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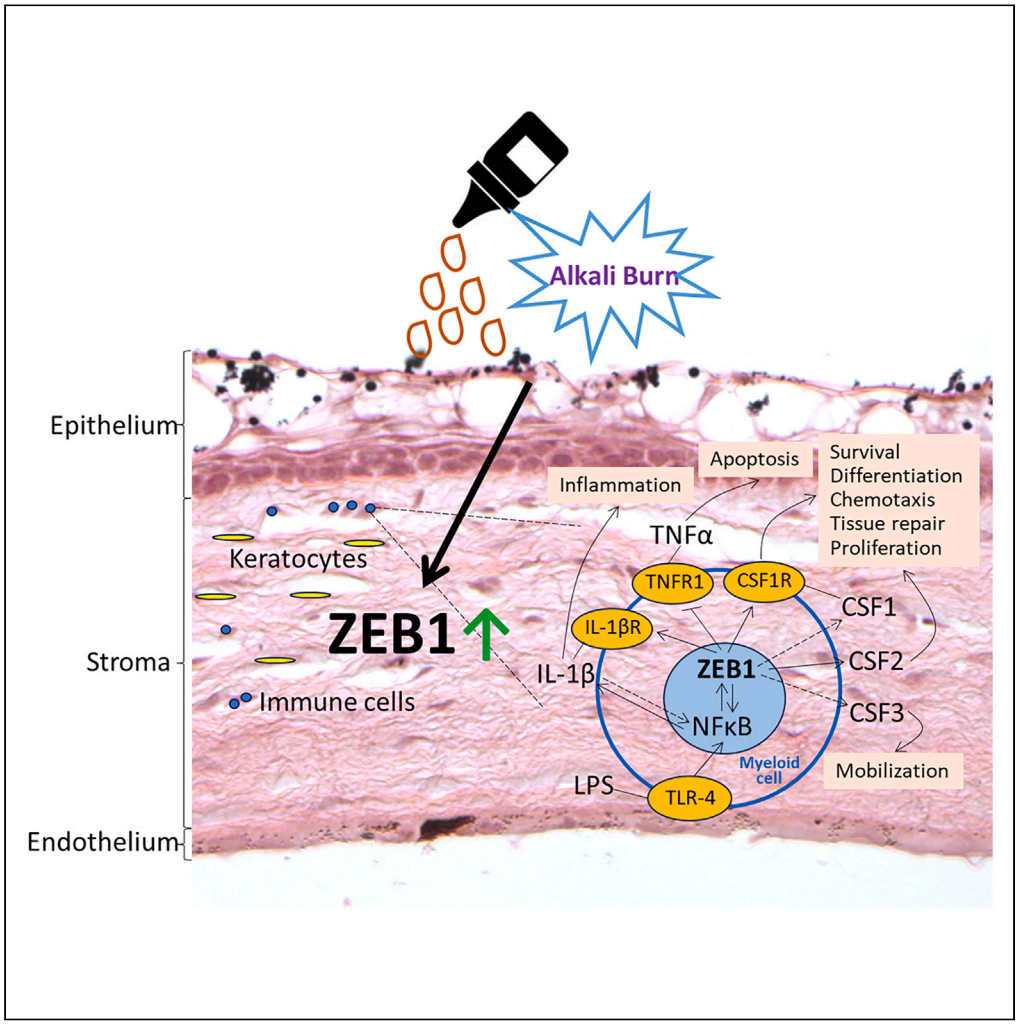

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Highlights

Zeb1 is an essential factor; lack of Zeb1 results in embryonic death in mice

In adults, Zeb1 promotes mouse corneal inflammation by retaining myeloid cell viability

Zeb1 represses Tnf/Tnfr1 signaling pathway leading to stress-induced myeloid cell death

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Conditional deletion of Zeb1 in Csf1r^{+} cells reduces inflammatory response of the cornea to alkali burn

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SUMMARY

ZEB1 is an essential factor in embryonic development. In adults, it is often highly expressed in malignant tumors with low expression in normal tissues. The major biological function of ZEB1 in developing embryos and progressing cancers is to transdifferentiate cells from an epithelial to mesenchymal phenotype; but what roles ZEB1 plays in normal adult tissues are largely unknown. We previously reported that the reduction of Zeb1 in monoallelic global knockout (Zeb1$^{+/-}$) mice reduced corneal inflammation-associated neovascularization following alkali burn. To uncover the cellular mechanism underlying the Zeb1 regulation of corneal inflammation, we functionally deleted Zeb1 alleles in Csf1r^{+} myeloid cells using a conditional knockout (cKO) strategy and found that Zeb1 cKO reduced leukocytes in the cornea after alkali burn. The reduction of immune cells was due to their increased apoptotic rate and linked to a Zeb1-downregulated apoptotic pathway. We conclude that Zeb1 facilitates corneal inflammatory response by maintaining Csf1r^{+} cell viability.

