin K and Apigenin-4´-O-α-L-rhamnoside inhibit the inflammatory mediators of AP-1 and indirectly suppress the activation of AP-1. Treatment with resveratrol directly suppresses bradykinin-mediated AP-1 and NF-κB activities and inhibits COX-2 production in RASFs (40). Melittin, the primary component of bee venom, exhibits inhibitory properties by suppressing MMP1 and MMP8 by blocking the phosphorylation of PI3-K/AKT and ERK/JNK and the translocation of c-fos (106). Thymoquinone is another natural compound found in *Nigella sative*, which shows anti-inflammatory properties in preclinical arthritis models, blocking multiple pathways that include AP-1 and NF-κB (127). The ethanolic extract of *Sigesbeckia orientalis* inhibits pannus formation and reduces cartilage damage and bone erosion in the collagen-induced arthritic model, while it leads to decreased expression of IL-1β, IL-6, IL-8, COX-2, MMP9, and NLRP3 by inhibiting MAPKs, AP-1, and NF-κB in *in vitro* studies carried out in synovial cells (125).

5 IRFs

5.1 Activation of IRFs

In humans, the interferon regulatory factor (IRF) family of transcription factors consists of nine members, IRF1 to IRF9. They share a homology region found in the N-terminal DNA binding domain, which binds to the interferon stimulated response element (ISRE). The diverse C-terminal domain is unique to each member and binds to a wide range the proteins outside the IRF family. They are activated via signals received by activation of TLR and BCR (128). Upon activation, IRFs can form homo- or hetero dimers, which are translocated to the nucleus. Hyperactivated IRFs mainly produce IFNs and thus contribute to inflammatory diseases. Each member of the IRF family is regulated by a range of inflammatory mediators present in RA synovium and their mode of activation is discussed below (Figure 4).

5.1.1 IRF1

TLR-activated TAK1 can induce IRF1 transcription via the RelA/p50 complex (129). Furthermore, the JAK/STAT signaling cascade has also been shown to induce the expression of IRF1 (130). The following studies have also demonstrated a similar pathway of IRF1 activation. Pristane induces autophagy in macrophages and can induce IRF1 activation by activating STAT1 (66), and IKKβ regulates IRF1 transcription in conventional type 1 dendritic cells (129).

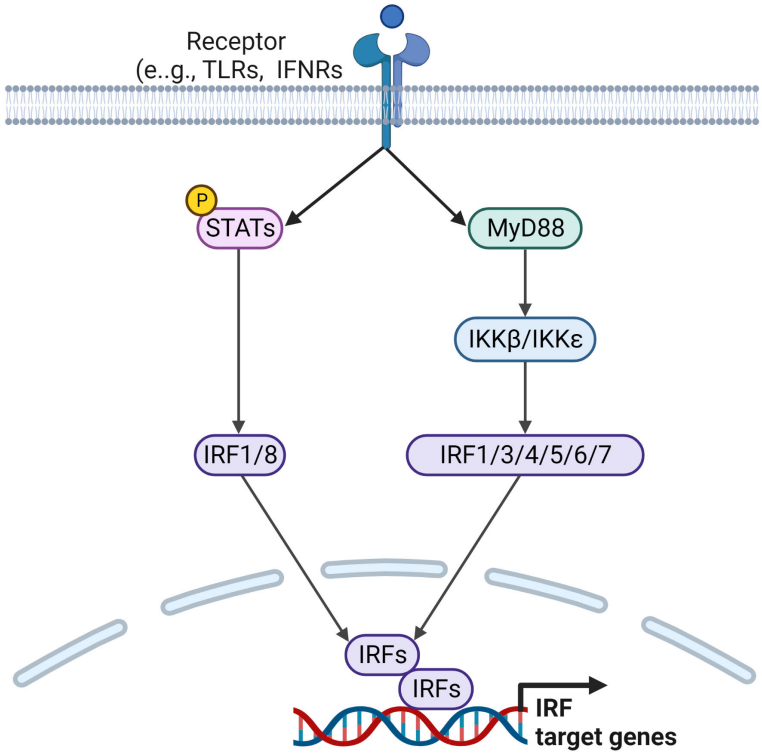

FIGURE 4
Signaling pathways leading to the regulation of IRF target genes. Ligands binding to TLRs and IFNRs initiate downstream signaling pathways via downstream activators and adaptor molecules, such as STATs and MyD88, which lead to the activation of IRF family transcription factors. Activated IRFs form homo or heterodimers before being translocated to the nucleus, where they regulate transcription of IRF target genes.

5.1.2 IRF3

IRF3 is activated by various signals received from intracellular receptors such as RIG-1, MDA5, TLR3, TLR4, and cytosolic double-stranded DNA (dsDNA) sensors (131). Activated TLR3 signals via TRIF to activate TBK1 and IKKε via TRAF3. dsDNA in the cytosol can trigger type 1 IFN through STING, which can activate IRF3 by stimulating its phosphorylation by activating TBK1. It is suggested that the C-terminal tail of the STING oligomer can recruit both TBK1 and IRF3 by binding to the IRF3 motif and delivers IRF3 to TBK1 (132). Finally, TBK1, along with IKKε, phosphorylate IRF3 to form dimers and then translocate to the nucleus (133).

5.1.3 IRF4

IRF4 is induced by activation of BCR and CD40 in B cells, TCR in T cells, and TLR in macrophages. However, it is not activated by type 1 or type 2 IFNs. The receptor activation leads to activation of c-Rel, which binds to the promotor region of IRF4 to induce transcription. The promotor region contains Foxp3, STAT4, STAT6, and IRF4 binding sites, suggesting that IRF4 is capable of autoregulating its expression (134).

5.1.4 IRF5

IRF5 activation is initiated upon binding of ligands to TLR7/8/9, wherein MyD88, IRAK1/4, and TRAF6 are recruited. Then autophosphorylation of IRAK4 activates TAK1 to phosphorylate IKKβ. Meanwhile, TRAF6 ubiquitinates IRF5, which is subsequently phosphorylated by IKKβ, thus forming homodimers. IRF5 homodimers are translocated to the nucleus to activate target genes (128).

5.1.5 IRF6, IRF7 and IRF8

IRF6 is regulated via TLR2 in epithelial cells and TLR3 in keratinocytes (135). In keratinocytes, RIPK4 phosphorylates and activates IRF6 (136). IRF7 is activated through TLR3 and TLR7 via two different pathways. TLR3 is activated by dsRNA and phosphorylates IRF7 via TRIF, while TLR7 signals via MyD88-TRAF6 signaling, which is induced by ssRNA (137). IRF8 is activated in macrophages, dendritic cells, T cells, and NK cells. Binding of IFNγ to its receptor activates IRF8 by activating STAT1. IRF8 is also activated following stimulation with IFNα/β and LPS (138).

5.2 IRF-regulated genes

IRFs play a major role in autoinflammation and autoimmunity (139). IRF1, IRF3, IRF5 and IRF7 are important in the induction of type 1 IFN, where IRF4, IRF5 and IRF8 regulate the development of myeloid cells and play a crucial role in inflammatory responses (140). A wide range of studies have suggested a role for each IRF in inflammatory diseases, including RA.

5.2.1 IRF1

IRF1 regulates several IFN-regulated genes (e.g., CXCL9, CXCL10, and CXCL1)1 in rheumatoid synovium and activates B cell activating factor (BAFF), which is highly expressed in RA (141). IRF1 regulates TNF-induced IFNβ expression and subsequently activates STAT1 to activate IFN-regulated genes (141). IRF1 can induce TLR3 expression in pristane-induced arthritis (66). A recent study demonstrated that the invasiveness of synovial fibroblasts is regulated by the expression of follistalin-like protein 1 induced by IRF1 (142).

5.2.2 IRF2 and IRF3

IRF2 negatively regulates IFN type 1 signaling and counterbalances the activity of IRF1. IRF2 activates IL-12p40 and VCAM-1, leading to the development of NK cells and Th1 cells, respectively (143). IRF2 stimulates inflammatory ROS levels, TNF, IL-1β, and IL-6 expression, and suppresses superoxide dismutase. The knockdown of IRF2 gene is shown to inhibit the JAK/STAT signaling pathway (144). IRF3 regulates the expression of IL-6, IL-8, MMP3, and MMP9 in RA FLS by activating c-Jun/AP-1 (145).

5.2.3 IRF4

IRF4 plays a diverse role in inflammation and arthritis. It is mainly involved in T cell differentiation. IRF4 responds to IL-4 and regulates Th1 and Th2 differentiation through interaction with T-bet and GATA3, respectively. IRF4 binds directly to RORγt and mediates the differentiation of Th17 cells (146) and regulates Glut1, IL-17 and IL-21 levels (147, 148). Furthermore, it interacts with BCL-6 and Foxp3 to produce T follicular helper cells and Tregs, respectively (146). In macrophages, IRF4 distorts macrophages into the M2 phenotype through JMJD3 competing for MyD88 with IRF5 while suppressing M1 polarization of M1 and inducing IL-4 and IL-10 secretion. Previous studies have shown that a pro-inflammatory cytokine GM-CSF can regulate CCL17 formation in monocytes/macrophages through JMJD3 and IRF4 (78). In addition to these functions, IRF4 binds to STAT3, STAT6, and NFATs to carry out transcription. IRF4 functions as a transcriptional repressor by forming a homodimer or heterodimer with IRF8, suppressing the expression of IFN-inducible genes and inhibiting IRF1 activity in macrophages and T cells (134).

5.2.4 IRF5

Studies show that IRF5 acts in T cells, monocytes, and macrophages. Increased expression of IRF5 induces M1 polarization while suppressing M2 polarization (128). IRF5 increases the expression of IL-12 in circulating monocytes in samples from OA patients without treatment and promotes Th1-related genes in resting T cells (149). Furthermore, IRF5 induces a wide range of pro-inflammatory cytokines such as IL-17, monocyte chemotactic proteins (MCP-1), TNF-α, RANTES, IL-6, IL-12p40, and IL-23p40 (150, 151). IRF5 up-regulates MMP3 production mediated via NF-κB (151).

5.2.5 IRF6 and IRF7

TLR3-induced activation of IRF6 leads to enhanced expression of IL-23p19, while negatively regulating IFNβ expression by competing with IRF3 in the IFNβ promotor region or by forming a heterodimer complex with IRF3 (135). In addition, IRF6 induces the expression of IL-8, CCL5, and CXCL11 (136, 152) and IRF7 mediates RANKL production in RA FLS (137).

5.2.6 IRF8

IRF8 is crucial for the development and maturation of myeloid cells. At the transcription level, IRF8 is co-recruited to form ternary complexes with other TFs. It forms a heterodimer with IRF1, STATs, AP-1, and PU.1 (138, 143) and induce the production of IL-6, IL-12p40 and TNF. On the other hand, IRF8 negatively regulates osteoclastogenesis by inducing IFNγ (153). Recently, it was found that IRF8 can promote the expression of MMP13 in OA (154).

5.3 Current treatments targeting IRFs

Since the IRF family of TFs is involved in the regulation of a wide range of inflammatory mediators, they can be potential treatment targets for RA. Currently a type 1 IFN inhibitor, anifrolumab, is subjected to phase 2 clinical trials (155). A study in RA patients by Juge et al. shows a IRF5 response to rituximab within 24 weeks (156). Certain JAKi, such as tofacitinib and baricitinib, are documented to suppress the activity of certain IRFs by inhibiting STAT1 activity (141).

6 Other TFs

In addition to the TFs discussed above, other TFs such as, hypoxia-inducible factor (HIF) and nuclear factor-erythroid 2-related factor-2 (Nrf2), are also implicated in the pathogenesis of RA. In RA synovium, HIF is activated during hypoxia, which aggravates angiogenesis, synovial hyperplasia, and pannus formation (157–159). TNF, IL-1β, and IL-33 can induce the expression of HIF in RASFs and resident macrophages (157). Production of HIF primarily induces the expression of VEGF, which promotes the synthesis of proteolytic enzymes in endothelial cells (160). Furthermore, it promotes the generation of M1-type macrophages and Th17 cells (161, 162). Knockdown of HIF-α in collagen-induced arthritis (CIA) mouse model has been shown to inhibit multiple inflammatory pathways and thereby, ameliorating arthritis (163). In recent years, several studies are focusing on HIF inhibitors as potential therapeutics for treating arthritis. Pharmacological HIF inhibitor, PT2977 has been shown to ameliorate arthritis in the CIA mouse model (164). Moreover, natural compounds, that include andrographolide, geniposide, dihydroarteannuin, and tylophorine-based compounds, can inhibit HIF and be effective in attenuating RA progression (163, 165–167).

Nrf2 is a redox regulator, which plays a protective role by exerting anti-inflammatory and antioxidant effects (168). Significantly, the protective role of Nrf2 has been linked to relieving severe symptoms in RA via detoxification, regulation of redox balance, and metabolism (34, 169). TNF and increased ROS levels can induce the expression of Nrf2 in RA synovium, which in return suppresses the proliferation and

MMPs production in RAFLS via inhibition of inflammatory mediators activated in RA (169). Due to this protective role of Nrf2, studies are focusing on synthetic drugs, including, dihydroartemisinin, and dimethyl fumarate, as well as natural compounds, including sinomenine, licochalcone, 7-deacetyl-gedunin, calycosin and resveratrol, that increase the expression of Nrf2 to treat RA (170–174; 90). While both HIF and Nrf2 have been identified as potential treatment targets for RA, further studies, utilizing the above-mentioned synthetic and natural compounds, are required to explore their therapeutic potential.

7 Network of TFs

In a complex disease, such as in RA, all the aforementioned TFs can form a network to cross-regulate each other or function cooperatively to activate or antagonize downstream target genes (175, 176). Examples of such interactions are STAT1-STAT2 with IRF9, STAT1 with NF-κB, STAT3 with Jun, STAT3 with IRF4, RORγt, and BAFT in T cells, and STAT1 with IRF1, IRF8 and PU.1 in macrophages (146, 177). Further, c-Fos/AP-1 and NFATc1 together control the osteoclast differentiation (110). IRF4 can bind to STAT3, STAT6, and NFATs to facilitate transcription of their downstream genes but it can also function as a transcriptional repressor by forming a heterodimer with IRF8, suppressing the expression of IFN-inducible genes and inhibiting IRF1 activity in macrophages and T cells (134). AP-1, NF-κB, and IRFs together are known to activate MMPs, (178). NF-κB, IRF4/8, PU.1, AP-1, and STAT1 induce the expression of IL-1β (179). STAT3 can activate HIF in RA synovium (180). While these studies highlight the complex network of TFs and their regulation of downstream inflammatory mediators, a careful approach is warranted when targeting them for therapeutic benefits. Since these TFs function both individually and cooperatively, targeting one or more TFs can effectively ameliorate RA. However, the key TFs involved in RA pathogenesis are also associated with biological processes involving homeostasis, and therefore inhibiting these TFs may lead to undesirable side effects. This challenge is currently being addressed by tissue-specific/joint-specific drug delivery via nanocarriers, which increase the specificity and efficacy, while minimizing potential adverse effects (17, 30, 31, 35, 121, 181–183).

8 Conclusion and prospect

RA is a chronic inflammatory autoimmune disease, causing pain and disability. Several drugs that are currently used for RA treatment are effective only delaying the progression of the disease or alleviating inflammatory symptoms. Many of these drugs have drawbacks, including disease recurrence and adverse effects due to long-term use. Therefore, there is a need to develop novel therapeutic strategies to address these shortcomings.

TFs play important roles in immune and nonimmune cells through regulation of gene expression. Studies emphasize the importance of the forementioned TFs in RA disease initiation and progression of RA disease. With the approval of JAK inhibitors in the treatment of RA, the pursuit of TFs or their signaling pathways as potential treatment targets has gained momentum. Currently, several inhibitors of TFs are being investigated, and they block TF function by inhibiting protein-protein interaction, translocation of TFs from the cytosol to the nucleus, or protein-DNA binding.

In summary, this review highlights key TFs and their signaling pathways that may become targets for future RA therapies; it also provides an update on several synthetic drugs and natural compounds that are in consideration for targeting such TFs or the signaling pathways that activate TFs.

Author contributions

All authors contributed to the writing and revision of the manuscript. TB and AA developed the concept, structure, and prepared tables and figures. TB drafted the original manuscript. AA, JH, and KL reviewed and edited.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Karsten Kretschmer,
Technical University Dresden, Germany

REVIEWED BY
Diana Dudziak,
University Hospital Erlangen, Germany
Daniel Hawiger,
Saint Louis University, United States

*CORRESPONDENCE
Shengbo Zhang
zhang.sh@wehi.edu.au

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Transcriptional regulation of dendritic cell development and function

Shengbo Zhang[1,2]*, Cindy Audiger[1,2], Michaël Chopin[3] and Stephen L. Nutt[1,2]

[1]Immunology Division, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia, [2]Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia, [3]Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia

Dendritic cells (DCs) are sentinel immune cells that form a critical bridge linking the innate and adaptive immune systems. Extensive research addressing the cellular origin and heterogeneity of the DC network has revealed the essential role played by the spatiotemporal activity of key transcription factors. In response to environmental signals DC mature but it is only following the sensing of environmental signals that DC can induce an antigen specific T cell response. Thus, whilst the coordinate action of transcription factors governs DC differentiation, sensing of environmental signals by DC is instrumental in shaping their functional properties. In this review, we provide an overview that focuses on recent advances in understanding the transcriptional networks that regulate the development of the reported DC subsets, shedding light on the function of different DC subsets. Specifically, we discuss the emerging knowledge on the heterogeneity of cDC2s, the ontogeny of pDCs, and the newly described DC subset, DC3. Additionally, we examine critical transcription factors such as IRF8, PU.1, and E2-2 and their regulatory mechanisms and downstream targets. We highlight the complex interplay between these transcription factors, which shape the DC transcriptome and influence their function in response to environmental stimuli. The information presented in this review provides essential insights into the regulation of DC development and function, which might have implications for developing novel therapeutic strategies for immune-related diseases.

KEYWORDS

dendritic cells, transcription factor, IRF8, cDCs, pDCs

1 Introduction

Our body is constantly exposed to danger in the form of pathogenic micro-organisms that seek to break through the skin and the mucous membranes that provide the first barrier of defense. The acquisition of mutations in our own cells resulting in their transformation into malignant clones represents another form of danger to which the

body must respond in order to avoid the development of cancer. A rare group of heterogeneous immune cells known collectively as dendritic cells (DCs) are central to sensing these dangers and orchestrating the appropriate response, while at the same time ignoring normal healthy cells and commensal micro-organisms.

DCs are a diverse group of cell types that are widely dispersed throughout the body. They act as sentinels to capture exogenous antigens that are processed and presented via either major histocompatibility complex class II (MHC-II) to $CD4^+$ T cells (direct presentation) or shuttled through a specialized pathway to MHC-I to engage $CD8^+$ T cells (cross-presentation) (1–5). Antigen uptake alone is insufficient to fully activate DCs, thus allowing DCs to remain tolerant to harmless antigens derived from healthy tissue or commensal microbes (6–12). However, DCs express an array of pattern-recognition receptor (PRRs) and C-type lectin receptors (CLRs) whose engagement induces maturation and migration, key steps in promoting their interaction with antigen specific T cells and thereby initiating adaptive immunity (13–15).

To face this variety of immune challenges, DCs have evolved into a variety of phenotypically and functionally distinct cellular subsets in both mouse and human (5, 16–19). DCs can be broadly separated into conventional dendritic cells (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (moDCs), the latter becoming prevalent during inflammation. Conventional DCs can be further divided into type 1 cDC (cDC1s) and type 2 cDCs (cDC2s). Of note Langerhans Cells that were traditionally classified as DCs due to their morphological and phenotypic similarities with DCs and their ability to prime T cell response, are now recognized to be a specialized population of tissue macrophages (20, 21), and therefore their ontogenetic and homeostatic properties differ greatly from DC (22, 23).

Generally, mouse cDCs and moDCs are defined by high cell surface expression of the integrin CD11c (encoded by *Itgax*) and MHC-II. Beyond the expression of CD11c and MHC-II, additional cell surface markers can be used to distinguish mouse DC subsets. cDC1s co-express the cell surface molecules XCR1, CD24, DEC205, CD8a and CLEC9A (24, 25) (Figure 1). In the peripheral lymphoid and non-lymphoid organs such as the lung, gut and LN, cDC1s also can also be identified as $CD103^+CD11b^-$ cDCs (26, 27). The splenic cDC2 subset is defined by the presence of CD11b, Sirpα (CD172a) and CD4 on the cell surface (28, 29). Adding to that cDC2s can co-express $CD103^+CD11b^+$ in non-lymphoid organs (27, 30). Although the cDC2 compartment has been described as a discrete subset, the advent of single cell technology has revealed a high degree of diversity within this population and some additional markers have been proposed to define the basis of this heterogeneity (discussed later). Under inflammatory conditions, moDCs can respond to the chemokines such as CCL2 and CCL7 and upregulate cell surface expression of MHC-II, CD11c and CD11b, and thus can be easily mistaken as cDC2s (31). Additional markers such as CD64 and MAR-1 can be used to discriminate moDCs from cDC2s (32). pDCs are distinct from the other DC subsets in that they exhibit a lower level of expression of CD11c and MHC-II. pDCs also express a variety of unique markers (compared to cDCs and moDCs), including BST2, B220, and SiglecH (33). Whether pDC belongs to the DC lineage remains at present a matter of debate given that pDCs express some lymphoid markers and overall have a limited capacity to present antigens to T cells compared to the cDC or moDC compartments (34–36).

Given their critical role in orchestrating adaptive immune responses, high dimensional and throughput techniques such as single cell RNAseq and Cytometry by time of flight (CyTOF), have

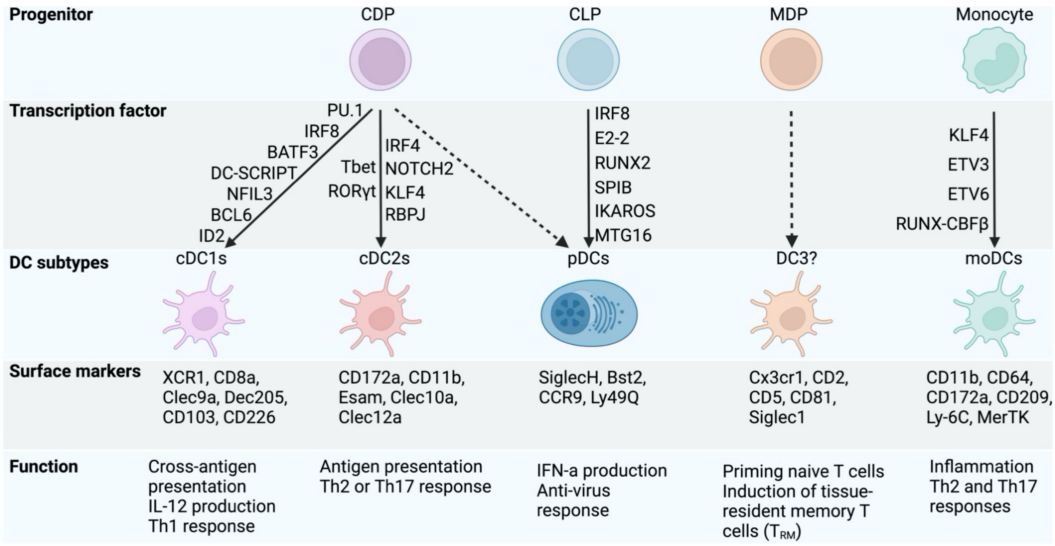

FIGURE 1
Transcription factors controlling DC specification and function. The figure highlights the development of cDCs subtypes and pDCs from the shared common dendritic cell progenitors (CDP). Some other DCs subtypes (DC3) have also been reported recently in mouse and human and derive from monocyte-dendritic cell progenitors (MDP). The common lymphoid progenitor (CLP) generates pDCs but lack cDC potential. Under inflammation, monocytes can differentiate into monocyte-derived (mono)DCs. Each DC subtype has unique surface markers and attributes in regulating immune response. The transcription factors governing DC lineage specification and function are shown.

been applied to the DC lineages. These approaches have revealed unexpected heterogeneity within the DC subsets in both mouse and human, especially the cDC2s (37–39). Single-cell analysis of human mononuclear phagocytes also identified an inflammatory subset of $CD5^{-}CD163^{+}CD14^{+}$ inflammatory DC3s that were distinct from cDC2s and able to prime Th2 responses (40). The integration of these newly identified subsets into the overall picture of DC development is a very active area of current research (41–43). In this review we will focus on the recent insights on both the transcriptional programming and the ontogeny of the DC lineages and discuss how these findings inform our understanding of the functional specialization of the DC subsets.

2 cDC1 development and function

2.1 Transcriptional regulation of cDC1 development

cDC1s differentiate principally from the common dendritic cell progenitor (CDP), a population that also gives rise to cDC2s (44, 45). A CDP subset committed to cDC1 fate has been characterized through the expression of $CD11c^{-}$MHC-$II^{-/int}CD117^{int}$Zbtb46-GFP^{+} in the bone marrow (46) and pre-cDC1s ($CD11c^{+}$MHC-$II^{-/int}CD135^{+}CD172^{-}$Siglec-$H^{-}Ly6C^{-}$) (47) in the bone marrow and spleen (44, 48, 49). However, cellular barcoding and fate mapping studies have challenged this linear model of differentiation, given that cDC1 imprinting could be detected as early as the hematopoietic stem cell (HSC) (50–52).

Despite the challenges surrounding their origin, there is a very good understanding of the transcriptional mechanisms controlling cDC1 differentiation. cDC1 commitment is dependent on the expression of specific transcription factors (TFs), including BATF3 (Basic Leucine Zipper ATF-Like Transcription Factor 3) (53), IRF8 (Interferon Regulatory Factor 8) (54), PU.1 (55), NFIL3 (Nuclear Factor, Interleukin 3 Regulated) (56, 57), and ID2 (Inhibitor of DNA Binding 2) (58), where the specific inactivation of any of these TFs is associated with a strong defect in cDC1 development (Figure 1). However, this cDC1 deficiency can be rescued by short-term bone marrow reconstitution (59) or over-expressing IRF8 in absence of BATF3 (60), highlighting the significant role of IRF8 and the fine network of TFs allowing cDC1 differentiation.

cDC1 differentiation is intimately linked to optimal expression of IRF8 which is tightly regulated by the spatio-temporal coordinated action of key TFs (Figure 2A). Indeed, its expression is initiated in early DC progenitors, including Lymphoid Primed Multipotent Progenitors (LMPPs) and is dependent on PU.1-induced chromatin remodelling (61). At the LMPP stage, RUNX and CBFβ induce the activation of the distal +56Kb *Irf8* enhancer that is essential for the initiation of IRF8 expression (62). Further down the path toward DC differentiation the activity of two additional enhancers have been shown to be pivotal in dictating cDC1 vs pDC fate: +41Kb and +32kb *Irf8* enhancers. In progenitors, E protein controls the activation of +41Kb *Irf8* enhancer, which results into the commitment of DC progenitors to the pDC lineage. As alluded to earlier IRF8 expression in progenitors is central for cDC1 differentiation, therefore it has been proposed that the upregulation of ID2 can counteract the action of E protein on the +41Kb *Irf8* enhancer, which results in the activation of the +32Kb *Irf8* enhancer whose accessibility is tightly regulated by BATF3, DC-SCRIPT and IRF8 itself to maintain adequate IRF8 level in pre-cDC1 and cDC1 (46, 63, 64). This key decisional step is also controlled by additional transcription factors, namely ZEB2 (Zinc finger E-box binding homeobox 2) and NFIL3. ZEB2 inhibits ID2 expression of in CDPs thereby promoting pDC differentiation(65, 66). In contrast, NFIL3 acts upstream of ID2 and ZEB2 to control cDC1 differentiation as its binding in CDPs to the -165Kb *Zeb2* enhancer prevents ZEB2 expression in CDPs, promoting the transition from a $ZEB2^{hi}ID2^{lo}$ CDPs to $ZEB2^{lo}ID2^{hi}$ CDPs (57, 63). This concomitant reduction in ZEB2 expression and increase in ID2 expression drive the differentiation of cDC1s (63). Beyond the important role for IRF8 in controlling DC fate in progenitors, a role for IRF8 in maintaining cDC1 survival has been postulated (67). However, recent studies suggested that rather than being essential for their survival, IRF8 as well as BATF3 control cDC1 identity in fully differentiated cells as their deletion, in both cases, enables the appearance of cDC1-like cells expressing cDC2 features (68, 69).

2.2 Key attributes and function of cDC1s

The importance of cDC1s in the immune system has been highlighted by the interrogation of cDC1-deficient mouse models (53, 70). The absence of cDC1s is associated with a reduction in the control of tumor growth (71–76) and impaired control of viral (53) or parasitic (77) infections. The major role of cDC1s in these contexts is inferred from their capacity to activate naïve $CD8^{+}$ T cells. Indeed, cDC1s can confer the 3 signals required for the efficient activation of naïve T cells: 1) the presentation of antigen-derived peptides mainly via cross-presentation, 2) co-stimulatory signals and 3) cytokines.

cDC1 are not only important for the activation of naïve $CD8^{+}$ T cells (78–80) but also for the re-activation of memory $CD8^{+}$ T cells which confer a faster and higher control of secondary infection, as for example in the case of *Listeria monocytogenes* (53). In this setting, cDC1s are the main producer of IL-12 and CXCL9 which facilitate the recruitment and activation of memory $CD8^{+}$ T cells (81). In the tumor context, the production of prostaglandin E2 (PGE2) by tumor cells leads to cDC1 dysfunctionality marked by the downregulation of IRF8, and key effector cytokines such as CXCL9 and IL-12, resulting in poor $CD8^{+}$ T cell tumor infiltration and ultimately in tumor immune evasion (82, 83). Moreover, cDC1s play a major role in licensing $CD4^{+}$ T cells for $CD8^{+}$ T cells activation (84, 85). The cDC1/$CD4^{+}$ T cell interaction through CD40/CD40L signaling increases expression of CD70 and BCL2L11 in the cDC1, allowing an increase in cDC1 survival and the differentiation and expansion of tumor-specific memory $CD8^{+}$ T cells (84, 86, 87).

In addition to their role in the initiation of the $CD8^{+}$ T cell response, cDC1s restrain progenitor of exhausted T (Tpex) cells in the white pulp niche of the spleen in an MHC-I dependent manner.

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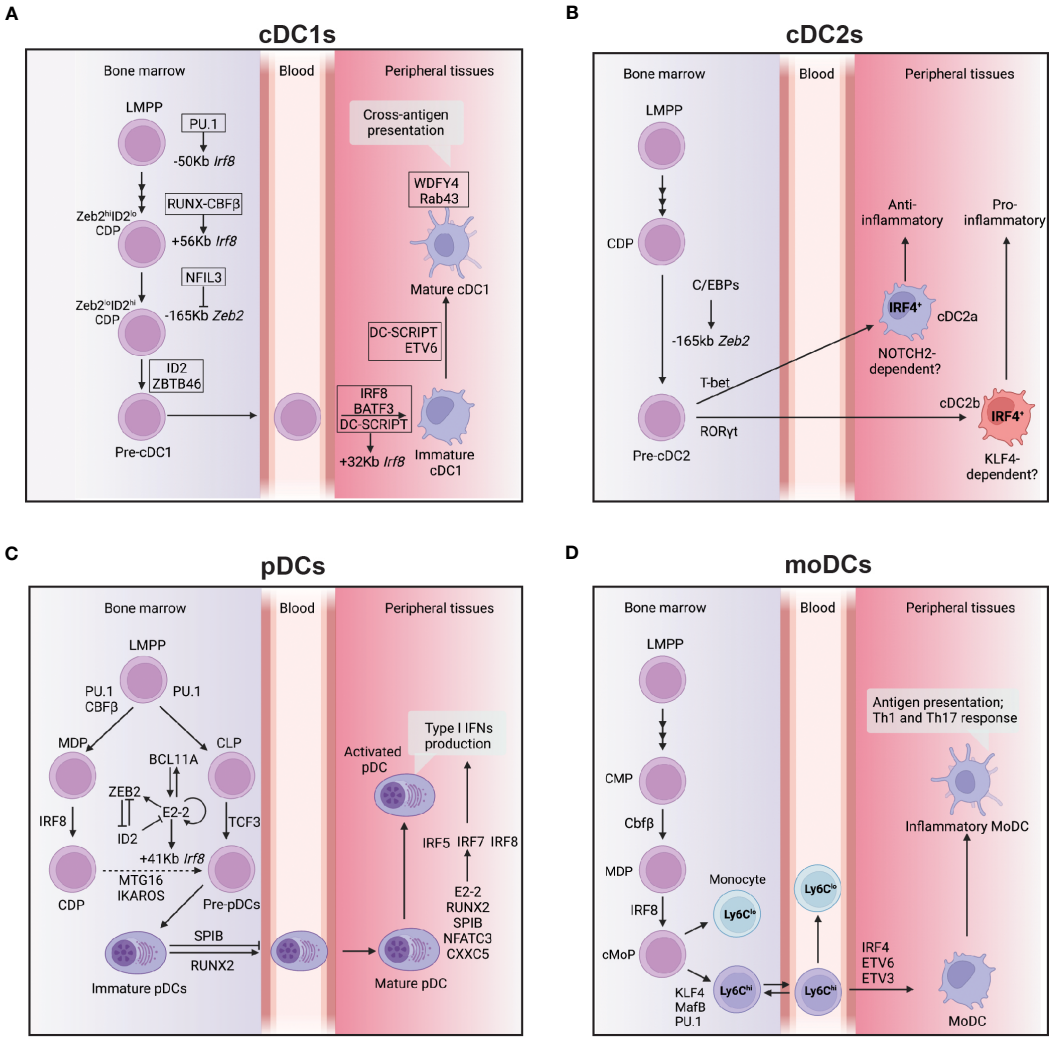

FIGURE 2
Transcriptional network controlling the development and function of DCs subsets. **(A)** This figure depicts the transcriptional network that regulates the development and function of cDC1s from bone marrow progenitors to peripheral tissues. The transcription factor IRF8 plays a crucial role in cDC1 development, and its expression is regulated by several enhancers located at -50 kb, +56 kb, and +32 kb relative to the *Irf8* gene locus. The transcription factors PU.1, RUNX-CBFβ, BATF3, and DC-SCRIPT activate these enhancers at different stages of cDC1 development. In addition, NFIL3 is required for cDC1 development, and it suppresses ZEB2 expression via binding at-165kb *Zeb2* enhancer during the CDP stage. ZBTB46 expression marks the commitment to the cDC1 lineage, while DC-SCRIPT and ETV6 promote the maturation process of cDC1s. WDFY4 is a co-activator that primarily controls the cross-antigen presentation ability of mature cDC1s. **(B)** cDC2s express IRF4 and can be further divided into two subtypes: cDC2a, which have an anti-inflammatory function, and cDC2b, which have a pro-inflammatory function. The development of cDC2a requires T-bet, while the development of cDC2b requires RoRγt. Both cDC2a and cDC2b develop from a CDP and this process is controlled by C/EBP binding at the -165kb zeb2 enhancer. **(C)** This figure illustrates the transcriptional network that controls the development and function of pDCs from bone marrow to peripheral tissues. The development of pDCs from multiple lineages requires the transcription factors PU.1, CBFβ, IRF8, and TCF3. The primary regulator of pDC development is E2-2, controlled by a network of transcription factors, including BCL11A, ZEB2, and ID2. E2-2 also controls the expression of IRF8 via binding to the *Irf8* + 41kb enhancer region at the CDP stage, possibly through complex formation with other transcription factors such as MTG16. The function of SPIB is to retain immature pDCs in the bone marrow, while RUNX2 expression promotes the egress of pDCs from the bone marrow. Type I IFN production, a significant function of pDCs is mainly controlled by IRF5, IRF7, and IRF8. Other transcription factors, such as E2-2, RUNX2, SPIB, NFATC3, and CXXC5, can directly control IRF7 expression and regulate type 1 IFN production. **(D)** moDCs develop from Ly6C^{hi} monocytes under the control of several transcription factors, including KLF4, MafB, and PU.1, as well as low levels of IRF8. The final differentiation of moDCs also requires the activity of IRF4, ETV6, and ETV3. Arrows indicate positive regulation, while bars indicate negative regulation.

