Trends in Immunology

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Review

ZEBs: Novel Players in Immune Cell Development and Function

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ZEB1 and ZEB2 are zinc-finger E homeobox-binding transcription factors best known for their role in driving epithelial to mesenchymal transition. However, in recent years our understanding of these two transcription factors has broadened, and it is now clear that they are expressed by a variety of immune cells of both myeloid and lymphoid lineages, including dendritic cells, macrophages, monocytes, B, T, and NK cells. In these cells, ZEBs function to regulate important transcriptional networks necessary for cell differentiation, maintenance, and function. Here, we review the current understanding of ZEB regulation across immune cell lineages, particularly in mice, highlighting present gaps in our knowledge. We also speculate on important questions for the future.

Highlights

ZEB proteins are expressed by various immune cells, where they can mediate the differentiation, maintenance, and function of these cells.

ZEB2 can regulate terminal differentiation in murine lymphocytes via a regulatory program that includes T-bet and ID2.

ZEB2 is required by murine plasmacytoid DCs (pDCs), tissue-resident macrophages, and monocytes for their development and/or survival; in pDCs, ZEB2 represses ID2 activity.

The precise role for ZEB2 in conventional dendritic cells type 2 (cDC2s) remains unclear because deletion of ZEB2 has only a minor effect on murine cDC2 numbers.

ZEB1 can regulate cytokine expression from cDC1s upon activation with CpG.

ZEB1 and ZEB2 can play reciprocal roles in $CD8^+$ T cells.

ZEBs: A Brief History

The zinc-finger E homeobox-binding (ZEB) protein family of **transcription factors** (TFs, see Glossary) consists of two members; ZEB1 and ZEB2. ZEBs primarily function in mammals as transcriptional repressors via cooperation with activated SMAD proteins and by recruitment of either the corepressor C-terminal binding protein (CtBP) or histone deacetylase complexes, such as nucleosome remodeling and deacetylase (NuRD) [1] (Figure 1). They are best known to drive **epithelial to mesenchymal transition** (EMT) through repression of epithelial genes [2]. Although partial or complete EMT is a reversible but crucial embryonic process, aberrant activation of EMT is also a trigger for tumor metastasis [3]. In addition to their role in EMT, ZEBs are thought to be involved in other important cellular processes such as maintaining **stemness**, promoting survival and longevity, and inducing cell-cycle arrest [4]. In recent years investigations of the role of ZEBs in the hematopoietic compartment have been initiated, and it is now clear that ZEB2 is crucial for mammalian embryonic development [5]. Moreover, loss of ZEB2 specifically in the murine adult hematopoietic system results (after 10–12 months) in splenomegaly with the enlarged spleens containing a significant infiltration of hematopoietic stem cells and megakaryocyte/erythroid progenitor cells, indicative of extramedullary hematopoiesis [6,7]. In addition, within the murine immune system, ZEBs have been implicated in the development, differentiation, and maintenance of various cell types. We review here these latest developments and our current understanding of the roles played by ZEBs across immune cell lineages. Because *Zeb2* deficiency is lethal, we focus on murine studies using conditional knockouts (KOs), unless stated otherwise.

ZEB1 and ZEB2 in Myeloid Cells

Zeb1 and *Zeb2* are widely expressed by murine myeloid immune cells. *Zeb2* is expressed by dendritic cells (DCs), macrophages, monocytes, and eosinophils, while *Zeb1* is primarily expressed by DCs and neutrophils (Figure 2). In this section, we discuss what is currently known regarding these TFs and their role in modulating the development, maintenance, and function of myeloid immune cells.

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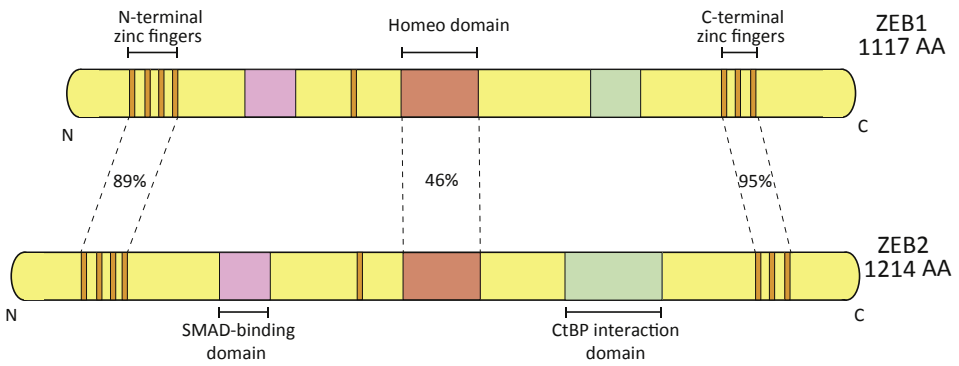

Figure 1. The ZEB Family of Transcription Factors. Schematic representation of the two ZEB family members in humans, ZEB1 and ZEB2. ZEB1 consists of 1117 amino acids (AA) whereas ZEB2 has 1214. These proteins are highly homologous, and both are characterized by clusters of zinc fingers at both the N- and C termini. In addition, both proteins share a homeo (DNA-binding) domain as well as a SMAD-binding domain and a C-terminal binding protein (CtBP) interaction domain; % indicates the extent of amino acid homology [66].

ZEB2 in Dendritic Cells

DCs form the crucial link between innate and adaptive immunity, and can be divided into two main groups, **plasmacytoid dendritic cells** (pDCs) and **conventional dendritic cells** (cDCs). pDCs are major producers of type I interferons (IFNs) and function in modulating immune responses to viruses [8]. Conversely, cDCs are professional antigen-presenting cells, sampling antigen in the periphery and subsequently migrating to draining lymph nodes where they present antigen to naïve T cells, inducing appropriate T cell responses. cDCs are further subdivided into two subtypes; cDC1s and cDC2s [9]. cDC1s are the main cDC subset involved in crosspresentation of antigens on MHC class I to naïve $CD8^+$ T cells, but can also drive **type 1 T helper cell** (Th1) differentiation of naïve $CD4^+$ T cells [10]. cDC2s are primarily associated with the induction of **type 2 T helper cell** (Th2) and **type 17 T helper cell** (Th17) responses [10]. In 2012, the **ImmGen Consortium** first reported that *Zeb2* was expressed by murine pDCs and cDC2s, but was absent from cDC1s [11]. These findings were then extended to show that *Zeb2* expression was downregulated during cDC1 development, with its expression decreasing as cells transitioned from common DC progenitors (CDPs) to cDC1-committed precursors (pre-cDC1s), and finally to mature cDC1s [12]. Conversely, *Zeb2* expression was consistently maintained throughout cDC2 development and maturation [13].

Stimulated by these findings, we and others, used various conditional genetic approaches, including the *Itgax–Cre* (CD11c–Cre) and ***Mx1–Cre*** models crossed to $Zeb2^{fl/fl}$ mice, to examine the requirement for ZEB2 expression by DCs [7,14]. pDCs were found to require ZEB2 in a dose-dependent manner for their development and/or maintenance in tissues (Figure 3), and loss of one or both copies of *Zeb2* resulted in a reduction or loss of splenic pDCs, respectively, compared with wild-type (WT) controls [7,14]. In addition, retrovirus-driven overexpression of *Zeb2* in Kit^{hi} bone marrow (BM) progenitors increased pDC numbers following *in vitro* treatment with FMS-like tyrosine kinase 3 ligand (Flt3L), relative to control cultures [7] (Figure 3). In terms of mechanism, *Zeb2* deletion in both models resulted in increased expression of inhibitor of DNA binding 2 (*Id2)* compared with control pDCs [14]. ID2 is known to block pDC development by antagonizing the E-protein E2-2 [transcription factor 4, (TCF-4)] [15–17], suggesting that ZEB2 may regulate pDC development by repressing ID2. Direct repression of ID2 by ZEB2 was confirmed using chromatin immunoprecipitation (ChIP) [14].