INTRODUCTION

The cornea is the outmost part of the eye and plays a critical protective role in preventing physical, pathogenic, and chemical insults. Chemical insults can cause mortality and morbidity on the battle field or in a terrorist attack when a large-scale release of high toxic chemicals such as mustard gas is intentiona.[1] Understanding the underlying mechanisms of the chemical damage to the cornea is critical in developing medical countermeasures that must be prepared to protect from sudden vision loss and immediately relieve acute ocular symptoms to maintain sufferer's visual capability.

ZEB1 is an essential transcription factor in embryonic developmen[2]; homozygous Zeb1 knockout (KO) results in fetal death before birt.[2] In adults, Zeb1 has been widely reported to play a role in tumorigenesis and fibrotic disease.[3] However, its biological functions in normal tissues are not as clear. Previously, we reported that the monoallelic deletion of Zeb1 decreased corneal neovascularization (NV) by reducing vascular endothelial cell proliferation and migration in response to an alkali bur.[4] Also, we found that monoallelic Zeb1 KO reduced the infiltration of immune cells into the cornea after alkali burn, thereby contributing to the delayed initiation of corneal N.[5] However, monoallelic global Zeb1 KO should reduce Zeb1 in all tissues and cells, so it is not clear in what cells loss or reduction of Zeb1 would result in or contribute to an observed abnormality. To dissect how Zeb1 specifically affects different cell types, which is critical in understanding the mechanisms underlying the Zeb1 regulation of the corneal response to chemical insults, we sought to delete Zeb1 in a specific group of cells, in particular in immune cells, to check if Zeb1 loss disturbs their normal biological functions. During embryonic development, it is known that Zeb1 KO results in T cell deficiency[6] which has been ascribed to the reduced thymus size.[7] Using Zeb1 conditional KO Guan et al. successfully deleted Zeb1 in adult mouse hematopoietic stem cells (HSCs) and confirmed that Zeb1 KO significantly reduced thymus size and T cells in adult animal.[8] Here, we also utilized a conditional knockout (cKO) strategy to genetically erase Zeb1 in Csf1r^{+} myeloid cells and uncovered that Zeb1 enhanced the corneal inflammatory response to alkali burn by maintaining their viability.

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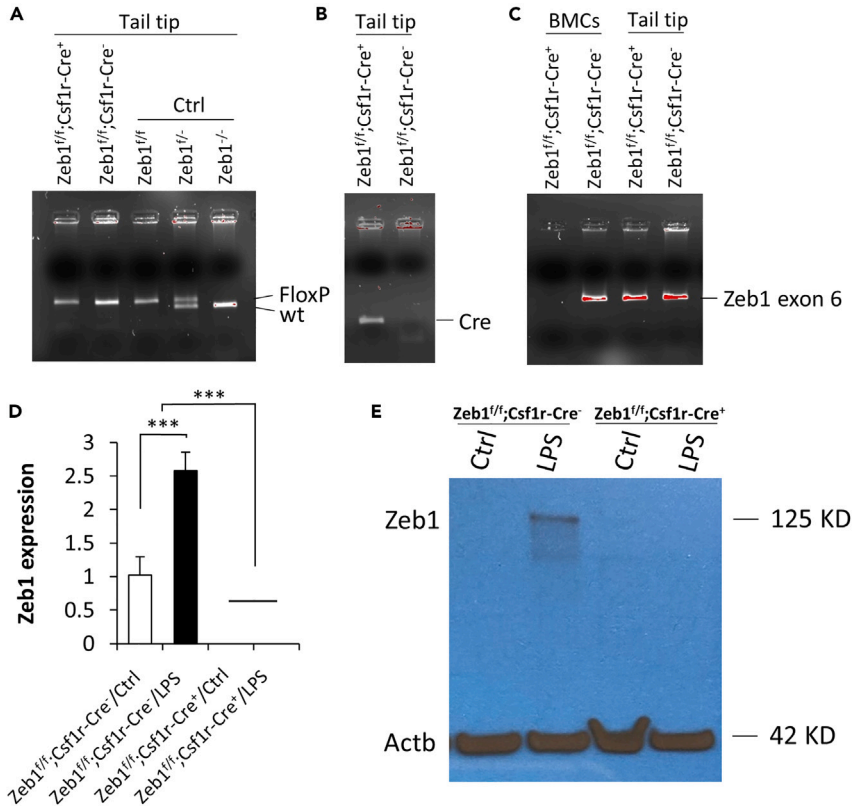

Figure 1. Zeb1 was induced to express in LPS-treated BMCs

(A) Homozygous *floxP* sites and (B) *Cre*$^{+}$ allele of Zeb1$^{f/f}$;Csf1r-Cre^{+} pups were assessed by PCR genotyping on their tail tip DNA samples.