This improves the control of infection by limiting Tpex migration to the red pulp and their differentiation into exhausted T cell (88). How this mechanism can be transposed to the control of tumor growth is still not clear despite evidence of the localization of Tpex in distinct niche in the tumor (89, 90).

3 cDC2 development and function

3.1 Transcriptional control of cDC2 development

Similar to cDC1s, cDC2s also develop from the CDP, although the transcriptional circuitry controlling cDC2 development is less well understood (Figure 2B). As opposed to cDC1s, cDC2s express low amounts of IRF8 and instead highly express IRF4 (Interferon Regulatory Factor 4). Conditional ablation of IRF4 in $CD11c^+$ cells has shown impaired, but not the complete loss of cDC2s (91). A potential explanation for the observation that some cDC2s develop in absence of IRF4 could be that the cDC2 population represents a heterogeneous mix of IRF4-dependent and independent subsets. In line with this possibility, a body of work has highlighted a certain degree of diversity within that compartment and the involvement of different TFs (39, 92).

The first report describing cDC2 diversity revealed that the conditional ablation of NOTCH2 (Neurogenic locus notch homolog protein 2) in CD11c-expressing cells resulted in the reduction of $ESAM^+$ splenic cDC2s and lamina propria $CD103^+CD11b^+$ DCs (93). Subsequently, the transcription factor KLF4 (Kruppel-like factor 4) was found to be important for the development of $ESAM^-$ cDC2s (43, 94). This evidence indicates that NOTCH2 and KLF4 independently control the development of functionally distinct cDC2 subsets (94, 95).

Yet, a study addressing cDC2 heterogeneity at a single cell level has put forward an alternative model to the one proposed here above (39). Brown et al. suggested that cDC2 could be separated instead into T-BET (T-box expressed in T cells) and RORγt (RAR-related orphan receptor gamma) cDC2s, cDC2a and cDC2b respectively. Importantly, in the aforementioned study, neither the expression of *Klf4* or *Irf4* enable the discrimination of cDC2a from cDC2b. Instead, the authors proposed the use of additional cell surface markers, namely CLEC10A and CLEC12A, to separate cDC2a and cDC2b. Interestingly, the interrogation of chromatin accessibility revealed that open chromatin regions in cDC2a showed an enrichment for RBPJ (Recombination signal binding protein for immunoglobulin kappa J region) motifs. As RBPJ is the DNA-binding component of the NOTCH TF complex, this finding is compatible with the earlier reported role for NOTCH2 signaling in controlling cDC2 heterogeneity (39, 93).

In addition to the aforementioned role for ZEB2 in controlling pDC differentiation, a role for ZEB2 in controlling cDC2 development has been shown. However, its function remains controversial as conflicting results have been reported. One study showed that conditional deletion of ZEB2 in $Itgax^{cre}Zeb2^{fl/fl}$ mice led to reduced number of splenic cDC2s (65), but a subsequent study failed to confirm this observation (66). This latest study is somewhat contrasting with the development of a novel mouse model lacking cDC2s and other myeloid lineages (57). In this study, a triple mutation of all three NFIL3-C/EBP sites within the -165Kb enhancer of *Zeb2* ablated its expression exclusively in the myeloid compartment and led to the complete loss of pre-cDC2 specification and mature cDC2 development *in vivo* (57). Whilst the nature of this discrepancy warrants further investigation, these studies also highlighted ZEB2 as a critical regulator of pDC development through its repressive activity on ID2, as well as its important role for monocytes commitment as these 2 populations were strongly affected in this mouse model (57).

3.2 Diversity and function of cDC2s in mice and human

Compared to cDC1s, cDC2s appear more efficient in presenting antigens via MHC-II molecules to $CD4^+$ T cells (1, 96). However, cDC2s are not equally able to present soluble versus cell associated antigens. $CD4^+$ T cell proliferation in response to soluble antigen was unperturbed in mice lacking cDC1s ($Xcr1^{DTR}$ mice or $Batf3^{-/-}$ mice), demonstrating that cDC2s compensate for the lack of cDC1s in this setting (53, 97). In contrast, cDC2s are far less efficient than cDC1s in the uptake and processing of cell-associated antigens, and thus display a limited capacity to prime $CD8^+$ T cells through this route (98).

As alluded earlier, mice lacking IRF4 were originally used to define the function of cDC2s (91). These studies led to define a key role for cDC2s in the regulation of Th2 and Th17 immune responses aiming to eliminate extracellular pathogens (*Nippostrongylus brasiliensis*) and parasites (*Aspergillus fumigatus*), respectively (91, 99). At that time, it remained unclear how cDC2s could direct such distinctive responses. Some clarification for this division of labor came from studies highlighting the distinct roles for NOTCH2 dependent and KLF4 dependent cDC2s. For example, in the gut NOTCH2-dependent cDC2s were the critical source of IL-23 that were required for clearance of extracellular pathogens such as *Citrobacter Rodentium* though the induction of a Th17 biased immune response (100, 101). In addition, NOTCH2-dependent splenic cDC2s were required to promote T follicular helper (T_{FH}) cell and germinal center (GC) B cell formation in response to *Listeria monocytogenes* (102, 103). In contrast, it was found that conditional deletion of *Klf4* in DCs was detrimental for Th2, but not Th17, immune responses in mice (94). In line with the above, a STAT6/KLF4 dependent $CD11b^{low}$ cDC2 population localized in the skin has been shown to mediate Th2 immune responses (43).

cDC2s are also important for the T cell response to viral infection. Following PV (single-stranded RNA pneumonia virus) infection, cDC2s can acquire a hybrid phenotype characterized by increased IRF8 expression and the capacity to prime both $CD4^+$ and $CD8^+$ T cells. The acquisition of these cDC1-like properties by cDC2s was dependent on the signaling via Toll-like receptors and the type 1 interferon receptor (104). Additionally, the induction of T_{FH} cell differentiation was dependent on the presentation of viral antigens at the T/B border by migratory cDC2s (102). Furthermore,

LN resident cDC2s are strategically positioned to capture the influenza A virus (105) and other blood born antigens (106) resulting in the rapid initiation of T cell responses, independent of migratory DCs influx. While moDCs were also reported to activate T cells under similar conditions (107, 108), some studies have suggested that inflammatory cDC2s can acquire moDC like features, such as the expression of MAR-1 and CD64, and the moDCs will express cDC2 signature genes including CD11b and CD172a, suggesting that the antigen presentation capacity of moDCs may actually be due to contamination by inflammatory cDC2s (104, 105). In agreement with this conclusion, the use of CD26 as an additional marker to differentiate inflammatory cDC2 from moDCs, highlighted the limited antigen presentation capacity of $CD26^-$ moDCs (104).

Collectively, these studies highlight the functional specificities of the various cDC2 subtypes within different organs. Deciphering the molecular mechanisms underpinning this diversity is a prerequisite to define the role of these different subsets of cDC2s in initiating adaptive immune responses in the context of pathogens, virus infection and tumor clearance, as this knowledge will provide a rational framework for their use in clinical settings.

4 DC3: a unique DC subtype or the DCs with different cells state?

The application of single-cell RNAseq technology to DCs has led to many reports of novel DC subtypes (38, 40, 92, 109). The use of different annotation strategies to define populations with otherwise very similar transcriptomic features has created a good deal of confusion in the field (110). The status of the DC3 population represents an example of this issue.

DC3s were initially identified in the blood of humans through single-cell RNA sequencing (38). The subsequent studies phenotypically characterized the DC3 population as $CD163^+CD14^+$ DCs that accumulate in the blood of patients with systemic lupus erythematosus (SLE) (40). DC3s display an intermediate phenotype and function between cDC2s and monocytes and are characterized by low expression of IRF8 (111). Unlike cDC1s and cDC2s, the development of DC3s relies on GM-CSF, but not FLT3L, and it is developmentally independent of the CDP (92). Functionally, these cells have been proposed to promote the differentiation of naïve $CD8^+$ T cells into tissue-homing $CD103^+$ T cells (92).

The AXL^+ DC subpopulation was also reported in the blood of humans, alongside the DC3 population, displaying an intermediate phenotype between cDC2s and pDCs (38). This population was characterized by the expression of Siglec6 and AXL. Similarly, in mice, transitional DCs (tDCs), also referred to as "pDC-like" cells, with characteristics spanning between cDC2s and pDCs, were observed during steady-state and influenza infection, and appear to be the equivalent to the AXL^+ DCs in humans (109). It has been recently proposed that these "pDC-like" cells are pre-cDC2s and require KLF4 for both their development and function (112).

Other similar single-cell transcriptomic studies have identified another DC population that exhibits an "activated" DC phenotype and is referred to as "DC3" in both mouse and human (113). This DC population lacks the canonical cDC1s and cDC2s gene signature but expresses the matured cDC1 and cDC2 signatures (113). Similar population have also been described as $CCR7^+LAMP3^+$ DCs, Mreg DCs or ISG^+ DCs within tumors (114–116). It is important to note that these "activated" DC populations represent developmental states of both cDC1s and cDC2s and therebefore they are not to be confounded with $CD163^+CD14^+$ DCs (DC3s) reported by Dutertre, Cytlak, Bourdely and Villani et al. Currently, it is recommended to designate this "activated" DC population as "$CCR7^+$ DCs" due to the consistent detection of CCR7, a common marker for DC activation and maturation, in various contexts except ISG^+ DCs (110, 116).

Sorting out the cellular relationships between the cDC1, cDC2, DC3 and $CCR7^+$DCs populations is one of the key goals for the DC field moving forward. Regardless of their development origins, identifying the environmental cues and the molecular mechanisms driving DC3 and $CCR7^+$ DC phenotype and functional attributes also warrants further investigation.

5 pDC development and function

5.1 pDC ontogeny

pDCs are a distinct cell type first identified through their capacity to rapidly produce large amounts of type I interferons (IFNα/β) (117–120). Whether pDCs developed from lymphoid or myeloid progenitors has remained a controversial question for more than two decades (34, 121). Similar to the development of cDCs, Flt3 signaling is required for optimal pDC development (122). Yet as opposed to cDCs, that can only originate from the myeloid progenitors, $Flt3^+$ CMPs, CDPs and CLPs have all been shown to retain pDC potential both *in vitro* and *in vivo* following adoptive transfer (44, 45, 48, 49, 123–125). These findings led to the concept that pDC have a dual origin: myeloid and lymphoid (Figure 2C). However, the myeloid origin of the pDCs is being disputed by different groups (35, 36, 125, 126). This issue has been revisited with $IL\text{-}7R^+$ lymphoid progenitors being proposed to be the main source for pDCs *in vivo* (126). A predominantly lymphoid origin for the pDCs is also supported by their expression history of the recombination activating gene 1 (*Rag1*) and the rearrangement of the D-J regions of the *Igh* locus (125, 127). In an effort to distinguish the properties of myeloid- vs lymphoid-derived pDCs, it was found that the myeloid-derived $Zbtb46^+$ pDCs have a distinct transcriptome that resulted in them being more efficient than lymphoid-derived pDCs in their capability to present antigens to T cells (125). While this study is accordance with earlier reports pointing to the dual origin of pDC (127), these findings were subsequently challenged by a study that proposed that a $CD115^-$ $Ly6D^+$ lymphoid progenitors are the sole source of pDCs *in vivo* (126). Crucially, the definition of a lymphoid or myeloid origin of

pDCs largely depends on the markers used to track the development history of pDCs. For example, Dress et al. used CD2 as a lymphoid lineage marker to trace the development history of pDCs, and conclude that the pDCs are of lymphoid origin (41, 126). However, CD2 expression is not restricted to the lymphoid lineage as 20% of the cDC are fate mapped in the hCD2-iCre$^{+/-}$R26-stop-EYFP^{+} mouse model (128), thus this model cannot completely rule out the participation of myeloid biased progenitor to the pDC pool. Adding to that, clonal tracing of HSC and CX3CR1^{+} progenitors using *FlipJump* system and single-cell transcriptome and phenotype analysis (CITE-seq) suggested that cDCs and pDCs share a common progenitor (129). Further characterization of the pDCs specific transcriptional program will be helpful to improve our understanding of pDC ontogeny and the heterogeneity of this population.

5.2 Transcriptional control of pDCs development

The development of pDCs requires the TF E2-2 (E protein encoded by *Tcf4*) (Figure 2C). E2-2 deficient mice die *in utero*, but transfer of *Tcf4*$^{-/-}$ fetal liver cells into irradiated WT recipients results in the complete loss of pDCs from the BM and all peripheral lymphoid organs, but has no impact on the development of other myeloid or lymphoid cell types (33). E2-2 is a member of the basic helix-loop-helix superfamily of TFs that has long (E2-2$_L$) and short (E2-2$_S$) isoforms (130). E2-2$_S$ is expressed in all hematopoietic progenitors and different types of mature immune cells, but E2-2$_L$ is preferentially expressed in pDCs and binds to the pDC specific 3' enhancer of *Tcf4* to maintain E2-2s expression via a positive feedback loop (130). E2-2s expression initiates in HSCs and is further upregulated during pDC development. E2-2s forms a complex with Mtg16 (myeloid translocation gene on chromosome 16) to directly control the expression of key genes involved in pDC development and function, including CCR9, TLR9, Bst2 and B220 (131). In DC progenitors, ID2 as an E protein inhibitor binds E2-2s preventing its binding to DNA, and thereby inhibits their pDC potential (63). In contrast, ZEB2 expression in progenitors prevents ID2 expression, enabling E2-2s to promote pDC development. In line with the above, constitutive deletion of -165kb *Zeb2* enhancer featuring a cluster of E box motifs, results in lack of ZEB2 expression, increased ID2 expression that prevents pDC differentiation (132). Thus, the coordinate action of E2-2$_L$, E2-2s, ID2 and ZEB2 dictates pDCs development at steady state.

Other TFs have been implicated in the cellular fate of BM progenitors. PU.1 is highly expressed in myeloid and lymphoid BM progenitors, but its expression level is substantially reduced following the commitment of progenitors to the pDC lineage (55, 122, 133, 134). High expression of PU.1 in cDC was shown to be essential to maintain their identity as PU.1 deficient cDCs gained pDC like features (55). Thus, it is conceivable that downmodulation of PU.1 in progenitors constitutes a key instrumental step in allowing pDC differentiation (135). In line with this, the expression of PU.1 is negatively regulated by BCL11A (B-cell chronic lymphocytic leukaemia/lymphoma 11A), a critical regulator of pDC development (136). Adding to that, loss of PU.1 in CD11c^{+} cells resulted in an increased differentiation of progenitor toward the pDC lineage, although PU.1 deficient pDCs were dysfunctional, as IFNα production was reduced in PU.1 deficient pDCs (55). In contrast to the down-modulation of PU.1 following pDCs commitment, IRF8 expression is increased markedly during pDC development (67). Thus, it is somewhat surprising, that IRF8 deficiency in CD11c^{+} cells has no impact on the development of pDCs. This is in fact due to a compensatory mechanism provided by IRF4 as double knockout mice lack pDCs (67). Although IRF8 is dispensable for pDC differentiation, it is essential for their IFNα production, thus indicating a nonredundant role for IRF8 in controlling pDC function.

Spi-B is another ETS family TF that is highly expressed in pDCs (137). In contrast to the decreased PU.1 expression following pDCs development, Spi-B expression is substantially increased from progenitors to mature pDCs. Germline deletion of *SpiB* results in decreased pDC numbers in the BM but their numbers are increased in peripheral organs (138). These data suggests that Spi-B is dispensable for pDC differentiation but a critical regulator of pDC homeostasis. Having said that, its role and its mode of action in pDCs remains under investigated. In contrast to BM, the TF RUNX2 (RUNX family transcription factor 2) promotes pDC their egress, as germline ablation or tamoxifen induced deletion of RUNX2 result in reduced number of peripheral pDCs, whilst RUNX2 is dispensable for their differentiation in the BM (139, 140). Two mechanisms were proposed. Sawai et al. showed that RUNX2 was required for the expression of chemokine receptors on the cell surface of pDCs including CCR2 and CCR5 that were required for the migration of pDCs from BM into the periphery in response to their ligands (139). In contrast, Chopin et al. demonstrated that RUNX2 deficiency resulted in increased expression of CXCR4, a key chemokine receptor associated with BM tropism (140). Spi-B and RUNX2 are not only critical regulators of pDC homeostatic in the periphery but also have been both shown to be critical for IFNα production by pDC, though the regulation of *Irf7* (138, 140).

BCL11A is a zinc-finger TF and is known to regulate lymphoid development (141). Both BCL11A and PU.1 control *Flt3* expression in early hematopoietic progenitors (142), which is required for pDC development and their homeostasis. ChIP-seq data showed that BCL11A bound to the *Tcf4* proximal promoter and knockdown of BCL11A strongly reduced E2-2 expression (136). Interestingly, downregulation of *Bcl11a* occurred after *Tcf4* deletion in BM derived pDCs (143), indicating a positive feedforward loop between BCL11A and E2-2 in controlling pDC development.

IKAROS (encoded by *IKAROS Family Zinc Finger 1* (*Ikzf1*)) is a zinc-finger DNA-binding protein that homo- or hetero-dimerizes with other IKAROS family members to suppress the gene

expression. IKAROS prevents premature cDC gene expression in CDPs and promotes pDC development (144, 145). The relationship between IKAROS with other TFs that control the development and function of pDCs has not been studied.

Collectively, these studies have revealed a dynamic TF network that regulates the development of pDCs within the hematopoietic system. These studies also highlight a critical point in the current debate about whether pDCs and cDCs share a common ancestor. These findings suggest that the lineage trajectories of DCs are dictated by mutual antagonism between transcription factors (E2.2/ZEB2 vs ID2/NFIL3 or PU.1 vs BCL11A), thus inferring a close relationship between pDCs and cDCs.

5.3 The function of pDCs in mouse and human

Unlike cDCs, pDCs have limited capacity to present antigens. Instead, their key feature is the rapid production of type I IFNs (IFNα/β) after exposure to the ligands for TLR7 (recognize ssRNA) and TLR9 (recognize CpG), especially after the viral infection (33, 146, 147). The early production of type I IFNs by pDCs initiates the anti-viral gene expression program in many cell types and promotes the expansion of NK cells and virus specific CTLs for viral clearance (146, 147). This type I IFN production results in the apoptosis of activated pDCs, potentially limiting the scale of inflammatory response and preventing pathology associated with an overly active anti-viral immune response (148). This control appears important as aberrant type I IFN production by pDCs is strongly linked to the development of autoimmune diseases like SLE and systemic sclerosis in both mouse models and human (149, 150).

6 moDCs development and function

6.1 Transcriptional control of moDCs development

The ambiguous nature of moDCs has hampered our capacity to define some of the key TFs associated with their differentiation. Lineage tracing experiments have demonstrated that moDCs derive from a separate myelopoiesis branch distinct from the one producing cDCs and pDCs (151). In contrast to the requirement of high dose IRF8 for cDC1 development, moDCs develop in a relatively low concentration of IRF8. This expression of IRF8 is driven by *Irf8* + 56kb enhancer whose activation is controlled by RUNX-CBFβ (62). The differentiation of Ly6C$^+$ monocytes into moDCs or macrophages is controlled by the TFs IRF4 and MafB (MAF BZIP Transcription Factor B), and PU.1 (Figure 2D) (152–154). The differentiation of mouse monocyte into moDCs in presence of GM-CSF and IL-4 requires IRF4. In its absence, the cells differentiate into macrophages (155). It also had been reported that MafB expression will push the human monocytes into the macrophage pathway, while high concentration of PU.1 will suppress MafB and thus promote differentiation into moDCs (152, 156). Apart from PU.1, a most recent study found that ETV3 and ETV6 are able to repress macrophages development potential in monocytes by suppressing MafB expression in both mouse and human (154). Thus, moDCs use a distinct repertoire of TFs compared to those that promote cDC development.

6.2 The function of moDCs

Monocytes represent a major cell population in the circulation, from which they are recruited into the tissues by inflammatory cues and give rise to both macrophage and moDCs. Normally, monocytes express Ly6C and macrophage colony stimulating factor receptor (M-CSFR/CD115) and respond to GM-CSF (157). The moDCs can be easily confounded for cDCs in tissues as they share a variety of cell surface markers including the "canonical DC markers" MHC-II and CD11c, as well as the cDC1 marker CD24 and the cDC2 marker CD172a (158). In addition to sharing cDC phenotypic features, moDCs can present antigen to both CD4$^+$ and CD8$^+$ T cells. Notably, moDCs can cross-present antigen released from certain microorganisms to CD8$^+$ T cells under acute inflammation condition and might replace some (41), but not all anti-infection functions of cDCs (77). As per their cDCs counterpart, moDCs express costimulatory molecules that support the differentiation of CTLs (159) and present antigen directly to CD4$^+$ T cells promoting their differentiation into Th17 cells (160). Furthermore, moDCs are strong producers of proinflammatory cytokines including IL-1β, TNFα, IL-23 (161), and IL-12 in cancer (162). Collectively, although moDCs arise from a distinct myeloid branch compared to cDCs, both subsets share a substantial number of overlapping phenotypic and functional characteristics after activation.

7 Concluding remarks

Recent advances in the field of DC research have provided new insights into the heterogeneity and functional diversity of DC subsets. Studies on the transcriptional regulation of DC development and function have led to the identification of key TFs and their targets that shape the transcriptome and function of DCs. In-depth phenotyping of DCs has also identified novel DC subtypes, such as DC3, highlighting the need for continued investigation into the ontogeny of DCs. While much progress has been made, much is still to be learned about the intricate connections between different TFs and their doses regulating the differentiation and activation of DCs.

Whilst we try to build a comprehensive map of the transcriptional network governing DC heterogeneity, which will be essential for their clinical application, there is an urgent need to understand how DC functionalities, independently of their origin, are shaped by environmental signals. To fulfill the long-recognized potential of DC based therapy to treat malignancies, we believe that an in-depth characterization of the signals that drive their diversity and a better under understanding of the environmental cues that shape their functional attributes is urgently required.

Author contributions

SZ, CA and MC contributed to original draft preparation. SZ contributed to the figures. SN, MC, CA and SZ contributed to review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Ashley Ng,
The University of Melbourne, Australia

REVIEWED BY
Jean-Christophe Bories,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Jacob T. Jackson,
The University of Melbourne, Australia

*CORRESPONDENCE
Catherine L. Carmichael
catherine.carmichael@hudson.org.au

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An "unexpected" role for EMT transcription factors in hematological development and malignancy

Karthika Radhakrishnan[1], Lynda Truong[1] and Catherine L. Carmichael[1,2]*

[1]Centre for Cancer Research, Hudson Institute of Medical Research, Clayton, VIC, Australia, [2]Monash University, Faculty of Medicine, Nursing and Health Sciences, Clayton, VIC, Australia

The epithelial to mesenchymal transition (EMT) is a fundamental developmental process essential for normal embryonic development. It is also important during various pathogenic processes including fibrosis, wound healing and epithelial cancer cell metastasis and invasion. EMT is regulated by a variety of cell signalling pathways, cell-cell interactions and microenvironmental cues, however the key drivers of EMT are transcription factors of the ZEB, TWIST and SNAIL families. Recently, novel and unexpected roles for these EMT transcription factors (EMT-TFs) during normal blood cell development have emerged, which appear to be largely independent of classical EMT processes. Furthermore, EMT-TFs have also begun to be implicated in the development and pathogenesis of malignant hematological diseases such as leukemia and lymphoma, and now present themselves or the pathways they regulate as possible new therapeutic targets within these malignancies. In this review, we discuss the ZEB, TWIST and SNAIL families of EMT-TFs, focusing on what is known about their normal roles during hematopoiesis as well as the emerging and "unexpected" contribution they play during development and progression of blood cancers.

KEYWORDS

EMT, hematopoiesis, leukemia, blood cells, stem cells, malignancy

1 Introduction

The Epithelial to Mesenchymal Transition (EMT) is a physiological process whereby epithelial cells transform into a more mesenchymal phenotype, enabling them to migrate away from their epithelial layer of origin. Typically, epithelial cells are arranged side by side through strong intercellular junctions and are attached to the basement membrane with a clear apical-basal polarity. The cells are held together and to the basement membrane through various cell adhesion molecules such as claudin and E-cadherin. In contrast, mesenchymal cells are generally motile with only transient polarity and intercellular junctions. Depending on the biological context, EMT can be classified into three types.

Type I EMT occurs during normal embryonic development and was first described in chicken embryos (1, 2), Type II EMT occurs during tissue repair, wound healing and fibrosis (reviewed in (3, 4) and Type III EMT occurs during pathogenic processes – most notably cancer metastasis (5).

Several key transcription factors, hereafter termed EMT-transcription factors (EMT-TFs), play fundamental roles in regulating the initiation and progression of all three types of EMT. These EMT-TFs belong to three distinct families, the ZEB (ZEB1 and ZEB2), TWIST (TWIST1 and TWIST2) and SNAIL (SNAI1, SNAI2 and SNAI3) families. During embryogenesis, these EMT-TFs are critically important for regulating essential developmental processes such as gastrulation, mesoderm specification, neural crest formation and skeletal development (6–11). In the malignant context, EMT-TFs also play fundamental roles in critical aspects of cancer cell function and survival including tumor progression and metastasis, resistance to therapy, immune evasion and stemness (12–18).

There has been an increasing interest in the role EMT-TFs play in the development and functioning of the hematopoietic system, even though there is no obvious EMT process involved. Even more surprisingly, these EMT-TFs are now also emerging as significant contributors to the pathogenesis and development of malignant hematological disease. However, the underlying mechanisms of their involvement are not yet fully understood. In this review, we discuss the ZEB, TWIST, and SNAIL families of EMT-TFs and outline their "unexpected" functions in regulating normal and malignant blood cell development.

2 EMT transcription factors (EMT-TFs)

2.1 ZEB family

The Zinc-finger E-box binding homeobox (ZEB) family of transcription factors were first discovered in *Drosophila melanogaster* by Fortini et al. (19). Fortini described two highly conserved homologous genes, *zfh1* and *zfh2 (*now known as *ZEB1* and *ZEB2)* that encode for large proteins containing multiple N- and C- terminal DNA-binding C_2H_2 zinc-fingers separated by a homeodomain region (19). Lai et al. found *zfh1* to be expressed in the early embryonic mesoderm, along the dorsal vessel and in the developing central nervous system (CNS). Expression of *Zfh2*, on the other hand, was largely localized to the CNS and hindgut of developing embryos (20). Chicken *Zeb1 (Zfh1)* was later identified during embryonic lens development as a transcriptional repressor of the δ1-crystallin enhancer core (21). This study subsequently found ZEB1 to be primarily expressed during the post-gastrulation period in mesodermal tissues, neuroectoderm, neural crest and lens (21). Murine *Zeb1* was first cloned from a mouse brain cDNA library in 1996 (22), while mouse *Zeb2* was initially named *Sip1* (for SMAD-interacting protein 1) following its identification in a yeast two-hybrid system using the MH2 domain of Xenopus Smad1 as bait (23).

The vertebrate ZEB1 and ZEB2 proteins share a high degree of structural similarity, with both carrying C_2H_2 zinc-finger clusters at their N- and C-terminal ends that bind E-box and E-box-like DNA motifs (5'-CACCTG-3') (23, 24). Around 85% of protein sequence identity within ZEB1 and ZEB2 is shared at the zinc-finger domains, whereas only 30-50% sequence identity is shared in the intervening region containing the SMAD interaction domain (SID), homeodomain (HD) and C-terminal binding repressor protein (CtBP) interaction domain (CID) (25, 26). ZEB proteins primarily act as transcriptional repressors, through interaction with SMAD proteins, the CtBP and histone remodeling complexes such as the nucleosome remodeling and deacetylase complex (NURD) (27, 28). One of the best characterized targets of ZEB proteins is the CDH1 gene, encoding E-cadherin, a key epithelial gene that is downregulated during the EMT process (27, 29).

Zeb1 knockout mice display skeletal and craniofacial defects and die shortly after birth due to a failure to respirate (30, 31). Homozygous *Zeb1* mutant mice, lacking the C-terminal zinc-finger domain, also experience perinatal lethality with ~80% of mice dying within two days of birth. However, in contrast to full knockout mice, *Zeb1* mutant mice are morphologically normal with the exception of a significantly reduced thymus (32). In the adult ZEB1 has been shown to be a critical regulator of bone development, with *Zeb1* expression found to be downregulated as mesenchymal stem cells (MSCs) differentiate down the osteoblastic lineages in the presence of BMP-2 (33). *In vitro* knockdown of *Zeb1* in MSCs resulted in enhanced osteogenesis, while *in vivo* osteoblast knockdown of *Zeb1* increased bone mass in the ovariectomized mouse model of osteoporosis (34). Interestingly, Fu et al. reported that *Zeb1* deletion in endothelial cells reduced bone associated angiogenesis and subsequently impaired bone formation (35). These findings indicate that ZEB1 has differential functions within endothelial and osteoblastic cells which coordinately contribute to bone development and maintenance. How the expression of ZEB1 is controlled in these different cell types and what level of crosstalk is involved remains to be elucidated.

Zeb2 KO mice die around E9.5, exhibiting growth retardation as well as failure of neural tube closure and neural crest delamination (36). Various conditional *Zeb2* deletion models have demonstrated a critical role for ZEB2 in neurological, gastrointestinal, craniofacial and CNS development (reviewed in (37). Germline *de novo ZEB2* mutations or deletions cause a dominant syndromic form of Hirschsprung disease (HSCR) called Mowat-Wilson Syndrome. Patients with this syndrome exhibit microcephaly, mental retardation, submucous cleft palate among other distinct facial features (38–40).

2.2 TWIST family

The Twist family consists of two members, TWIST1 and TWIST2 (DERMO-1), which exist as a sub-class of the basic helix-loop-helix (bHLH) superfamily of transcriptional repressors. This superfamily is characterized by the presence of a bHLH motif, which is a short chain of basic amino acids followed by two amphipathic α-helices separated by a more divergent loop (41–43). The basic region of the bHLH motif serves to recognize and

bind E-box sequences in the DNA, while the HLH region is responsible for forming homo/heterodimers with other bHLH proteins (44, 45). Through recognition of distinct E-box sequences, heterodimerization with different bHLH proteins allows significant heterogeneity in the target DNA sequences bound by TWIST proteins.

Twist1 was first discovered in *Drosophila melanogaster* by Simpson et al. who identified that embryonic lethal *twi* mutations resulted in abnormal gastrulation, impaired dorso-ventral patterning and failed mesoderm differentiation, resulting in an embryo with a 'twisted' phenotype (46). The Drosophila *twist* gene was subsequently cloned in 1987 (7) and mouse *Twist1* in 1991 (47). *Twist1* KO mouse models are embryonic lethal at E11.5, and show a failure of neural tube closure and developmental defects impacting somite formation, cranial mesenchyme and limb development (10). The human *TWIST1* gene displays 92% sequence identity with murine *Twist1* and was mapped by Wang et al. to chromosome 7p21 (48). Haploinsufficiency of the *TWIST1* gene in humans results in Saethre-Chotzen syndrome, a congenital anomaly characterized by craniosynostosis as well as facial and limb anomalies (49–51).