Glossary

B-1 cells: an atypical class of self-renewing B cells with limited receptor repertoire that are found in peritoneal and pleural cavities.
Bone marrow-derived dendritic cells (BMDCs): DCs generated from culturing bone marrow (BM) progenitors *in vitro* with growth factors such as Flt3L.
Central memory T cells (T_{CM}): long-lived memory $CD62L^+CCR7^+CD8^+$ T cells that traffic to lymph nodes and exhibit high proliferation capacity upon reactivation.
Conventional dendritic cells (cDCs): professional antigen-presenting cells that drive T cell responses to antigens acquired in the periphery.
CSF1R: colony-stimulating factor 1 receptor; binds macrophage growth factors (CSF1, IL-33) which can induce macrophage proliferation.
Epithelial to mesenchymal transition (EMT): the process by which epithelial cells become multipotent mesenchymal stem cells. EMT is important in development, wound healing, fibrosis, and cancer metastasis.
E-box: a DNA consensus motif, CANNTG, that is targeted by E-proteins.
Effector memory T cells (T_{EM}): memory $CD62L^-CCR7^-CD8^+$ T cells that circulate through the blood and non-lymphoid tissue, and can elicit direct effector function.
Immgen Consortium: a consortium of immunologists investigating the transcriptional profiles of distinct immune cells (www.immgen.org).
Innate lymphoid cells (ILCs): innate lymphocytes derived from common lymphoid progenitor (CLP) cells that lack B or T cell receptors. ILCs are further divided into subsets based on cytokine production and TF expression.
Kupffer cells (KCs): liver-resident macrophages.
MicroRNAs: small noncoding RNAs that bind specifically to target mRNAs, thereby silencing expression.
Memory precursor cells (MP cells): an effector $KLRG1^{low}IL7R^{high}CD8^+$ T cell subset exhibiting longevity, self-renewal, and multipotency.

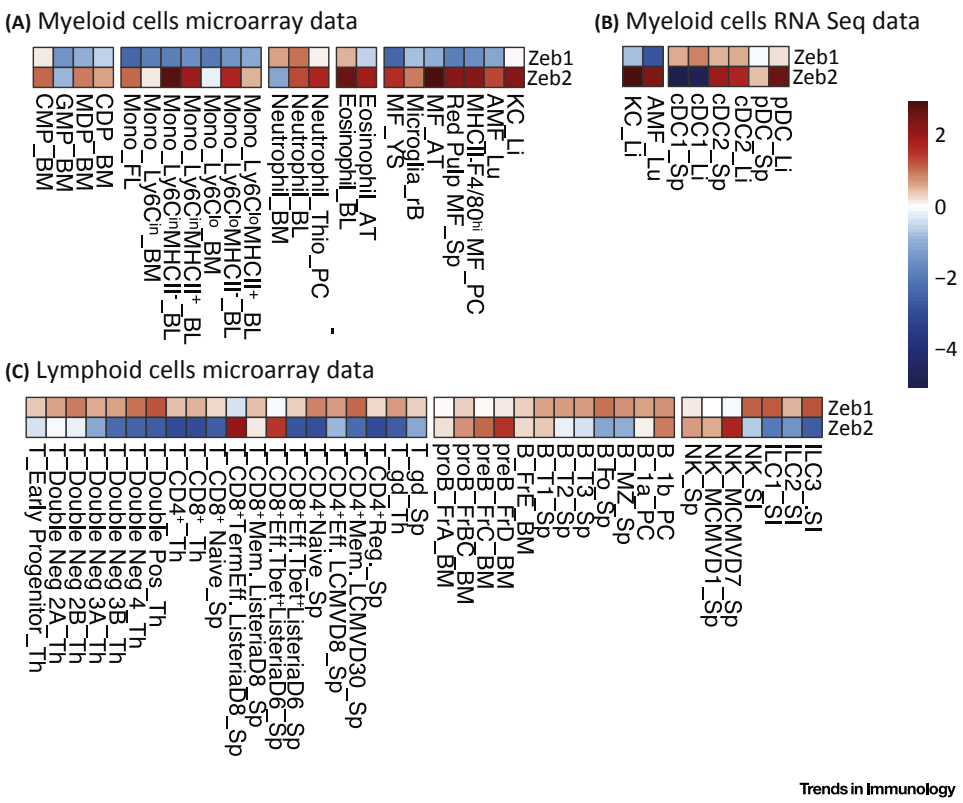

Figure 2. ZEB Expression in the Murine Immune System. For illustrative purposes in this review, a heatmap is shown detailing relative transcript expression levels of *Zeb1* and *Zeb2* across mouse immune cells. Data are a compilation of that generated by the ImmGen Consortium (www.immgen.org) or in-house [14,25,35]. Microarray and bulk RNA-Seq data were processed separately. Microarray: data were analyzed using the limma R package (Bioconductor). The robust multi-array average (rma) procedure was used to normalize data within arrays (probeset summarization, background correction, and log2 transformation) and between arrays (quantile normalization). Only probesets that mapped uniquely to one gene were retained, and for each gene the probeset with the highest expression level was retained. Bulk RNA-Seq: normalization was performed using the calcNormFactors function of the edgeR package (Bioconductor) with default parameters. Normalized counts were subsequently transformed to log2 values using the voom function of the limma R package. Values shown were obtained by calculating the mean expression per gene and subtracting this from each log2-normalized value of that gene. Separate heatmaps were created for microarray and bulk RNA-Seq data. Both heatmaps use the same color legend.

In cDCs, the exact role played by ZEB2 appears to be slightly more complex. As observed for pDCs, conditional KOs of *Zeb2* led to reduced numbers of splenic cDC2s relative to WT; however, this reduction was minor compared with the almost complete ablation of pDCs in the absence of *Zeb2* [7,14]. Nevertheless, although *Zeb2* loss only resulted in a minor reduction in cDC2 numbers, ZEB2 was found to be intrinsically required for the development of cDC2s, with fewer cDC2s originating from $Zeb2^{-/-}$ versus $Zeb2^{+/+}$ BM in a competitive BM chimera setting [7,14]. One possible explanation for this is that ZEB2 may not regulate the entire cDC2 lineage, but instead regulates an undefined subset (Figure 3 and Box 1), although this remains to be tested. Fitting with this idea, the impact of *Zeb2* deficiency (in terms of cDC2 numbers) varied between tissues in this study. For example, in the liver, *Zeb2* deletion resulted in fivefold fewer cDC2s relative to WT controls, but only a 1.5-fold and 2.5-fold reduction in the spleen and lung, respectively [14]. Moreover, when cDC2s in the small-intestine lamina propria were further subdivided based on CD103 expression, only the $CD103^{-}$ cDC2s were found to partially depend on ZEB2, being reduced in numbers in *Zeb2*-deficient mice compared with WT

Mowat–Wilson syndrome: a rare human genetic disorder caused by *de novo* loss-of-function mutations or monoallelic deletion of *ZEB2*. Major symptoms include distinctive facial features, intellectual disability, delayed development, and in some cases an intestinal disorder termed Hirschsprung disease.
MutuDC: a cDC1-like cell-line derived from splenic tumors from CD11c:SV40LgT transgenic C57BL/6 mice.
***Mx1–Cre*:** an inducible Cre line activated by interferons or double-stranded RNA such as poly(I:C).
Necroptosis: a programmed form of inflammatory cell death.
Plasmacytoid dendritic cells (pDCs): a DC subset that produces large quantities of IFN in response to viruses.
Pre-pro-B cell: the earliest B cell progenitor; expresses B220 and has germline Ig genes.
Pro-B cell: the second step in B cell development. These cells express CD19 and rearrange their IgM genes.
Stemness: a cellular gene expression profile that underlies the potential for self-renewal and multipotency.
Tγδ cells: a subset of 'unconventional' T cells activated in an MHC-independent manner; they express heterodimeric TCRs composed of γ and δ chains.
Terminal effector cells (TE): effector $KLRG1^{high}IL7R^{low}CD8^{+}$ T cells with reduced longevity and proliferative capacity, as well as restricted plasticity.
Tissue-resident memory T cells (T_{RM}): memory $CD8^{+}$ T cells residing in non-lymphoid tissues; they provide a first-line of defense upon pathogen reinfection.
Transcription factors (TFs): bind to specific DNA sequences mediating gene transcription.
T regulatory cells (Tregs): $FOXP3^{+}CD4^{+}$ T cells that suppress inflammation and maintain tolerance.
Type 1T helper cells (Th1): $T\text{-}BET^{+}IFN\text{-}\gamma^{+}CD4^{+}$ T helper cells that defend the body against intracellular pathogens.
Type 2T helper cells (Th2): $GATA3^{+}IL\text{-}4^{+}IL\text{-}13^{+}CD4^{+}$ T helper cells that defend the body against extracellular pathogens.