(C) *Zeb1* loss of function mutation was further validated by PCR on their cultured BMC DNA samples.

(D) Zeb1 message RNA was detected by qPCR.

(E) while its protein was detected by WB in Zeb1$^{f/f}$;Csf1r-Cre^{-} (Ctrl) and Zeb1$^{f/f}$;Csf1r-Cre^{+} BMCs treated with and without LPS. LPS, lipopolysaccharides; BMCs, bone marrow-derived cells; Ctrl, control. two-way ANOVA was used to assign significant differences between cell samples. ***, $p \leq 0.001$.

RESULTS

Zeb1 cKO in Csf1r^{+} myeloid cells diminished lipopolysaccharide (LPS)-induced expression of Zeb1 in bone marrow-derived cells (BMCs)

We demonstrated previously that the monoallelic deletion of Zeb1 in mice could decrease corneal inflammation-associated neovascularization (NV) with alkali burn injur.[4,5] However, it was a whole-body deletion of Zeb1 and therefore it is not clear in what cells loss or reduction of Zeb1 would result in or contribute to the reduced corneal NV. To validate if immune cells are specifically targeted by Zeb1 knockout, Dr. Kaech kindly provided us the Zeb1$^{f/f}$ mice where the Zeb1 locus is flanked by *LoxP* site.[9] The Csf1 (M-CSF) receptor gene *Csf1r* is reported to specifically express in progenitor, precursor and mature myeloid cell.[10] To specifically delete Zeb1 in Csf1r^{+} myeloid lineages, we created homozygous Zeb1$^{f/f}$;Csf1r-Cre^{+} conditional knockout (cKO) mice by crossing and backcrossing Csf1r-Cre^{+} mice that we purchased from Jackson Laboratory in which the *LoxP* site-specific recombinase *Cre* gene is under the myeloid cell-specific *Csf1r* gene promoter contro[11] to the Zeb1-floxed mic[9] (see STAR methods). Homozygous *LoxP* sites (Figure 1A) and the *Cre*$^{+}$ allele (Figure 1B) of Zeb1$^{f/f}$;Csf1r-Cre^{+} pups were assessed by PCR genotyping with tail-tip DNA samples (see STAR methods) while Zeb1 loss of function mutation, i.e., the deletion of the exon 6 of *Zeb1* gene, was further validated by PCR (Figure 1C) using BMC DNA samples (see STAR methods). The expression of Zeb1 in monolayer-cultured BMCs of Zeb1$^{f/f}$;Csf1r-Cre^{-} mice (Zeb1 wildtype or wt) was not detected by qPCR or WB (Figures 1D and 1E). However, Zeb1 expression was induced by lipopolysaccharides (LPS) (Figures 1D–1E), a bacterial toxin often used to stimulate inflammatory responses in cultured BMCs. It appears that Zeb1 does not express in normal BMCs; but can be induced by a proinflammatory stimulus and thereby might play a role in inflammation. More importantly, little LPS-induced Zeb1 was detected in the BMCs of Zeb1$^{f/f}$;Csf1r-Cre^{+} mice (Zeb1 cKO, Figures 1D and 1E), thereby confirming an effective cKO of Zeb1 in the monolayer-cultured BMCs.

Zeb1 conditional knockout reduced CD45^{+} leukocyte accumulation in the cornea following alkali burn

During embryonic development, ZEB1 is needed for T cell and pulmonary cell maturation,[4,7] the function of ZEB1 in normal adult tissues is not as clear. It seems that in adults Zeb1 is not required for Csf1r^{+} myeloid cell homeostasis under normal physiological conditions as it is not