The *Twist2* gene was first discovered in mice by Li et al. using a yeast two-hybrid system to screen for binding partners of the bHLH protein, E12. This study identified a novel bHLH dimerization partner, which was named *Dermo-1* due to its expression in the embryonic murine dermis (52). Like TWIST1, TWIST2 is also detectable throughout embryonic development and during the neonatal period, however it is downregulated in adult tissues (52). An early study by Sosic et al. revealed that unlike *Twist1* KO, *Twist2* KO mice were viable and born at expected mendelian ratios. *Twist2* KO mice did, however, display significant post-natal abnormalities including growth retardation, cachexia and elevated levels of pro-inflammatory cytokines. KO mice also experience perinatal lethality with 60% of homozygous KO mice dying within three days of birth (53). A later study by the same group identified germline nonsense homozygous mutations in the *TWIST2* gene in patients with autosomal recessive Setleis syndrome, an inherited developmental disorder under the branch of Focal Facial Dermal Dysplasia (FFDD) (54, 55).

2.3 SNAIL family

The Snail family of transcription factors consists of three members, SNAI1 (Snail), SNAI2 (Slug) and SNAI3 (Smuc) characterized by the presence of a highly conserved C_2H_2 zinc-finger C-terminal region containing four to five zinc fingers and a more diverse amino-terminal region. The C_2H_2 zinc-fingers allow Snail family transcription factors to recognize and bind E-box elements in target DNA sequences (56, 57). All Snail family members also contain a highly conserved eight amino acid (MPRSFLVK) N-terminal SNAG repressor domain (58, 59). Studies have shown that the Snail family predominantly act as transcriptional repressors across a plethora of developmental and EMT-related pathways (60–62).

SNAI1 was the first and founding member of the Snail family, originally identified in *Drosophila melanogaster*. Embryos with loss of function mutations in the *Sna* gene show defects in gastrulation, mesoderm specification and embryo patterning resulting in an embryo resembling a Snail (46, 63). The murine *Snai1* gene was cloned in 1992 and found to be expressed in mesoderm and primitive ectoderm during gastrulation, as well as in the pre-somitic mesoderm, neural crest, developing lung, gut and kidney and early stages of cartilage differentiation (64). Mouse *Snai1* KO is embryonically lethal at E7.5-8.5 due to defects in gastrulation and mesoderm formation (65). SNAI1 is a major driver of the EMT process, playing a key role in repressing the epithelial specific cadherin, E-Cadherin, through binding to E-box sequences in its promoter (66). Other EMT related genes regulated by SNAI1 include epithelial markers such as claudins, occludins and desmoplakins and mesenchymal markers such as vimentin and fibronectin (60, 67, 68).

The *Snai2* gene, also known as *Slug*, was first identified by Nieto et al. in chickens as a homolog for the Xenopus *snai1* gene (69). Using antisense oligos towards *snai2*, Nieto et al. further identified a role for this gene in EMT processes associated with neural tube development and mesoderm emergence from the primitive streak (69). The mouse homolog of *Snai2* was subsequently cloned from mouse cDNA using chicken *Snai2* oligos, and found to initiate EMT when ectopically expressed in a rat carcinoma cell line (70). In sharp contrast to *Snai1* KO mouse models, *Snai2* KO mice are viable however, exhibit severe growth retardation and eyelid malformations as well as pigmentation, gonadal and hematopoietic defects post birth (71, 72). Germline homozygous SLUG deletions have been identified in Waardenburg disease, a congenital disorder characterized by hearing loss and pigmentation changes in hair, skin and eyes (73).

The third member of the Snail superfamily, *Snai3* (also known as *Smuc*), was the last to be identified and is the least well understood. In 2000, Kataoka et al. isolated a *Snai1*-related gene from mouse tissues, initially named *Smuc*, which was highly expressed in the skeletal muscle and thymus (74). The human *SNAI3* gene was later identified using *in silico* analysis, and determined to contain the conserved SNAG domain as well as five DNA-binding zinc fingers (75). Murine *Snai3* KO mice do not exhibit any obvious abnormalities [Bradley et al., 2013 (76); Pioli et al., 2013 (77)], suggesting a possible redundant role for *Snai3* alongside its other family members.

3 An emerging role for EMT-TFs in hematopoiesis

Hematopoiesis is not readily associated with EMT, although an exception to this could be the emergence and generation of primitive HSCs in the embryo (78). In vertebrates, hematopoiesis occurs in two waves: primitive hematopoiesis, which occurs during early embryogenesis, and definitive hematopoiesis, which occurs during later stages of development (79, 80). Unlike primitive HSCs, definitive HSCs can give rise to the entire hematopoietic system and

persist throughout life. Definitive HSCs arise from a population of hemogenic endothelial cells in the embryonic AGM (embryonic aorta, gonad and mesonephros region) (81). This process of Endothelial to Hematopoietic transition (EHT) closely resembles EMT and is characterized by a loss of endothelial characteristics and increased migratory capabilities (82, 83). EMT-TFs have thus far not been implicated in EHT and HSC emergence in the embryo, however there is increasing evidence that these factors are expressed in hematopoietic cells and play important roles in regulating normal blood cell development and function (Figure 1; Tables 1, 2).

3.1 ZEB family

ZEB1 is expressed widely throughout hematopoiesis, with the greatest expression observed in hematopoietic stem and multipotent progenitor cells (HSPCs) as well as in more differentiated myeloid, erythroid, and lymphoid cells. Conversely, ZEB1 expression is significantly lower in committed myeloid-restricted and lymphoid-restricted progenitors (99, 100). *Zeb1* mutant embryos, lacking the C-terminal zinc-finger domain, experience perinatal lethality with ~80% of mice dying within two days of birth (32). Homozygous mutant embryos are morphologically normal; however, they show significant thymic atrophy and drastically reduced thymocyte number, a phenotype that persists in the 20% of mice surviving the perinatal lethality period (32). Thymocyte analysis in surviving mice revealed a significant reduction in both immature and mature T cells, with the majority of detectable thymocytes being double positive (DP) $CD4^+CD8^+$ or single positive (SP) $CD4^+$ or $CD8^+$ T cells. A concurrent reduction of mature T cells was also observed in the peripheral lymphoid organs of these mice (32). B and myeloid cell development appeared unaffected, with numbers of these cells in the spleen and bone marrow of *Zeb1* mutant mice reported to be normal. A second *Zeb1* mutant mouse with a C-terminal truncation generated through ENU mutagenesis, termed $Zeb1^{Cellophane}$, also displayed thymic atrophy and impaired T cell development. The thymus similarly contained a significantly enhanced proportion of immature double negative (DN) T cells and mature SP T cells, alongside a reduced proportion of intermediate DP T cells (85). Despite these thymic abnormalities, the $Zeb1^{Cellophane}$ mice had normal T cell numbers in the spleen. B cell development was largely normal, although they had a slightly reduced percentage of marginal zone B cells in the spleen and significantly reduced proportion peritoneal B1 cells. These mice also had significantly reduced natural killer (NK) cell numbers, however this phenotype was not described further. Myeloid cell development was not explicitly analyzed in any of these mutant *Zeb1* mouse models.

Almotiri et al. has more recently employed an interferon-inducible Mx1-Cre based approach to conditionally knockout (KO) *Zeb1* in adult hematopoietic cells (100). In this system, two weeks after Mx1-Cre induced *Zeb1* deletion, all KO mice developed reduced monocytic cell numbers but retained normal numbers of granulocytic and lymphoid cells. In line with the constitutive mutant *Zeb1* mice, these inducible

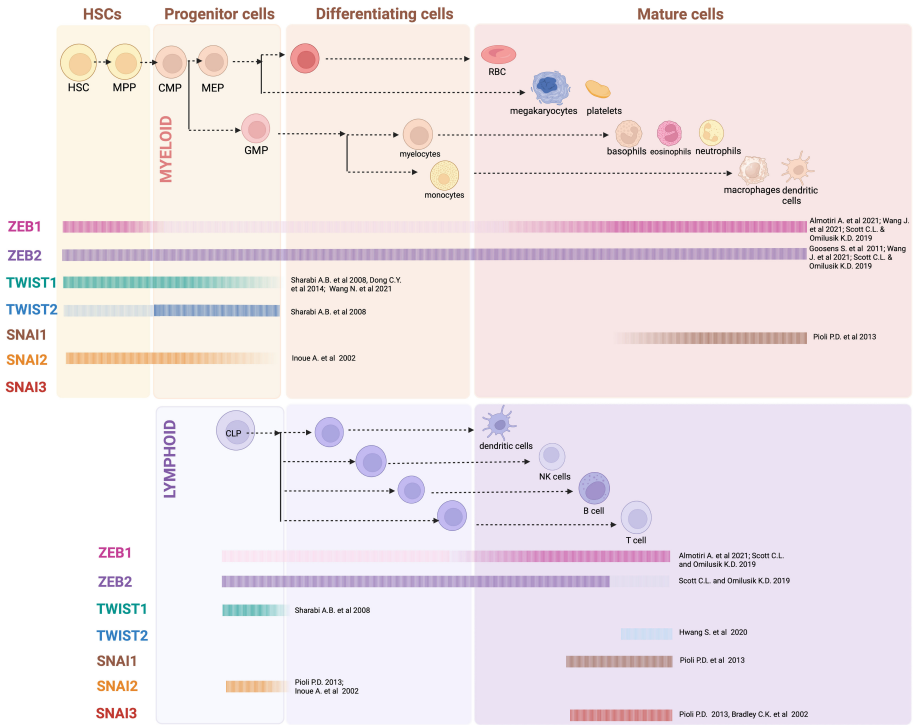

FIGURE 1
EMT-TF expression in hematopoiesis: Generalized overview of EMT-TFs expression throughout the hematopoietic hierarchy, as outlined in referenced articles. In many cases expression of EMT-TFs has not been thoroughly assessed experimentally, and current knowledge relies on gene expression datasets obtained from sorted mouse and/or human cells. Created with BioRender.com.

TABLE 1 Summary of EMT-TF knockout or mutant mouse model hematopoietic phenotypes.

KNOCKOUT/DEFICIENCY						
GENE	MODEL	STEM and PROGENITOR	MYELOID	LYMPHOID	OTHER PHENOTYPE	REFERENCE
ZEB1	Constitutive C-terminal deletion			Reduced thymus size, reduced T-cells in thymus and periphery. Majority T cells detected in thymus were mature DP and SP	CD4 + ATLL	Higashi Y 1997 (32), Hidaka T 2008 (84)
	Constitutive C-terminal deletion			Reduced thymus size with reduced DF T-cells but enhanced DN and mature SP T cells. Normal T cells but abnormal B cells in the spleen		Arnold C, 2012 (85)
	Inducible KO (MX1-Cre)	Normal HSC numbers at steady state but have defective competitive transplantation ability, reduced self-renewal and impaired differentiation. Normal HSC homing and migration	Reduced monocytes, normal numbers of granulocytes	Normal lymphoid cells in blood Reduced thymus size with reduced DP T-cells but enhanced DN and mature SP T cells. DN1 to DN2/3 block also evident Reduced effector and central memory CD8+ T cells in periphery		Almotiri A, 2021 (100)
	Conditional KO (haematopoietic cells): inducible KO (RosaERT2Cre)	Reduced HSPC number	Reduced monocytes	Reduced thymic cellularity, reduced % DN4 with increased CD8+. B-cells normal		Wang J, 2021 (86)
ZEB2	Constitituve KO	Impaired HSPC differentiation all lineages and migration in embryos				Goossens S, 2011 (87)
	Inducible KO (MX1- Cre)	Increased HSCs and MEPs. but reduced GMPs	Reduced monocytes and erythroid cells, enhanced granulocytes and immature megakaryocytes	Reduced B-cells and a block in development from pre-pro-B to pro- B. Reduced T cells	Myeloproliferative disease splenomegaly bone marrow fibrosis	Li J, 2017 (88)
TWIST1	Constitutive KO	Reduced GM-CFU. M-CFU, BFU-E formed from AGM 10.5		E10.5 AGM cells show impaired B cell development on OP9 co-culture		Kulkeaw K, 2017 (89)
	Constitutive KO	HSCs have reduced repopulating capacity				Dong CY, 2014 (90)
	Inducible KO (MX1- Cre)	Reduced HSPCs with impaired self-renewal and reduced quiescence. Reduced lymphoid and meg/eryth differentiation with enhanced granulocyte/macrophage differentiation. Loss of lymphold biased HSCs. Reduced engraftment in competitive transplantation. HSC homing normal			HSCs sensitive to irradiation induced DNA damage and apoptosis. 5FU treatment also led to rapid HSC exhaustion and haematopoletic failure	Wang J, 2021 (91)
TWIST2	Constitutive KO	Enhanced GMPs with increased proliferative capacity and enhanced differentiation *in vitro*	Enhanced myeloid cell numbers, increased macrophages, neutrophils and basophils. Normal erythrocytes and platelets	Normal lymphoid numbers	Meylodysplasia/ myeloproliferation?	Sharabi A B, 2008 (92)

(Continued)

TABLE 1 Continued

KNOCKOUT/DEFICIENCY						
GENE	MODEL	STEM and PROGENITOR	MYELOID	LYMPHOID	OTHER PHENOTYPE	REFERENCE
SNAI1	Conditional haematopoietic specific KO		Normal	Normal		Carmichael C, 2017 (93)
	Constitutive KO	Normal HSC numbers. Reduced BFU-E and CFU-E in spleen, normal in BM	Normal myeloid cell numbers macrocytic anaemia	Reduced thymus size, reduced DP T cells. B cells normal increased T cell apoptosis	Stress erythropoesis impaired	Perez-Losada J, 2002 (72)
	Constitutive KO	Slightly reduced CFU-GM CFU-M, BFU-E CFU-E	Normal myeloid cell numbers	Normal lymphoid numbers	HSCs increased sensitiviy to DNA damage and increased apoptosis, unable to recover haemat system after irradiation LD50 dose.	Inoue A, 2002 (94)
SNAI2	Constitutive KO	HSCs show enhanced repopulating capacity in competitive transplants. HSCs show normal homing and differentiation but increased self-renewal and proliferation capacity	Normal	Normal	5FU induced enhanced HSC cycling and proliferation leading to enhanced haematopoietic recovery	Sun Y, 2010 (95)

TABLE 2 Summary of EMT-TF overexpression mouse model hematopoietic phenotypes.

OVEREXPRESSION						
GENE	MODEL	STEM and PROGENITOR	MYELOID	LYMPHOID	OTHER PHENOTYPE	REFERENCE
ZEB1	Transgenic mouse, Vav-iCre		Expanded monocytic development, increased myeloid, extramedullary haematopoiesis, splenomegaly			Wang J, 2021 (86)
ZEB2	Transgenic mouse, Vav-iCre		Expanded monocytic development, increased myeloid, extramedullary haematopoiesis, splenomegaly	Impaired T cell development, DN block, expanded DN population	ETP-ALL, extramedullary haematopoiesis, splenomegaly, myeloproliferation?	Wang J, 2021 (86)
TWIST1	Retroviral overexpression and transplant	Enhanced quiescnece and self renewal, enhanced repopulating capacity, myeloid-erythroid differentaition bias				Dong CY, 2014 (90)
	Transgenic mouse, Vav-iCre	Increased ST-HSCs, increased GMPs.	Enhanced myelopoiesis, increased immature myeloid cells with enahcned self-renewal and proliferative capacity		Myeloproliferation, AML	Carmichael C, 2020 (96)
SNAI1	CombiTA-SNAI1 transgenic				AML, B-lymphomas	Perez-Mancera PA, 2005 (97)
SNAI2	CombiTA-SNAI1 transgenic				AML, B-ALL	Perez-Mancera PA, 2005 (97)
SNAI3	Retroviral overexpression and transplant	Normal HSCs from retroviral SNAI3+ cells	Normal myeloid output from retroviral SNAI3+ cells	Reduced lymphoid (B and T) cell output from retroviral SNAI3+ cells		Dahlem T, 2012 (98)

Zeb1 KO mice also displayed reduced thymic cellularity with an increase in the proportion of immature DN T cells and more mature SP T cells, and a concomitant reduction in the proportion of intermediate DP T cells. Within the DN population, a further differentiation block was apparent between the DN1 and DN2/3 stages of maturation. Overall, the authors concluded that the reduced thymocyte cellularity in *Zeb1* conditional KO mice was likely due to enhanced apoptosis in the more mature DP and SP T cells, suggesting *Zeb1* loss impairs thymocyte survival at these later stages of maturation (100). Almotiri et al. also observed reduced $CD8^+$ central and effector memory T cells in the blood and bone marrow of their *Zeb1* conditional KO mice. This finding correlates with earlier published data showing ZEB1 expression to be important for the development and maintenance of $CD8^+$ T-cell memory (101).

HSCs were present in normal numbers following induction of *Zeb1* KO, however upon competitive transplantation with wild type cells they displayed severe self-renewal and differentiation defects leading to rapid engraftment failure. Bone marrow homing 18 hours post-transplant was normal, demonstrating the migration and invasion capability of *Zeb1* KO HSCs was not impacted. Gene expression analysis of *Zeb1* KO HSCs identified altered expression of EMT related genes, such as those involved in cell adhesion, cell polarity and the cytoskeleton as well as alterations in genes important for both myeloid and lymphoid differentiation (100). In particular, increased expression of the epithelial adhesion molecule EPCAM1 in *Zeb1* KO HSCs was found to enhance their survival by supporting a pro-survival gene expression program, including increased expression of anti-apoptotic BCL-XL, leading to reduced apoptosis. As EPCAM1 is usually downregulated as HSCs differentiate, this increased expression in *Zeb1* KO HSCs also likely contributes to an imbalance between self-renewal and differentiation *in vivo* (100).

Wang et al. independently generated a hematopoietic-restricted KO of *Zeb1* using Tie2-Cre, Vav-iCre or the tamoxifen inducible RosaERT2-cre crossed onto a *Zeb1* floxed background (99). They also generated an inducible *Zeb1/Zeb2* double knockout (DKO) model using the tamoxifen inducible RosaERT2-cre approach. They used these models in combination with bone marrow transplantation studies to examine the role of ZEB1 in hematopoietic differentiation, both alone as well as in collaboration with ZEB2. In these animal models, *Zeb1* KO led to decreased HSPC populations, impaired myeloid cell output (particularly monocytic cells) and reduced thymic cellularity. While absolute numbers were not provided, characterization of T-cell proportions in the thymus revealed a reduced percentage of DN4 T cells and increased percentage of $CD8^+$ SP T cells. Differences in the T-cell phenotype described by Wang et al. and Almotiri et al. may reflect the different models utilized, such as the use of bone marrow transplantation models in the Wang et al. study and the potential immune modulating impacts of polyI:polyC treatment in the Mx1-Cre model utilized by Almotiri et al. Nevertheless, *Zeb1* loss clearly impacts T-cell development in the thymus and it will be important for future studies to clarify the role it plays using more sophisticated lineage restricted knockout models.

Wang et al. further demonstrated that *Zeb1* KO HSPCs had impaired self-renewal potential, as evidenced by decreased hematopoietic colony formation in serial replating assays and reduced capacity to give rise to all mature hematopoietic cells in competitive BM repopulation assays. These HSPC defects were more severe in *Zeb1/2* double knockout (DKO) mice, with mice rapidly succumbing to anemia and cytopenia following tamoxifen induced deletion of both genes. Interestingly, a single wildtype allele of *Zeb2* was sufficient to rescue the hematopoietic defects observed in the DKO mice, indicating that ZEB2 might play a more dominant role in regulating hematopoietic lineage differentiation (99).

In other studies, ZEB1 expression has been detected across all dendritic cell (DC) subsets and neutrophils (102) and *in vitro* culture systems have identified a role for this protein in DC activation and subsequent induction of T cell responses (103). Further research, however, is needed to clarify the role/s of ZEB1 in mature myeloid and lymphoid cell subsets.

ZEB2 is also broadly expressed throughout hematopoiesis, with reduced expression in T cells relative to myeloid and B lineage cells (87, 102). A role for ZEB2 during normal hematopoiesis has been studied using a variety of conditional *Zeb2* loss of function mouse models. Hematopoietic-restricted (Vav-Cre) and combined hematopoietic and endothelial-restricted (Tie2-Cre) KO of *Zeb2* was utilized by Goossens et al. to study the role of ZEB2 in HSC formation and differentiation during embryonic hematopoiesis (87). While ZEB2 was not required for HSC cluster formation in the embryonic AGM region, it played a crucial role in HSPC differentiation and migration. *Zeb2* KO embryos displayed a severe block in hematopoietic differentiation in all lineages, as evidenced by reduced development of mature blood cells *in vivo* and impaired differentiation in *in vitro* methylcellulose cultures. In addition, *Zeb2* KO embryos showed significant alterations in the localization of HSPCs in the fetal liver, a significant reduction in circulating HSPCs as well as decreased homing of hematopoietic cells to the bone marrow compared with wildtype controls (87). This was attributed to an aberrant increase in the expression of β1 integrin and CXCR4, previously shown to be crucial for HSC mobilization and homing (104–106). Moreover, *Zeb2* KO fetal livers contained increased numbers of HSCs, pointing toward a possible feedback loop compensating for the hematopoietic differentiation block and/or enhanced retention of HSPCs. Interestingly, *Zeb2* deficiency also resulted in high embryonic/neonatal lethality due to intracephalic hemorrhaging. This was proposed to be due to significantly reduced angiopoietin-1 expression and subsequently impaired pericyte coverage of vasculature (87). A similar lethality was not observed in *Zeb1* deficiency models described earlier that were generated using the same approach by Wang et al. (86).

Li et al. generated conditional *Zeb2* KO in adult hematopoietic cells using the interferon-inducible Mx1-Cre approach. *Zeb2* deletion using this model resulted in an increased frequency of HSPCs in the BM and an expansion of megakaryocyte-erythroid progenitors (MEPs) with concomitant reduction of granulocyte-monocyte progenitors (GMPs). Bone marrow in these mice also displayed a reduction in B cells (due to a block in transition from pre-pro-B to pro-B), monocytes and mature erythroid cells along with a significant expansion of granulocytes and immature megakaryocytes (88). The mice also developed splenomegaly, extramedullary hematopoiesis and bone marrow fibrosis suggestive of a myeloproliferative phenotype. Bone marrow

transplantation assays provided evidence that *Zeb2* KO did not alter HSC self-renewal but confirmed their impaired differentiation capacity. These assays also demonstrated that hematopoietic abnormalities in *Zeb2* KO mice were not a consequence of an impaired BM niche (88). Mechanistically, Li et al. identified impaired responsiveness of ZEB2 KO bone marrow cells to IL-3 and IL-6 cytokine signaling but enhanced responsiveness to G-CSF stimulation. This latter finding likely contributing to the predominant granulopoiesis observed in ZEB2 KO mice.

Studies looking at *Zeb2* KO or overexpression during DC development have demonstrated that ZEB2 is required for the development of a subset of DCs and is thought to play a role in maintaining their cell fate or identity (107–109). Mechanistically, Zeb2 was shown to directly repress expression of *Id2*, which negatively impacts plasmacytoid DC (pDC) development. These data implicate *Id2* repression as a mechanism by which ZEB2 drives pDC development (108). Similar roles for ZEB2 in maintaining monocytic (109) and tissue-resident macrophage cell identity have also been identified (110) although the key mechanisms involved remain to be elucidated. While *Zeb2* KO mice do not display overt T cell abnormalities, ZEB2 has been shown to be upregulated following $CD8^+$ T cell activation and is important for promoting $CD8^+$ T effector cell differentiation and survival (111, 112). Interestingly, again here ZEB2's role in $CD8^+$ T effector cell regulation has been contributed, at least partially, to *Id2* repression which is important for $CD8^+$ effector memory differentiation (113, 114). See Figure 2 for an overview of ZEB family roles in hematopoiesis.

3.2 TWIST family

TWIST1 is highly expressed in embryonic HSPCs in the AGM region at E9.5 and E10.5 with significantly lower expression in HSPCs in the E14.5 fetal liver (89, 115). *Twist1* KO is embryonically lethal due to vascular and cranial neural tube defects around E11.5 (10). Kulkeaw et al. found that while *Twist1* deficiency did not affect formation of embryonic HSPCs themselves, it instead impaired embryonic HSPC differentiation (89). This was evidenced by reduced numbers of myeloid and erythroid colonies in *in vitro* colony assays using *Twist1* KO E10.5 AGM-derived cells, as well as impaired B lymphoid differentiation following culture on an OP9 cell layer. Mechanistically, TWIST1 controls embryonic HSPC differentiation, at least partially, through direct regulation of MYB and GATA2 expression (89).

In the adult hematopoietic compartment, TWIST1 expression is most abundant in long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), with its expression declining during differentiation (90, 91). Enforced expression of TWIST1 in HSCs enhanced their ability to repopulate the bone marrow long term following competitive transplantation alongside wild type HSCs, while loss of TWIST1 led to a reduced ability of HSCs to engraft in a similar experiment. TWIST1 overexpressing HSCs also displayed enhanced quiescence and increased self-renewal potential, as well as a specific myeloid/erythroid differentiation bias. These phenotypes were associated with activation of the myeloid lineage-determining factors PU.1 and GATA-1 and downregulation of the lymphoid factor GATA-3 and HSC regulator RUNX1 (90). Conditional

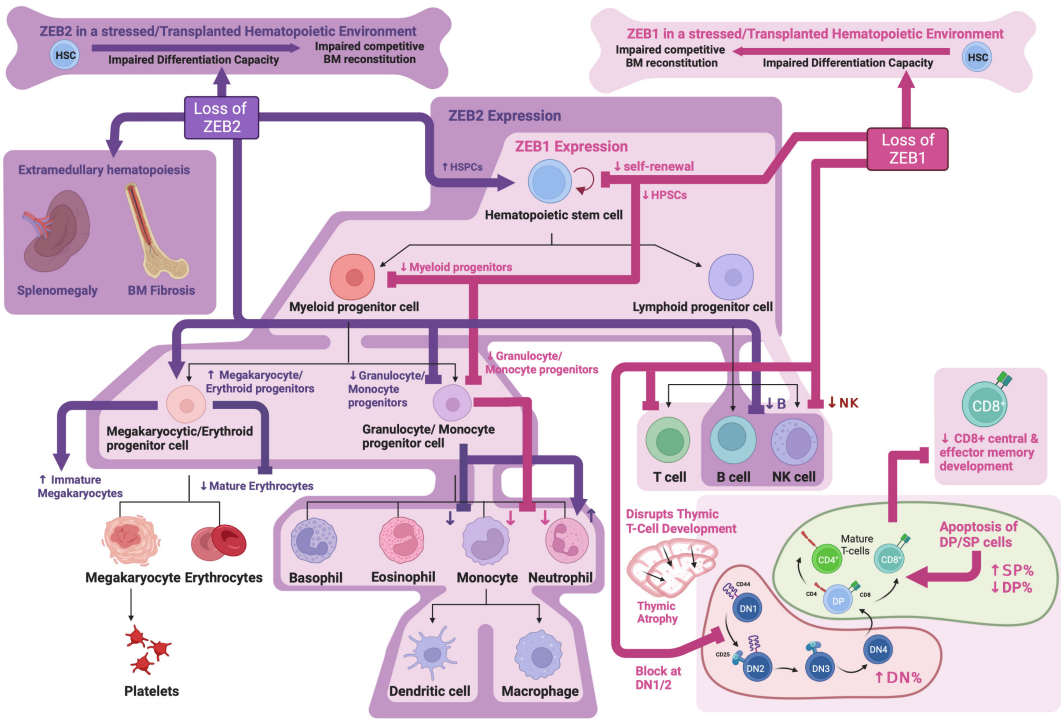

FIGURE 2
ZEB family roles in hematopoiesis: Schematic showing known functions of ZEB1 and ZEB2 during normal hematopoiesis as determined through analysis of knockout mouse models. Created with BioRender.com.

Twist1 KO using an Mx1-Cre based approach in the adult hematopoietic system resulted in reduced HSC numbers with impaired quiescence and self-renewal capacity. Furthermore, *Twist1* KO HSCs had reduced lymphoid and megakaryocyte/erythroid differentiation ability with a concomitant increase in granulocyte/macrophage differentiation capacity (91). *Twist1* KO HSCs also had significantly reduced engraftment capacity in a competitive bone marrow transplant setting, which was not due to any observable homing defect. The impact of *Twist1* deletion during stress hematopoiesis was examined following irradiation or treatment with the chemotherapeutic drug, 5-Fluorouracil (5-FU). This analysis revealed an important role for TWIST1 in protecting HSCs from irradiation-induced apoptosis, senescence and DNA damage. Treatment with 5-FU also led to significantly reduced bone marrow cellularity and impaired HSC recovery in *Twist1* KO mice after a single dose, and rapid HSC exhaustion and mouse death following serial 5-FU treatments. Mechanistically, *Twist1* KO resulted in enhanced mitochondrial calcium levels and subsequently increased production of reactive oxygen species (ROS) in lymphoid-biased HSCs but not myeloid-biased HSCs following irradiation induced stress. Importantly, blockage of voltage-gated calcium channels was largely able to reverse irradiation induced death in *Twist1* KO mice as well as rescue HSC levels, demonstrating a key role for enhanced mitochondrial calcium influx in driving the stress induced hematopoietic phenotype in these mice (91).

TWIST1 is also known to play a role in mesenchymal stem cell (MSC) proliferation, survival and differentiation (116–119). Interestingly, *Twist1* KO in the bone marrow niche compartment (including MSCs) resulted in reduced homing of wild type HSCs following irradiation and transplantation. Wild type HSCs in a *Twist1* deficient bone marrow microenvironment also displayed reduced quiescence and self-renewal potential with enhanced proliferation and a clear myeloid lineage bias. There was also reduced retention of wild type HSCs in *Twist1* deficient bone marrow, with enhanced mobilization to the spleen and blood - likely due to an observed reduction in expression of CXCL12 and VCAM1 (118). Interestingly, increased TWIST1 expression in bone marrow-derived mesenchymal stem/stromal cells (BMSC) enhanced their ability to maintain $CD34^+$ hematopoietic stem cells (HSC) in long-term *in vitro* cultures (116). This was likely mediated, at least partially, by direct activation of the *Cxcl12* gene by TWIST1. These findings demonstrate a clear role for TWIST1 expression in bone marrow niche support of HSCs likely through regulation of CXCL12 expression, a protein known to be important for supporting HSC survival and self-renewal and also involved in protecting HSCs from oxidative stress (120, 121)

TWIST2 is also expressed in the hematopoietic compartment, preferentially in myeloid progenitors (92), where it plays a key role in suppressing myeloid differentiation. *Twist2* silencing in embryonic stem cells leads to enhanced generation of myeloid lineage cells during *in vitro* hematopoietic differentiation (122), while *Twist2* deficient mice show significantly increased numbers of immature and mature myeloid cells across all hematopoietic organs, including macrophages, neutrophils and basophils (92). The significant basophilia as well as the presence of hyper-segmented neutrophils and atypical monocytes were suggestive of a myelodysplastic/myeloproliferative phenotype. No significant alteration in the numbers of lymphocytes, red blood cells or platelets was observed in these mice. The increase in total myeloid cells likely resulted from an overall expansion of myeloid progenitors in the bone marrow of *Twist2* KO mice, particularly the granulocyte/macrophage progenitor (GMP) which showed increased proliferation and differentiation capability in *in vitro* assays. The myeloid skewed and enhanced differentiation of *Twist2* KO progenitors was also observed in both non-competitive and competitive bone marrow transplant experiments, demonstrating a cell-intrinsic effect of *Twist2* KO as well as a strong competitive advantage against wild type cells. Mechanistically, TWIST2 was found to inhibit the activity of known regulators of myeloid differentiation, RUNX1 and C/EBPα, as well as suppress the production of pro-inflammatory cytokines and chemokines (92).

Interestingly, *Twist2* KO mice also develop an inflammatory syndrome shortly after birth due to enhanced pro-inflammatory cytokine production that results in perinatal death within 3-4 weeks after birth (123). A possible role for TWIST2 in the regulation of inflammation is further supported by its high expression in chronically activated T helper (Th) lymphocytes (124), and ability to repress the expression of key pro-inflammatory cytokines such as TNFα, IL1β and IFNγ (123, 125–128).

While no obvious T or B lymphoid phenotype was identified in *Twist2* KO mice, TWIST2 has been documented to play a role in regulating T cell selection and apoptosis in the developing thymus (129–131). Furthermore, Hwang et al. found that TWIST2 expression is important for regulating the CD4/CD8 thymocyte lineage determination downstream of TCR activation (132). See Figure 3 for an overview TWIST family roles in hematopoiesis.