Type 17 T helper cells (Th17): RORγT^{+}IL-17^{+}CD4^{+} T helper cells that play a role in the induction of tissue inflammation/destruction.

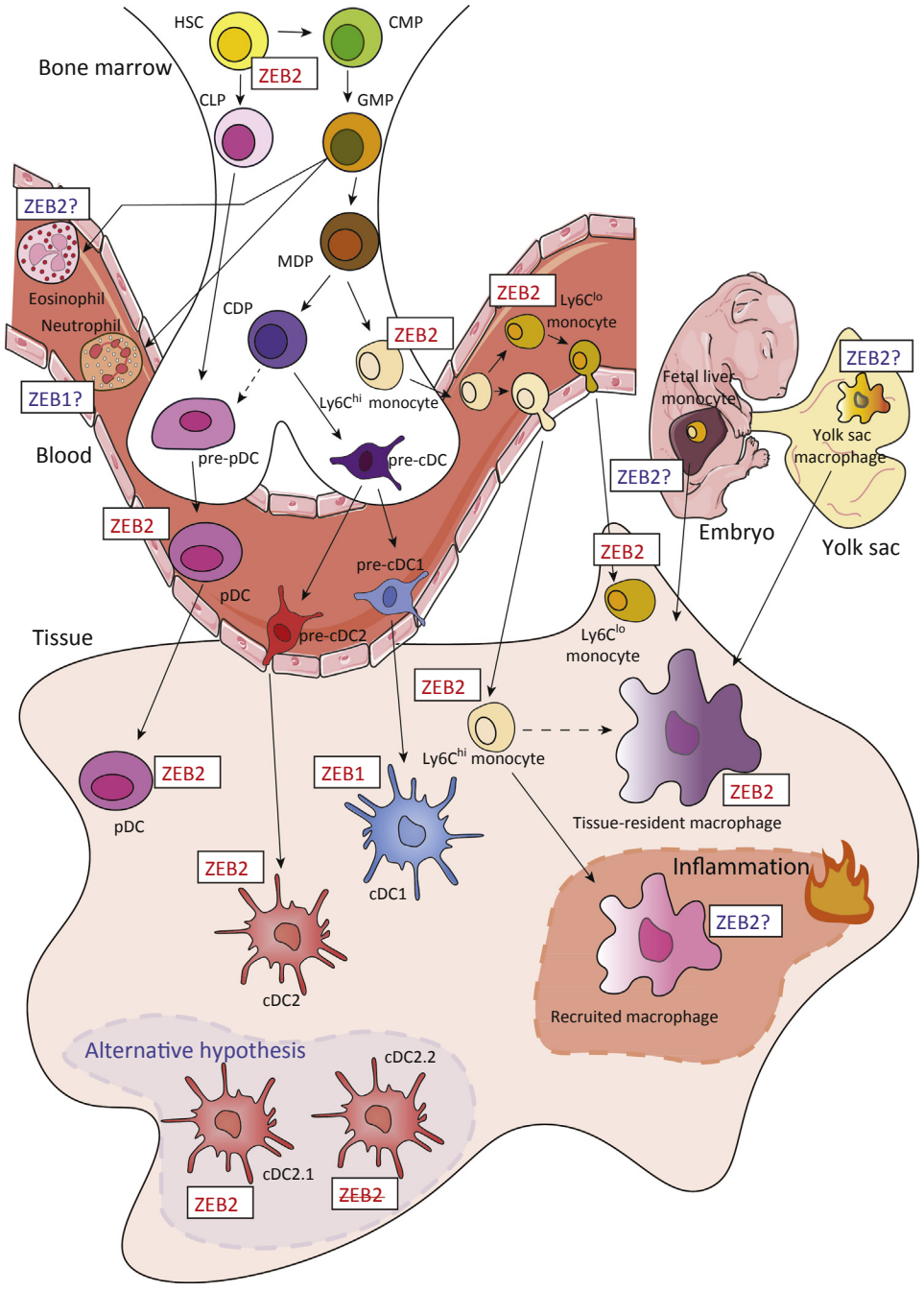

Figure 3. Role of ZEBs in Murine Myeloid Immune Cells. Myeloid immune cells develop primarily in the bone marrow from HSCs, and subsequently migrate into the blood and tissues where they exert their functions or further differentiate into mature cells which then carry out their specific roles. The main exceptions to this are tissue-resident macrophages that primarily develop from either yolk-sac macrophages or fetal liver monocytes during embryogenesis, with a contribution from BM monocytes in adulthood in some tissues such as the gut and skin. The role of ZEBs in this differentiation process and in the maintenance/function of mature myeloid cells has recently begun to be elucidated, and demonstrated roles (red font) for ZEB2 have been identified in monocytes, pDCs, cDCs, and macrophages. On the one hand, ZEB2 must be absent for cDC1 development, and is downregulated from CDP to pre-cDC1 to cDC1 stages. On the other, ZEB2 is required by

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Box 1. ZEB2 and DCs: A Cellular Fate Switch?

Despite largely overlapping data, different conclusions regarding the role of ZEB2 in murine DCs have been reached. Our laboratory proposed that, in addition to being important for pDCs, ZEB2 can act as a fate switch between cDC1s and cDC2s [14]. However, the laboratory of Ken Murphy concluded that ZEB2 does not play a role in cDC2 biology but instead that it can act as a fate switch between pDCs and cDC1s [7]. In line with previous reports suggesting that pDC development is regulated by TFs at the expense of cDC1s [17,63]. Our hypothesis was based on observations that cDC1s or cDC2s acquired features of one another upon overexpression or loss of *Zeb2*, respectively and because the minor effects of *Zeb2* deficiency in cDC2s were cell-intrinsic [14]. The latter hypothesis was based on the idea that pDCs and cDC1s arise from a common dendritic cell progenitor (CDP), however, the validity of this model is now being questioned [64]. Thus, it is clear that we do not yet fully understand how ZEB2 controls the DC lineage, and further investigation is necessary. In line with this, one key question concerns why there is only a minor reduction in cDC2s upon loss of ZEB2. One possibility is that ZEB2 is only required by a subset of cDC2s. In T cells, ZEB2 has been suggested to function primarily in the later stages of differentiation; thus it might be tempting to speculate a similar occurrence within cDC2s. However, our results suggest that ZEB2 is required early in cDC2 development, rather than late, given that loss of ZEB2 later in cDC2 development appears to have no effect on cDC2 numbers (when ZEB2 loss is driven by the 'late-*Itgax–Cre*', where CRE expression is only detected after the pre-cDC stage) [14]. By contrast, it is possible that distinct subsets of cDC2s might already be present at the pre-cDC stage. If this is the case, what are these subsets and which factors induce them? In terms of the role of ZEB2 in cDC1s, key questions include when and how is *Zeb2* down-regulated during progression from the CDP to cDC1s?

controls. Intestinal CD103$^+$ cDC2s were unaffected (in terms of number) by the absence of *Zeb2* [14]. Concomitantly, intestinal CD103$^-$ cDC2s also displayed higher *Zeb2* expression than their CD103$^+$ counterparts [14]. Similarly to the effects observed for pDCs, increased *Id2* expression was also reported in *Zeb2*$^{-/-}$ cDC2s and their immediate precursors relative to WT controls [7,14]; this suggested that ZEB2 might also function in cDC2s to repress ID2, which is required for cDC1 but not cDC2 development [16,18]. Further studies should clarify this possibility.