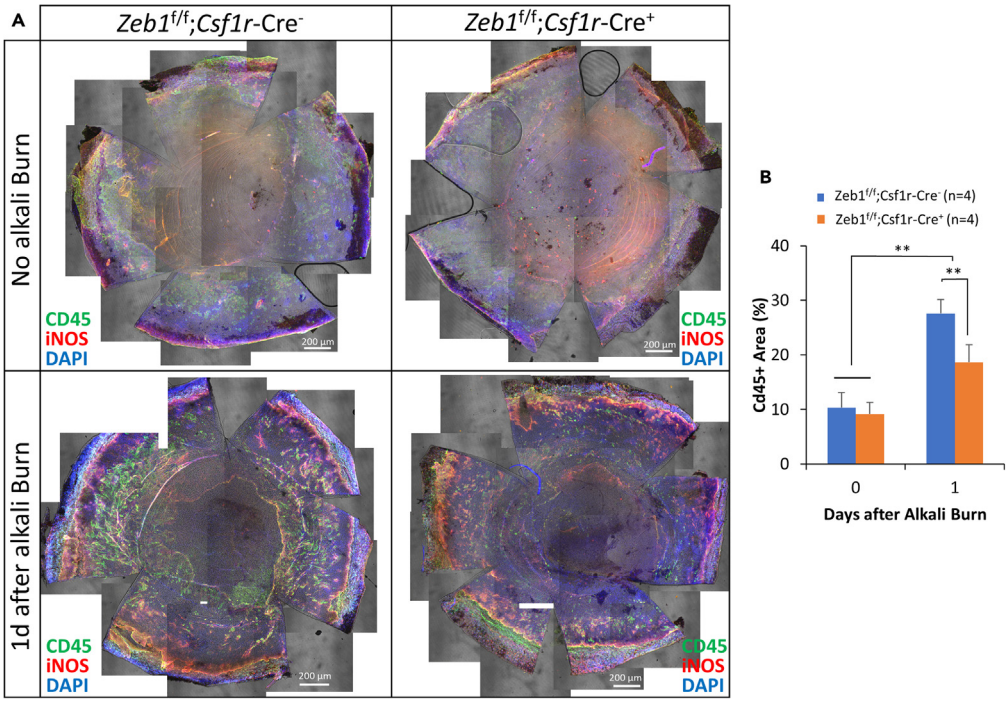

Figure 2. Zeb1 cKO in Csf1r$^+$ myeloid cells diminished the infiltration of CD45$^+$/iNOS$^+$ immune cells in the cornea following alkali burn

(A) Representative images of the corneal wholemount immunostaining with the leukocyte marker CD45 and the macrophage marker iNOS.

(B) Zeb1 cKO significantly reduced Cd45$^+$ leukocytes in the 1 day alkali burned corneas detected by immunostaining and analyzed by ImageJ. Data are represented as mean +SD. two-way ANOVA was used to assign significant difference between corneal samples. **, $p \leq 0.01$.

detected in these cells by WB (Figure 1E) and Zeb1$^{f/f}$;Csf1r-Cre$^+$ mice not only survived the loss of Zeb1 in Csf1r$^+$ cells but also appeared normal with no obvious defect. It has been noticed that ZEB1 is highly expressed in many cancer[12,13] and diseased tissues and cell,[14] and reducing ZEB1 in a diseased tissue often slows down disease progression.[3,4] To determine whether the loss of Zeb1 in Csf1r$^+$ myeloid cells would affect the accumulation of CD45$^+$ leukocytes in the cornea after an alkali burn, we immunostained both Zeb1 wt and Zeb1 cKO corneas before (0days) and after the alkali burn (1day) with the leukocyte marker CD45 antibody and the macrophage marker iNOS. We chose 1 day alkali burned corneas because the immune cell infiltration reached its peak in the cornea at this time.[5] As a result, we found that the alkali burn immediately resulted in an augmentation of CD45$^+$/iNOS$^+$ leukocytes and that the number of CD45$^+$ cells in the Zeb1 cKO corneas was significantly diminished as compared to that in Zeb1 wt corneas (Figures 2A and 2B, S1A–S1D), verifying that Zeb1 promotes inflammation by facilitating the infiltration of CD45$^+$ leukocytes, mostly iNOS$^+$ myeloid cells into the wounded cornea following the alkali burn.

Zeb1 conditional knockout bone marrow-derived cells displayed a reduced proliferation rate after corneal alkali burn

ZEB1 induces the proliferation of many malignant tumor cells and other mitotic cells such as stem cell[8]; Y.[13,15] To determine whether the elimination of Zeb1 in the Csf1r$^+$ cells would disturb their reproduction in the bone marrow (BM), we directly counted the numbers of BMCs isolated from both Zeb1$^{f/f}$;Csf1r-Cre$^-$ (wt) and Zeb1$^{f/f}$;Csf1r-Cre$^+$ (cKO) mice. The result showed that the total number of Zeb1 wt BMCs was less than that of Zeb1 cKO BMCs before the alkali burn (0 days) though the total number of BMCs of both genotypes was immediately plunged following the alkali burn (1 day) (Figure 3A). Thereafter, the number of Zeb1 cKO BMCs stayed in low levels when the number of Zeb1 wt BMCs was gradually recovered almost to the level prior to the alkali burn (Figure 3A), suggesting that the corneal wound by the chemical burn immediately exhausts a large number of BMCs and the loss of Zeb1 in the Csf1r$^+$ cells significantly disturbs the production of total BMCs in response to the alkali burn. Using flow cytometry together with the CD11b$^+$ antibody for myeloid cells (i.e., monocytes), we also checked the dynamic change of CD11b$^+$ cells throughout the recovery course of the injured corneas following the alkali burn (Figure S2) and found that the CD11b$^+$ cells were eventually restored to the prior level in the Zeb1 cKO corneas while exceeded the prior level in the Zeb1 wt corneas (Figure 3B), suggesting (1) CD11b$^+$ cells are likely required for corneal wound healing; (2) loss of Zeb1 in Csf1r$^+$ cells reduces the production of CD11b$^+$ cells in the BM (Figure 3B) and thereby total leukocytes in the wounded cornea (Figure 2).