3.3 SNAIL family

In the hematopoietic compartment SNAI1 and SNAI3 are expressed in mature T and B cells, with SNAI1 also expressed in mature myeloid lineage cells (76, 77), whereas SNAI2 has only been detected in hematopoietic stem and progenitor cells (77, 94). *Snai1* KO is embryonically lethal at E7.5-8.5, thus precluding studies being undertaken into the role of SNAI1 during hematopoiesis (65). A hematopoietic specific *Snai1* KO showed no overt phenotype, suggesting that SNAI1 is not required for normal hematopoiesis, or alternatively that other family members may be able to compensate for SNAI1 loss (93). A deeper investigation of this mouse model, however, is still required. Hematopoietic specific *Snai1* transgenic mice, on the other hand, develop a myeloproliferative phenotype characterized by an expanded population of both immature and mature myeloid cells (particularly granulocytes), disrupted bone marrow and spleen architecture and evidence of extramedullary hematopoiesis. Interestingly, some of these mice developed Acute Myeloid Leukemia (AML), which will be discussed more later.

Snai2 KO mice display normal B and myeloid cell development, however they show macrocytic anemia as well as abnormal T cell development characterized by reduced thymus size and reduced numbers of $CD4^+CD8^+$ DP T cells (72). The reduced thymus size and thymocyte numbers correlated with increased T cell apoptosis

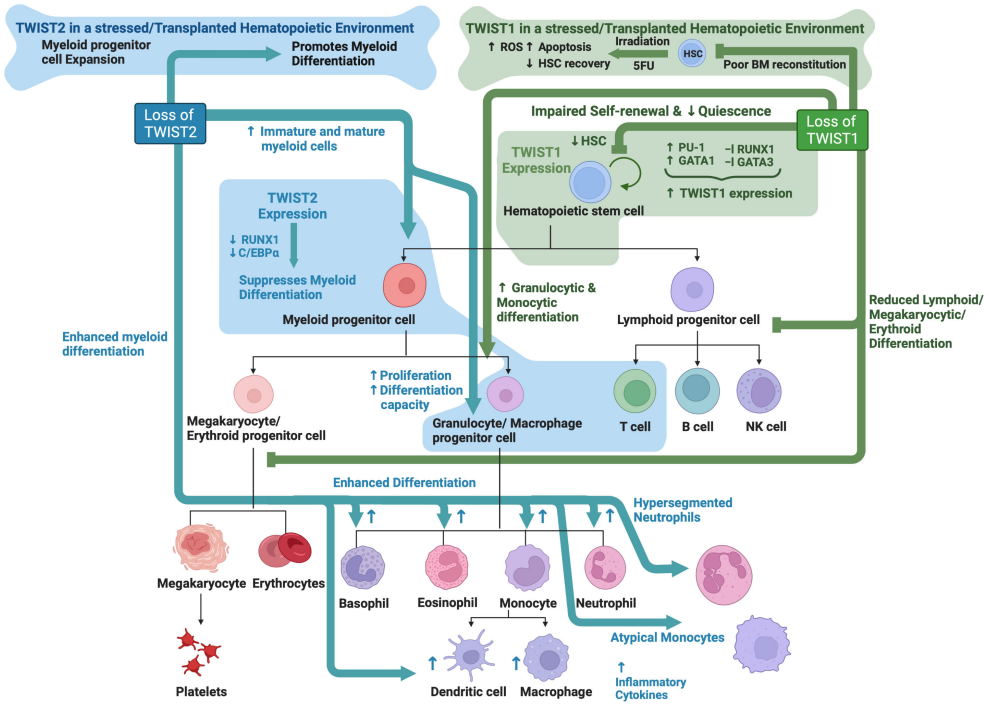

FIGURE 3
TWIST family roles in hematopoiesis: Schematic showing known functions of TWIST1 and TWIST2 during normal hematopoiesis as determined through analysis of knockout mouse models. Created with BioRender.com.

as demonstrated by an increase in apoptotic bodies and TUNEL positive cells in histological sections. In addition to the macrocytic anemia observed at steady state, stress erythropoiesis was also perturbed in *Snai2* KO mice as demonstrated by reduced erythroid recovery following *in vivo* hematopoietic stress driven by either phenylhydrazine (PHZ)-induced hemolytic anemia or pregnancy-induced anemia. This impaired stress erythropoietic response was likely due to reduced numbers of BFU-E and CFU-E in the spleen of *Snai2* KO mice at steady state, and a significant reduction in their ability to expand under stress conditions (72). A role for SNAI2 downstream of SCF/cKIT signaling in HSCs was postulated based on similar phenotypes observed between *Snai2* KO mice and *cKit* or *Scf* mutant mice, supported by data showing induction of *Snai2* expression upon *Scf* stimulation of *cKit in vitro* and anemia-induced activation of *cKit* signaling *in vivo* (72). In a follow up study these authors further found that, similarly to *cKit* or *Scf* mutant mice, *Snai2* KO bone marrow cells were also significantly radio-sensitive. Impaired hematopoietic recovery following low dose irradiation resulted in death in the majority of *Snai2* KO mice as compared to 100% survival in wild type controls. Importantly, intraperitoneal injection of a TAT-SNAI2 fusion protein that readily enters cells was able to rescue irradiation induced death not only in *Snai2* null mice but also in *cKit* mutant mice demonstrating a key role downstream of cKit/SCF signaling in radioprotection of HSCs (133).

A separate study by Inoue et al. performed an extensive analysis of the hematopoietic system of an independently generated *Snai2* KO mouse model (94). *Snai2* KO mice had normal peripheral blood cell counts, however the number of *in vitro* colony-forming progenitors (BFU-E, CFU-E, CFU-GM, and CFU) was slightly increased relative to wild type mice. In contrast to their relatively normal steady state hematopoietic development, *Snai2* KO mice were severely impaired in their ability to reconstitute their bone marrow following total body irradiation (TBI). *Snai2* KO mice showed increased sensitivity to DNA damage induced by irradiation and all *Snai2* KO mice died by day 13 post irradiation due to severe pancytopenia. By comparison, wild type and *Snai2* heterozygous mice survived longer, with around 50% surviving to at least 30 days post irradiation. In response to irradiation, *Snai2* KO HSPCs displayed significantly increased apoptosis as compared with wild-type HSPCs, suggesting a role for SNAI2 in protecting against DNA damage induced cell death (94). In a follow up study, Wu et al. found that wild type mice previously reconstituted with *Snai2* KO bone marrow were just as sensitive to irradiation induced death as *Snai2 KO* mice, demonstrating that the increased sensitivity of *Snai2* KO HSPCs to irradiation was cell intrinsic. Importantly, the authors also discovered that this radio-sensitivity of *Snai2* KO HSPCs could be rescued by transgenic expression of the antiapoptotic protein BCL2 or deletion of TP53. Further, it was demonstrated that SNAI2 is upregulated by TP53 following irradiation, and in turn it can transcriptionally repress the BH3-only pro-apoptotic protein, PUMA leading to an antagonism of TP53 induced apoptosis. These data indicate that SNAI2 plays a key role in mediating the DNA damage response downstream of the TP53 pathway in HSPCs (134).

In a third study, Sun et al. further examined the functional capacity of *Snai2* KO HSCs (95). Using competitive bone marrow transplantation experiments these authors demonstrated that *Snai2*

KO HSCs had increased proliferative capacity and enhanced ability for hematopoietic reconstitution, with an approximately 8-fold higher repopulation efficiency as compared to *Snai2* heterozygous HSCs. Importantly, this enhanced reconstitution ability was not due to an altered differentiation or homing capacity. *Snai2* KO HSCs also displayed increased self-renewal capacity as demonstrated by limiting dilution and serial transplantation experiments. Following treatment with the chemotherapeutic drug 5-fluorouracil (5FU), which kills proliferating cells and drives quiescent HSCs into cell cycle, *Snai2* KO HSCs showed enhanced proliferation and expansion compared to WT cells both *in vitro* and *in vivo*. This enhanced HSC proliferation and expansion of *Snai2* KO HSCs following 5FU treatment also lead to superior repopulating ability upon competitive transplantation with wild type cells into irradiated recipient mice (95). The percentage of *Snai2* KO HSCs in S phase was also significantly higher than for wild type HSCs, supporting the idea that quiescent *Snai2* KO HSCs were induced into cell cycle more effectively by 5FU than wild type HSCs. No difference in the level of 5FU-induced apoptosis was observed in *Snai2* KO HSCs. Together, these data suggest that SNAI2 acts as a negative regulator of HSC self-renewal and proliferation, and a positive regulator of HSC quiescence.

While the above studies suggested that the hematopoietic defects in *Snai2* KO mice were hematopoietic cell intrinsic, Wei et al. identified a potential extrinsic role for SNAI2 in the bone marrow niche (135). Following exposure to a lethal dose of irradiation (12Gy), *Snai2* KO mice could not be rescued from irradiation-induced death *via* transplantation of wild type bone marrow cells, with the majority of mice dying by three weeks post irradiation and transplantation. In contrast, 100% of wild type mice receiving either wild type bone marrow or *Snai2* KO bone marrow survived. These findings are somewhat contradictory to those of Wu et al. who previously found that *Snai2* KO mice could in fact be rescued from irradiation-induced death by transplantation of wild type bone marrow cells (134). This discrepancy may be explained by the use of a lower dose of irradiation by Wu et al. (7Gy) or different genetic backgrounds of the *Snai2* KO mice between the two studies. Interestingly, Wu et al. had also demonstrated that following complete bone marrow reconstitution, a second dose of irradiation (7Gy) still induced bone marrow failure and death in wild type mice with *Snai2* KO bone marrow, whereas *Snai2* KO mice with wild type bone marrow were protected (134). Combined these data suggest that extrinsic SNAI2 in the bone marrow niche is crucial for enabling HSPC engraftment and hematopoietic reconstitution following irradiation, whereas intrinsic SNAI2 expression in the HPSC compartment is important for protecting against irradiation-induced cell death.

The first evidence of a role for SNAI3 in the hematopoietic system came from a study that examined the negative regulatory element of the mouse *Itgb2l*, which is preferentially expressed in maturing neutrophils (136). Using an electrophoretic mobility shift assay (EMSA) it was demonstrated that SNAI3 could bind to the negative regulatory element on the *Itgb2l* gene and block the transcriptional activator, PU.1, from binding and driving transcription. Another study by Dhalem et al. examined hematopoietic lineage differentiation and derivation of mature hematopoietic cells upon retroviral mediated over-expression of SNAI3 in mouse HSPCs (98). Mice transplanted with SNAI3 expressing HSPCs (marked by GFP expression) had an almost complete loss of GFP^+ T and B lymphoid cells, with the GFP^+ cells being primarily myeloid. Interestingly, the GFP^+ HSPC compartment appeared relatively normal in these mice as compared to control mice receiving HSCPs transduced with an empty vector control retrovirus (98). These data demonstrate that aberrant expression of SNAI3 significantly perturbs lymphoid differentiation but has minimal if any impact on early HSPC development and myeloid differentiation.

Snai3 KO mice are completely viable with no obvious phenotypic defects, demonstrating that SNAI3 is not essential for embryogenesis or steady state adult development (76). Pioli et al. generated a conditional Cre-mediated *Snai3* knockout mouse model and performed a T cell specific *Snai3* deletion using Lck-Cre. Deletion of *Snai3* in the T cell lineage had no effect on T cell development in the thymus and no T cell abnormalities were observed in the peripheral lymphoid organs. To test for possible functional redundancy between SNAI2 and SNAI3 in T cells, Pioli et al. further generated *Snai2/Snai3* double knockout (DKO) mice. These DKO mice had a more severe phenotype as compared to either single KO mouse, with severe growth retardation, infertility and almost complete lethality by 15 weeks of age (77). Analysis of lymphoid organs revealed that DKO mice had a significantly reduced thymus size (even when normalized for the reduced body weight), a decreased proportion of DP ($CD4^+CD8^+$) thymocytes with a concomitant increase in $CD4^+$ and $CD8^+$ SP cells. Surprisingly, the distribution of $CD4^+$ and $CD8^+$ SP T cells in peripheral organs was relatively normal. DKO mice also displayed significantly reduced B cell numbers and increased myeloid cells in the marrow, spleen and blood (77). Whether the increase in myeloid cells was a direct result of *Snai2* and *Snai3* loss in these cells or was rather due to the striking loss of B cells still remains to be elucidated. No analysis was performed on the primitive HSPC compartment of these mice and so it is not known what impact combined loss of *Snai2* and *Snai3* might have at earlier stages of hematopoietic development (77). This study however did clearly indicate potentially redundant functions for SNAI2 and SNAI3 during later stages of hematopoiesis. Similar studies using *Snai1* knockout in combination with either *Snai2* and/or *Snai3* would provide important additional knowledge in this area. See Figure 4 for an overview of SNAIL family roles in hematopoiesis.

4 EMT transcription factors in hematological malignancy

While there is still much to be learned regarding the exact mechanisms involved, it is becoming increasingly evident that EMT-TFs are important regulators of normal blood cell development and function. It is perhaps not surprising, therefore, that aberrant expression and/or function of EMT-TFs is also now emerging as a novel and important contributor to the malignant hematopoietic phenotype.

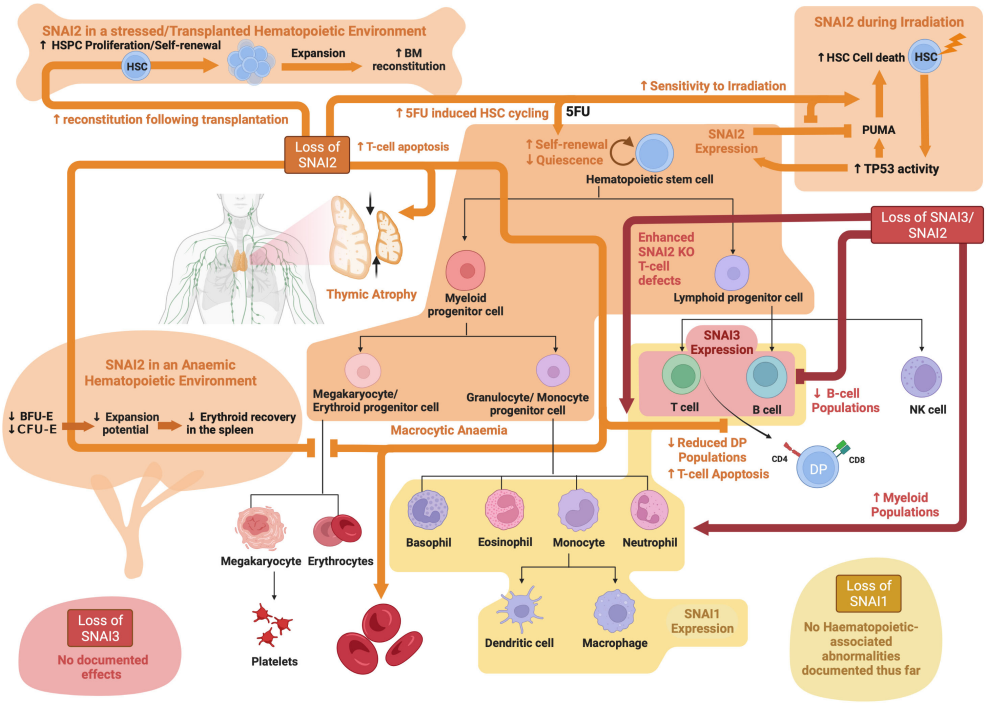

FIGURE 4
SNAIL family roles in hematopoiesis: Schematic showing known functions of SNAI1, SNAI2 and SNAI3 during normal hematopoiesis as determined through analysis of knockout mouse models. Created with BioRender.com.

5 Myeloid malignancies

5.1 ZEB family

Using publicly available RNA-sequencing data from the GEPIA database (http://gepia.cancer-pku.cn), Li et al. identified high *ZEB1* expression in AML patients and found it to be associated with a worse overall survival (137). A similar association between high ZEB1 expression and worse overall survival had also been shown by Stavropoulou et al. in their own AML patient cohort, and indeed ZEB1 expression was significantly higher in AMLs with a more stem-cell like and aggressive phenotype (138). Shousha et al. identified a 1.8 fold increase in *ZEB1* mRNA expression in more than half of their AML patients as compared to control subjects, using qRT-PCR analysis on peripheral blood samples (139). In contrast to the above studies, Almotiri et al. used publicly available Affymetrix microarray data to describe *ZEB1* expression as being lower in AML patient samples compared to normal cells (100). The use of datasets generated using alternative gene expression analysis technologies may explain the discrepant results between these studies, however additional investigation is warranted to clarify whether aberrant *ZEB1* expression is indeed a significant finding in AML.

ZEB1 appears to play important roles in AML cell biology, with siRNA mediated knockdown of *ZEB1* in human AML cell lines leading to reduced cell proliferation and induced myeloid cell marker expression *in vitro*, and subsequently delayed tumor onset in *in vivo* xenograft models (137). Extending these studies to primary mouse AML models, Stavropoulou et al. demonstrated that shRNA mediated knockdown of *Zeb1* in an MLL-AF9 driven AML model resulted in impaired tumor cell invasion *in vitro* and reduced *in vivo* infiltration into the bone marrow 1-week post-transplant (138). In contrast, Almotiri et al. found that Cre-mediated knockout of *Zeb1* in either a MLL-AF9 or Meisa1/Hoxa9 mouse model of AML actually enhanced tumor development *in vivo* (100). These stark differences may be due to the use of distinct models of *Zeb1* perturbation, with Stavropolou et al. and Li et al. using a stable shRNA knockdown approach, where the cells already had reduced ZEB1 expression prior to transplant, and Almotri et al. using an Mx1-Cre model to induce *Zeb1* knockout after AML was established *in vivo*. It is also important to note that Stavropoulou et al. did not extend their animal studies to study tumor development post 1-week and thus no data on disease progression and latency is available. These data do, however, pose the question as to whether ZEB1 may play opposing roles in driving tumor cell engraftment on one hand, while impairing tumor cell proliferation on the other. This would not, however, agree with the observed negative impact of *ZEB1* knockdown on cell proliferation in AML cell lines *in vitro*. Stavropoulou et al. further determined that high ZEB1 expression was particularly associated with a more immature and stem cell like AML phenotype generated by transducing the MLL-AF9 oncogene virally into long term repopulating HSCs (LT-HSCs) as opposed to more differentiated granulocyte/macrophage progenitors (GMPs). These HSC-derived AMLs were also more invasive with higher numbers of leukemia initiating cells (LICs) *in vivo* (138).

Mechanistically, Li et al. found ZEB1 expression in AML to be linked to altered TP53 protein levels, with knockdown of *Zeb1*

leading to enhanced TP53 protein levels and overexpression resulting in reduced TP53 protein levels (137). Whether this is due to direct effects on TP53 transcription, translation or protein stability remains to be determined. The authors further suggested that this ZEB1 mediated regulation of TP53 may occur *via* the PTEN/PI3K/AKT signaling pathway, but again clear mechanistic insight remains to be elucidated.

Expression of *ZEB2* does not appear to be specifically increased in AML cells, with its expression level in AML being similar to that of normal HSPCs. Similarly, no correlation has yet been demonstrated between *ZEB2* expression and survival in AML. Despite ZEB2 not being specifically upregulated in AML cells, its expression was found to be significantly increased following transduction of the *AML-ETO* oncogene into a mouse hematopoietic progenitor cell line. Furthermore, high ZEB2 expression was specifically associated with an invasive phenotype and EMT-like gene expression signature in these cells (140). In human AML cell lines, Li et al. were able to show that shRNA mediated knockdown or CRISPR mediated knockout of ZEB2 reduced cell growth and induced aberrant myeloid differentiation *in vitro* (141). Furthermore, shRNA knockdown of *Zeb2* in mouse MLL-AF9 AML cells led to reduced leukemia cell proliferation *in vitro* (141). A similar finding was obtained by Wang et al. using a RosaERT2Cre-mediated knock out of *Zeb2* in the MLL-AF9 driven mouse AML model (86). Interestingly, when the authors introduced a double knockout of *Zeb2* and *Zeb1* in this same MLL-AF9 model they did not observe any further delay in tumor onset suggesting that *Zeb1* loss was could not compound the effect of *Zeb2* loss alone.

Strikingly, hematopoietic specific expression of either a *Zeb1* or *Zeb2* transgene in mice led to a significantly expanded myeloid compartment (predominantly monocytic) and development of extramedullary hematopoiesis (86). No AML was observed in these mice up to 1.5 years of age suggesting that while these genes may contribute to AML pathogenesis, they are not strong drivers of AML and likely act in concert with other AML mutations or oncogenes. Somewhat surprisingly, loss of *Zeb2* during adult hematopoiesis was also found to drive development of a myeloproliferative-like phenotype characterized by splenomegaly, extramedullary hematopoiesis and bone marrow fibrosis (88). In contrast to *Zeb2* transgenic mice, where enhanced myeloid development favored the monocytic lineage, these *Zeb2* knockout mice showed enhanced granulocyte production. Mechanistically, Li et al. identified deficient JAK/STAT signaling responses in *Zeb2* KO bone marrow cells when stimulated with IL6 or IL3, but enhanced signaling when stimulated with the granulocyte cytokine G-CSF (88). Furthermore, Pellman and colleagues determined that ZEB2 expression in AML regulates genes important for granulocytic differentiation, likely through interaction with key epigenetic proteins such as LSD1 and HDACs (141). Combined, these data suggest that correct dosage of ZEB transcription factors is important for normal myeloid development and their expression levels may impact different lineages variably – possibly through regulation of key lineage specific cytokine signaling pathways and gene expression networks. See Figure 5 for an overview of ZEB family in malignant hematopoiesis.

5.2 Twist family

TWIST1 expression is highly upregulated in malignant HSCs from Myelodysplastic syndrome (MDS) patients, with its expression increasing with more advanced disease (142). In contrast, there appears to be reciprocally lower levels of TWIST1 expression in the surrounding bone marrow mesenchymal cells in MDS patients, reducing with disease severity. Li et al. further found that levels of TWIST1 in MDS cells resulted in enhanced resistance to TNFα driven apoptosis, TNFα being a pro-inflammatory cytokine that is highly expressed in the MDS bone marrow microenvironment. Knockdown of *TWIST1* in MDS cell lines rendered them more sensitive to TNFα induced cell death, with this thought to be at least in part driven by coordinated regulation of apoptosis by TWIST1, miRs10a/b, NFkB and TP53 (142, 143).

A more recent study by this same group found that TWIST1 expression was actually higher in MDS patients that were non-responsive to treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine, compared to those that were responsive (144). The level of responsiveness was also correlated with increased DNA methylation and expression of the *de novo* DNA methyltransferase, DNMT3A. A direct interaction between TWIST1 and DNMT3A was identified, with evidence provided to suggest this complex can methylate and repress expression of the cyclin dependent kinase inhibitors CDKN1A and CDKN1C. As 5-aza-2'-deoxycytidine treatment induces cell cycle arrest in MDS cells, TWIST1 driven loss of cell cycle inhibition and reduced G0/G1 arrest may contribute to an enhanced resistance to 5-aza-2'-deoxycytidine treatment upon TWIST1 expression. Furthermore, augmented *de novo* DNA methylation through increased DNMT3A levels in TWIST1 expressing cells likely also contributes to reduced sensitivity to the demethylating activity of 5-aza-2'-deoxycytidine.

TWIST1 expression is also upregulated in AML samples, however the impact of this expression on prognosis in AML remains somewhat controversial. One study has found that patients with high TWIST1 expression were more likely to achieve remission following standard AML induction chemotherapy (cytarabine and daunorubicin combination therapy) than those with lower TWIST1 expression, and subsequently achieved a greater overall survival (145). The authors further found that enforced TWIST1 expression in a single AML cell line (KG1a) led to enhanced sensitivity to cytarabine but no change in response to daunorubicin. In contrast, a second study determined that enforced TWIST1 overexpression in two independent AML cell lines (U937 and K562) led to increased resistance to daunorubicin, mitoxantrone or imatinib, and subsequently found that high TWIST1 in AML samples was associated with a worse overall survival (146). The reason for these discordant findings remains unclear, however it may relate, at least partially, to the different ways of stratifying AML patients for survival analysis. For example, Chen et al. included only patients that had received standard of care chemotherapy, while Wang et al. included all AML patients in their analysis.

Wang et al. went on to further show that TWIST1 was most highly expressed in the putative leukemia stem cell (LSC)

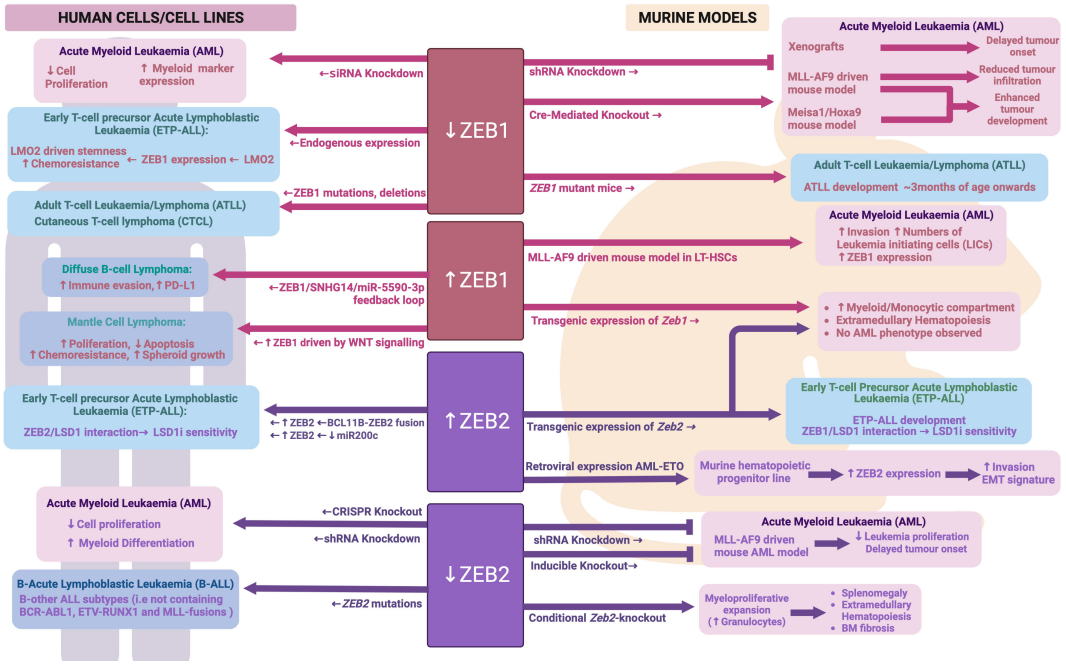

FIGURE 5
ZEB family during malignant hematopoiesis: Schematic outlining ZEB family roles in malignant hematopoiesis as determined through human and mouse model analyses. Created with BioRender.com.

compartment in AML ($CD34^+CD38^-$) and that its expression in LSCs was higher than in normal $CD34^+CD38^-$ HSCs. They also found that enforced TWIST1 expression could drive increased cell proliferation and enhanced colony formation along with reduced apoptosis in AML cell lines. *TWIST1* knockdown, on the other hand, led to reduced cell proliferation and colony formation and increased apoptosis (146). *TWIST1* knockdown in the K562 AML cell line delayed AML onset in *in vivo* xenograft experiments, while knockdown in LSCs isolated from AML patients led to significantly reduced colony forming potential *in vitro*. These data implicate TWIST1 in regulation of LSC function, which mechanistically may relate to the direct regulation of BMI1 expression, a critical regulator of HSC self-renewal, and indirect regulation of RUNX1 and MPL expression, both important modulators of HSC function and proliferation (146).

TWIST1 expression has been particularly associated with the M3 subtype of AML, also termed Acute Promyelocytic Leukemia (APL), which is driven by the t(15;17) translocation (146, 147). Knockdown of *TWIST1* in the NB4 APL cell line or in a mouse model of APL resulted in apoptosis and differentiation of AML blasts *in vitro* and enhanced survival of transplanted mice *in vivo* (147). In other non-APL subtypes of AML, an association between TWIST1 expression and DNMT3A mutation (a key driver mutation identified in around a third of AMLs) has also been identified, with TWIST1 expression being higher in AML cells carrying mutant DNMT3A (148). Furthermore, mutant DNMT3A but not wild type was able to upregulate TWIST1 when ectopically expressed in an AML cell line. Knockdown of TWIST1 in a DNMT3A mutant AML cell line (OCI-AML3) led to reduced invasion of these cells into the central nervous system of xenografted mice.

While TWIST1 appears to have a clear tumor promoting role in AML cells, its expression in the bone marrow microenvironment seems to have a more tumor inhibiting impact on AML cells. Liu et al. found that deletion of *Twist1* specifically in the bone marrow microenvironment resulted in enhanced engraftment and increased dissemination/infiltration of wild-type murine MLL-AF9 leukemia cells (118). Mechanistically, activated Notch signaling was observed within the *Twist1* deleted niche, which has been shown to contribute to enhanced LSC expansion and self-renewal.

In Chronic Myeloid Leukemia (CML), TWIST1 expression is also upregulated compared to normal samples, with expression increasing further during more advanced phases of the disease (146, 149). More than 90% of CML cases are driven by the BCR-ABL fusion, which is uniquely sensitive to tyrosine kinase inhibitors (TKI) such as imatinib. In samples from CML patients that did not respond to TKI treatment, TWIST expression was 100X greater compared to patient samples that did respond (149). TWIST1 expression was also higher in an imatinib resistant CML cell line compared to a sensitive cell line (149). Furthermore, knockdown or overexpression of TWIST1 in CML cell lines led to enhanced sensitivity and increased resistance to TKI treatment respectively (146, 149). These data strongly implicate TWIST1 in driving TKI resistance in CML, however the mechanism/s involved remains to be determined.

While TWIST1 has been studied in much greater detail than its family member TWIST2 in the context of malignant hematopoiesis, the data currently available suggest opposing roles for these two

proteins in AML. Whereas TWIST1 is upregulated in AML, TWIST2 is hypermethylated in ~30% of AML patients resulting in significantly reduced expression (150). Knockdown of TWIST2 in AML cells led to enhanced growth and colony forming capacity, while enforced TWIST2 expression in AML cells inhibited their growth and clonogenic capacity as well as protected mice from AML in a subcutaneous xenograft model. Mechanistically, TWIST2 expression was found to repress a number of known tumor suppressor genes as well as directly activate expression of the cell cycle regulator CDKN1A. Interestingly TWIST2 was not able to alter expression of known TWIST1 targets in AML, such as BMI1, suggesting different interacting partners and/or DNA binding sites for these two family members in AML cells (150). See Figure 6 for an overview of TWIST family in malignant hematopoiesis.

5.3 Snail family

In keeping with the findings for ZEB proteins and TWIST1, SNAI1 is also highly expressed in AML cells compared to normal HSPCs (96, 139, 151), and is associated with worse overall survival (96) and chemotherapeutic resistance (151). To study the role of SNAI1 expression in AML, Carmichael et al. generated hematopoietic restricted SNAI1 transgenic mice. These mice all developed a myeloproliferative phenotype, which could transform into AML after a long latency of 12 months or greater (96). Analysis of *Snai1* transgenic mice at the pre-leukemic stage identified a significant skewing toward granulocyte/macrophage lineage development, with increased numbers of immature myeloid cells possessing increased self-renewal and mildly impaired differentiation capacity (96). Mechanistically, this SNAI1-driven hematopoietic phenotype was dependent on the histone lysine demethylase, LSD1, with physical interaction between the two proteins leading to impaired LSD1 function, altered DNA binding and aberrant target gene regulation. HSPCs ectopically expressing SNAI1 subsequently displayed altered gene expression programs related to normal myeloid differentiation, cytokine signaling, migration/invasion/adhesion and inflammatory pathways (96).

These findings suggest that hematopoietic restricted SNAI1 expression can predispose to malignant transformation of hematopoietic cells but does not directly drive it. Interestingly, Perez-Mancera et al. found that expression of a tetracycline regulatable *Combi-tTA-Snai1* transgene was able to induce tumor development in mice from 5 months onwards, with 40% of tumor being AML and 50% being lymphomas (152). The earlier onset and greater penetrance of AML development in the *Combi-tTA-Snai1* mice, as well as the lack of lymphoma formation in the hematopoietic-restricted model generated by Carmichael et al., suggest that either expression level differences between the two models (which is unknown at this time) or the non-hematopoietic expression of transgenic SNAI1 in the *Combi-tTA-Snai1* mice contributes to AML and/or lymphoma development.