Reciprocally, in terms of cDC1s, deletion of *Zeb2* resulted in a slight increase relative to WT in the proportion and number of these cells in a cell-intrinsic manner [7,14] (Figure 3). Moreover, overexpression of *Zeb2* in CD11c-expressing cells using the Rosa-26 promoter but not the retroviral transduction system resulted in a decrease in cDC1s relative to WT controls [7,14]. The cause of the different results between laboratories is unclear, but it is notable that, in the retroviral transduction system, cDCs in general were largely lacking compared with controls [7]. In addition to reduced cDC1s, *Zeb2* overexpression in CD11c$^+$ cells resulted in a novel population of splenic cDCs with a mixed cDC1 and cDC2 phenotype expressing XCR1 and SIRPα [14], suggesting that expression of *Zeb2* in cDC1s might induce a switch to a more cDC2-like phenotype, a finding corroborated by RNA-Seq [14]. Although the data presented are consistent between the two laboratories, our group suggested that ZEB2 might act as a fate switch between cDC1s and cDC2s [14]; however, a role for ZEB2 as a fate switch between pDCs and cDC1s has also been suggested [7], and further work will therefore be necessary to fully understand the role played by ZEB2 in cDC biology (Box 1 and Outstanding Questions). A final key question left unanswered concerns whether ZEB2 regulates DC function during an immune response. Although the studies described above demonstrate a role for ZEB2 in DC development, these studies did not assess DC function in the absence of *Zeb2*.

cDC2s, but its deletion causes only a partial reduction in cDC2s. This could be because cDC2s have a minimal requirement for ZEB2 or because there are multiple subsets of cDC2s that derive from distinct pre-cDC2s, and only one of these subsets is ZEB2-dependent. Further work will be necessary to understand this. ZEB1 has been implicated (red font) in the function of cDC1s, although its role in maintaining this population is unclear. A role for either ZEB1 or ZEB2 remains to be investigated (blue font) for some precursors as well as for neutrophils, eosinophils, and monocyte-derived macrophages recruited during inflammation/infection. Abbreviations: cDC, conventional dendritic cell; CDP, common dendritic cell progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; HSC, hematopoietic stem cell; MDP, macrophage/dendritic cell progenitor; pDC, plasmacytoid dendritic cell.

The models used were not sufficiently specific to limit *Zeb2* deletion to a particular DC subset or even to DCs in general; it is therefore very difficult to ascribe a DC or DC subset-specific function to ZEB2. In the future, *ex vivo* studies using fluorescence-activated cell sorting (FACS)-purified cells or *in vivo* studies using novel, specific CRE lines may help to understand any consequences of *Zeb2* loss on DC function.

ZEB1 in Dendritic Cells

Although considerable progress has been made in understanding the role of ZEB2 in DCs, our knowledge regarding the role of ZEB1 is still limited. Unlike *Zeb2*, *Zeb1* is expressed across most DC subsets in mice (Figure 2). Recently, one study examined the role of ZEB1 in the murine tumor-derived DC cell line (**MutuDC**) [19] previously reported to resemble splenic cDC1s [20]. This study found that knocking down *Zeb1* in MutuDCs led to their impaired activation upon stimulation with the toll-like receptor 9 (TLR9) agonist CpG, and CpG-activation of *Zeb1*-deficient MutuDCs skewed T cell differentiation towards a Th2 rather than a Th1 response; relative to controls, this was evidenced by a slight increase in $GATA3^{+}$interleukin (IL)-13^{+} Th2 cells and reduced T-$bet^{+}IFN\text{-}\gamma^{+}$ Th1 cells following DC:T cell coculture [19] (Figure 3). Transient *Zeb1*-deletion in mouse Flt3L-derived $CD11c^{+}CD24^{+}$ **bone marrow-derived dendritic cells** (BMDCs) also resulted in impaired cytokine secretion upon stimulation with CpG compared with WT controls [19]. Although these results suggest that ZEB1 may play a role in modulating DC activation and the subsequent induction of T cell responses, in this study the effect of deleting *Zeb1* on the development of cDC1s was not addressed and remains to be determined. In addition, with the caveats associated with the use of *in vitro* DCs – that do not fully recapitulate *bona fide* tissue cDC1s – it will be intriguing to see if these results can be reproduced either *in vivo*, or by using alternative *in vitro* 'cDC1' culture systems [21] including those culturing human cDC1s [22].

ZEB2 in Monocytes and Neutrophils

Monocytes represent a highly heterogeneous population with many distinct functions, both under homeostatic and inflammatory conditions [23]. They are commonly characterized by the expression of CD11b, CD115 (macrophage colony-stimulating factor receptor MCSFR, also known as **CSF1R**), and varying expression of Ly6C (mouse) or CD14 and CD16 (human). Monocytes develop in the BM from common myeloid progenitor (CMP) cells, which subsequently differentiate into granulocyte/macrophage progenitor (GMP) cells, giving rise to either granulocytes or macrophage/dendritic cell progenitors (MDPs); MDPs in turn generate monocytes or DCs (Figure 3). In mice, the two best-characterized subsets of monocytes are referred to as $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes, with the latter deriving from the former [23]. Notably, both subsets express and require *Zeb2* (Figures 2 and 3), as evidenced from experiments where induced deletion of *Zeb2* (*Mx1–Cre*) resulted in a cell-intrinsic prolonged reduction of both subsets in the BM, blood, and spleen relative to WT mice [6,7]. Mechanistically, this was attributed to reduced expression of *Csf1r* upon loss of *Zeb2*, perhaps through upregulation of *Id2* [7]. This would be consistent with a report suggesting that ID2 can repress PU.1-mediated induction of *Csf1r* [24]. Also in agreement with this hypothesis, downregulation of *Csf1r* expression was also observed in *Zeb2*-deficient tissue-resident macrophages across tissues, relative to WT controls [25] (Box 2 and below). However, the reduced expression of *Csf1r* in *Zeb2*-deficient **Kupffer cells** (KCs) was not sufficient to prevent CSF1–Fc-mediated proliferation [25,26]; this suggested that the ability of these macrophages (and possibly monocytes) to signal through CSF1R might not be completely abrogated upon loss of *Zeb2*. Similarly to the DCs, the functional consequences of *Zeb2* loss in monocytes could not be formally examined owing to non-specificity of the CRE.

Despite expressing *Zeb2* in blood and tissues, neutrophils have not been shown to be affected by the deletion of *Zeb2* (Figure 2) [7]. At first glance, this seems to contrast with data from

Box 2. Common Features and Networks of ZEB2 across Murine Immune Cells

Deletion of *Zeb2* results in many cell/tissue-specific aberrations; however, some conserved features have been identified. One of these is the downregulation of *Csf1r* expression in *Zeb2*-deficient myeloid cells [7,14,25]. In addition, many *Zeb2*-deficient macrophages exhibit upregulated expression of *Siglecf*, *Cd101*, *Epcam*, and *Ms4a1*, or some combination thereof [25]. Expression of *Siglecf* and *Ms4a1* is also upregulated in *Zeb2*-deficient monocytes [7], whereas *Siglecf*, *Epcam*, and *Cd101* are upregulated in *Zeb2*-deficient cDC2s [14]. Moreover, small-intestinal CD103$^+$ cDC2s (ZEB2-independent) also express *Siglecf*, *Epcam*, and *Cd101* compared with their CD103$^-$ (partially ZEB2-dependent) counterparts [14,65]. Taken together, this suggests a putative common ZEB2 gene signature in myeloid cells. Of note, altered expression of these surface receptors might be potentially considered as a good surrogate for identifying *Zeb2*-deficient myeloid cells because antibody staining for ZEB2 is often unreliable [25].

Studies into the role of ZEB2 in lymphoid cells have indicated that ZEB2 exists within a larger conserved regulatory network in partnership with T-bet [48,49,59]. T-bet is required for the expression of *Zeb2*, and gene expression profiling or extensive flow cytometry in effector T cells and NK cells has shown that ZEB2 and T-bet can regulate the expression of a common set of genes or proteins in these cells [48,49]. T-bet ChIP-Seq analyses in CD8$^+$ T cells have indicated that T-bet exhibits enriched binding at genes coregulated by ZEB2, and furthermore that *Zeb2* deficiency can alter such T-bet binding [49]. Moreover, retroviral overexpression of T-bet in ZEB2-deficient T cells responding to LCMV-Arm infection only partially rescued the KO phenotype, suggesting that ZEB2 and T-bet might be non-redundant, and might cooperate to repress MP genes, but also upregulate TE genes [49]. Taken together, T-bet appears to regulate gene expression early, inducing *Zeb2* expression, and this presumably helps to reinforce T-bet actions; this suggests that a feed-forward loop might exist to induce terminal differentiation [49,59].