Zeb1 conditional knockout did not affect cell proliferation in the cornea

The sudden drop of BMCs on day 1 following the alkali burn was supposed to be due to the mobilization of cells from the BM to the blood stream and then to the affected cornea. The number of BMCs was significantly reduced in Zeb1 cKO mice more than that in Zeb1 wt mice

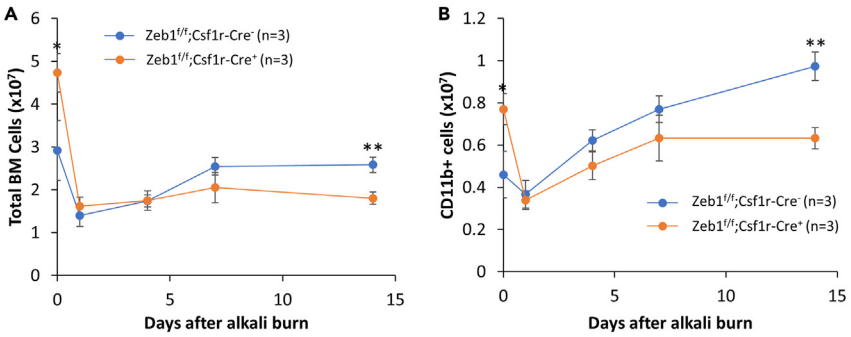

Figure 3. Knockout of Zeb1 in Csf1r$^+$ cells decreased production of CD11b$^+$ myeloid cells in the bone marrow after corneal alkali burn

Loss of Zeb1 in Csf1r$^+$ cells reduced (A) total and (B) CD11b$^+$ BMCs in the BM following the corneal alkali burn.

Data are represented as mean ± SD. Zeb1$^{f/f}$;Csf1r-Cre$^-$, Zeb wt; Zeb1$^{f/f}$;Csf1r-Cre$^+$, Zeb1 cKO; BMCs, bone marrow-derived cells; BM, bone marrow; *, $p \leq 0.05$; **, $p \leq 0.01$.

(Figure 3A), indicating more BMCs were circulated to Zeb1 cKO corneas. However, the number of CD45$^+$ cells in Zeb1 cKO corneas detected by the immunostaining was actually less than that in Zeb1 wt corneas (Figure 1B), suggesting that CD45$^+$ cells in Zeb1 wt corneas either self-renewed more frequent, or died less frequently than in Zeb1 cKO corneas, or both. To clarify the first possibility, we immunostained paraffin sections of both Zeb1 wt and cKO corneas before (0 days) and after the alkali burn (4 days) with the proliferative cell marker Ki67 antibody. The alkali bure did significantly increase the number of Ki67$^+$ cells in the corneas of both genotypes, including the epithelium and the stroma (Figures 4A–4D). Surprisingly, Zeb1 cKO did not significantly alter the number of Ki67$^+$ cells in the corneas either before or after the alkali burn compared to Zeb1 wt genotype (Figures 4A–4D), indicating that the Csf1r$^+$ cells circulated to the cornea are mostly not proliferative as reporte[16] and the number of resident Csf1r$^+$ immune cells are likely very limited compared to the infiltrated Csf1r$^+$ cells though resident macrophages have been reported to be of self-renewability.[17]

Zeb1 conditional knockout lowed myeloid cell viability in the cornea

As reported above, if the reduction of CD45$^+$ cells in the cornea by Zeb1 cKO after the alkali burn (Figure 2) was not due to less immune cells circulating to and/or proliferating in the cornea (Figure 4), then it was likely because more CD45$^+$ cells died in the Zeb1 cKO cornea than in the Zeb1 wt cornea. To clarify this possibility, using TUNEL method we checked the apoptosis of corneal cells before (0 days) and after the alkali burn (4 days). As predicted, we noted that more apoptotic cells were discovered in the stroma of the Zeb1 cKO corneas compared to that in the Zeb1 wt corneas (Figures 5A–5D). It appeared that immune cells were able to infiltrate into not only the stroma but also the epithelium, and that the myeloid cells displayed a significant decrease in viability after loss of Zeb1 and subjected to a stressed environment.

Zeb1 conditional knockout modulated the expression of pro-inflammatory cytokine genes

The alkali burn prompts an acute corneal inflammation with a sudden inflow of immune cells (Figure 2) usually by inducing the expression of pro-inflammatory cytokine.[18] As we showed earlier, LPS treatment increased the expression of not only Zeb1 (Figure 1), but also number of inflammatory cytokines such as *Csf1/2/3, Il-1b* and *Tnfa* in Zeb1 wt BMCs (Figures 6A and 6B). While Zeb1 cKO in Csf1r$^+$ cells significantly reduced the expression of *Csf1/3* and eliminated *Csf2* expression though it had a moderate negative effect on *Il-1b* expression and a positive effect on *Tnfa* expression (Figures 6A and 6B). It is of note that among the colony-stimulating factors (Csf) Csf3, also known as G-CSF, is an essential cytokine for hematopoietic stem cell mobilization which increases circulating levels of BMCs at different stages of maturation.[19] It appears that Zeb1 cKO in Csf1r$^+$ cells not only reduced the inflammation-associated production of BMCs in the BM (Figure 3B), but also likely lowed their capability of mobilization to the circulation.