SNAI2 expression is also significantly increased in human AML samples compared to normal bone marrow (153). This increased expression may be directly driven by AML oncogenes, as SNAI2 was found to be significantly upregulated in HSCs following viral transduction with *MLL-AF9*, *MEIS1* or *HOXA9* oncogenes. Furthermore, *Snai2* knock out was able to reduce the ability of *MLL-AF9* and *NUP98-HoxA9* oncogenes to transform mouse HSCs *in vivo*, while limiting dilution assays demonstrated reduced LSC/LIC frequencies in *Snai2* knockout MLL-AF9 leukemia. Homing of *Snai2* deficient MLL-AF9 AML cells was normal, however increased

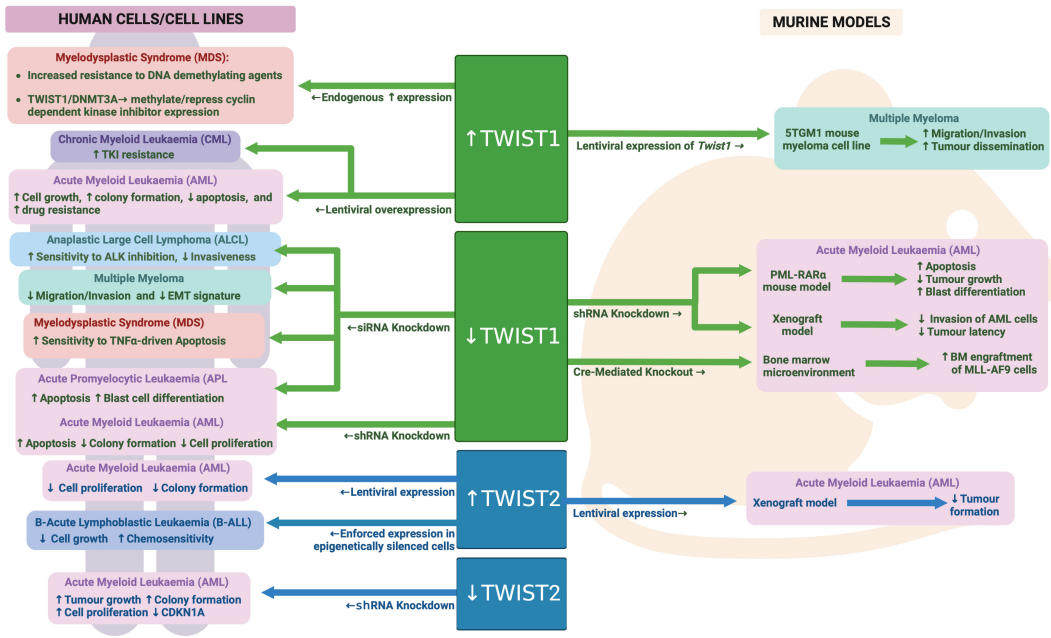

FIGURE 6
TWIST family during malignant hematopoiesis: Schematic outlining TWIST family roles in malignant hematopoiesis as determined through human and mouse model analyses. Created with BioRender.com.

apoptosis and impaired cell cycle progression were apparent. These data suggest that upregulation of SNAI2 is important for the transforming ability of AML oncogenes (153).

Zhang et al. further confirmed these data in human AML, with *SNAI2* knockdown in AML cell lines resulting in reduced proliferative capacity and reduced LIC/LSC frequency. Use of a cell permeable peptide (TAT-SNAG), predicted to interfere with SNAI2 protein-protein interactions mediated by the SNAG domain, was also able to impair AML cell growth and colony formation as well as synergize with Cytarabine treatment *in vitro* to induce AML cell death. It is important to note, however, that the SNAG domain is highly conserved across SNAI family members as well as the GFI family of hematopoietic transcription factors (154). Therefore, this TAT-SNAG peptide may also inhibit the function of other SNAG-domain proteins and so these particular results cannot be conclusively linked to inhibition of SNAI2.

As with SNAI1, a similar *Combi-tTA-Slug* (*Snai2*) transgene model was generated by Perez-Mancera et al. and also found to drive development of Acute Leukemias, of which 40% were AML (the other 60% being B-lymphoid) (97). Perez-Mancera et al. subsequently went on to show that expression of SNAI2 is upregulated in CML patient cells as compared to normal controls and is directly upregulated by the *BCR-ABL* fusion oncogene that drives the majority of CML cases. Strikingly, knockout of *SNAI2* was able to completely block CML development in a *BCR-ABL* transgenic mouse model, suggesting a key role for SNAI2 expression downstream of BCR-ABL (97). Furthermore, SNAI2 overexpression driven by BCR-ABL was shown by Mancini et al. to be reversed upon TKI treatment, leading to a release of SNAI2 driven repression of the pro-apoptotic protein PUMA and subsequent induction of apoptosis. In contrast, in CML samples carrying a TKI resistant BCR-ABL mutation, SNAI2 expression was even more enhanced than in TKI sensitive samples and helped to drive cell survival in response to TKI (155). These data demonstrate a clear role for oncogene driven SNAI2 expression in driving CML cell survival and therapy resistance. It is likely that similar mechanisms are at play in AML, where AML oncogenes can also upregulate SNAI2 expression. See Figure 7 for an overview of SNAIL family in malignant hematopoiesis.

6 Lymphoid malignancies

6.1 Zeb family

Enhanced expression of ZEB2 has been identified in patients with early T-cell precursor Acute Lymphoblastic Leukemia (ETP-ALL), a particularly poor outcome subtype of T-ALL. Goossens et al. discovered a novel *BCL11B-ZEB2* fusion in rare cases of ETP-ALL, which is thought to drive increased ZEB2 expression through the 5' BCL11B fusion partner (156). In other ETP-ALL cases, high expression of ZEB2 may result from downregulation of miR200c, a microRNA known to suppress ZEB2 protein expression (156). A direct functional link between ZEB2 expression and ETP-ALL was clearly demonstrated by the development of an ETP-ALL like disease in hematopoietic-restricted *Zeb2* transgenic mice from 5 months of age (156, 157). This same group also discovered that ZEB2 could physically interact with LSD1 in transgenic ETP-ALL

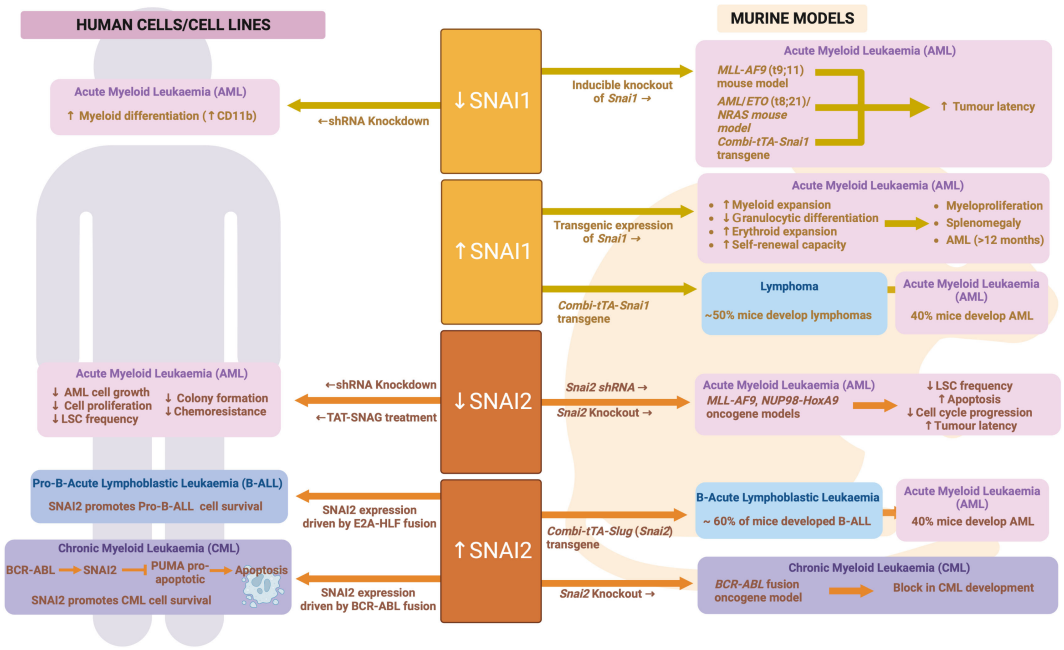

FIGURE 7
SNAIL family during malignant hematopoiesis: Schematic outlining SNAIL family roles in malignant hematopoiesis as determined through human and mouse model analyses. Created with BioRender.com.

cells, and intriguingly this interaction appeared to infer sensitivity to LSD1 inhibition (158). This sensitivity was further confirmed in human ETP-ALL cell lines with high ZEB2 levels, but not found in T-ALL lines without high ZEB2. A recent follow-up study by this group identified upregulation of the IL7R in *Zeb2* transgenic ETP-ALL cells as driving IL7-mediated activation of JAK/STAT signaling and upregulation of the pro-survival protein BCL2. This ZEB2/LSD1 interaction appears to repress pro-apoptotic genes such as BIM, making ETP-ALL cells susceptible to combined treatment with LSD1 inhibitor and the BCL2 inhibitor ABT-199 or the JAK/STAT inhibitor Ruxolitinib (159).

ZEB2 has also been implicated in B-cell Acute Lymphoblastic Leukemia (B-ALL) with likely pathogenic mutations identified in a small proportion of B-ALLs (160–162). Interestingly, in one study these *ZEB2* mutations were associated with a significantly worse overall survival and an increased likelihood of relapse, being found in nearly 30% of relapsed cases compared to only 2-3% of diagnosed cases (161). However, the significance of these results remains to be confirmed as the authors' own subsequent work found this link to be less evident in a second cohort of patients. It is still unclear how the identified mutations affect ZEB2 function and how mutant ZEB2 contributes to B-All pathogenesis, however these mutations do appear to be associated exclusively with the "B-other" ALL subtype, which lacks common B-All associated fusion proteins such as BCR-ABL1, ETV-RUNX1 and MLL-fusions (163).

Interestingly, while ZEB2 expression is upregulated in ETP-ALL, ZEB1 appears to be reduced suggesting opposing roles for these two family members in this disease. The *LMO2* oncogene, which is specifically associated with the ETP-ALL phenotype, can directly repress *ZEB1* at the transcriptional level, and ZEB1 expression is negatively correlated with LMO2 expression in ETP-ALL cells. LMO2 can also physically interact with ZEB1 and block its DNA binding ability (164, 165). Wu et al. provided additional evidence to suggest that downregulation of ZEB1 is essential for the LMO2 driven stemness phenotype in T-ALL cells as well as resistance to methotrexate treatment, a chemotherapeutic drug used to treat T-ALL (165).

ZEB1 is also downregulated in other malignant T-cell diseases, specifically Adult T-cell Leukemia/Lymphoma (ATLL), driven by infection with HTLV-1, and cutaneous T cell lymphoma (CTCL). In ATLL, Hidaka et al. discovered that the *ZEB1* gene is frequently impacted by focal deletion of the 10p11 chromosomal region (~1/3 of cases) (84). However, other epigenetic mechanisms also likely lead to reduced ZEB1 expression in ATLL, as demonstrated by the ability of demethylating and deacetylating agents to restore ZEB1 expression in ATLL cell lines lacking a 10p11 deletion. A direct functional link between ZEB1 downregulation and ATLL development is evident from *ZEB1* mutant mice, which develop a $CD4^+$ ATLL from as early as 3 months of age (84). In CTCLs, which consist of Mycosis Fungoides (early stage disease) and Sezary Syndrome (late stage disease), up to 65% of patients display focal deletion or somatic inactivating mutations in the *ZEB1* gene. A clear pathogenic role for these mutations in CTCL, however, has yet to be elucidated.

In contrast to T-cell malignancies, ZEB1 expression is increased in B-cell malignancies, specifically Mantle Cell Lymphoma (MCL) and Diffuse B Cell Lymphoma (DLBCL). Sanchez-Tillo et al. identified ZEB1 protein expression in 50% of MCL cases studied histologically, and found it to be directly correlated with b-catenin expression (166). ZEB1 expression was subsequently found to be driven by activated WNT-signaling in MCL cell lines, and was linked to enhanced proliferation, reduced apoptosis and resistance to chemotherapy (166). Expression of ZEB1 in MCL cells also enhanced their lymphoma spheroid growth potential and increased their resistance to Bortezomib – suggestive of a cancer stem cell promoting role for ZEB1 in MCL (167). High ZEB1 expression has also been observed in DLBCL patient samples, both through immunohistochemical staining (168) and qRT-PCR analysis (169). Lemma S et al. further determined that high nuclear ZEB1 expression is associated with adverse three year overall survival (168), while Zhao et al. linked ZEB1 expression with increased immune evasion of DLBCL cells *via* a feedback loop involving ZEB1/SNHG14/miR-5590-3p that ultimately drives upregulation of PD-L1 expression (169).

6.2 Twist family

Thus far, TWIST1 expression has not been investigated in the context of T- or B- ALL, however it is significantly expressed in CTCL (170–172). Increased TWIST1 expression appears to be due to either gain of the chromosomal region 7p21 (171) or promoter hypomethylation (172). Goswami et al. further determined that TWIST1 expression in CTCL increases with disease stage from the more indolent Mycosis Fungoides stage through to the advanced Sezary syndrome stage (173). TWIST1 is also upregulated in ALK+ Anaplastic Large Cell Lymphoma (ALCL), a common pediatric lymphoma driven by the t(2;5) NPM-ALK fusion. TWIST1 knockdown in ALK+ ALCL cell lines reduced their invasiveness and enhanced their sensitivity to an ALK inhibitor, suggesting TWIST1 may contribute to therapeutic resistance (174).

TWIST1 has also been implicated in Multiple Myeloma (MM). In ~15% of MM patients the t(4;14) translocation leads to enhanced expression of the NSD2 gene (175). Gene expression profiling by Cheong et al. identified EMT gene signatures correlated specifically with NSD2 high MM patient samples. They further demonstrated that TWIST1 expression is upregulated in t(4;14) MM cell lines but not in MM cell lines lacking this fusion. Knockdown of TWIST1 in NSD2+ MM cell lines led to downregulation of the EMT gene signature and reduced invasiveness *in vitro*. Conversely, enforced TWIST1 expression in a mouse MM cell line enhanced its migration *in vitro* and its dissemination/invasiveness *in vivo*, but did not impact on overall tumor growth and proliferation (176).

Promoter hypermethylation of the *TWIST2* gene is frequently observed in both childhood and adult ALLs (both B and T lineage) and is associated with loss of TWIST2 protein expression. Interestingly, while *TWIST2* hypermethylation was found in approximately half of diagnostic ALL cases, it was present in nearly all relapsed samples analyzed - suggesting a role for reduced TWIST2 expression in disease relapse and therapy resistance. Indeed, enforced expression of TWIST2 in B-ALL cell lines led to reduced cell growth and increased sensitivity to

chemotherapy (177). *TWIST2* hypermethylation has also been observed in some chronic lymphocytic leukemia (CLL) patients, however no further investigation has been performed into possible associations or implications (177, 178). Similarly to TWIST1, TWIST2 was also found to be expressed highly in a Sezary syndrome (CTCL) cell line compared to a T-ALL cell line in one study, however no further evidence to support a role for TWIST2 in this disease has been published to date (129).

6.3 Snail family

To date, no studies have looked specifically at SNAI1 in lymphoid malignancies, however the *Combi-tTA-Snai1* transgenic mice do develop lymphomas in 50% of cases suggesting SNAI1 should be considered in the context of human lymphomas as well (152). SNAI2 was originally identified as a downstream target of the t(17; 19) E2A-HLF oncoprotein in human pro-B-ALL (179) implicating it in this disease. Concordantly, Inukai et al. found SNAI2 to be expressed in B-ALL cells expressing the E2A-HLF oncoprotein and their preliminary studies suggested SNAI2 plays an anti-apoptotic role downstream of this oncogene (179). Furthermore, in the *Combi-tTA-Slug* transgenic mice, 60% of the leukemias that developed were B-cell derived (97). These same authors further found SNAI2 to be highly expressed in cell lines and samples from B-ALL patients, however it remains unclear exactly how SNAI2 expression contributes to B lineage transformation.

7 Conclusion

The importance of EMT-TFs during hematopoietic development and their subsequent contribution to malignant hematological disease is an emerging area of research. The ZEB, TWIST and SNAIL families play distinct *and* overlapping roles throughout hematopoiesis, including regulating HSC self-renewal, quiescence and survival as well as differentiation along various myeloid and lymphoid lineages. The functions of EMT-TFs in hematopoiesis seem to be largely separate from the classical EMT processes they control during development. Instead, they regulate the expression and/or activity of key hematopoietic transcription factors, epigenetic modifiers, cytokine signaling pathways and regulators of cell survival and apoptosis.

In myeloid malignancies, increased expression of EMT-TFs has been identified and linked to worse overall survival and poor therapeutic response. In lymphoid malignancies, they have been implicated in disease development through either increased or decreased expression as well as mutations, deletions or fusions. Pathologically, in leukemia and lymphoma EMT-TFs contribute to enhanced LSC self-renewal and resistance to apoptosis, augmented tumor cell invasion and dissemination as well as the aberrant differentiation, cell growth and proliferation of tumor cells. It remains unclear, however, exactly how coordinated and discrete expression of these EMT-TFs is regulated during malignant transformation of hematopoietic cells, as well as what determines their oncogenic or tumor suppressive roles in different hematopoietic contexts.

It is intriguing to speculate about a potential role/s for EMT-TFs in regulating the immune response to cancer. While EMT-TFs have been implicated in controlling the cancer immune microenvironment from a cancer cell perspective, they may also play an intrinsic role in regulating the immune cells themselves. It is clear that EMT-TFs contribute to the normal differentiation, development and function of immune cells such as macrophages, DCs and T lymphocytes. They also regulate the expression of various inflammatory cytokines and chemokines, as well as genes involved in DC and T-cell activation. How immune cell intrinsic functions for EMT-TFs may contribute to cancer development, progression and outcome remains an important future question to address.

Despite significant progress in understanding the role of EMT-TFs in blood cell development and malignant transformation, there is still much to uncover about their complex mechanisms of action as well as their future promise as therapeutic targets. Further research in this area has the potential to reveal new insights into the underlying biology of leukemia and lymphoma and to identify novel approaches for the treatment of these aggressive hematological diseases.

Author contributions

KM, LT and CC wrote the paper, reviewed and approved the final version.

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Conflict of interest

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.

Publisher's note

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EDITED BY
Li Wu,
Tsinghua University, China

REVIEWED BY
Lidia Yshii,
KU Leuven, Belgium
Andreas B. Wild,
Universtätsklinikum Erlangen, Germany

*CORRESPONDENCE
Hong-Yan Qin
hyqin@fmmu.edu.cn

†These authors have contributed equally to this work

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Myeloid-specific blockade of notch signaling alleviates dopaminergic neurodegeneration in Parkinson's disease by dominantly regulating resident microglia activation through NF-κB signaling

Shi-Qian Liang[1†], Peng-Hui Li[1,2†], Yi-Yang Hu[1†], Jun-Long Zhao[1], Fang-Ze Shao[1], Fang Kuang[3], Kai-Xi Ren[4], Tiao-Xia Wei[1], Fan Fan[1], Lei Feng[1], Hua Han[5] and Hong-Yan Qin[1*]

[1]State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, School of Basic Medicine, Fourth Military Medical University, Xi'an, China, [2]Department of Orthopedics, Xijing Hospital, Fourth Military Medical University, Xi'an, China, [3]Department of Neurobiology, School of Basic Medicine, Fourth Military Medical University, Xi'an, China, [4]Department of Neurology, Tangdu Hospital, Fourth Military Medical University, Xi'an, China, [5]Department of Biochemistry and Molecular Biology, School of Basic Medicine, Fourth Military Medical University, Xi'an, China

Yolk sac–derived microglia and peripheral monocyte–derived macrophages play a key role during Parkinson's disease (PD) progression. However, the regulatory mechanism of microglia/macrophage activation and function in PD pathogenesis remains unclear. Recombination signal–binding protein Jκ (RBP-J)–mediated Notch signaling regulates macrophage development and activation. In this study, with an 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride-induced acute murine PD model, we found that Notch signaling was activated in amoeboid microglia accompanied by a decrease in tyrosine hydroxylase (TH)–positive neurons. Furthermore, using myeloid-specific RBP-J knockout (RBP-J^{cKO}) mice combined with a PD model, our results showed that myeloid-specific disruption of RBP-J alleviated dopaminergic neurodegeneration and improved locomotor activity. Fluorescence-activated cell sorting (FACS) analysis showed that the number of infiltrated inflammatory macrophages and activated major histocompatibility complex (MHC) II^+ microglia decreased in RBP-J^{cKO} mice compared with control mice. Moreover, to block monocyte recruitment by using chemokine (C-C motif) receptor 2 (CCR2) knockout mice, the effect of RBP-J deficiency on dopaminergic neurodegeneration was not affected, indicating that Notch signaling might regulate neuroinflammation independent of $CCR2^+$ monocyte infiltration. Notably, when microglia were depleted with the PLX5622 formulated diet, we found that myeloid-specific RBP-J knockout resulted in more TH^+

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neurons and fewer activated microglia. *Ex vitro* experiments demonstrated that RBP-J deficiency in microglia might reduce inflammatory factor secretion, TH^+ neuron apoptosis, and p65 nuclear translocation. Collectively, our study first revealed that RBP-J–mediated Notch signaling might participate in PD progression by mainly regulating microglia activation through nuclear factor kappa-B (NF-κB) signaling.

KEYWORDS

notch signaling, Parkinson's disease, microglia, monocyte-derived macrophages, neuroinflammation

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by insidious deterioration of motor control, which often occurs in the older population with emotion, sleep, and cognition disturbances (1). A prominent pathological symptom of PD is the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and less loss in the ventral tegmental area and other middle brain regions. Although the cause of PD is not fully understood, a large amount of evidence indicates that neuronal degeneration is always accompanied by neuroinflammation (2–5), presented by reactive morphology of microglia and astrocytes, infiltration of monocytes/macrophages, and increased cytokine levels such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in cerebrospinal fluid (CSF) and blood (6–10). Therein, innate immune activation, especially microglial activation, represents the major immunologically activated cell population. However, the underlying mechanisms of microglial activation remain unclear.

Microglia, as one kind of classical tissue-resident macrophage in the central nervous system (CNS), originate from the precursors of the embryonic yolk sac and play a pivotal role in cerebral tissue development and neuronal integrity maintenance under physiological conditions (11). However, in several neurogenerative diseases, including PD, microglia are exposed to non-physiological immune activators and become abnormally activated microglia that may promote the pathogenesis and progression of disease (9, 12, 13). Recently, with single-cell RNA sequencing applications, microglia have been shown to be highly heterogeneous, especially during disease progression, and have also been named disease-associated microglia (DAM) (14, 15). DAM is composed of tissue-resident microglia and monocyte-derived macrophages that migrate into the brain *via* the blood–brain barrier. During disease progression, infiltrated monocyte-derived macrophages are intermingled with tissue-resident microglia to speed up or impede disease progression (11). There are at least two subtypes of monocytes in mouse blood: $Ly6C^{hi}$ classical inflammatory monocytes and $Ly6C^{lo}$ non-classical patrolling monocytes, both of which can contribute to infiltrating inflammatory macrophages (IMs) (16, 17). However, how monocyte-derived macrophages and microglia contribute to PD pathogenesis and progression as well as the underlying mechanism are not well defined.

Many studies, including our studies, have shown that recombination signal–binding protein Jκ (RBP-J)–mediated Notch signaling participates in regulating monocyte differentiation and macrophage activation under physiological and pathological conditions (18–20). The Notch signaling pathway is a highly conserved developmental pathway in evolution that regulates cell fate by mediating cell–cell communication. The mammalian Notch signaling pathway consists of four transmembrane receptors, five Notch ligands, the Notch intracellular domain (NICD), and the key transcription factor RBP-J. Once the Notch receptor is activated by its ligand presented by an adjacent cell, the NICD is cleaved by γ-secretase and translocated to the nucleus, where it can associate with RBP-J and then recruit coactivators to trigger downstream gene transcription, such as *Hes1* and *Hes5*, leading to cell proliferation or differentiation (21, 22). Using RBP-J conditional knockout mice combined with some disease models, one of our previous studies suggested that Notch activation in myeloid cells could aggravate spinal cord injury by promoting M1 macrophage polarization and upregulating inflammatory cytokine expression (23). Recently, our study with a mouse experimental autoimmune neuritis model further demonstrated that myeloid-specific Notch signaling activation could alleviate immune-mediated neuropathies by regulating $Ly6c^{hi}$ monocyte conversion through the RBP-J/NR4A1 axis (24). However, how Notch signaling regulates microglial activation and monocyte-derived macrophage infiltration during PD progression remains unknown.

In the present study, we found that the Notch pathway was activated in activated amoeboid microglia in an MPTP-induced PD mouse model. Furthermore, with myeloid-specific RBP-J–deficient ($RBP\text{-}J^{cKO}$) mice, we found that myeloid-specific Notch deficiency resulted in more TH^+ DA neurons and improved movement ability compared with the control PD mice. Meanwhile, the number of resident microglia showed no changes, whereas the number of activated MHC II^+ microglia and infiltrated monocyte-derived macrophages decreased significantly in $RBP\text{-}J^{cKO}$ PD mice. Then, utilizing $CCR2^{-/-}$ mice to block $CCR2^+$ monocyte recruitment or a PLX5622-formulated diet to deplete microglia, we found that blockade of $CCR2^+$ monocytes contributed negligibly to the attenuated DA neuron degeneration in $RBP\text{-}J^{cKO}$ PD mice, whereas microglia depletion enhanced the number of TH^+ DA neurons and reduced the inflammatory response in $RBP\text{-}J^{cKO}$ PD mice. Further mechanistic studies showed that Notch signaling

might regulate microglial activation through NF-κB signaling. In summary, our results are the first to reveal that Notch signaling might participate in PD progression by regulating resident microglial activation through NF-κB signaling.

Method and materials

Mice and PD models

Wild-type mice with the C57BL/6 background were maintained under specific pathogen–free conditions in the animal facility of the Fourth Military Medical University. For myeloid-specific RBP-J knockout (RBP-J^{cKO}) mice, Lyz2-cre (namely, LysM-Cre) transgenic mice (stock #019096, Jackson Laboratory, Bar Harbor, ME, USA) were mated with RBP-J floxed (RBP-$J^{f/f}$) mice (25). After genotype detection, Lyz2-$cre^{+/-}$:RBP-$J^{+/f}$ mice were obtained as the control mice, and Lyz2-$cre^{+/-}$:RBP-$J^{f/f}$ mice were treated as RBP-J^{cKO} mice. The RBP-J knockout efficiency in infiltrated macrophages and microglia was detected with genomic DNA by Real-time Quantitative PCR (qPCR), respectively. CCR2 knockout ($CCR2^{-/-}$) mice (stock #004999, Jackson Laboratory, Bar Harbor, ME, USA) exhibit a defective monocyte recruitment during immune responses and were crossed with RBP-J^{cKO} mice to obtain $CCR2^{-/-}$ RBP-J^{cKO} or $CCR2^{-/-}$ control mice. $CX3CR1^{GFP}$ (stock #005582, Jackson Laboratory, Bar Harbor, ME, USA) mice, which can label $CX3CR1^{+}$ microglia by Green fluorescent protein (GFP) signal, were adopted. In some cases, $CX3CR1^{GFP}$ mice were mated with RBP-J^{cKO} mice. The mouse genotype was determined by polymerase chain reaction (PCR) with mouse genomic DNA. All PCR primers are listed in Table S1.

Acute PD models were used in this study. Briefly, MPTP hydrochloride (MPTP-HCl; Sigma Co., St. Louis, MO, USA) was blended in 0.9% sterile saline and then administered to the animals intraperitoneally every 2 h for four times at 20 mg/kg body weight. An equal volume of saline was injected into the control mice. All mouse experiments were approved by the Animal Experiment Administration Committee of Fourth Military Medical University. All animals were treated according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Open-field test

Mice were placed in an open-field arena (40 cm × 40 cm × 40 cm) made of white acrylic and monitored using video for 5 min. Four mice in four independent fields were simultaneously recorded. Total distance moved and total time spent in three zones (10 cm × 10 cm, 20 cm × 20 cm for center, and 40 cm × 40 cm for the peripheral zone excluding the center area) were calculated using ETHOVISION 9.0 software (Noldus). After each test, feces were eliminated, and the floor was cleaned with 75% ethanol and then dried completely. The locomotor activity was measured with average speed and moved distance. Exploratory behavior was evaluated as the distance moved in the central area.

Elevated plus-maze test

The elevated plus-maze apparatus contained four aims (30 cm × 5 cm): two open and two closed arms with the same size, in which 16-cm-high black walls were elevated 45 cm over the floor and weak red light was used as an illuminator. Each mouse was placed in the central square of the plus-maze apparatus and stood facing the open arm, and, then, their behavior was recorded for 5 min. The total number of entries into the open and closed arms, as well as immobility time, was recorded as overall locomotor activity. Meanwhile, the degree of anxiety was calculated according to the percentage of entrance into the open arms.

Single-cell suspension preparation and FACS

Single-cell suspensions of the brain were prepared according to a previous report (26, 27). Mice were deeply anesthetized in a CO_2 chamber and transcranially perfused with 20 mL of phosphate buffer saline (PBS). Brains were carefully removed from the skull and ground by Dounce homogenizers. Mononuclear cell isolation was performed by density gradient centrifugation with Percoll (70%/37%). After that, the interphase containing mononuclear cells was collected and washed with 1× HBSS. Myelin was removed by high-speed centrifugation at 850g in a 0.9 M solution of sucrose in 1× Hank's Balanced Salt Solution (HBSS). Mononuclear cells were then rinsed in HBSS. After that, the cells were resuspended completely in PBS containing 0.5% bovine serum albumin (BSA) and 2 mM Ethylenediaminetetraacetic acid (EDTA) and then incubated with antibodies. Each antibody for FACS is listed in Table S2.

FACS was performed by BD FACSCanto II. Cell sorting was done using BD FACSAria III. All FACS data were analyzed using FlowJo software (FlowJo LLC).

Cell culture

The murine microglia cell line N9, the hippocampal cell line HT-22, and the human neuroblastoma cell line SH-SY5Y were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 2 μM glutamine, 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were cultured in a saturated humidified incubator in 95% air and 5% CO_2 at 37°C. To induce microglial activation, 5×10^4 N9 cells were cultured in 24-well plates and stimulated with Lipopolysaccharide (LPS) (1 μg/mL; *Escherichia coli* 0111: B4, l4391, Sigma-Aldrich, MO, USA) for 24 h.

For primary microglia culture, neonatal mixed culture was slightly modified on the basis of the previous literature (28). postnatal 0–3 (P0–3) mouse pups were anesthetized with hypothermia, the meninges were removed, and the cortices were minced in PBS containing 5% FBS. Cells were then collected by centrifugation and dissociated with trypsin for 20 min at 37°C. After filtration with a 40-μm cell strainer (BD Biosciences, San Diego, CA, USA), the mixed cells were inoculated

in 75-mm flasks at a density of 1.5×10^7 and cultured with DMEM containing 10% FBS and Granulocyte-macrophage colony stimulating factor (GM-CSF) (25 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 2 weeks, mixed cells were separated by oscillation (125 Revolutions Per Minute (rpm), 37°C), and, then, microglia were harvested and inoculated in 24-well plates overnight. The next day, the cells were treated with LPS (100 ng/mL) or PBS for 24 h. GM-CSF (25 ng/mL) was added during the whole microglia culture process. The cultured medium and cells were harvested for subsequent enzyme-linked immunosorbent assay (ELISA) detection and RNA preparation. For the detection of p65 translocation, microglia were inoculated on a slide in a 24-well plate and treated with LPS (100 ng/mL) for 6 h followed by immunofluorescence staining.

For BMDM culture, bone marrow cells were isolated from mouse femurs and were cultured in DMEM containing 10% FBS for 16h, and then the suspension cells were inoculated in 24-well plate at a density of 2 × 106 and cultured with DMEM containing 10% FBS and GM-CSF (25 ng/mL) for 7 days to obtain BMDMs.

Coculture experiments

LPS-treated primary microglia with neuron were cocultured. Primary microglial cells (3×10^5) were cultured in 24-well plates and then stimulated with LPS (100 ng/mL) for 6 h. After washing with fresh DMEM, the activated microglia were cocultured with 5×10^4 HT-22 cells for 36 h, and, then, the mixed microglia and HT-22 cells were collected and stained with CD45 and Annexin V/Propidium Iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) for FACS analysis.

Coculture primary microglia with N-Methyl-4-Phenylpyridinium Iodide (MPP)(+) induced DA neurons. MPP(+) neuron/microglia coculture experiment was slightly modified on the basis of the previous literature (29). In brief, SH-SY5Y (8×10^4) cells were cocultured with primary microglia cells (3×10^5) in 24-well plates. MPP^+ iodide (Selleck, Houston, TX, USA) (1 μM) was applied directly to the mixed cultures for 48 h, and, then, the mixed microglia and SH-SY5Y cells were collected and stained with CD45 and Annexin V/PI (Invitrogen, Carlsbad, CA, USA) for FACS analysis.

Immunofluorescence

Mice were sacrificed and transcranially perfused with 30 mL of PBS plus 30 mL of 4% paraformaldehyde (PFA). Brains were fixed again in 4% PFA for 4 h followed by 30% sucrose dehydration overnight. Frozen sections were made using a cryostat microtome (Leica, Nussloch, Germany). A series of coronal sections (14 μm) containing the midbrain were cut and attached to gelatine-coated slides. After drying at room temperature, sections were blocked with blocking buffer (1% bovine serum albumin plus 0.3% Triton X-100 in PBS) for 2 h at room temperature. Primary antibodies were incubated with sections at 4°C overnight. The next day, secondary antibodies were incubated with the sections for 1 h at 37°C. Hoechst 33258 was counterstained (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Therein, the DA neurons in the SNpc were stained using anti–tyrosine hydroxylase (TH) (1:10,000) (Sigma-Aldrich, St. Louis, MO, USA) antibody, and microglia in the SNpc were stained using anti–ionized calcium-binding adaptor molecule-1 (IBA-1) (1:1,000) (Wako, Kyoto, Japan) antibody or anti-Transmembrane Protein 119 (TMEM119) (1:200) (Abcam, Cambridge, UK) antibody. All sections were observed and photographed using a fluorescence microscope (BX51, Olympus, Tokyo, Japan) or a laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan). TMEM119 antibody information is listed in Table S2.