Altered *Id2* expression has been deemed to be a common feature of *Zeb2*-deficient immune cells [7,14,25,48,49,59]. In myeloid cells, ZEB2 can repress ID2, whereas in lymphoid cells ZEB2 can cooperate with ID2. ZEB2 shares a DNA-binding motif with E-proteins, and in lymphoid cells is predicted to work in partnership with ID2 to inhibit E-protein activity [48]. However, pDCs require the E-protein E2-2 for their development ereas and ID2 antagonizes this [15]. *Id2* expression is also upregulated in *Zeb2*-deficient cDC2s, monocytes, and tissue-resident macrophages [7,14,25], although the significance of this remains unclear. It will be interesting to delineate which are the cooperative versus competing roles of ZEB2 and ID2 in different immune cells to better understand their functional roles.

another study reporting an increase in Ly6G$^+$CD11b$^+$ granulocytes and Ly6G^{int}CD11b$^+$ 'immature granulocytes' 8 weeks after inducing *Zeb2* loss, relative to WT controls [6]. However, this was only observed when the relative percentages of these cells were examined [6]. In BM chimera settings (competitive and noncompetitive), *Zeb2*-deficient progenitors did not show any increased intrinsic potential to generate granulocytes [6], thus leading to the hypothesis that ZEB2 might not be intrinsically required by neutrophils, but instead that the increase in neutrophil subsets observed may have been due to altered proportions of other cells. Notably, the remaining monocytes in *Zeb2*-deficient mice were found to upregulate the expression of several neutrophil- or GMP-related genes. These include *Cebpe* and *Zeb1* (also present in neutrophils) and other genes including *Camp*, *Mmp9*, and *Ltf* (expressed by neutrophils) as well as *Mpo*, *Elane*, and *Prtn3* (expressed by GMPs) [7]. It is thus possible that, similarly to its role in other myeloid cells, ZEB2 may act in monocytes as a regulator of cell identity, with its deletion skewing monocytes towards an alternative fate, such as the neutrophil lineage, or perhaps preventing GMP maturation and differentiation (Figure 3). However, this remains speculative at this point. Moreover, the function of ZEB1 in monocytes and neutrophils also remains to be studied. Investigating these possibilities thus represents an interesting goal for the future (see Outstanding Questions).

ZEB2 in Macrophages

Macrophages are present in almost every tissue in the body, and have unique tissue-specific transcriptional and epigenetic profiles, or 'tissue identities' [25,27–31]. Although best known for their role in immunity, particularly as scavengers of foreign material, the unique profiles of tissue-resident macrophages also enable them to carry out what have been dubbed 'accessory

functions' for their specific tissues, such as synaptic pruning by brain microglia [32] or the clearance of excess surfactant by lung alveolar macrophages [33–35]. Despite arising from a limited number of possible progenitors (either yolk-sac macrophages, fetal liver monocytes, or BM monocytes [36]), tissue imprinting of tissue-resident macrophages results in very few genes being conserved across the macrophage lineage. Comparison of the transcriptional profiles of different murine tissue-resident macrophages with other immune cells yielded a list of only 67 genes whose expression was conserved across the entire tissue-resident macrophage lineage, and one of these genes was *Zeb2* [25]. Moreover, *Zeb2* expression does not appear to be limited to mature tissue-resident macrophages but is also expressed at the pre-macrophage stage during embryonic development [28] and, possibly, in monocyte-derived macrophages recruited under nonhomeostatic conditions, although this remains to be further validated (Figure 3). Deletion of *Zeb2* from five different tissue-resident macrophage populations (liver, lung, brain, spleen, and colon) driven by either *Fcgr1–Cre*, *Clec4f–Cre*, or *Itgax–Cre*, resulted in unique tissue-specific changes in macrophage transcriptomes [25]. *Zeb2*$^{-/-}$ macrophages were lost from tissues over time, likely through **necroptosis**, and were replaced by *Zeb2*-expressing macrophages, either having escaped *Zeb2* deletion or having been newly recruited to accessible niches [25,36]. Despite reduced expression of *Csf1r* by *Zeb2*-deficient macrophages relative to controls, macrophage loss was not a result of the inability of these macrophages to proliferate in response to CSF1; indeed, proliferation was observed following administration of CSF1–Fc [25,26]. Compared with WT macrophages, *Zeb2* haploinsufficiency did not result in macrophage death but did change the transcriptomic profiles of these cells [25]. Because *ZEB2* haploinsufficiency in humans through *de novo* loss of function mutations or deletion of the dominant *ZEB2* allele leads to **Mowat–Wilson syndrome** [37], it will be interesting to further study the precise effects of the monoallelic loss of *Zeb2* specifically in macrophages (see Outstanding Questions).

Exactly how ZEB2 maintains tissue-resident macrophage identities across different tissues remains unclear. Further analysis of KCs revealed that loss of the core KC TF, liver X receptor α (LXRα) [25], was a major driving force behind identity loss. Deletion of LXRα (*Nr1h3*) specifically from KCs also resulted in loss of KC identity, with many of the same genes being downregulated compared with *Zeb2* deficiency [25]. However, this overlap was not 100%, and deletion of *Zeb2* had a greater effect than removing LXRα alone; this suggested that ZEB2 might have additional roles in KCs instead of solely modulating LXRα expression; however, this remains to be studied further. Similarly, the loss of *Zeb2* from alveolar macrophages (AM) of the lung led to downregulation of *Cebpb* (encoding CCAAT/enhancer binding protein β; CEBPβ), a TF proposed to be important for the maintenance of murine AMs [38]. However, whether this is a general mechanism whereby ZEB2 regulates expression of tissue-specific TFs in different macrophage populations remains to be seen. Another outstanding question is whether ZEB2 can directly regulate the expression of these tissue-specific TFs, for example LXRα in KCs. As mentioned, ZEB2 is primarily considered to be a transcriptional repressor; however, if ZEB2 can directly regulate LXRα expression, it might point to a role in transcriptional activation. This is an interesting future question because it may reveal further functional roles for ZEB2 (see Outstanding Questions).

Finally, the function of ZEB2 in macrophages during an immune response remains unclear. In the conditional KO models described to date, a *Zeb2*-sufficient population of macrophages was maintained in almost all tissues studied [25]. Therefore, it remains to be established whether there are any *in vivo* experimental conditions that will allow a functional assessment of *Zeb2*-deficient macrophages without compensation from *Zeb2*-expressing cells. Examining resident macrophage functions in the absence of *Zeb2*, as well as the consequences of loss of

Zeb2 in macrophages recruited during inflammation, represent important goals for future investigations (Figure 3 and see Outstanding Questions).

ZEB1 and ZEB2 in Lymphoid Cells

The generation of naïve lymphocytes and their subsequent differentiation to effector cells is driven by complex and precisely regulated transcriptional networks. *Zeb1* and *Zeb2* expression is dynamically regulated through this process (Figure 2); however, their roles in developing cells committed to the lymphocyte lineage are only beginning to be uncovered. In this section, we highlight the role of ZEBs in B, T, and NK cell development, focusing on recent studies describing ZEBs as transcriptional regulators of effector lymphocyte populations.