Most pro-inflammation cytokines are under the control of the inflammation master regulator Nfkb (Liu et al.,[20] while endogenous molecules released from disrupted cells, known as damage-associated molecular patterns (DAMPs), can activate Nfk.[21] Indeed, the expression of Nfkb was detected to be significantly upregulated by the alkali burn and the upregulation levels of Nfkb were reduced in Zeb1 cKO corneas (Figures 6C–6F). Zeb1 binds to and directly transactivates Nfkb gene expression as previously reported,[5,22] thereby leading to an upregulation of the pro-inflammation cytokine genes. Taken together, we conclude that the lower expression of the pro-inflammatory cytokines Csf in Zeb1 cKO BMCs (Figure 6A) is part of the cause for less infiltration of immune cells into Zeb1 cKO corneas after the alkali burn (Figure 2).

Zeb1 conditional knockout in myeloid cells increased the expression of the pro-apoptosis cytokine Tnfa

Corneal damage or removal of corneal epithelium by various physical or chemical means would cause epithelial and stromal cell (keratocyte) deat.[23,24] Such damage triggers a release of inflammatory cytokines from epithelial cells and/or tears, mainly IL-1 (α and β[25] and Tnfa (Zhang et al.,[26] that cause not only rapid infiltration of immune cells but also apoptosis through Tnfa/Tnfr1-associated signaling and later, necrosis of mainly anterior keratocyte.[27] IL-1β and TNFa are the two common pro-inflammatory cytokines in tissue wound healing,[28] though the cytokine

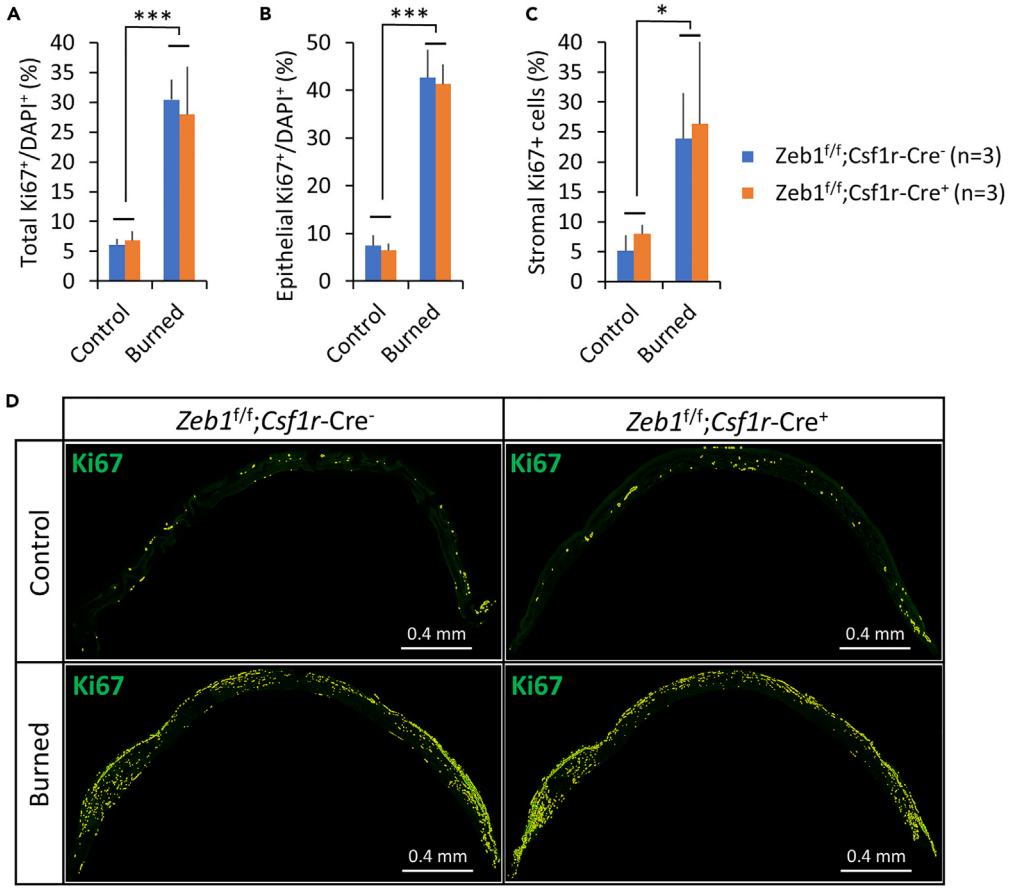

Figure 4. Knockout of Zeb1 in Csf1r$^+$ cells did not significantly affect cell proliferation in the cornea after alkali burn