Quantification of TH^+ neurons and $CX3CR1^+$/NICD microglia

We identified the SN regions according to the mouse atlas of Franklin and Paxinos (30) and quantified the SN regions corresponding to −3.64 to −2.92 on the bregma axis. The total number of TH^+ neurons of the SN was determined on the basis of the stereological methods that are described in published literature (31). In brief, a total of 10 sections were taken at intervals of 5 after consecutive sections, and, then, TH immunohistochemical staining was performed. The total number of TH^+ neurons in the SNpc from the 10 tissue sections was counted to quantitatively analyze the whole number of DA neurons in the midbrain of the right hemisphere. For microglia counting, the section at bregma of −3.08 was selected, in which there was the most prominent microglia activation. All $CX3CR1^+$ cells in the photographed field were counted, and, then, the mean value was analyzed.

Real-time PCR

Total RNA was extracted according to the protocol using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using an SYBR Premix EX TaqTM II kit (Takara Bio, Dalian, China) and the ABI PRISM 7500 real-time PCR system, and β-actin was used as an internal control. The primers used for qPCR are listed in Table S1.

Enzyme-linked immunosorbent assay

The concentrations of TNF-α, IL-1β, IL-6, IL-10, and transforming growth factor-β (TGF-β) in mouse serum and cell culture supernatant were determined with ELISA kits (eBioscience, San Diego, CA, USA) according to the recommended procedures. Each sample was measured in triplicate.

Western blot

Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer buffer containing the protease inhibitor Phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Shanghai, China), and, then, nucleic and cytoplasmic protein extraction kits were applied (Beyotime, Shanghai, China). Protein concentrations were quantitated with a Bicinchoninic Acid Assay (BCA) Protein Assay kit (Pierce, Waltham, MA, USA). Samples were run by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) and the membrane was blocked with 5% skim milk for 1 h and then incubated with the primary antibodies and secondary antibodies. Protein was determined with the Ultra High Sensitivity ECL Kit. All antibodies are listed in Table S2.

PLX5622 administration

Mice were fed PLX5622 formulated in the AIN-76A diet for microglia depletion (1,200 parts per million (ppm); Plexxikon) according to a previous description (32, 33). A standard AIN-76A diet was provided as a control diet [standard diet (SD)]. Mice were fed PLX5622 for 7 days to deplete microglia and fed SD as a control. After that, the mice were treated with MPTP and fed the PLX5622 diet or Standard Deviation (SD) for 7 consecutive days.

Statistics

Data were analyzed with GraphPad Prism version 9 (San Diego, CA, USA). Image-Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA) was used for quantification analysis. The statistical analyses were performed with Student's t-test or one-way ANOVA with Tukey's multiple comparisons test. The results are shown as the mean ± Standard Deviation. $P < 0.05$ was statistically significant.

Results

Notch signaling could be activated in microglia of PD mice. Many studies, including ours, have reported that Notch signaling can regulate macrophage activation and function (34–38). To assess whether Notch signaling can be activated in microglia in PD, we first established an acute PD model with MPTP treatment using $CX_3CR\text{-}1^{GFP/+}$ mice, in which $CX3CR1^+$ microglia can be traced by GFP signal (Figure 1A). Then, immunofluorescence staining of brain sections was performed to observe the pathological phenotype of MPTP mice. As shown in Figures 1B, C, TH^+ DA neurons were reduced significantly in the SNpc, where microglia ($CX3CR1^+$ or $TMEM119^+$) adjacent to TH^+ neurons transformed to amoeboid activated microglia as previously reported (11) (Figures 1B, D, Figure S1A). Meanwhile, FACS analysis further identified that $CD11b^+CD45^{lo}$ microglia were indeed tissue-resident microglia by staining with CX3CR1, TMEM119, F4/80, and Ly6C (Figures S1B, C). Furthermore, more proliferated $CX3CR1^+$ microglia in PD mice were confirmed by Ki-67 staining (Figures 1D, E). Enthusiastically, compared with the control mice, more NICD translocated into the nuclei of microglia in PD mice accompanied by the reduced TH^+ DA neurons, suggesting that Notch signaling was activated in microglia of PD mice (Figures 1F, G, Figure S1E). This result was further supported by a higher expression of Notch signal–related molecules, such as Notch1, Hes1, and Hes5, in the brains of PD mice (Figure 1H). Collectively, these results indicated that Notch signaling was activated in microglia of MPTP-induced acute PD mice.

Myeloid-specific RBP-J deficiency alleviated dopaminergic neurodegeneration in MPTP mice

Next, to address whether activated Notch signaling in microglia/macrophages could influence PD progression, Lyz2-$cre^{+/-}$:RBP-$J^{f/f}$ (RBP-J^{cKO}) mice, in which Notch signaling was specifically blocked in myeloid cells, were adopted. In some cases, RBP-J^{cKO} mice were crossed with $CX3CR1^{GFP}$ mice. After MPTP treatment, the number of TH+ DA neurons in the SNpc was recorded by immunofluorescence staining in $CX3CR1^{GFP/+}$RBP-J^{cKO} and $CX3CR1^{GFP/+}$ PD mice. The results showed that the number of TH-positive cells in myeloid-specific RBP-J–deficient mice was greater than that in control mice (Figures 2A, B). Correspondingly, the mean density of TH-positive axon fibers in the striatum of RBP-J^{cKO} PD mice was higher (Figures 2C, D). Moreover, the movement behaviors of the mice were examined using the open-field test and the elevated plus-maze test. In the open-field test, RBP-J^{cKO} mice showed better movement ability, as reflected in the moved distance, average speed, and the moved distance in the central area (Figures 2E-H). In the elevated plus-maze test, RBP-J^{cKO} mice also presented better movement behaviors based on the total time of entries and immobility count, but there was no obvious difference in the percentage of open arm entries compared with that of the control PD mice (Figures 2I-L). Together, these results demonstrated that the disruption of RBP-J in myeloid cells could alleviate DA neurodegeneration in PD mice.

Myeloid-specific RBP-J deficiency inhibited microglial activation and reduced the inflammatory response in PD mice

Because of the role of inflammation in neurodegeneration (3, 11, 12, 39), we next analyzed the phenotype of immune cells, especially myeloid cells, in RBP-J^{cKO} and control PD mice by FACS (Figure 3A, Figure S2A). Meanwhile, as shown in Figure S3, the RBP-J knockout efficiency in sorted $CD11b^+CD45^{hi}$-infiltrated IMs could reach more than 50%, whereas that in sorted $CD11b^+CD45^{lo}$ microglia was around 25%. Consequently, the FACS results indicated that the number of $CD11b^+CD45^{hi}$ IMs was significantly reduced in myeloid-specific RBP-J–deficient PD mice compared with control PD mice (Figures 3A, B). The total cell number of $CD11b^+CD45^{lo}$ microglia showed no difference between the two groups (Figure 3C, Figure S2), although the microglia numbers increased both in RBP-J^{cKO} and control mice following MPTP treatment (Figures S2B, C). Expectedly, Notch signal blockade in myeloid cells showed no effect on microglial proliferation and apoptosis, as demonstrated by Ki-67 and Annexin V staining (Figure S3). Because IMs in the brain have been reported to originate from blood monocytes, we further

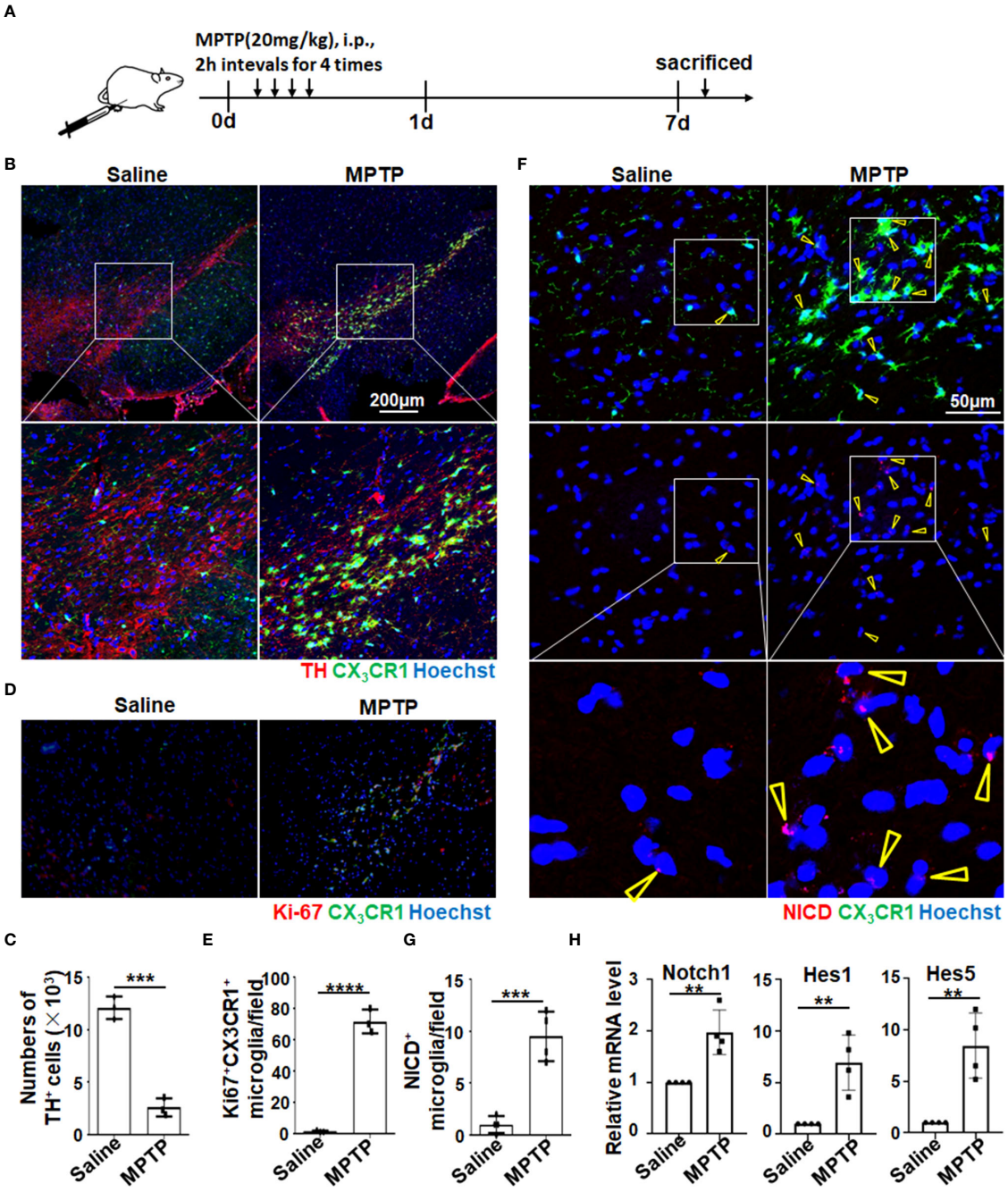

FIGURE 1
Notch signaling was activated in amoeboid microglia in MPTP-induced PD mice. **(A)** Mice were treated intraperitoneally with MPTP-HCl (20 mg/kg) or PBS every 2 h for four times and then sacrificed on day 7 for subsequent analysis. **(B)** Representative immunofluorescence images of tyrosine hydroxylase (TH) staining in the SNpc of CX3CR1$^{GFP/+}$ mice suffering from PD and control mice. **(C)** The TH$^+$ neurons in **(B)** were quantitatively compared (n = 3). **(D)** Representative immunofluorescence images of Ki-67 staining in the SN of CX3CR1$^{GFP/+}$ PD mice and control mice. **(E)** The Ki67$^+$ CX3CR1$^+$ microglia in **(D)** were measured using Image-Pro Plus and then quantitatively compared (n = 3). **(F)** Representative immunofluorescence images of Notch intracellular domain (NICD) expression in the SN of CX3CR1$^{GFP/+}$ PD mice and control mice. **(G)** The NICD-activated microglia in **(F)** were counted and quantitatively compared (n = 4). **(H)** Mononuclear cells of the whole brain were isolated by gradient centrifugation using 70%/30% Percoll. The mRNA expression of Notch-related molecules (Notch1, Hes1, and Hes5) was determined by qRT-PCR (n = 4). The Student's t-test was used for the statistical analyses. Bars = mean ± SD; **P < 0.01; ***P < 0.005 ****p< 0.0001..

confirmed their phenotype with more cell surface markers, such as Ly6C and CX3CR1 (16, 26, 40–42). The FACS analysis showed that the number of Ly6C$^+$CX3CR1$^+$ IMs showed no difference between the two groups, but the Ly6C^{lo}CX3CR1$^+$ IMs decreased obviously in myeloid-specific RBP-J–deficient mice (Figures 3D–F), suggesting that RBP-J deficiency in myeloid cells could mainly affect the differentiation of Ly6C^{lo} IMs during PD progression. Because more activated microglia occurred during PD progression (Figure 1D) and RBP-J deficiency in myeloid cells did not influence the total cell number of microglia, we proposed that Notch signaling might

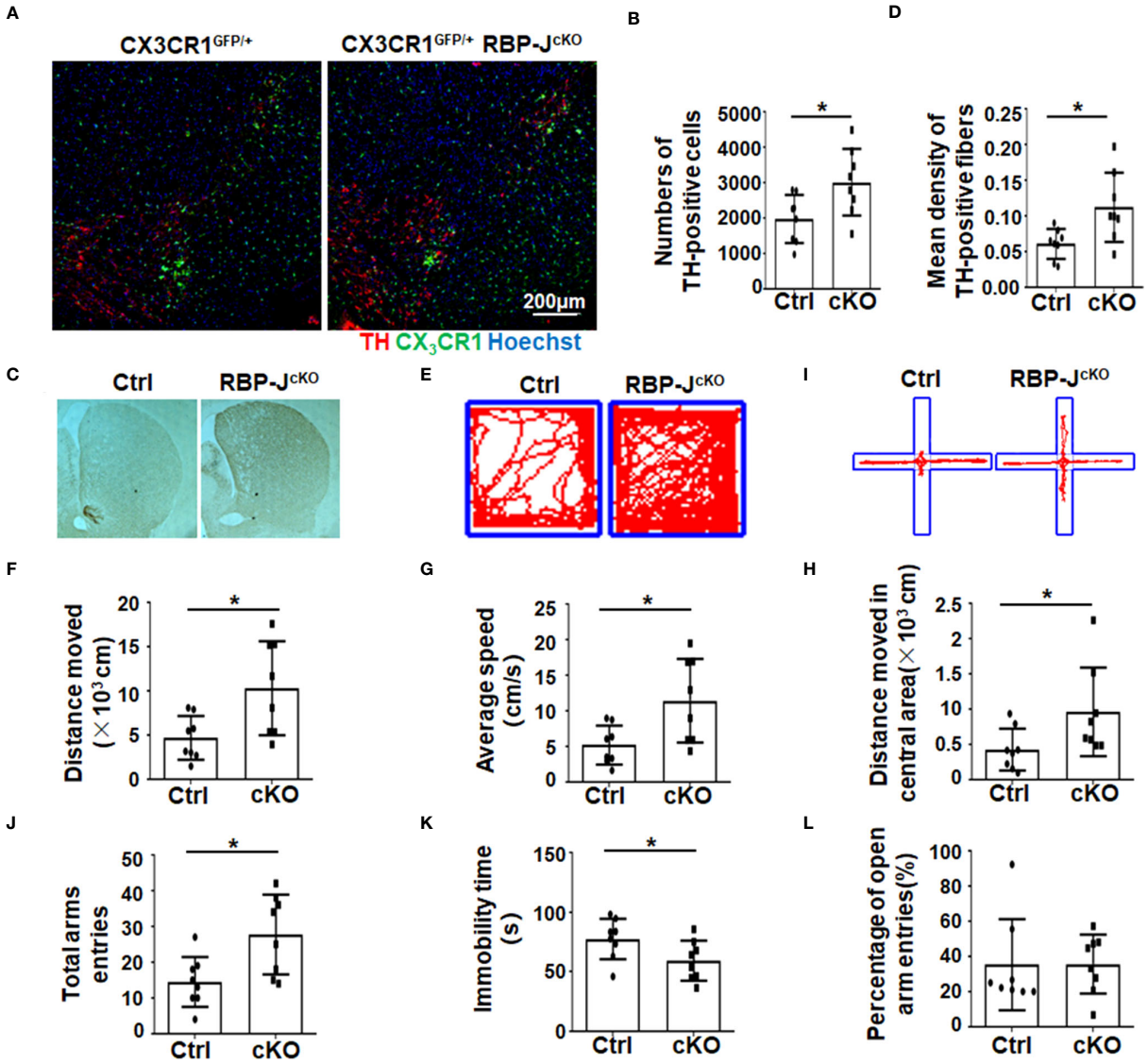

FIGURE 2
The disruption of RBP-J in myeloid cells alleviated dopaminergic neurodegeneration after MPTP treatment in mice. **(A)** Representative immunofluorescence images of TH staining in the SN of CX3CR1$^{GFP/+}$RBP-J^{cKO} and CX3CR1$^{GFP/+}$ (Ctrl) mice after MPTP treatment. **(B)** The number of TH$^+$ neurons was counted and quantitatively compared (n = 8). **(C)** Representative immunohistochemistry staining of TH in the striatum of RBP-J^{cKO} and control PD mice (n = 8). **(D)** The density of TH$^+$ axon fibers in striatum was measured by IOD/area with Image-Pro Plus and quantitatively compared (n = 8). **(E-H)** The open-field experiment was performed **(E)**. Locomotor activity—namely, distance moved **(F)**, average speed **(G)**, and distance moved in the central area—was counted and quantitatively analyzed between RBP-J^{cKO} and control (Ctrl) PD mice (n = 8). **(I-L)** The elevated plus-maze test was performed **(I)**. The total arm entries **(J)**, immobility time **(K)**, and percentage of open arm entries **(L)** were calculated and quantitatively compared. The Student's t-test was used for the statistical analyses. Bars = mean ± SD; *P < 0.05.

regulate microglial activation. As expected, the MHC II$^+$ activated microglia in RBP-J^{cKO} PD mice indeed showed a marked decrease (Figures 3G–I). Consistently, proinflammatory cytokines, including TNF-α and IL-6, in the serum of RBP-J^{cKO} PD mice showed a remarkable decrease, whereas anti-inflammatory cytokines such as TGF-β showed no difference between the two groups (Figure 3J), and IL-10 was undetectable (data not shown). Collectively, these results indicated that myeloid-specific RBP-J deficiency alleviated neuroinflammation and DA neurodegeneration, which might be attributed to the decreased number of infiltrated IMs and less activated microglia during PD progression.

CCR2 depletion contributed less to the attenuated dopaminergic neurodegeneration in myeloid-specific RBP-J–deficient mice

Ly6C^{hi} monocytes are recruited to the CNS in a CCR2-dependent manner (43–45). Combined with our previous studies, in which myeloid-specific Notch signaling blockade ameliorated murine renal fibrosis and lung fibrosis by regulating CCR2$^+$ monocyte-derived macrophage recruitment (37, 38), we wondered whether the attenuated DA neurodegeneration in RBP-J^{cKO} mice also depended

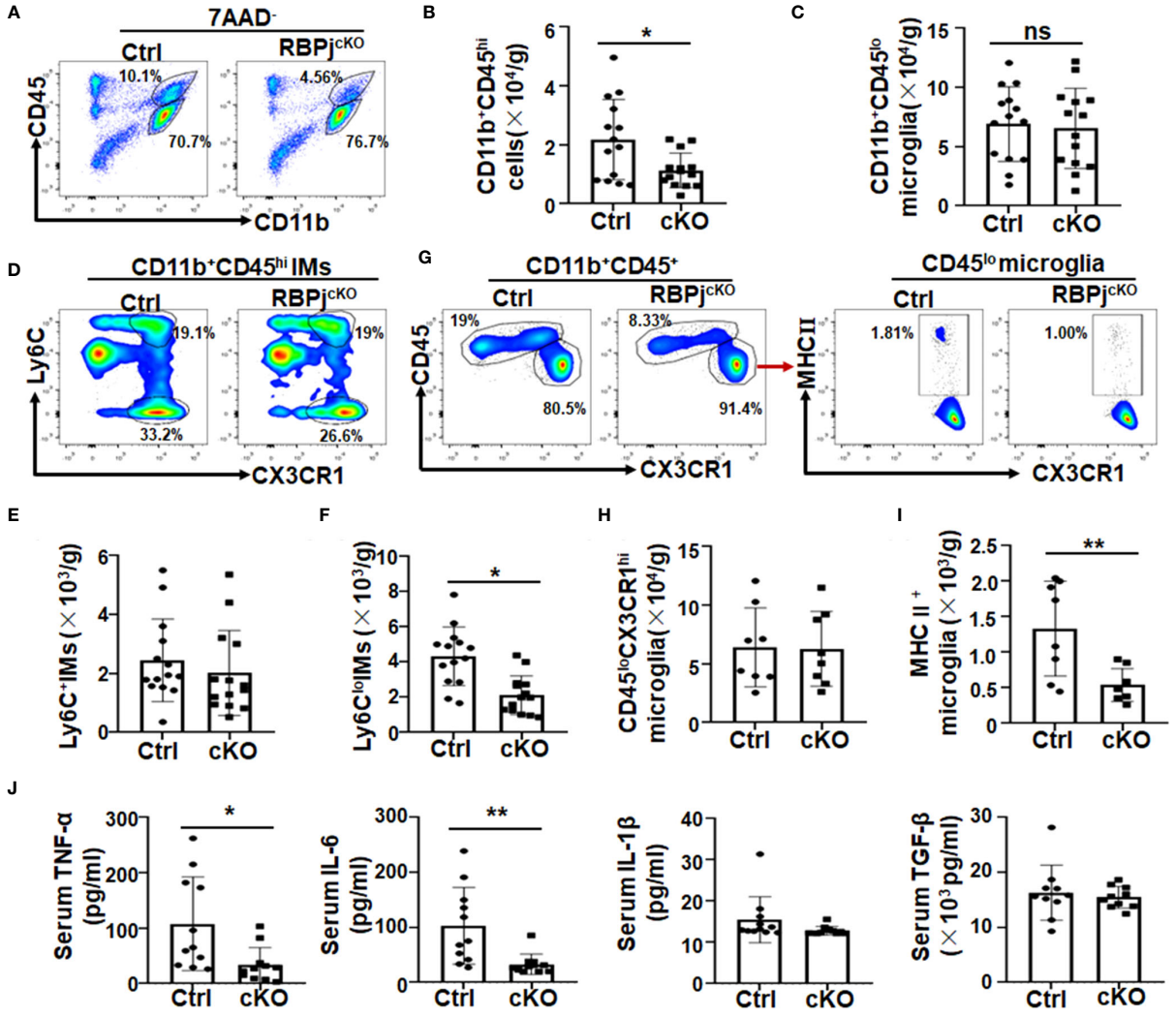

FIGURE 3
Myeloid-specific RBP-J deficiency inhibited microglial activation and reduced the inflammatory response in PD mice. Single-cell suspensions were prepared from the brains of RBP-J^{cKO} and control (Ctrl) PD mice. **(A)** The myeloid cell population were analyzed by FACS. **(B, C)** The number of CD11b^{+}CD45^{hi}-infiltrated inflammatory macrophages (IMs; B) and CD11b^{+}CD45^{lo} microglia **(C)** in **(A)** were analyzed and quantitatively compared, respectively (n = 14). **(D)** Infiltrated IMs were further analyzed with Ly6c and CX3CR1 staining by FACS. **(E, F)** The total cell number of CD11b^{+}CD45^{hi}CX3CR1^{+}Ly6c^{+} **(E)** and CD11b^{+}CD45^{hi}CX3CR1^{+}Ly6c^{lo} **(F)** IMs in brain was quantitatively compared (n = 14). **(G)** The microglia (CD11b^{+}CD45^{lo}CX3CR1^{hi}) and the activated microglia (MHCII^{+}CD11b^{+}CD45^{lo}CX3CR1^{hi}) were analyzed by FACS. **(H, I)** The total cell number of microglia **(H)** and activated microglia **(I)** in **(G)** was quantitatively compared; **(J)** The levels of IL-6, TNF-α, IL-1β, and TGF-β in serum of RBP-J^{cKO} and Ctrl PD mice were detected using ELISA (n = 11 in IL-6, TNF-α, and IL-1β; n = 10 in TGF-β). The Student's t-test was used for the statistical analyses. Bars = mean ± SD; *P < 0.05; **P < 0.01 ns, no significance.

on the reduction in CCR2^{+} monocyte recruitment. To address this question, we crossed RBP-J^{cKO} mice with CCR2$^{-/-}$ mice to gain RBP-J and CCR2 double-knockout mice (CCR2$^{-/-}$RBP-J^{cKO}), in which the migration of CCR2^{+} monocytes was blocked. After MPTP treatment, FACS assays showed that the Ly6C^{hi} monocytes in peripheral blood were dominantly diminished in CCR2$^{-/-}$ and CCR2$^{-/-}$RBP-J^{cKO} mice when compared with the Ly6C^{hi} monocytes in control and RBP-J^{cKO} mice, whereas Ly6C$^{int\text{-}lo}$ monocytes increased in CCR2$^{-/-}$ and CCR2$^{-/-}$RBP-J^{cKO} mice (Figures S4A, B), indicating that CCR2 deficiency can successfully deplete blood Ly6c^{hi} monocytes but not Ly6C$^{int\text{-}lo}$monocytes. Next, immunofluorescence staining showed that CCR2 deficiency could not enhance the numbers of TH^{+} DA neurons in the SNpc between RBP-J^{cKO} and CCR2$^{-/-}$RBP-J^{cKO} mice (Figures 4A, B). Meanwhile, compared with the control PD mice, the numbers of TH^{+} DA neurons were not changed in CCR2$^{-/-}$PD mice, which was consistent with previous reports in which CCR2 blockade does not prevent striatal dopamine loss in the MPTP-induced PD model (46–48). Moreover, although CD11b^{+}CD45^{hi}-infiltrated IMs decreased significantly in CCR2$^{-/-}$ PD mice compared with control mice, CCR2 knockout did not result in significantly decreased IMs in RBP-J^{cKO} PD mice (Figures 4C, D), indicating that myeloid-specific RBP-J deficiency alleviated DA neurodegeneration independent of CCR2^{+} monocyte recruitment. More importantly, regardless of whether CCR2 was knocked out, the total number of MHC II^{+} activated microglia was decreased significantly in the RBP-J^{cKO} mice (Figures 4E–G). Meanwhile, the expression of some inflammatory

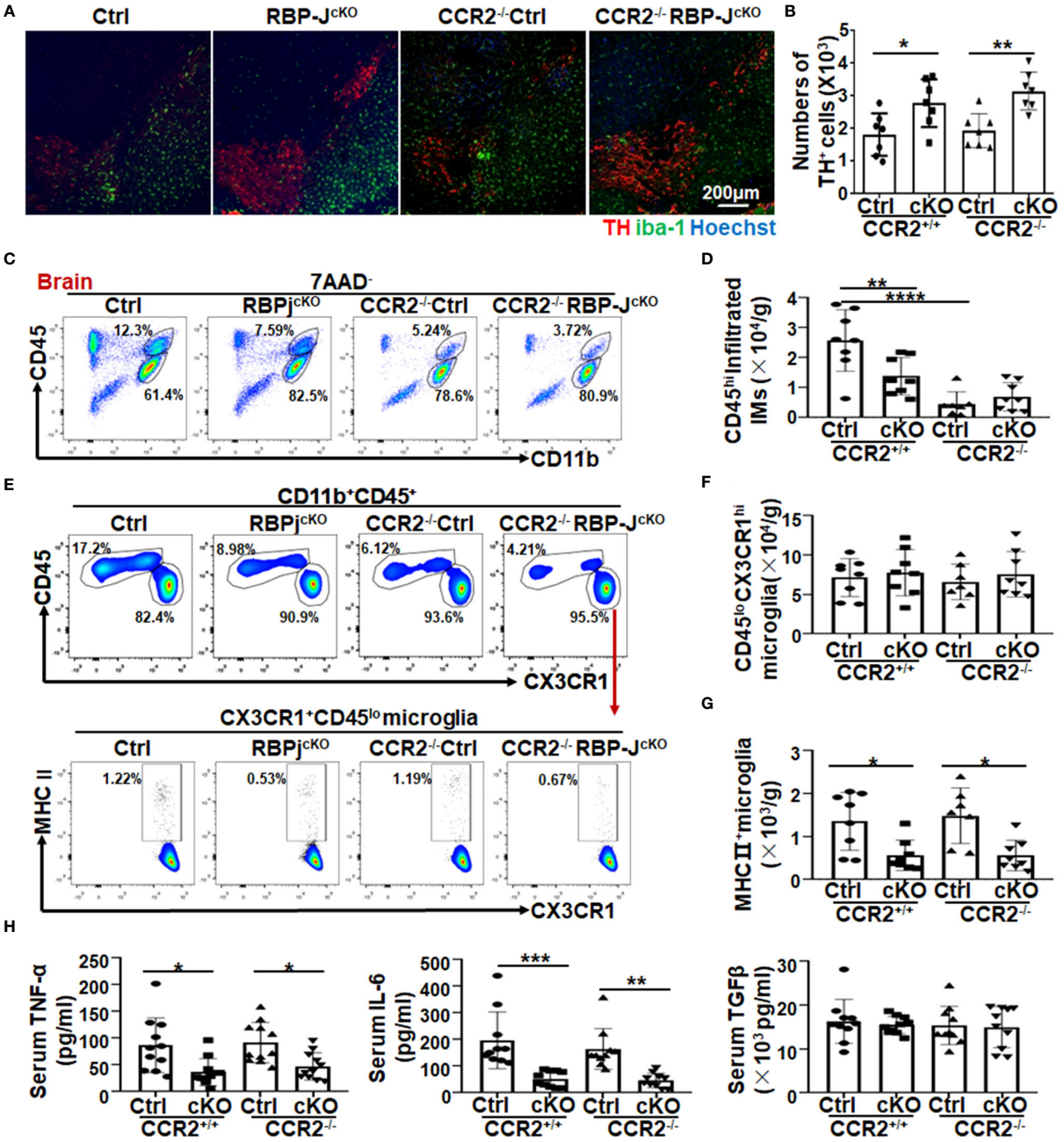

FIGURE 4
CCR2 depletion contributed less to the attenuated dopaminergic neurodegeneration in myeloid-specific RBP-J–deficient PD mice. **(A)** Ctrl, RBP-J^{cKO}, CCR2$^{-/-}$, and RBP-J^{cKO}/CCR2$^{-/-}$ mice were treated intraperitoneally with MPTP-HCl as mentioned above to induce the acute PD model. Brains were dissected and stained with TH, IBA-1, and Hoechst on tissue sections using immunofluorescence staining. **(B)** The numbers of TH$^+$ neurons were counted in five areas and quantitatively compared among each group (n = 8). **(C)** Single-cell suspensions were prepared from the brain, and infiltrated IMs were analyzed by FACS. **(D)** The CD11b$^+$CD45^{hi}-infiltrated IMs in **(C)** were quantitatively compared (n = 7 in the CCR2$^{-/-}$group; n = 8 in the other groups). **(E)** The total microglia and activated microglia were analyzed by FACS. **(F, G)** The total cell number of microglia **(F)** and MHCII$^+$ activated microglia **(G)** in **(E)** was quantitatively compared (n = 7 in the CCR2$^{-/-}$group; n = 8 in the other groups). **(H)** The levels of TNF-α, IL-6, and TGF-β in serum among each group were measured using ELISA (n = 11 in TNF-α; n = 10 in IL-6 and TGF-β). One-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. Bars = mean ± SD; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

factors, including TNF-α and IL-6, was also decreased significantly in the serum of RBP-J^{cKO} mice or CCR2$^{-/-}$RBP-J^{cKO} mice. However, the level of the anti-inflammatory factor TGFβ showed no difference among the groups (Figure 4H). Together, these results indicated that CCR2$^+$ monocytes might not contribute to the attenuated DA neurodegeneration in RBP-J^{cKO} PD mice.