ZEB2 in B Cells

B cells are a crucial component of the adaptive immune system that provides humoral and cellular protection against a wide array of pathogens. Although the precise role ZEB2 plays over the lifespan of a B cell is undefined, it is clear that ZEB2 is necessary for the development of mature populations. Specifically, induced deletion of *Zeb2* in adulthood has not been shown to affect common lymphoid progenitor (CLP) cells in the BM, but led to near-complete loss of mature B cells in blood [6]. Closer examination revealed a block in B cell differentiation at the transition from a **pre-pro-B cell** to a **pro-B cell** [6]. IL-7 signaling is known to be important for this transition [39,40], and pre-pro-B cells lacking *Zeb2* exhibited severely reduced expression of *Il7r* relative to WT controls. Stimulation of these cells with IL-7 yielded no detectable activation of the JAK/STAT pathway [6], demonstrating that IL-7 signaling was impaired. Thus, dysregulation of *Il7r* expression is likely – and at least in part – to be responsible for the block in B cell maturation [6].

The role of ZEB2 in the maintenance and function of mature B cell populations could not be studied using this model. Nevertheless, increased *Zeb2* expression is observed in mouse peritoneal cavity **B-1 cells** compared with other subsets (www.immgen.org) (Figure 2). Thus, future experiments examining the transcriptional requirements for mature B cells subsets at steady-state, following infection, or in cases of autoimmunity would prove informative. Furthermore, *Zeb1* is also expressed across B cell populations, although less dynamically than *Zeb2* (www.immgen.org) (Figure 2). Although no studies examining its role in the B cell lineage have been described, it will be important to understand how ZEB family members participate in the differentiation of mature B cells (see Outstanding Questions).

ZEB1 and ZEB2 in T Cells

T cells are central arbiters of cell-mediated immunity against bacterial and viral infections as well as against tumors. T cell development and thymic selection is a complex process involving multiple transcriptional regulators [41]. Analysis of ImmGen datasets revealed dynamic expression of *Zeb1* and *Zeb2* in T cells (www.immgen.org) (Figure 2). Early studies in mice demonstrated a crucial role for ZEB1 in mediating T cell development, likely through the repression of **E box**-containing genes such as *Cd4*, *Il2*, *Gata3*, and *Itga4* [42–45]. By contrast, in the absence of *Zeb2* expression, murine adult hematopoiesis was minimally affected and blood T cell numbers were comparable with those of WT controls [6]. However, in a competitive BM transplant setting, *Zeb2*-deficient hematopoietic stem and progenitor cells (HSPCs) exhibited an impaired ability to reconstitute most cell lineages, including circulating T cells, 16 weeks after transplant [6]. This may suggest either that ZEB2 is dispensable for T cell development or that compensatory mechanisms for loss of *Zeb2* may exist. After development in the thymus, naïve, effector, and memory T cells engage transcriptional networks to integrate environmental stimuli and to shape and maintain their cellular state. As described in the next section, recent work has highlighted an important role for ZEB proteins in the differentiation of mature T cell populations.

ZEB2 in Mature CD8$^+$ T Cells

In response to infection or malignant transformation, naïve antigen-specific CD8$^+$ T cells become activated and differentiate to form a pool of effector T cells that provide acute protection [46]. Although the majority of effector CD8$^+$ T cells are considered to be terminally differentiated (**terminal effector cells**, TEs) and die at the resolution of an immune response, a small proportion of less-differentiated effector T cells act as **memory precursor cells** (MPs) and survive to seed the long-lived memory population [46]. CD8$^+$ memory T cells can broadly be divided into three subsets: **effector memory T cells** (T_{EM}), **central memory T cells** (T_{CM}), and **tissue-resident memory T cells** (T_{RM}). T_{EM} and T_{CM} are found in circulation where the shorter-lived, more differentiated T_{EM} can elicit direct effector function to provide immediate protection upon reinfection (e.g., with a virus), whereas long-lived T_{CM} exhibit more stem-like properties [46]. T_{RM} are permanently lodged in non-lymphoid tissues and provide sentinel immunosurveillance [46]. Unique transcriptional signatures regulating effector and memory T cell differentiation have been described, and in both humans and mice, high *Zeb2* expression has been observed in the most-differentiated terminal effector and T_{EM} populations relative to other subsets (Figure 2) [47].

Using conditional mouse models where *Zeb2* was deleted in the developing T cell population, or shortly after T cell activation, we and others investigated the function of ZEB2 in effector CD8$^+$ T cell differentiation during acute lymphocytic choriomeningitis virus (Armstrong strain; LCMV-Arm) infection. At the peak of the response, *Zeb2*$^{-/-}$ effector CD8$^+$ T cells failed to accumulate in the blood, spleen, or peripheral tissues [48,49]. However, by day 60 following infection, *Zeb2*-deficient and WT T cell numbers in the spleen were comparable [49]. Moreover, despite normal functionality, *Zeb2*-deficient effector T cells generated significantly fewer of the most differentiated (KLRG1^{hi}IL7R^{lo}) TE cells relative to WT controls. The TE cells that did form, exhibited reduced expression of effector-signature molecules and increased amounts of pro-memory molecules relative to WT controls [48,49]. In contrast to what has been observed in myeloid cells (Box 2), *Zeb2* expression has been found to correlate with the expression of *Tbx21* and *Id2*–both key drivers of the KLRG1^{hi} TE subset [50–54]. Thus, *Zeb2* likely acts in a larger transcriptional network with T-bet and ID2, regulating genes that are necessary for the formation of the TE effector and effector-memory CD8$^+$ T cell state, while restricting memory cell potential [48,49].

Furthermore, in the acute LCMV infection model, *Zeb2*-deficient memory T cells produced more IL-2, and expressed more CD62L (L-selectin) and BCL-2, but less KLRG1, than WT controls, suggesting that in the absence of *Zeb2* the formation of less differentiated T_{CM} was accelerated; this appears to further support a role for ZEB2 in the programming of a more differentiated cell state [49] (Figure 4). Homeostatic turnover and cytokine production by antigen-specific memory *Zeb2*$^{-/-}$ CD8$^+$ T cells was comparable with that of WT cells [49]. However, *Zeb2*$^{-/-}$ antigen-specific memory CD8$^+$ T cells [endogenous or T-cell receptor (TCR) transgenic], were shown to generate fewer secondary effector CD8$^+$ T cells following acute virus or bacteria rechallenge [48]. In contrast, another study found that, when equal numbers of memory CD8$^+$ T cells were transferred to naïve mice subsequently infected with LCMV (clone 13, chronic infection model), *Zeb2*-deficient CD8$^+$ T cells expanded to similar numbers and controlled viral burden to the same extent as WT memory CD8$^+$ T cells [49]. This discrepancy may be the result of differing experimental protocols, and needs to be further analyzed. Nevertheless, based on these overall findings, we speculate that ZEB2 might regulate memory differentiation but not memory homeostasis or function. Further experiments are warranted to assess this possibility.

ZEB1 in Mature CD8$^+$ T Cells

Whereas *Zeb2* is upregulated upon CD8$^+$ T cell activation, and is most highly expressed in T cells at the effector phase following viral infection in mice (e.g., LCMV), *Zeb1* expression