Loss of Zeb1 in Csf1r$^+$ cells did not significantly change (A) the numbers of total proliferative cells in the cornea including (B) the epithelium and (C) the stroma. (D) Representative images of Ki67$^+$ cells in both Zeb1 wt and cKO corneas before (Control) and 4days after the alkali burn (Burned). Zeb1$^{f/f}$;Csf1r-Cre$^-$, Zeb wt; Zeb1$^{f/f}$;Csf1r-Cre$^+$, Zeb1 cKO. Data are represented as mean +SD. two-way ANOVA was used to assign significant difference between corneal samples. *, $p \leq 0.05$; ***, $p \leq 0.001$.

TNFa has multifaceted biological functions in addition to promoting inflammation, it is also a well-known pro-apoptosis cytokin.[29] We did detect an upregulation of these two cytokines in the affected corneas after the alkali burn whereas the monoallelic Zeb1 KO reduced this upregulation of *Il-1β*, but not *Tnf*,[5] which is consistent with the results of the BMCs treated with LPS above (Figure 6A). As opposite to Il-1β, Zeb1 cKO increased the expression *Tnfa* in BMCs (Figure 6A), suggesting that Tnfa may play an additional role other than pro-inflammation in the cornea after the alkali burn, i.e., the induction of apoptosis, as previously reported.[26]

ZEB1 may directly and indirectly regulate the expression of the indicated cytokines and their receptors involved in the according signaling transduction pathways leading to tissue inflammation. Similarly, Zeb1 cKO in the Csf1r$^+$ myeloid cells also downregulated the expression of *Csf* receptors while upregulated *Tnfr1* expression though *Csf2r* message was not detected (Figure 7A). We previously reported that ZEB1 could directly bind to and thereby might transactivate *IL-1b* and *NF-κB* to increase their expression; but it was not detected to bind to *TNFa* promoter and thereby might indirectly to increase the expression of other pro-inflammatory genes through the up-regulation of *NFκ*.[5] To clarify whether ZEB1 binds and thereby directly regulates CSF factors and their receptors, we performed ChIP-PCR assays using human chromatin as previously describe[30] because all CSF factor and their receptor putative promoters contain ZEB1 binding consensus (i.e., CANNTG) sequences. As a result, we found that ZEB1 binds to the promoters of *CSF2* and *CSF1R* genes (Figure 7B), indicating that ZEB1 might directly regulate CSF2-involved myeloid cell proliferation, differentiation, survival, and tissue damage repai.[31] It is of note that although ZEB1 was not detected to bind *TNFa* it appeared to be binding to *TNFR1* (Figure 7B), a TNFa receptor gene leading to apoptosis pathwa.[32]

DISCUSSION

Csf1r is expressed at a low level in hematopoietic stem cell (HSC); it is gradually increased in myeloid progenitors and precursors such as myelocytes and monocytes both in the BM and in the peripheral blood, and reached its highest levels in matured myeloid cell types though it has almost no expression in lymphoid cell.[31] In mature myeloid cells, macrophage expresses Csf1r almost 5 times higher than neutrophi.[31]