Microglia depletion increased TH$^+$ neuron cells slightly in myeloid RBP-J deficient PD mice

As mentioned above, Notch signaling might regulate microglial activation during PD progression, and we further depleted

microglia by feeding mice commercial food containing a small-molecule inhibitor of CSF1R signaling, namely, PLX5622, to assess its contribution (32, 33, 49, 50). First, we confirmed the efficiency of microglia depletion by feeding mice a PLX5622-formulated AIN-76A diet (PLX) or AIN-76A diet [standard diet (SD)] for 7 days. The results showed, *via* immunofluorescence staining and FACS assays, that PLX5622 administration effectively depleted Iba1^{+} microglia (CD11b^{+}CD45^{low}) (Figures S4C–F). Next, as shown in the scheme in Figure 5A, RBP-J^{cKO} and control mice were fed the PLX5622 diet or standard diet for 7 days and then treated with MPTP to induce the PD model and fed the PLX5622 diet or SD for another 7 consecutive days. Immunofluorescence staining showed

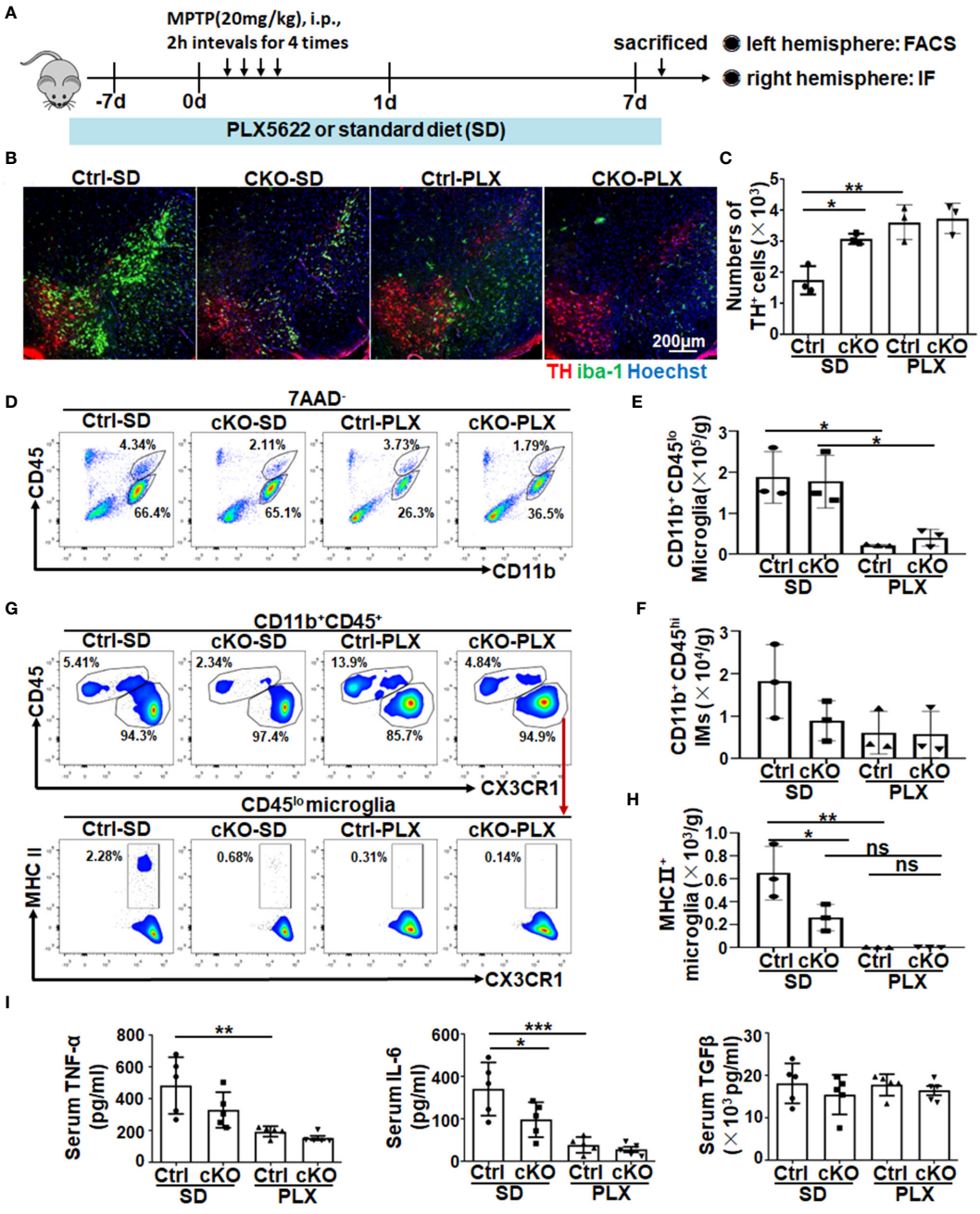

FIGURE 5
The depletion of microglia showed a resistance role in neurodegeneration of myeloid-deficient RBP-J PD mice. **(A)** Mice were fed a commercial PLX5622 diet or standard diet for 7 days and then treated with MPTP to induce a PD model accompanied by a PLX5622 diet for another 7 days. **(B)** Brain sections were made from RBP-J^{cKO} or control mice fed the above diet and then subjected to immunofluorescence staining with anti-TH and IBA-1 antibodies. Nuclei were stained with Hoechst (n = 3). **(C)** The number of TH^{+} neurons in **(B)** was counted and quantitatively compared among each group (n = 3). **(D)** Single-cell suspensions from the brains of PD mice in **(A)** were prepared and analyzed by FACS (n = 3). **(E, F)** The number of microglia **(E)** and infiltrated myeloid cells **(F)** in **(D)** was quantitatively compared among each group (n = 3). **(G)** MHC II^{+} microglia from different groups were analyzed by FACS (n = 3). **(H)** The number of MHC II^{+} microglia was quantitatively compared among each group (n = 3). **(I)** The levels of TNF-α, IL-6, and TGF-β in serum among each group were detected using ELISA (n = 5). One-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. Bars = mean ± SD; *P < 0.05; **P < 0.01; ***p< 0.001; ns, no significance.

that mice with continuous administration of the PLX5622 diet showed a remarkable decrease in the number of microglia and exhibited improved DA neurodegeneration in both RBP-J–deficient and control PD mice (Figures 5B, C). In addition, FACS results showed that $CD11b^{+}CD45^{lo}$ microglia and $CD11b^{+}CD45^{hi}$-infiltrated IMs, especially $Ly6C^{lo}CD11b^{+}CD45^{hi}$-infiltrated IMs, decreased significantly in the PLX5622-treated groups (Figures 5D–F, Figures S5). As expected, MHC II^{+} activated microglia were decreased remarkably in PLX5622-treated RBP-J–deficient and control mice (Figures 5G, H). Correspondingly, the serum TNF-α and IL-6 but not TGF-β were decreased obviously in PLX5622-treated RBP-J–deficient and control mice (Figure 5I). Collectively, these results demonstrated that myeloid-specific RBP-J deficiency could ameliorate DA neurodegeneration by reducing $MHCII^{+}$ microglial activation.

RBP-J–deficient primary microglia exhibited reduced proinflammatory cytokine secretion through NF-κB signaling

To further explore the mechanism of Notch signaling-regulated microglial activation in PD progression, primary microglia were isolated from $RBP\text{-}J^{cKO}$ and control mice according to the described protocol (28, 51). The purity of isolated primary microglia was approximately 96.7%, as determined by immunofluorescence staining and FACS assay (Figures S6A, B). Then, the primary microglia were stimulated with LPS for 24 h, and the mRNA and protein levels of proinflammatory factors, including TNF-α, IL-6, and IL-1β, as well as anti-inflammatory cytokines, including TGF-β and IL-10, were measured by qRT-PCR, ELISA, and immunofluorescence staining. The results showed that the protein levels of TNF-α and IL-6 decreased markedly, whereas the IL-10 level increased significantly in $RBP\text{-}J^{cKO}$ microglia (Figures 6A, B, Figures S6C, D). Furthermore, we first observed the effect of LPS-stimulated microglia on dopamine neurons by coculture experiments. The results showed that $CD45^{-}AnnexinV^{+}PI^{+}$ apoptotic neurons decreased remarkably in RBP-J–deficient microglia, which might be due to fewer proinflammatory cytokines and more anti-inflammatory cytokines secreted by $RBP\text{-}J^{cKO}$ microglia after LPS stimulation (Figures 6C, D). Because of the cytotoxin effect of LPS on primary microglia, we further performed the coculture experiments using primary microglia and MPP(+)-induced DA neurons as shown in Figures 6E, F. The results were consistent with the coculture experiment using LPS-treated microglia and neuron. Meanwhile, bone marrow–derived macrophages (BMDMs) were cultured as previously described (34) and stimulated with LPS for 24 h; then, the mRNA and protein levels of inflammatory factors were detected, and the results showed that RBP-J deficiency in BMDMs could not alleviate inflammatory response significantly (Figures S7A, B); in line with this, the experiment that cocultures BMDMs with DA neurons showed a little effect on the apoptosis of DA neurons (Figures S7C, D). These results were consistent with the previous reports that microglia and BMDM showed different gene profiles in neurodegeneration (52, 53). As Notch signaling can cooperate with Toll-like receptors (TLR) signaling to defend against pathogen infection through NF-κB signaling, we further examined the downstream molecules of NF-κB signaling using immunofluorescence staining and Western blotting with an anti-p65 antibody. All results showed that the nuclear expression of p65 was significantly reduced in RBP-J–deficient microglia after LPS stimulation (Figures 6G-J). Together, these results indicated that myeloid-specific blockade of Notch signaling could participate in PD progression by mainly affecting the microglia-mediated neuroinflammation through NF-κB signaling.

Discussion

Microglia are the most abundant innate immune cells in the CNS that can mediate synaptic pruning and remodeling by interacting with neurons in physiological and pathological conditions (54–56). In the past few decades, the heterogeneity of microglial phenotype and function in neurodegenerative diseases such as PD has received great attention, but the mechanisms that regulate microglia from the physiological state to the pathological state are still unclear. Notch signaling has been reported to play a critical role in regulating microglial activation and neuroinflammation-related disorders such as cerebral ischemia and epilepsy (57, 58). Our previous studies have further shown that inhibition of Notch signaling in myeloid cells could significantly alleviate spinal cord injury or Guillain-Barré syndrome by reducing proinflammatory macrophage polarization or promoting inflammatory monocyte conversion (23, 24). In this study, we found that Notch signaling can be greatly activated in microglia of the MPTP-induced acute PD mouse model accompanied by decreased TH^{+} neurons in the SNpc. As expected, myeloid-specific blockade of Notch signaling inhibited DA neuron death and improved mouse motor behavior by reducing $MHCII^{+}$ microglial activation and IM infiltration. Moreover, we demonstrated that myeloid-specific RBP-J deficiency could attenuate PD progression by reducing inflammatory factor secretion through NF-κB signaling. On the basis of our findings, targeting Notch signaling in myeloid cells might be a potential therapeutic strategy for neuroinflammation-related diseases, including PD, in the future.

The brain macrophage population demonstrates increasing heterogeneity and plasticity following the application of single cell RNA sequencing (scRNA-seq), which includes tissue-resident microglia, border-associated macrophages, and recruited monocyte-derived macrophages (14, 15, 59). Although the contribution of both activated microglia and infiltrated monocytes in neuroinflammation has been widely studied, the conclusion remains controversial (46–48, 60). Here, using myeloid-specific RBP-J–deficient mice combined with an acute PD model, we found that Notch signaling blockade in myeloid cells could ameliorate the symptoms of murine PD. On the one hand, Notch signaling blockade could regulate microglial activation as the total microglia number was not affected; on the other hand, Notch signaling blockade could reduce monocyte infiltration. In general, the CCR2–CCL2 axis is a popular chemokine axis for recruiting peripheral monocytes into the CNS during neuroinflammation (61, 62). Our recent studies have shown that myeloid-specific Notch signaling disruption could alleviate renal or lung fibrosis progression by

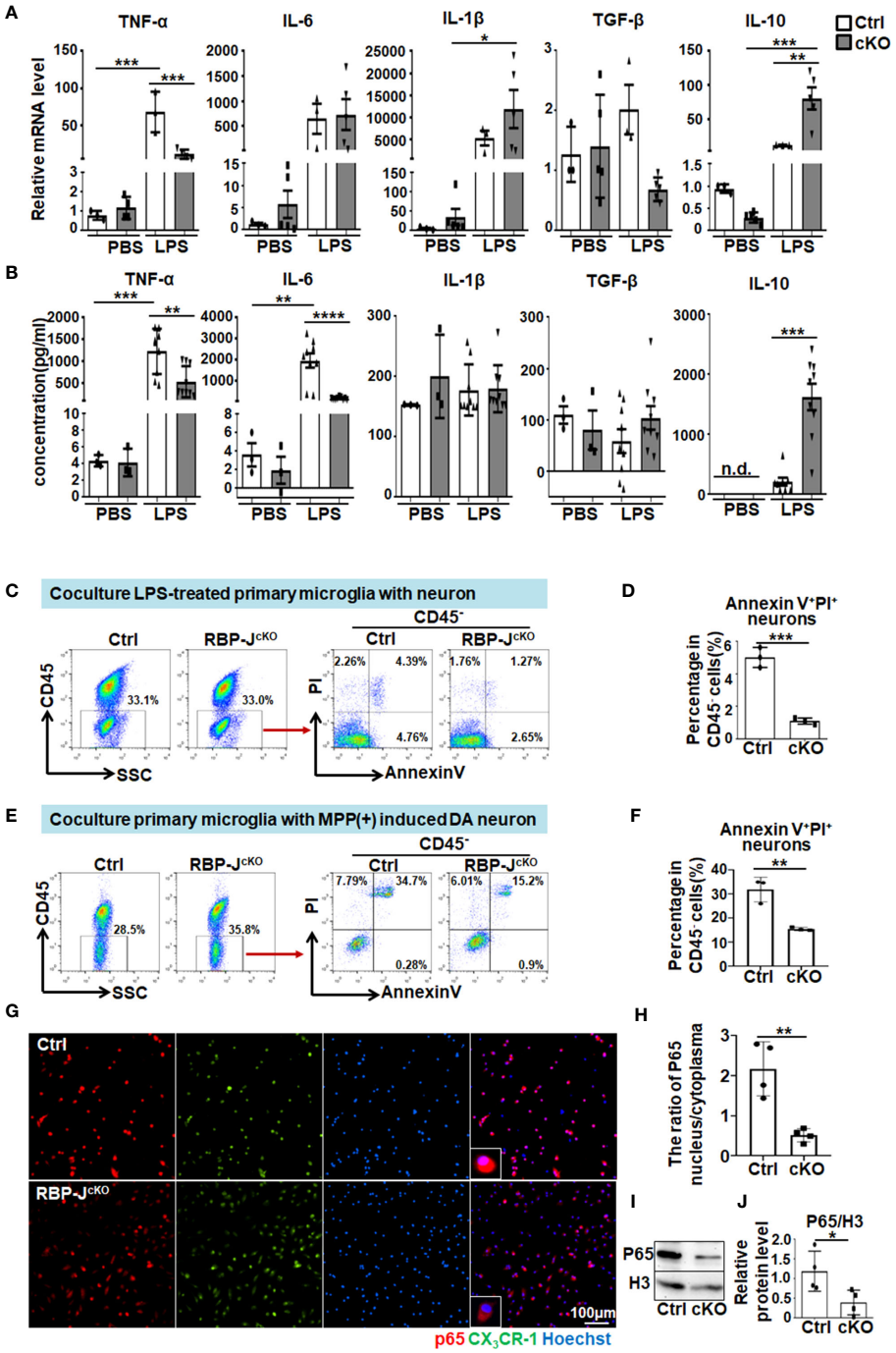

FIGURE 6
RBP-J–deficient primary microglia exhibited reduced proinflammatory cytokine secretion through NF-κB signaling. **(A)** Primary microglia were isolated from RBP-J^{cKO} and control mice and then cultured and stimulated with LPS or PBS (100 ng/mL) for 24 h. After that, cells were collected for RNA extraction, and the relative mRNA expression levels of TNF-α, IL-1β, IL-6, IL-10, and TGF-β were determined by RT-PCR [primary microglia from Ctrl mice: n = 3; primary microglia from cKO mice: TNF-α (n = 4), IL-6 (n = 5), IL-1β (n = 5), TGF-β (n = 5), and IL-10 (n = 5)]. **(B)** The protein levels of TNF-α, IL-1β, IL-6, IL-10, and TGF-β in cultured medium collected from primary microglia in **(A)** were detected by ELISA (n = 3 in groups treated with PBS, n = 9 in groups treated with LPS except IL-10, in which n = 8 in LPS-Ctrl group). **(C)** LPS-stimulated primary microglia from RBP-J^{cKO} or control mice were cocultured with HT-22 cells for 24 h, and, then, the apoptotic HT-22 cells in CD45-negative cells were examined by Annexin V/PI staining. **(D)** The AnnexinV^{+}PI^{+} apoptotic HT-22 cells in **(C)** were quantitatively compared (n = 3). **(E)** Cocultured primary microglia from RBP-J^{cKO} or control mice with MPP+ (1 μM)–treated SH-SY5Y for 48h, and, then, the apoptotic SH-SY5Y cells in CD45-negative cells were examined by AnnexinV/PI staining. **(F)** The Annexin V^{+} PI^{+} apoptotic HT-22 cells in **(E)** were quantitatively compared (n = 3). **(G)** Primary microglia were isolated from RBP-J^{cKO}/CX3CR1$^{GFP/+}$ and Ctrl/CX3CR1$^{GFP/+}$ mice and then cultured on coverslips overnight. After stimulation with LPS (100 ng/mL) for 6 h, cells on coverslips were subjected to immunofluorescence staining with anti-p65 antibody and Hoechst. **(H)** The ratio of P65 nucleus/cytoplasm was calculated depending on the fluorescence intensity (n = 4). **(I)** Primary microglia in **(A)** were lysed, and, then, the nucleic and cytoplasmic proteins were extracted. The expression of P-p65 in the nucleic was measured by Western blot, with H3 as an internal control (n = 4). **(J)** The relative protein level of nuclear p65 was quantitatively compared (n = 4). One-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. Bars = mean ± SD. n.d., not detectable. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

regulating monocyte-derived macrophage recruitment *via* the CCR2–CCL2 axis (37, 38). Unexpectedly, in the current study, CCR2 knockout did not result in significantly decreased IMs in RBP-J^{cKO} PD mice and contributed less to the increased numbers of TH^{+} DA neurons in RBP-J^{cKO} PD mice, suggesting that Notch signaling blockade in myeloid cells alleviated DA neurodegeneration independent of CCR2^{+} monocyte recruitment. However, Ly6C^{lo} IMs decreased significantly in RBP-J^{cKO} PD mice, which was reminiscent of the contribution of Ly6c^{lo}CX3CR1^{hi}CCR2^{lo} patrolling monocytes to infiltrated macrophages in MPTP-treated mice (48, 63). Whether Notch signaling in myeloid cells could regulate these patrolling monocytes involved in PD progression still needs to be investigated.

Microglial activation has been demonstrated to be a key regulator of PD pathogenesis (10). MHC II is a hallmark of microglial activation and was first reported by McGeer et al. in 1988. They found large numbers of Human leukocyte antigen DR (HLA-DR)–positive reactive microglia (macrophages) along with Lewy bodies in the substantia nigra of patients with PD (64). Recently, Williams et al. further suggested that targeting MHC II expression by shRNA against *CIITA* in microglia could attenuate inflammation and neurodegeneration in an α-synuclein model of PD (65). In our study, we also found that, regardless of whether CCR2 was knocked out, the total number of MHC II^{+} activated microglia decreased significantly in RBP-J^{cKO} PD mice. Moreover, RBP-J–deficient microglia exhibited less proinflammatory factor secretion and neuronal apoptosis, suggesting that Notch signaling might dominantly regulate microglial activation involved in PD pathogenesis rather than CCR2-depenedent monocyte recruitment.

To address the role of microglia under pathological conditions, researchers usually adopt microglia depletion experiments using clodronate liposomes, anti-colony stimulating factor 1 receptor (CSF1R) antibodies, or CSF1R inhibitors. Intracerebral administration of clodronate liposomes into the brain parenchyma can cause macrophage apoptosis but can also damage other brain cells, including blood vessels (66). CSF1R is expressed on microglia/macrophages and is responsible for their survival and proliferation (67, 68). Although genetic deletion of CSF1R can be used to deplete microglia, other cells expressing CSF1R are often affected (69). Recently, one CSF1R inhibitor, PLX5622, has been largely assumed to be microglia-specific with few off-target effects, which have beneficial effects on motor and non-motor symptoms in a PD model (70). On the basis of 90% microglia depletion efficiency, we utilized a PLX5622-formulated diet to evaluate the contribution of RBP-J–deficient microglia to PD progression and found that microglia depletion could inhibit DA neurodegeneration. However, in addition to microglia depletion, in our system, we also found that CD11b^{+}Ly6C^{lo} monocytes were depleted (data not shown). This phenomenon is consistent with a previous report in which CX3CR1^{hi}ly6C^{lo} monocytes could be severely depleted by CSF1R inhibition, whereas CX3CR1^{lo}ly6C^{hi} monocytes could not be depleted in the peripheral immune system (69). Because myeloid-specific RBP-J deficiency reduced the infiltration of Ly6c^{lo}CXCR1^{hi} IMs in MPTP-treated mice (Figures 3D, F), the effect of PLX5622 on this population may not be an ideal method for investigating microglial function under a Notch signaling disruption background. In addition, the study of Orthgiess et al. founded that, targeting microglia *in vivo* using the LysM promoter is less efficient than that using the CX3CR1 promoter and neurons that exhibit LysM promoter activity (71). Therefore, in the future, more genetically modified mice, such as microglia-specific Notch-activated mice (CX3CR1-Cre or TMEM119-Cre combined with NICD $^{stop\text{-}flox}$), should be used to clarify the importance of Notch signaling in regulating microglial activation in PD progression.

In summary, our present study first demonstrates that myeloid-specific Notch signaling blockade can alleviate DA neurodegeneration in PD mice. The underlying cellular mechanism may be attributed to reduced MHC II^{+} activated microglia and infiltrated Ly6c^{lo}CX3CR1^{hi} macrophages. Meanwhile, the molecular mechanism may be related to the alleviation of neuroinflammation regulated by NF-κB signaling.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

S-QL, P-HL, and Y-YH performed experiments and analyzed the results. J-LZ, F-ZS, and FK took part in various aspects of the study and revised first draft. K-XR, T-XW, and LF participated in all animal experiments. FF performed data statistics and analysis. HH discussed the data. H-YQ designed the project, analyzed the data, and supported the study. S-QL and H-YQ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

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Supplementary material

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EDITED BY
Stephen Nutt,
The University of Melbourne, Australia

REVIEWED BY
Paolo Casali,
MD, The University of Texas Health Science Center at San Antonio, United States

*CORRESPONDENCE
Koushik Roy
koushik.roy@path.utah.edu

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The NFκB signaling system in the generation of B-cell subsets: from germinal center B cells to memory B cells and plasma cells

Koushik Roy[1*], Mainak Chakraborty[2], Ashok Kumar[1], Asit Kumar Manna[1,3] and Neeladri Sekhar Roy[4]

[1]Division of Microbiology and Immunology, Department of Pathology, School of Medicine, University of Utah, Salt Lake City, UT, United States, [2]Division of Immunology, Indian Council of Medical Research-National Institute of Cholera and Enteric Diseases, Kolkata, India, [3]Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, UT, United States, [4]Department of Biochemistry, School of Medicine, Emory University, Atlanta, GA, United States

Memory B cells and antibody-secreting cells are the two prime effector B cell populations that drive infection- and vaccine-induced long-term antibody-mediated immunity. The antibody-mediated immunity mostly relies on the formation of specialized structures within secondary lymphoid organs, called germinal centers (GCs), that facilitate the interactions between B cells, T cells, and antigen-presenting cells. Antigen-activated B cells may proliferate and differentiate into GC-independent plasmablasts and memory B cells or differentiate into GC B cells. The GC B cells undergo proliferation coupled to somatic hypermutation of their immunoglobulin genes for antibody affinity maturation. Subsequently, affinity mature GC B cells differentiate into GC-dependent plasma cells and memory B cells. Here, we review how the NFκB signaling system controls B cell proliferation and the generation of GC B cells, plasmablasts/plasma cells, and memory B cells. We also identify and discuss some important unanswered questions in this connection.

KEYWORDS

B cell, memory B cell, plasma cell, NFκB, and cell signaling

Introduction

Following an infection or vaccination, secondary lymphoid organs undergo profound structural changes to form extrafollicular foci and germinal centers (GCs) (1, 2). Antigen-activated B cells within extrafollicular foci proliferate and differentiate into GC-independent plasmablasts (PBs), which generate short-lived immunity and memory B cells (MBCs) (1, 2). Antigen-activated B cells that enter the GC proliferate and undergo somatic hypermutation (SHM) of the B cell receptor (BCR) with an average of 10^{-3} mutations per base pair in each proliferative cycle to enhance affinity for antigens (3–5).

High-affinity B cells capture more antigen than low-affinity B cells, present the antigen to T cells, and subsequently receive strong T cell help. GC B cells that receive strong T cell help (mediated through the interaction of the CD40 receptor on GC B cell and the CD40 ligand on T cell) become affinity mature and differentiate into long-lived plasma cells (PCs). GC B cells that receive weak T cell help (weak activation through CD40) differentiate into MBCs, while others receiving a little/no T cell help undergo apoptosis (1, 2). However, this affinity-based selection model of PCs and MBCs generation has recently been challenged (1–5). Our single-cell lineage tracking study found that B cells show cell-to-cell variability in their proliferative capacity in response to BCR-independent stimulation, even when they express the same BCR (HEL transgenic BCR) (6). Both computational modeling and experimental results show that variable proliferative capacity is due to preexisting variation in the molecular networks, which is independent of BCR affinity. Hence, the selection of GC B cells may be a combinatorial effect of BCR affinity, preexisting variation in the molecular networks of the GC B cells during recruitment to the GC, and the complex environment of GC itself.

Mice deficient in T cells produce class-switched IgG antibodies upon viral infection and T cell-independent immunization (7, 8). A recent study has shown that "TLR-BCR linked co-engagement" with TLR-ligand and antigen generates T cell-independent class-switched and hypermutated high-affinity antibodies and GC-like structure (9). Another genetic fate mapping study shows that T cell-independent immunization develops transient GCs and generates GC-derived PCs and MBCs (10). Thus, both T cell-dependent and independent pathways generate GC and high-affinity antibodies, though the T cell-dependent pathway generates GC and high-affinity antibodies more efficiently.

GC B cells circulate between the two distinct anatomical zones of GC viz the light zone (LZ) and the dark zone (DZ). GC B cells undergo rapid proliferation (6-8 h) and SHM within the DZ to acquire affinity-improving mutations and return to the LZ, where they are tested for antigen affinity and the affinity-damaging mutation lead to apoptosis (2, 11, 12). Affinity-matured GC B cells differentiate into PCs within the LZ (2). Whether the generation of MBCs requires affinity maturation or not is controversial (1, 2, 5). The diversity and affinity of antibodies generated in response to an immune challenge are largely GC-dependent. The success of vaccination and protection from re-infection depends on the longevity of the generated antibodies and MBCs. As a result, GC B cells play a key role in generating long-lasting protective humoral immunity. However, chronic infection and other pathological conditions may disrupt GC B cell differentiation and contribute to lymphoid malignancy and autoimmunity (13). Therefore, precise regulation of GC B cell differentiation is needed to generate effective humoral immunity without generating B cell lymphoma/autoimmunity. The accurate regulation of GC B cell differentiation is controlled by the coordination of cell signaling pathways (such as NFκB, PI3K/AKT, MAPK, and STAT) and transcription factors (such as NFκB, IRF, Myc, Bcl-6, OCA-B, Bach2, etc.) (1, 14). The transcription factor NFκB is a direct stimulus-responsive transcription factor. Stimulation leads to nuclear translocation of NFκB within a few minutes to an hour and activates the transcription of many key regulators essential for GC B cells, PCs, and MBCs (1, 14–21) (discussed below). It has been shown that NFκB and its upstream signaling (defined here as the NFκB signaling system) are dysregulated in many B cell lymphomas and immune disorders (13, 22–25).

Overview of the NFκB signaling system

In mammals, the transcription factor NFκB family comprises homo- and heterodimers formed combinatorially by five Rel family proteins RelA/p65, cRel, RelB, p50 (NFκB1), and p52 (NFκB2) (26–28). The five NFκB monomers can theoretically generate 15 possible dimers (29, 30). The three Rel family members, RelA, cRel, and RelB, have a DNA binding domain and function as transcriptional activators (30–33). The other two Rel family members, NFκB1 and NFκB2 have a DNA binding and an ankyrin repeat domain (ARD) (30, 33, 34). The ARD of NFκB1 and NFκB2 inhibits the activation of NFκB. Constitutive or stimulus-responsive proteolytic cleavage of the ARD generates p50 and p52 from NFκB1 and NFκB2, respectively. p50 and p52 contain a DNA binding domain but not a transcription activation domain and may inhibit transcription as homodimers (p50:p50 and p52:p52) (26, 29, 35, 36). However, p50:p50 and p52:p52 dimers may form a complex with co-activators (e.g., Bcl3 and IκBζ) to activate transcription (37, 38). The detail of NFκB signaling has been extensively studied and summarized in several excellent reviews (29, 39, 40). Here, we have briefly described the NFκB signaling system, primarily in the context of B-cells.

In the absence of extra-cellular stimuli, NFκB is associated with inhibitors of NFκB (IκBα, IκBβ, IκBε, and IκBsome) in the cytosol. The activation of B cells by exogenous (foreign materials: e.g., protein/peptide antigen, LPS, etc.) and endogenous (host-derived materials: e.g., CD40-ligand, BAFF, etc.) stimuli causes degradation of IκBs by proteolysis and releases IκB-bound NFκB dimers for their translocation to the nucleus where they activate transcription (15) (Figure 1) (details below). Naïve B cells are enriched for nuclear p50:p50 homodimer, which may function as a transcriptional inhibitor; B cell activation replaces inhibitor NFκB (p50:p50) with activator NFκB (cRel:p50/RelA:p50) (35, 44). IgM-mediated BCR signaling and TLR signaling in B cells predominantly activate RelA:p50, cRel:p50, and p50:p50 through the canonical pathway (15–18, 45–47). IgD-mediated BCR signaling induces expression of NFκB2 and generates p52, suggesting activation of the non-canonical NFκB pathway (41). CD40 signaling in B cells activates both canonical and non-canonical pathways, leading to nuclear translocation of RelA:p50, cRel:p50, and RelB:p52 (15, 48, 49). BAFF signaling alone predominantly activates the non-canonical pathway more than the canonical one (15, 50, 51) (Figure 1).

Canonical NFκB signaling is transduced by a NEMO-dependent kinase (IKK) complex composed of IKK1, IKK2, and NFκB essential modulator (NEMO). The activation of this IKK

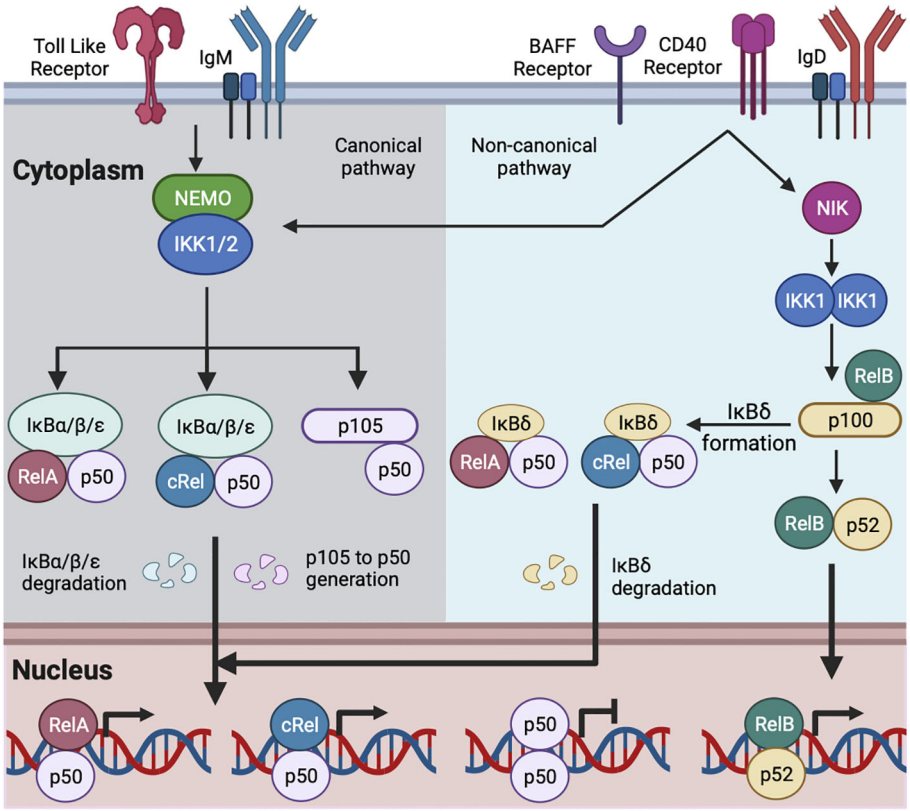

FIGURE 1
Schematic of canonical and non-canonical NFκB activation in B cell. TLR and IgM-mediated BCR signaling activate the canonical NFκB pathway (15, 35). IgD-mediated BCR signaling may activate both the canonical and non-canonical NFκB pathway (41, 42). CD40 and BAFF activate the canonical and non-canonical NFκB pathways (15). The canonical signaling activates NEMO and IKK1/2 containing complex. The activated IKK1/2 phosphorylates members of the IκBs (IκBα, IκBβ, and IκBε here referred as IκBα/β/ε) bound with NFκB, leading to the degradation of IκBα/β/ε. The degradation of IκBα/β/ε releases IκB-bound NFκB, which translocates to the nucleus. The activated IKK1/2 phosphorylates IκB-like molecule p105, and ubiquitin-mediated degradation of p105 generates p50 with the formation of RelA:p50, cRel:p50 and p50:p50 (15, 22, 35). RelA:p50 and cRel:p50 dimers are transcriptional activators. p50:p50 dimer may function as a transcriptional inhibitor and are present in naïve mature B cell (35). B cell activation by canonical pathway replaces p50:p50 with RelA:p50 and cRel:p50 (15, 35). The non-canonical signaling stabilizes NIK and subsequent activation of IKK1. The activated IKK1 phosphorylates IκB-like molecule p100 and generates p52. The degradation of RelB-bound p100 generates RelB:p52 dimer, and its nuclear translocation (15). The multimeric association of p100 generates IκBδ. IκBδ remains predominantly bound with cRel:p50 and RelA:p50 dimers. The activated IKK1 degrades IκBδ and releases cRel:p50 and RelA:p50 dimers to the nucleus (16, 43).

complex is NEMO-dependent and mediated by phosphorylation-dependent activation of IKK2 (29, 52, 53). The activated IKK2 phosphorylates IκBα, IκBβ, and IκBε, leading to their degradation and freeing NFκB dimers for nuclear translocation (26–28). The canonical NFκB signaling pathway in B cells predominantly activates RelA:p50, cRel:p50, and p50:p50 dimers (15–17, 35). Non-canonical NFκB signals are transduced in a NEMO-independent but NIK (NFκB inducing kinase) and IKK1-dependent manner (29, 52). The non-canonical pathway has dual functions. The first function is to process the p100 monomer to p52, leading to the formation of RelB:p52 dimer (43, 54). Unprocessed p100 oligomerizes and forms the IκBsome inhibitory complex (55). The second function is to degrade the IκBsome and release IκBsome bound NFκB, including RelA:p50 and cRel:p50 (16, 43). The non-canonical NFκB in B cells predominantly activates RelB:p52; however, in a context-dependent (e.g., anti-IgM and BAFF co-stimulation, discussed below) and cell type-specific manner, the non-canonical pathway also activates RelA:p50, and cRel:p50 dimers (15, 16, 56).