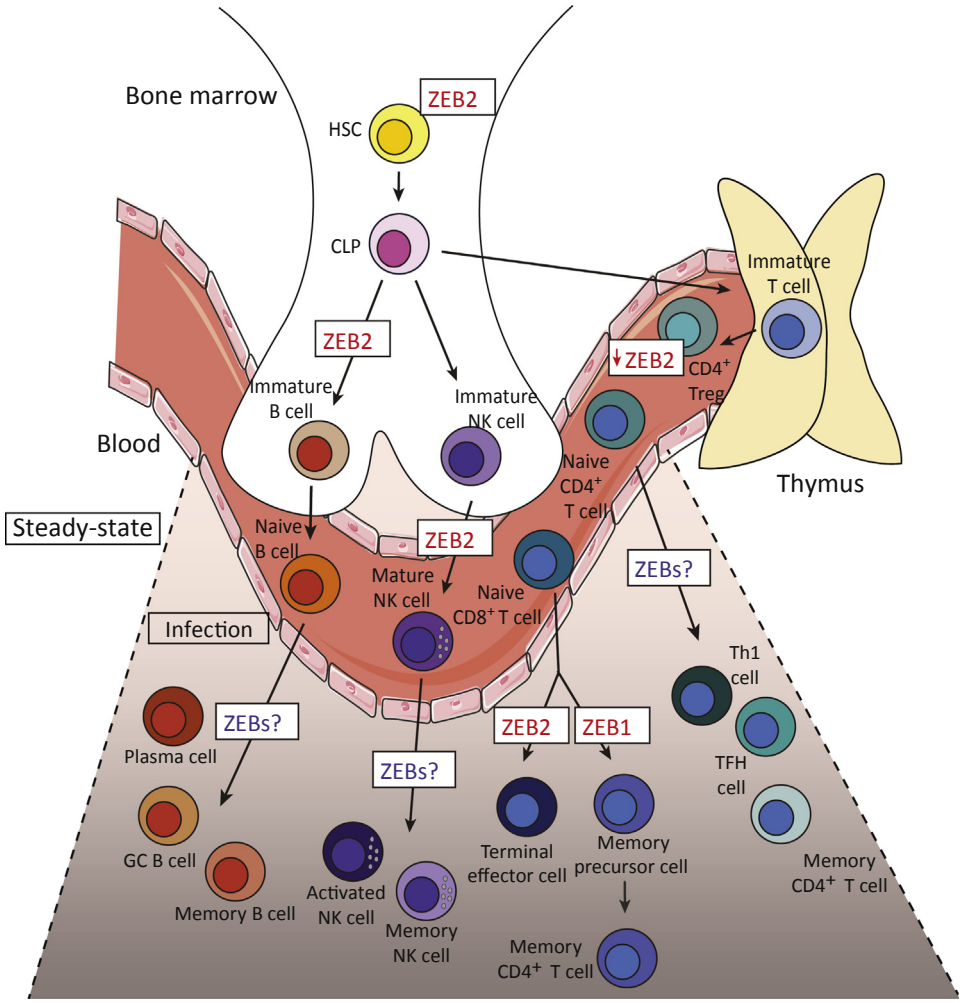

Figure 4. ZEB-Mediated Regulation of Murine Lymphocyte Differentiation States. Although ZEB2 is crucial for murine B cell development in the bone marrow, it is primarily the mature lymphocytes that depend on ZEB transcription factors (TFs) to regulate the acquisition and maintenance of distinct cellular states at steady-state or following infection (e.g., viral; lymphocytic choriomeningitis virus, LCMV). ZEB TFs have a demonstrated (red font) or speculated (blue font) key role in modulating the differentiation of most lymphocyte populations. Abbreviations: CLP, common lymphoid progenitor; GC, germinal center; HSC, hematopoietic stem cell; NK cell, natural killer cell; TFH, T follicular helper cell; Treg, T regulatory cell.

appears to be inversely regulated [55] (Figure 4). Naïve CD8$^+$ T cells express high *Zeb1* that decreases following T cell activation, and which is then upregulated again in memory T cells [55]. Eight days following acute LCMV-Arm infection, murine MP T cells exhibited increased expression of *Zeb1* relative to TE T cells, in a reciprocal manner to *Zeb2* [55]. Similarly, *Zeb1* expression was increased in *Zeb2*-deficient bulk effector CD8$^+$ T cells relative to WT controls, and *Zeb2* expression was elevated in *Zeb1*-deleted memory CD8$^+$ T cells, suggesting that ZEB1 and ZEB2 might repress the expression of each other. Accordingly, ChIP-qPCR demonstrated direct ZEB1 binding to the murine *Zeb2* promoter in WT naïve CD8$^+$ T cells, although direct ZEB2 binding to ZEB1 could not be confirmed because a ZEB2 antibody was unavailable [55]. In contrast to EMT, where ZEB1 and ZEB2 can function together to drive EMT [4], here,

ZEB family members appear to be expressed during distinct phases of the immune response to modulate transcription (Box 3).

Related to this, using a mouse model of *Zeb1* deletion specifically in activated T cells, *Zeb1*-deficient $CD8^+$ T cells displayed an opposite phenotype to that of *Zeb2*-KO T cells [55]. Specifically, $CD8^+$ T cells lacking *Zeb1* underwent clonal expansion following LCMV-Arm infection, but declined in numbers over time, in comparison with WT controls. Both survival and homeostatic proliferation in memory $CD8^+$ T cells were impaired in the absence of *Zeb1* compared with the WT population [55]. Moreover, *Zeb1*-deficient T cells produced less IL-2 and expressed less CD62L, CD122, or eomesodermin than WT controls – all properties of T_{CM}; this suggested that a more differentiated cell type appears to be favored in the absence of *Zeb1*. Following this, equal numbers of WT and *Zeb1*-deficient LCMV-specific memory $CD8^+$ T cells were adoptively transferred to naïve hosts and examined after secondary infection with *Listeria monocytogenes* expressing the LCMV $GP_{33–41}$ epitope; mice receiving *Zeb1*-deficient cells exhibited impaired expansion of $CD8^+$ T cells, reduced expression of cytotoxic granzyme B, and diminished control of bacterial burden, compared with mice receiving WT $CD8^+$ T cells [55]. This demonstrated that ZEB1 can play a role in the establishment, maintenance, and execution of some functions in long-lived $CD8^+$ T cell memory [55].

Taken together, these results appear to highlight reciprocal roles for ZEB1 and ZEB2 in the regulation of $CD8^+$ T cell responses. Although ZEB2 can promote TE cell differentiation and survival, ZEB1 is required for normal maintenance of memory $CD8^+$ T cells and protective immunity. A key question then arises – how is this division of labor controlled? In a recent study, **microRNA** 200 (miR-200) family members and the cytokine, transforming growth factor (TGF)-β, two factors important for regulating ZEB1 and ZEB2 expression in EMT, were positioned as

Box 3. Repurposed Regulation of ZEBs

In EMT, ZEB family members operate together in a well-described double-negative feedback loop [4]. Stromal factors including TGF-β stimulate EMT by activating transcriptional regulators including ZEBs, whereas miR-200 family members can repress these drivers to maintain epithelial identity. In mammals, both ZEB1 and ZEB2 contain miR-200 binding sites in their 3′-untranslated regions (UTRs) and, in turn, ZEB1 and ZEB2 can directly repress miR-200 expression [4].

During mature $CD8^+$ T cell differentiation, this regulatory network appears to become uncoupled: ZEB1 and ZEB2 seem to work on two different branches of $CD8^+$ T cell differentiation. TGF-β signaling leads to increased *Zeb1* but decreased *Zeb2* expression, whereas the expression of miR-200 members mirrors that of *Zeb1* in $CD8^+$ T cells responding to LCMV-Arm infection; this in turn indicates that *Zeb1* can escape miR-200-mediated transcriptional repression [55]. Retroviral overexpression of miR-200a or miR-200c can reduce *Zeb2*, but not *Zeb1*, expression in LCMV-specific effector $CD8^+$ T cells, resulting in substantially reduced numbers of TE cells at day 8 post-infection relative to controls [55]. In this study, miR200-deficient antigen-specific memory $CD8^+$ T cells showed increased *Zeb2* expression and decreased *Zeb1* expression relative to controls, thus resulting in a substantial decrease in the formation of T_{CM} cells 45 days post-infection [55]. Consequently, TGF-β signaling and miR200 repression in $CD8^+$ T cells during viral infection (at least for LCMV) seem to uncouple *Zeb1* and *Zeb2* expression in such a way as to control effector and memory states.

Although only limited data are available for myeloid cells, it is tempting to speculate that a similar feedback loop may exist. For example, in monocytes, deletion of *Zeb2* leads to increased expression of *Zeb1* [7]. Moreover, with GMP differentiation, monocytes and neutrophils display differential dependence on *Zeb2* and expression of *Zeb1*. Likewise, in DCs, ZEB2 must be absent for normal cDC1 development to take place [7,14]. However, ZEB1 has been reported to be required for normal cytokine production by cDC1s following CpG stimulation [19]. Moreover, a role for TGF-β signaling and miR-200 family regulation remains be investigated in DCs, but as peripheral cDC1s are known to express CD103 (an integrin regulated by TGF-β signaling), hypothetically, this regulatory network might exist. Similarly, in the intestine, $CD103^+$ cDC2 development is ZEB2-independent, unlike $CD103^-$ cDC2s. Furthermore, because $CD103^+$ cDC2s require TGF-β for their development [65], it is also tempting to speculate that a similar regulatory network to that observed for T cells might be present in cDC2s.

key elements of this regulatory network in T cells [55] (Box 3). Future studies should focus on furthering our knowledge of such differential regulatory networks, and of how they might modulate the functions of ZEB family members.

ZEB2 in Mature CD4$^+$ T Cells

The generation of effector CD4$^+$ T cells is crucial for host protection. Following activation with antigen, naïve CD4$^+$ T cells undergo proliferation and differentiation into different T helper subsets depending on the context of infection or inflammation. Unlike CD8$^+$ T cells, the significance of ZEB2 in mature CD4$^+$ T cell populations remains to be studied in detail; however, regulation of *Zeb2* expression has been suggested to be important for the maintenance of **T regulatory cells** (Tregs) [56] (Figure 4). In mice and humans, FOXP3, the Treg lineage-defining TF, cooperates with miRNAs to repress target genes, reinforcing a suppressor phenotype [56]. Specifically, in mice, miR-155 with FOXP3 targets the effector-associated gene, *Zeb2* [57]. ZEB2 may also contribute to the generation of effector CD4$^+$ T cells following infection, and *Zeb2* expression has been reported to be somewhat increased in murine CD4$^+$ T cells at day 7 of LCMV-Arm infection relative to naïve cells (Figure 2). The significance of these findings remains to be discerned, but a closer examination of subset-specific transcriptional regulation following infection and inflammation might uncover specific roles for ZEBs in CD4$^+$ T cells (see Outstanding Questions).

ZEB2 in Innate Lymphocytes

NK cells, a predominant innate lymphocyte population, are an important defense against intracellular pathogens and cancers. Similarly to T cells, NK cells can secrete proinflammatory cytokines, IFN-γ, and tumor necrosis factor (TNF)-α, and also possess cytolytic function. In mammals, NK development takes place in the BM, and upon commitment to the cell lineage, NK cells undergo a series of developmental steps towards terminal maturation, losing their ability to proliferate while gaining effector function and unique trafficking ability [58]. High expression of *Zeb2* in the most mature human and mouse NK cell subsets has been observed (Figure 2) [59], and is remarkably similar to that described for effector CD8$^+$ T cell terminal differentiation (Box 3).

In line with this, the highest numbers of *Zeb2*-deficient NK cells have been described in the BM, with lower numbers in the blood and peripheral tissues; these mice exhibit a complete lack of mature CD27$^-$ NK cells in all organs analyzed [59] (Figure 4). Accordingly, overexpression of *Zeb2* has been reported to cause a reduction in BM NK cell numbers without altering spleen cell counts. However, in this study a significant increase in mature CD27$^-$ NK cells was observed in both BM and spleen relative to WT controls [59]. Careful examination of *Zeb2* deficiency has suggested that the absence of mature peripheral CD27$^-$ NK cells may be due to reduced viability and impaired cell trafficking [59]. ZEB2 has been directly or indirectly implicated in modulating IL-15-mediated survival as well as proper egress of developing NK cells from the BM; however, further *in vivo* analyses will be necessary to further understand these roles [59].

Of note, in a melanoma tumor model, mice harboring *Zeb2*-deficient NK cells were reported to be highly susceptible to B16 melanoma outgrowth, and displayed a substantially increased number of lung and liver nodules compared with controls [59]. Functionally, *Zeb2*-deficient NK cells were able to migrate in response to chemokines, degranulate, and produce IFN-γ; however, KO mice harbored only ~15% of normal WT NK numbers in the lungs. This suggested that low NK numbers – and not altered functionality – might have been responsible for the heightened susceptibility to melanoma [59].

Although warranting further investigation, such observations in NK cells parallel conclusions drawn from studies of effector CD8$^+$ T cell terminal differentiation that demonstrate a crucial

requirement for ZEB2 as part of a transcriptional network that directs terminal maturation. Of note, recent studies have ascribed features of adaptive immunity to NK cell responses – such as an antigen-driven expansion followed by a contraction phase, and the establishment of a long-lived and self-renewing population of NK memory cells [60]. Moreover, global profiling of Ly49H$^+$ NK cells over the course of mouse cytomegalovirus (MCMV) infection in mice has identified a common epigenetic and transcriptional signature shared by memory NK cells and memory CD8$^+$ T cells [61]. *Zeb2* was identified within this signature, suggesting that it may play a role not only in the regulation of CD8$^+$ T cell differentiation but, presumably, also in the generation of NK cell memory [61].

With the description of **innate lymphoid cell** (ILC) families, it will also be important to consider the role of the ZEBs in innate lymphocytes other than NK cells. Considerable similarities in transcriptional regulation have already been noted between ILCs and T cells [62]. However, although the expression of *Zeb2* in murine ILCs is somewhat low, *Zeb1* expression seems to occur reciprocally to that of *Zeb2*; consistent with that described above for CD8$^+$ T cell populations (Figure 2). Furthermore, ZEB functions in the γδ T cell (**Tγδ cell**) lineage remain to be examined. Thus, future studies defining the precise function of ZEB proteins in the differentiation and function of innate lymphocyte populations might allow a more thorough understanding of immune defense, as well as of any putative common regulatory pathways that may be shared between innate lymphocytes and T cells, and perhaps also B cells.

Concluding Remarks

In recent years our understanding of the roles of ZEB proteins has significantly increased. In addition to driving EMT, ZEBs are also expressed by many cells of the immune system where they play crucial roles in regulating development, differentiation, maintenance, and other functions. These findings have opened interesting avenues of research, raising many questions regarding the varied functions of ZEB proteins across immune compartments (see Outstanding Questions). It will be important to determine how our understanding of ZEBs from mouse models translates to the human immune system. This is particularly relevant in the context of Mowat–Wilson syndrome where patients harbor heterozygous expression of *ZEB2*. Thus, the functional relevance of ZEB protein haploinsufficiencies in immune defense requires further examination.

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Outstanding Questions

Why does ZEB2 deletion only have a minor, but intrinsic, effect on cDC2s? Are there undefined subsets of cDC2s with differential dependence on ZEB2? Is there a critical threshold of ID2 expression in these? If ZEB2 must be absent to generate cDC1s, at what stage of development can we define cDC1-committed progenitors?

What role does ZEB1 play in cDCs *in vivo*?

Given the upregulation of ZEB1 in ZEB2-deficient monocytes, coupled with expression of other neutrophil-associated genes, what is the role of ZEB1 in neutrophils?

Eosinophils express high levels of *Zeb2*; is there any role for ZEBs in eosinophil biology?

To date, most research on myeloid cells has focused on cell development/maintenance. What are the functional consequences of ZEB1/2 loss in myeloid cells?

Upon activation, naive B cells generate a heterogeneous population of progeny, including terminally differentiated plasma cells, germinal center B cells, and memory B cells; do ZEBs function in regulating these distinct paths of differentiation?

Because *Zeb1* is more highly expressed in CD4$^+$ T cell populations than *Zeb2*, what is the precise role (if any) of ZEB1 and ZEB2 in regulating the development and maintenance of CD4$^+$ T cells?

Given the considerable transcriptional overlap between mature differentiated CD4$^+$ and CD8$^+$ T cells following infection, do ZEBs participate in the transcriptional network regulating the formation and maintenance of CD4$^+$ T helper cell subsets?

How does ZEB2 exert such unique transcriptomic changes in different tissue-resident macrophages? Does ZEB2 bind directly to tissue-specific TFs to control identity?

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Given the apparent similarities between NK cell and T cell memory, can ZEB1 and ZEB2 play analogous roles in both lineages, promoting long-lived versus terminal effector states, respectively?

Are ZEBs crucial transcriptional regulators of ILC development and is their role in ILCs different to that described in NK cells?

What is the functional and regulatory overlap between ZEB1, ZEB2, TGF-β, and the miR-200 family in myeloid cells?

What are the functional consequences of an altered transcriptome in macrophages that are heterozygous for *ZEB2*? Does this play any role in the pathogenesis of Mowat–Wilson syndrome?

How do these findings translate to the human immune system?

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