The NFκB signaling system in B cell proliferation and survival

Naïve B cells are activated by antigen/ligand binding to cell-surface receptors, e.g., BCR signaling is activated by an antigen, TLR signaling is activated by TLR-ligand (e.g., LPS, CpG, etc.), CD40 receptor signaling is activated by CD40-ligand, and BAFF receptor signaling is activated by BAFF (Figure 1). BCR, CD40, and TLR signaling- all result in B cell activation, proliferation, and survival, while BAFF signaling, without co-stimulation, results in B cell survival (15, 22, 57). These signals activate NFκB, as discussed above (Figure 1). Activated B cells enter the growth phase and increase in cell size, and during the growth phase, they are protected from cell death (Figure 2) (60, 66). It has been shown that mature B cells stimulated for 24 hour activate the proliferative program, and these activated B cells are programmed to divide multiple times without further stimulation, suggesting that induction of key regulators within the first few hours may control division number (67). In line with this, Heinzel et al. showed that Myc expression

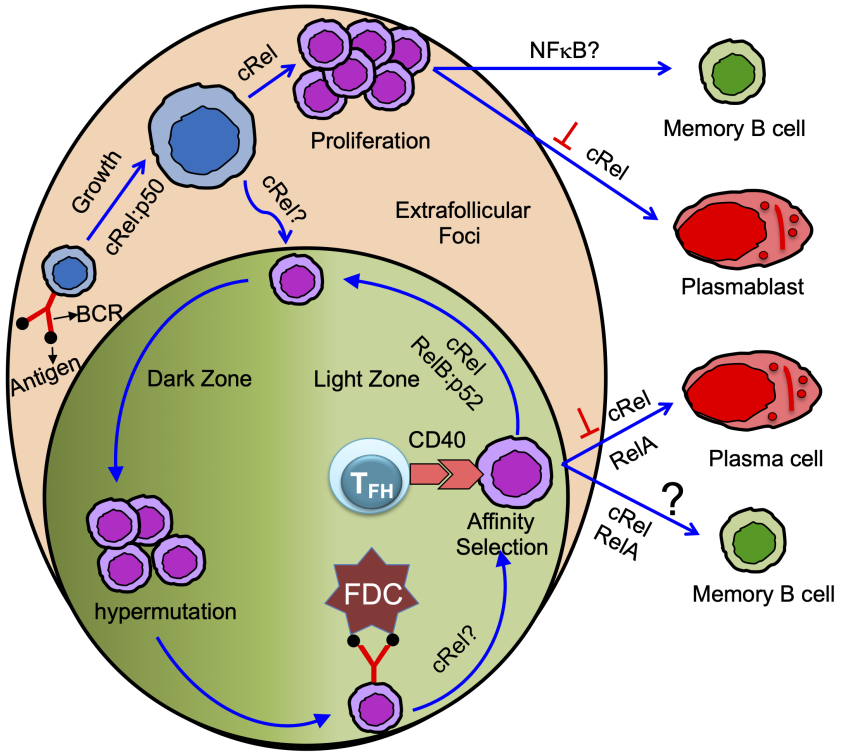

FIGURE 2
Antigen-specific naïve B cells following antigen binding activate and grow in size. cRel:p50 dimer control activation/B cell growth (58, 59). Activated B cells proliferate in the extrafollicular foci or differentiate in GC B cells. cRel is required for B cell proliferation (15, 58, 60, 61). Whether cRel is required in B cells for GC initiation or formation is not yet clear (62). Proliferating B cells in the extrafollicular foci differentiated into memory B cells and plasmablast. cRel inhibits plasmablast differentiation (21), and BAFF signaling needed for GC-independent memory B cell generation suggests NFκB could control GC-independent memory B cell generation (63). GC has anatomically two distinct zones: dark and light zone. Activated B cells that enter GC and differentiate into GC B cells undergo proliferation coupled with somatic hypermutation in the dark zone. GC B cells in the dark zone rapidly proliferate and undergo somatic hypermutation of B cell receptors. GC B cells in the light zone acquire antigen from follicular dendritic cell (FDC). Light zone B cells present the antigen to the T follicular helper (T_{FH}) cell and receive T cell help mediated by CD40 signaling (Note: T_{FH} cell also provides other modes of help such as IL21, IL4, etc. Here, we emphasize only the CD40 signal). Long-lived plasma cells are generated from affinity-selected GC B cells. It is controversial whether affinity-based selection is required for the generation of GC-derived memory B cells. cRel is required for GC maintenance and likely control the light to dark zone transition (20). cRel may control BCR-mediated GC survival as BCR-activated light zone B cells show NFκB target gene expression (64). RelA is required, but cRel inhibits, for GC-derived plasma cell generation (20). RelB:p52 is required for cell cycle entry of GC B cells and likely control the interaction of GC B cells with T_{FH} cells (19). Thus, RelB:p52 control re-entry of GC B cells from light to dark zone. Both cRel and RelA control GC-derived memory B cell generation (65), and the conclusion is based on an induced GC-B cells culture system.

before the 1st division determines the maximum division number (68). Myc is a cRel target gene (58). Therefore, it is possible, but as yet unproven, that the extent of NFκB activation before 1st division can control the maximum division number.

IKK2 deficiency leads to diminished NFκB activation. IKK2-deficient B cells show reduced mature B cell numbers and impaired B cell activation/proliferation upon mitogenic stimulation with LPS, anti-IgM, or anti-CD40 (69). NEMO-deficient B cells also show reduced NFκB activation and reduced generation of mature B cells, and the phenotype is similar to IKK2 deficiency (69). Constitutive activation of the canonical NFκB pathway, using constitutively active IKK2 (IKK2ca mutant), enhances B cell survival, leading to B cell hyperplasia (70). NIK-inactivating mutations impair the non-canonical NFκB pathway, leading to reduced p100 degradation and reduced generation of p52 (71, 72). It has been shown that NIK-inactivating mutant mice (aly/aly mice) have reduced B cell proliferation following LPS and anti-CD40 stimulation (71, 72), suggesting NIK activity is required for B cell proliferation. The activation of canonical NFκB results in the degradation of IκBα and IκBε, and the activation of non-canonical NFκB results in the degradation of IκBsome (IκBδ, p100 oligomer) (Figure 1). It has been shown that individual knockout of IκBα and IκBε enhances B cells proliferation and survival upon LPS and anti-IgM stimulation (17, 73, 74). Similarly, IκBsome reduction, caused by NFκB2 heterozygosity, enhances B cell proliferation and survival upon anti-IgM stimulation (16). Thus, hyper NFκB activation enhances B cell proliferation and survival. Both the canonical and non-canonical NFκB pathways are required for complete mitogen-induced B cell proliferation and survival.

All mitogens that activate the canonical NFκB pathway in B cells need cRel for proliferation, although the extent of cRel dependency varies, IgM-mediated signaling more dependent on cRel than LPS (Figure 2) (46). IgD-mediated signaling generates p52, although it fails to activate p65 (41). B cells deficient in NFκB2 show moderate defects in B cell proliferation in response to IgD signaling (75). B cells double deficient in p65 and p50 (p65-/-p50-/-) show impaired

proliferation in response to IgD signaling, although B cells deficient in either p65 or p50 have a little/no proliferative defects (42). Tonic BCR signaling mediated canonical pathway (cRel/RelA) activation is required to induce NFκB2 (16, 76). Thus, IgD signaling may activate both canonical and non-canonical NFκB (41, 42). NFκB1 is crucial for TLR4-dependent B cell proliferation (46, 77), presumably by enabling Tpl2-MAPK signaling (35). cRel deficient B cells show reduced expression of transcription factor IRF4, which is required for B cell proliferation (20, 78, 79). Although cRel deficient B cells still grow (enter the G1 phase), their transition from the G1 to S phase of the cell cycle is impaired (58, 60, 61). The failure to transit from the G1 to S phase has been attributed to impaired induction of transcription factor E2F3, which is required for G1 to S phase transition (80). Further, cRel-deficient B cells fail to upregulate the standard metabolic program associated with cell growth (20). The transcription factor Myc is required for B cell growth (81). cRel and NFκB1 double deficient B cells failed to grow in size (Figure 2) and have reduced Myc expression, suggesting both cRel and NFκB1 are required for G0 to G1 transition (58, 59). Transgenic Myc expression rescues B cell growth defects in cRel and NFκB1 double deficient B cells, though restoring Myc activity failed to drive proliferation upon stimulation (58). Therefore, NFκB plays multiple roles in the different phases of the cell cycle, and each NFκB subunit has distinct functions. cRel deficient B cells, upon BCR stimulation, failed to upregulate pro-survival regulators BclA1 and Bcl-xL, and Bcl2 transgenic expression inhibits BCR-induced cell death (80). Both cRel and NFκB1 are required to protect TLR4-stimulated B cells from apoptosis by blocking proapoptotic protein Bim (35, 82).

The NFκB signaling system in isotype switching

Immunoglobulin is also known as BCR when present on the cell surface. Class switching shifts immunoglobulin class, for example, from the isotype IgM to IgG. Naïve B cells express IgM and IgD (41). Class switching produces multiple isotypes of antibodies with the same variable domains but differing in the constant domains of heavy chains (83). LPS activates NFκB and promotes class switching to IgG3, while CD40L+IL4 promotes class switching to IgG1 and IgE, suggesting both canonical and non-canonical NFκB pathways could control class switching (84, 85). The deletion of NFκB1 in B cells, or the transactivation domain of cRel in B cells, leads to defects in the transcription of heavy chain constant region (86, 87). Class switch recombination (CSR) occurs within the region of the repeat sequence of the constant region, and mitogen-activated NFκB promotes transcription of the repeat sequence, thereby promoting isotype switching (88–90). It has also been shown that isotype switching depends on NFκB binding to the 3' IgH enhancer region (91, 92). CSR strictly depends on activation-induced cytidine deaminase (AID) (93–95). AID is also required for SHM (details below). AID expression must be tightly controlled as AID-mediated off-target activity poses a serious risk to the genome integrity and translocations, mutations, and oncogenesis (96). NFκB signaling is a key inducer of AID, mediated by the co-activation of TLR and BCR and by the interaction of CD40 receptor and ligand (41, 97). At least during CSR, p52 and RelA are recruited to the promoter and upstream enhancer regions of the *AICDA* gene, respectively, which encodes AID (98). Co-factors are also involved in NFκB-mediated AID activation, including HoxC4, SP1, and SP3 (93, 99). Xu et al. have shown that radiation-sensitive 52 (Rad52) is required to mediate IgD class switching through the downregulation of ZFP318, and Rad52 phosphorylation is strongly linked with high levels of IgD autoantibodies in mice models of lupus as well as SLE patients (100).

Cytokines released by T cells, such as IL-4 and TGF-β, act as secondary inducing stimuli directing isotype switching (101). Cytokines are crucial for class switching; for instance, IL-4 causes IgG1 and IgE synthesis (102, 103), while TGF-β causes IgA class switching (104, 105). The induction of the T cell-dependent IgA class switch requires TFG-β and CD40 ligand (106–108), while T cell-independent IgA class switch requires LPS along with TFG-β or BAFF and APRIL produced by DCs (108–110). CD40, LPS, and BAFF activate NFκB, suggesting NFκB could be essential for the IgA class switch. Mice lacking NIK produce less homeostatic IgA and exhibit defective SHM (111–113) and reduced synthesis of antigen-specific antibodies (72, 111, 114). Patients with B cell lymphopenia, who experience lower frequencies of class-switched MBCs and hypogammaglobulinemia, are frequently shown to carry a biallelic mutation of NIK (115). BAFF and APRIL promote the binding of MyD88 to TACI, which is necessary to activate NFκB and induce AID to promote CSR (116). Therefore, the picture emerges that NFκB is essential for CSR by directly controlling the transcription of immunoglobulin and then in an indirect way by controlling the transcription of AID.

The NFκB signaling system in germinal center B cells and somatic hypermutation

BCR functions as both a signaling molecule and an endocytic receptor to capture antigens for T cell help. BCR signaling in GC B cells is short-lived and attenuated by high phosphatase activity (117, 118). A recent study showed that IgA BCR transduces stronger BCR signaling than IgM BCR in intestine-generated GC B cells, and IgA BCR signaling is required for GC B cell survival (119). In line with this, BCR signaling in GC B cells has been shown to prolong survival and thus primes for selection (120). IgM BCR signaling fails to induce nuclear translocation of NFκB in GC B cells, although it induces nuclear translocation of NFκB in mature B cells (121, 122). CD40 signaling induces nuclear translocation of NFκB in both GC B cells and mature B cells (15, 121). However, a recent study revealed that both BCR and CD40 signaling induce the expression of NFκB target genes (such as *nfkbia* and *nfkbie*) in human tonsillar GC B cells, though the amplitude of NFκB target gene expression is much higher with CD40 signaling than BCR signaling (64).

A constitutively active IKK2 (IKK2ca mutant) leads to elevated constitutive NFκB activity and shows enhanced B cell survival. However, immunization of the IKK2ca mouse results in reduced

GC B cells, although PC numbers and antibody production remain unaltered (123). Deletion of Blimp1 (a master regulator of PC differentiation) in IKK2ca mice enhances the generation of GC B cells upon immunization but reduces the generation of PCs. Interestingly, IKK2ca mice develop PC hyperplasia at an older age, and deletion of Blimp1 in IKK2ca mutant mice leads to the development of activated B cell-like diffuse large B cell lymphoma (123). Similarly, adoptive transfer of IκBα knockout fetal liver cells (which have elevated constitutive NFκB activity) and subsequent immunization of the recipient mice results in impaired GC formation (73). Conversely, IκBε knockout enhances the generation of GC B cells (124). IκBα and IκBε inhibit cRel and RelA differentially, suggesting cRel and RelA could have distinct roles in controlling GC B cell formation (17, 125). Further, a recent study identified cRel- and RelA-specific target genes in BCR-stimulated B cells and found new cRel-specific target genes (Hhex/Bcl6b) that are known to play a critical role in GC B cells (126).

It is well established that cRel and RelA are critical for physiological B cell responses, and their misregulation leads to B cell-mediated diseases such as immune deficiencies, B cell lymphoma, and autoimmune disorders (13, 127). Mice with conditional deletion of cRel in B cells (CD19-Cre), upon TD immunization, fail to generate GC B cells 5 days after TD immunization (62), suggesting cRel is required for GC formation/initiation (Figure 2). Mice with conditional deletion of cRel in GC B cell (Cγ1-Cre), upon TD immunization, develop GCs (day 7, when the GC consists of predominantly DZ cells), which then start to involute and collapse (at 14 days). This study suggests that cRel in GC B cells is not required for the DZ establishment but is required for GC maintenance either by facilitating the recirculation of LZ to DZ or by priming LZ B cells through BCR signaling (Figure 2) (20). cRel-deficient B cells are known to have defective survival, and Bcl2 transgenic expression blocks BCR-induced cell death in cRel-deficient B cells (80). Therefore, it was anticipated that cRel-deficient GC B cells failed to maintain the GC due to impaired survival of GC B cells. However, cRel-deficient GC B cells do not show impaired expression of survival regulators (Bcl2, Bcl2L1, and Mcl1), and importantly, cRel-deficient GC B cells expressing the Bcl2-transgene fail to rescue GC collapse, suggesting GC collapse is not due to impaired survival in cRel deficiency. Interestingly, cRel-deficient GC B cells fail to upregulate the metabolic programming required for B cell growth, suggesting that cRel-dependent B cell growth could cause GC collapse (20). Myc is required for B cell growth (58). cRel-deficient B cells reduce Myc target gene expression signature (58), and cRel overexpression upregulates it (24), suggesting that Myc induction is cRel-dependent. It would be interesting to test whether transgenic expression of Myc in cRel-deficient GC B cells could rescue GC collapse. These effects are predominantly cRel-specific as RelA deficiency is associated with unaltered GC formation (20).

NFκB1 p105 has two functions. The first function is that the N-terminal domain of p105 generates p50, which forms a dimer with other NFκB family monomers, and the second function is that the C-terminal domain of p105 functions as an IκB and inhibits activation of NFκB (128) and Tpl2-MAPK signaling (35). Canonical pathway activation leads to proteolysis of the C-terminal domain of p105 and the formation of p50 hetero- or homodimers (129). To investigate the effect of p105 proteolysis on the GC and TD-dependent antibody production, Jacque et al. studied a signal-induced proteolysis-resistant mutant of p105 (*NFkB1*^{SSAA}, mutation of NFκB1 in the IKK2-target serine to alanine) which shows a block in p50 formation but retain a dominant IκB function (130). *NFkB1*^{SSAA} B cells show reduced nuclear p50, RelA, and cRel, whereas an unaltered level of RelB and p52 upon CD40 stimulation, suggesting *NFkB1*^{SSAA} is deficient in canonical NFκB activation but likely not in non-canonical NFκB activation. *NFkB1*^{SSAA} mice have a normal number of follicular B cells, although the number of marginal zone B cells is reduced. *NFkB1*^{SSAA} follicular B cells show impaired survival and proliferation upon IgM and CD40 stimulation. The TD immunization of *NFkB1*^{SSAA} mice shows reduced antigen-specific GC B cell formation and antibody production. Interestingly, increasing p50 levels in *NFkB1*^{SSAA} mice restores antigen-specific GC B cell and antibody generation upon TD immunization (130). The increased survival of *NFkB1*^{SSAA} B cells by Bcl-XL overexpression was unable to rescue TD antibody production. Therefore, the above study suggests that p50 (created by the proteolysis of p105) has multilayer functions in generating GC B cells and antibody production, beyond the role of p50-containing dimers in increasing B cell survival and proliferation.

NFκB2 (p100), similar to NFκB1, has two functions. The N-terminal domain of p100 generates p52, which predominantly forms a dimer with RelB (RelB:p52), and the C-terminal domain of p100 functions as an IκB (known as IκBδ) within the IκBsome and inhibits activation of NFκB (16, 43). Almaden et al. have shown that anti-IgM and BAFF co-stimulation leads to the degradation of IκBδ and enhances cRel activity with the subsequent enhancement of B cell proliferation (16). The authors have reduced the expression of IκBδ using NFκB2 heterozygosity, and the NFκB2 heterozygous B cells prolong stimulus-induced cRel activation and enhance B cell proliferation and antibody production upon TD immunization. The increased antibody production in NFκB2 heterozygosity could be due to increased GC formation. De-Silva et al. generated GC B cell-specific knockout of NFκB2 and RelB:p52 dimer to test the function of NFκB2 and RelB in GC B cell formation (19). NFκB2-deficient GC B cells show a partial defect in GC formation, though NFκB2 heterozygosity has no effect (19). Interestingly, the combined deficiency of NFκB2 and RelB in GC B cells led to the collapse of established GCs, whereas RelB deficiency alone shows no defect. However, precursor GC B cells in the peri-follicular region show higher RelB expression and nuclear translocation (131). The combined deficiency of NFκB2 and RelB in GC B cells results in reduced cell cycle entry and expression of Inducible T Cell Costimulator Ligand (ICOSL), which is required for the optimal interactions between B cells and T cells in the GC (Figure 2) (19). The increased antibody production in NFκB2 heterozygous mice could be due to the increased generation of antibody-producing cells from the GC-independent pathway. The above studies indicate that NFκB2 inhibits sustained cRel activation by forming IκBδ,

thereby reducing B cell proliferation and antibody production, while NFκB2-derived p52 generates RelB:p52 dimer, promoting GC maintenance. Thus, NFκB2 seems to have two opposite functions in humoral immunity. It is possible that the inhibitory function of NFκB2 (mediated by IκBδ) controls the GC-independent response, while transcription factor NFκB2 (mediated by RelB:p52) controls the GC-dependent response.

GC B cells undergo SHM to improve the affinity of the antibody to the cognate antigen and become affinity mature. SHM involves programmed mutations in variable regions, while CSR modifies the constant region of immunoglobulin genes (132). SHM occurs in DZ of GC, and nuclear translocation of NFκB has only been observed in LZ but not in DZ GC B cells (19, 133). However, both SHM and CSR are controlled by AID, which itself is controlled by NFκB (discussed above). cRel-deficient GC B cells show reduced affinity maturation and SHM of GC B cells (20). However, transgenic cRel expression in GC B cells does not significantly affect affinity maturation and SHM (24). The reduced SHM in cRel-deficient GC B cells could be either due to impaired AID expression or GC collapse. RelA and p52 contribute to AID expression in mature B cells (98). RelA-deleted GC B cells undergo normal affinity maturation, suggesting that RelA is not required for affinity maturation (20). It is possible that RelA controls AID expression in mature B cells, which is critical for CSR but not in GC B cells. Alternatively, it is possible that cRel compensates for RelA in RelA deficient GC B cells and facilitates SHM. Further investigations are needed to determine the role of NFκB systems in SHM and affinity maturation.

The NFκB signaling system in plasmablast/plasma cell development and survival

When stimulated by an antigen, activated B cells proliferate and differentiate into more specialized antibody-secreting cells. Antibody-secreting cells are generated by T cell-dependent and independent immunization and are heterogeneous in terms of their origin, secretory function, and lifespan (134). Antibody-secreting cells are broadly characterized in two types: PBs and PCs. PBs are cycling and short-lived antibody-producing cells, whereas PCs are terminally differentiated antibody-producing cells with life spans that can be short, long, or very long (135, 136). The gene regulatory network of short-lived PCs gradually changes to long-lived PCs over time (136–138). PCs reside in secondary lymphoid organs for a shorter duration and in the bone marrow for decades (139). A recent study showed that short-lived PCs were progressively differentiated into long-lived ones after arriving in bone marrow (140).

The expression of Blimp1, a master regulator for antibody-secreting cell generation, can distinguish cycling PBs and quiescent PCs. PBs express a low level of Blimp1, whereas PCs express a high level of Blimp1 in both mice and human (137, 141, 142). IRF4 is a key transcription factor for PC generation and enhances Blimp1 expression by creating a positive feedback loop with Blimp1 (143, 144). Both cRel and RelA induce IRF4 expression (21). RelA is required for Blimp1 expression and PC generation (Figure 2) (20, 145). RelA and IRF4 are induced during the early phase of B cell activation. However, activated B cells do not differentiate during early B cell activation, suggesting Blimp1 expression is inhibited during the early phase of B cell activation. It was not clear how Blimp1 expression was inhibited during B cell activation until Roy et al. discovered that cRel inhibits Blimp1 expression by Bach2 (21). It is well established that cRel promotes cell cycle progression, whereas Blimp1 inhibits cell cycle progression (59, 80, 146). Based on these observations and computational modeling of the molecular gene regulatory network, Roy et al. hypothesized that cRel inhibits Blimp1 expression. Indeed, cRel was found to be gradually downregulated from GC B cell> PB>PC, and the level of cRel expression was correlated with active cell cycle states (21). The expression of Blimp1 and cRel are inversely correlated, suggesting cRel downregulation may be a requirement for Blimp1 expression and PC generation. To determine whether cRel downregulation is a requirement to become PCs, cRel was overexpressed, and it was observed that cRel overexpression inhibits the generation of PCs by inhibiting Blimp1 expression, and cRel knockout enhances the generation of PCs and Blimp1 expression (Figure 2) (21). Further, Roy et al. investigated the mechanism of cRel downregulation in PCs and found that when Blimp1 was deleted, activated B cells failed to downregulate cRel. Mutation of Blimp1 binding site in cRel promoter impaired cRel downregulation, indicating that Blimp1 represses cRel by directly binding to cRel promoter (21). Our study showed that cRel inhibits PCs generation by repressing Blimp1, a RelA target gene, suggesting cRel and RelA antagonize B cell differentiation to PCs. A recent study also showed that functional antagonism of cRel and RelA in BCR stimulated B cells (126).

Studies have also revealed that human tonsillar PCs and precursor PCs in the GC express high levels of NFκB2 compared to other tonsillar lymphocyte populations (19). The deletion of NFκB2 leads to reduced antigen-specific antibody production in a mouse model (19, 147). NFκB2-deficient mice show IgA downregulation and significantly elevated IgM in the small intestine mucosa. The lamina propria of the small intestine of NFκB2 deficient mice had fewer $CD138^+$ PCs that produced IgA (148). Almaden et al. showed that germline NFκB2 heterozygosity enhanced antibody production and proposed that NFκB2 heterozygosity leads to disruption of IκBδ and sustains cRel activity leading to enhance B cell proliferation and subsequent antibody production (16). Overall, the above studies suggest NFκB2 deficiency reduces antibody production, whereas NFκB2 heterozygosity enhances antibody production. The role of NFκB2 in these under-expression systems is likely a complex combination of the effects of p100 and p52. It is possible that the inhibitory function of NFκB2 (mediated by IκBδ) controls extrafollicular antibody production, whereas the transcription factor NFκB2 (mediated by RelB:p52) controls GC-dependent antibody production. The role of RelB:p52 in GC response is discussed above in detail.

PCs may not be naturally long-lived; their ability to access and interact with particular niches is essential to their survival.

Specialized bone marrow niches support the survival of PCs by producing APRIL, BAFF, IL-6, CD44, and CXCL12 (149). PCs upregulate the expression of cell surface receptor BCMA, which provides survival signals upon binding with APRIL and BAFF (150). Both BAFF and APRIL activate NFκB signaling (151). T cell costimulatory receptor type CD28 is also essential for PC survival (152). The authors showed that CD28 selectively transmits pro-survival signaling to PCs. Reactive oxygen species (ROS) generation, mitochondrial mass/respiration, and glucose absorption were all elevated by CD28 signaling in PCs. In PCs, CD28 activation elevates the NFκB target gene IRF4, and IRF4 levels are associated with glucose absorption, mitochondrial mass, ROS, and CD28-mediated survival. Multiple myeloma, a plasma cell cancer, shows constitutive activation of both canonical and non-canonical NFκB pathways. The growth and survival of a subset of multiple myeloma depends on RelA alone, suggesting a RelA-mediated gene expression program could be critical for PC survival (13). Another study identified that tumor-promoting cytokines, such as tumor necrosis factor, activates RelB:p50 in multiple myeloma cell line. RelB:p50 is necessary and sufficient to provide pro-survival and anti-apoptotic signals in multiple myeloma (153). Inhibition of NIK results in apoptosis in multiple myeloma cells through reduced expression of anti-apoptotic proteins Bcl2L1, Bcl2A1, and Mcl1 (154). Overall, the NFκB pathway seems to play an important part in creating favorable conditions for PC survival, and the requirement of RelA/RelB in PC survival seems context-dependent.

The NFκB signaling system in memory B cells

MBCs develop both GC-dependent and -independent pathways. They constitute an essential part of the adaptive immune system as they circulate in the bloodstream for an extended time (155). MBCs remember the antigen and unleash a stronger secondary immune response upon exposure to the same antigen later in life (156). MBCs could mutate their immunoglobulin gene, differentiate into antibody-secreting cells, and produce an antibody with altered antigen specificity and affinity. Therefore, MBCs could protect against the same pathogen as well as antigen-drifted pathogens such as COVID-19 and influenza (157, 158). Despite the outstanding success of some vaccines, not all generate long-lasting humoral immunity; for example, influenza and COVID-19 vaccines require periodic administration (159). The vaccine goal for a highly mutating pathogen (e.g., influenza, SARS-Cov-2) is to generate higher numbers of MBCs. Influenza vaccine effectiveness drops even within a season due to both short-lived antibody production and higher antigen drift of the influenza virus (159, 160). Influenza vaccine development aims to produce more MBCs (159).

MBCs are comprised of phenotypically distinct MBC subsets with specialized functions. MBCs are present in the blood, lymphoid organs (e.g., tonsils), and barrier tissues, including the gut, lungs, and skin, in both human and mice (161, 162). MBC subsets can be characterized based on the expression of BCR isotypes, unswitched IgM/IgD MBCs, and switched IgG, IgE, and IgA MBCs. IgG transduces stronger BCR signaling than IgM in MBCs; thus, IgG lowers the activation threshold of MBC and enhances the propensity of PC generation than IgM (163). Antibody isotype-independent MBC subsets are characterized by differential expression levels of PDL2 and CD80 in mice. PDL2 +CD80+ MBCs preferentially differentiate into PCs upon rechallenge, and PDL2-CD80- MBCs preferentially seed in the GC (156, 164). Therefore, MBCs are reactivated by both BCR intrinsic and extrinsic pathways. Human MBCs can be identified based on the expression of CD27, a marker of antigen-experienced B cells (156, 165, 166). Interestingly, the number of human MBCs (CD27+ B cells) is higher than naïve B cells in the peripheral blood of aged individuals (167). Moroney et al. have identified the proportion of different human MBC subsets; IgD+CD27+ MBCs are about 10%, IgG+CD27+ MBCs are about 6.5%, and IgA+CD27+ MBCs are about 5% of total CD19+ B cells present in the peripheral blood of healthy human subject. The transcriptional signature of IgG+CD27+ and IgA+CD27+ MBCs are distinct from naïve B cells (165).

Lau et al. showed that B cell-intrinsic BAFF/BAFFR signaling is required for the GC-independent MBC generation, though BAFF/BAFFR signaling is not required for the GC-dependent MBC generation (63). BAFF is required for the survival of naïve mature B cells. The role of BAFF in MBC survival was unclear until Muller-Winkler et al. used a genetic knockout BAFF/BAFFR mouse model to study the function of BAFF/BAFFR signaling on the survival of MBCs (168). The authors found that knockout of BAFF/BAFFR leads to the loss of MBCs, and BAFF depletion by anti-BAFF monoclonal antibody treatment reduces lung-resident influenza-specific MBCs. BAFF predominantly activates the non-canonical (IKK1) NFκB pathway, though, under certain circumstances, it activates the canonical (IKK2) pathway. The author shows that IKK1 is partially required for IgM+ MBCs survival, and IKK2 is required for the survival of both IgM+ and IgG1+ MBCs. The combined BAFF and BCR signaling activates cRel in mature B cells (16). Studies have shown that RelB deficiency in humans results in impairment of B cell development, with an absence of CD27+ MBCs leading to severe B cell immunodeficiency and shortage in the secretion of antibodies (169). Overall, BAFF is required for GC-independent MBC generation, and MBC survival depends on the synergy of BCR- and BAFF-mediated activation of the NFκB pathway (170).

A recent study shows that CD40 signaling controls the generation of phenotypically defined MBCs, namely $CD80^{hi}$ and $CD80^{lo}$ MBC (65). A low CD40 signal leads to the generation of $CD80^{lo}$ MBCs, and a relatively high CD40 signal leads to the generation of $CD80^{hi}$ MBCs. CD40 signaling in GC B cells leads to the activation of cRel and RelA (121). Knockdown of cRel or RelA in "induced GC B cells" reduces the generation of $CD80^{hi}$ MBC (Figure 2). NFκB activation may depend on the dose of CD40. A high CD40 signal activates NFκB and promotes the generation of $CD80^{hi}$ MBCs, whereas a low CD40 signal fails to activate NFκB and promotes the generation of $CD80^{lo}$ MBCs. CD40 activates both

canonical and non-canonical NFκB pathways in B cells (Figure 1) (15, 48). It would be interesting to study whether the CD40 dose differentially activates canonical and non-canonical NFκB pathways in GC B cells and the impact of these pathways on MBC generation. The non-canonical NFκB pathway generates RelB:p52 dimer, although it could generate cRel:p50 and RelA:p50 dimers (discussed above). *In-vitro* "induced GC culture system" revealed that cRel and RelA are required for MBC generation (65). Further studies are required to identify the role of the NFκB system in the generation and reactivation of MBC subsets.

Conclusion and future direction

With the advancement of cell type-specific conditional knockouts, we know that the NFκB system is essential for generating healthy humoral immunity, and each NFκB monomer has a unique role in the generation of GC B cells and PCs. The basic understanding of the function of the NFκB system in the regulation of GC B cell and PC generation improved our understanding of the NFκB system function in B cell pathology (B cells lymphoma, autoimmunity, and immune deficiency). However, several questions remain unanswered and need to be addressed. (1) The role of NFκB system in the generation and reactivation of MBCs and their subsets are not known. (2) Antibody-secreting cells are highly heterogenous both phenotypically and functionally. The role of NFκB system in the generation of heterogenous antibody-secreting cells are not known. (3) It is unclear how GC B cells respond to receiving multiple cell surface receptor (BCR, CD40, ICOSL, etc.) signals sequentially and combinatorially. Understanding how these signals integrate into the NFκB system and push the cell fate decision towards PC, MBC, cell division, and cell death will be interesting. Integrating mathematical modeling with experiments will be essential to understand this process.

Author contributions

KR conceptualized the paper. KR and MC wrote the paper. AK, AM, and NR edited the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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