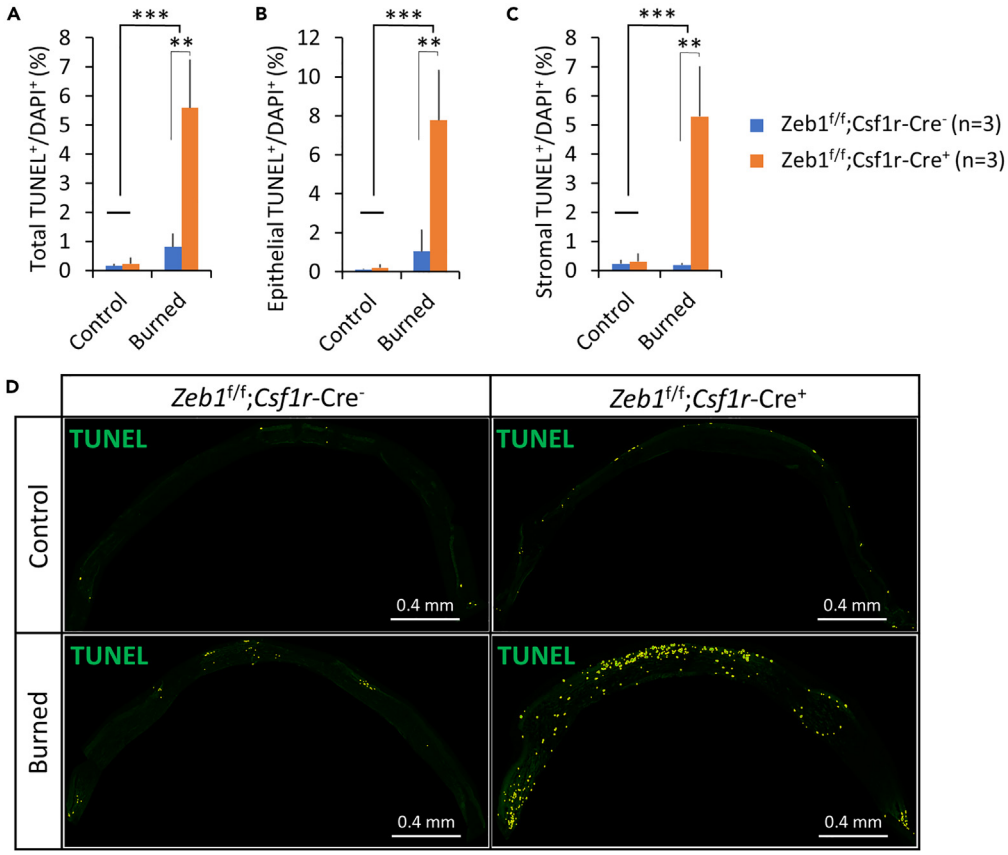

Figure 5. Knockout of Zeb1 in Csf1r$^+$ cells enhanced cell death in the cornea after alkali burn

Loss of Zeb1 in Csf1r$^+$ cells significantly enhanced apoptosis in (A) the cornea including (B) the epithelium and (C) the stroma following the alkali burn. (D) Representative images of TUNEL$^+$ cells in both Zeb1 wt and cKO corneas before (Control) and 4days after the alkali burn (Burned). Zeb1$^{f/f}$;Csf1r-Cre$^-$, Zeb wt; Zeb1$^{f/f}$;Csf1r-Cre$^+$, Zeb1 cKO; **, $p \leq 0.01$; ***, $p \leq 0.001$. Data are represented as mean +SD. two-way ANOVA was used to assign significant difference between corneal samples.

Thus, the Csf1r-Cre-defined cKO of Zeb1 is supposed to be mostly in monocyte and macrophage which are counted for about 3% of the entire immune cell population in the BM though more than 70% of BMCs are immature myelocyte.[33] Most of the BMCs surviving adherent culture with the macrophage stimulating factor (M-CSF or CSF1) would be adherent monocytes/macrophages, thus it is not surprising to find that the floxed Zeb1 exon 6 was completely removed by Cre recombinase activity (Figure 1C) and Zeb1 protein was not detected by WB even after the LPS treatment in such cultured cKO BMCs (Figure 1D). Before the alkali burn, Zeb1 detected by immunostaining (Figure S3), was mostly expressed in the epithelium and the endothelium where it is supposed to inhibit the expression of the cell adhesion molecule E-cadherin (CDH1)[15] and in cells scattered in the stroma, particularly those close to the limbal vasculature (Figure S3). After the alkali burn however, Zeb1 was highly expressed in most infiltrated immune cells in both Zeb1$^{f/f}$;Csf1r-Cre$^-$ (wt) and Zeb1$^{f/f}$;Csf1r-Cre$^+$ (cKO) corneas though the percentage was bit lower in cKO corneas than in wt corneas (Figure S3), indicating that only small proportion, i.e., less than 10% infiltrated immune cells actually had lost Zeb1 due to Zeb1 cKO, consistent with the percentage of monocytes, i.e., about 10–20% in the peripheral bloo.[34,35] It has been reported that Zeb1 expresses at higher levels in HSC and mature leukocyte, but not in myeloid progenitors and precursor,[36] indicating it may affect BMC reproduction and immune cell function such as differentiation in the BM, during the circulation and at peripheral tissues such as in the cornea. Indeed, Zhang et al., found that Zeb1 sustains hematopoietic stem cell function using Zeb1 KO mice[37] while Wang et al., determined that the over-expression of Zeb1 in mice expanded monocytic development and thereby myeloid cell.[38] Our experiment with Zeb1$^{f/f}$;Csf1r-Cre$^+$ animal confirms that Zeb1 cKO in myeloid cells affects their reproduction in the BM (Figure 3) and their viability in the cornea (Figure 5) after alkali burn.

Under a normal physiological condition, most resident macrophages are of embryonic origin, though heterogeneous but self-renewable.[39] These resident macrophages play important roles in maintaining tissue innate immunity before an inflammation is initiated and tissue damage repair after the inflammation is retreated, serving mostly as so-called M1 and M2 phenotype, respectively[39]; Upon a severe environmental insult such as alkali chemical burn, an immediate inflow of immune cells into the affected central cornea from the surrounding vasculature in the limbus to kill and clean up foreign invaders and to prevent further tissue damage. Initially, about 70–80% of them are T cells and B

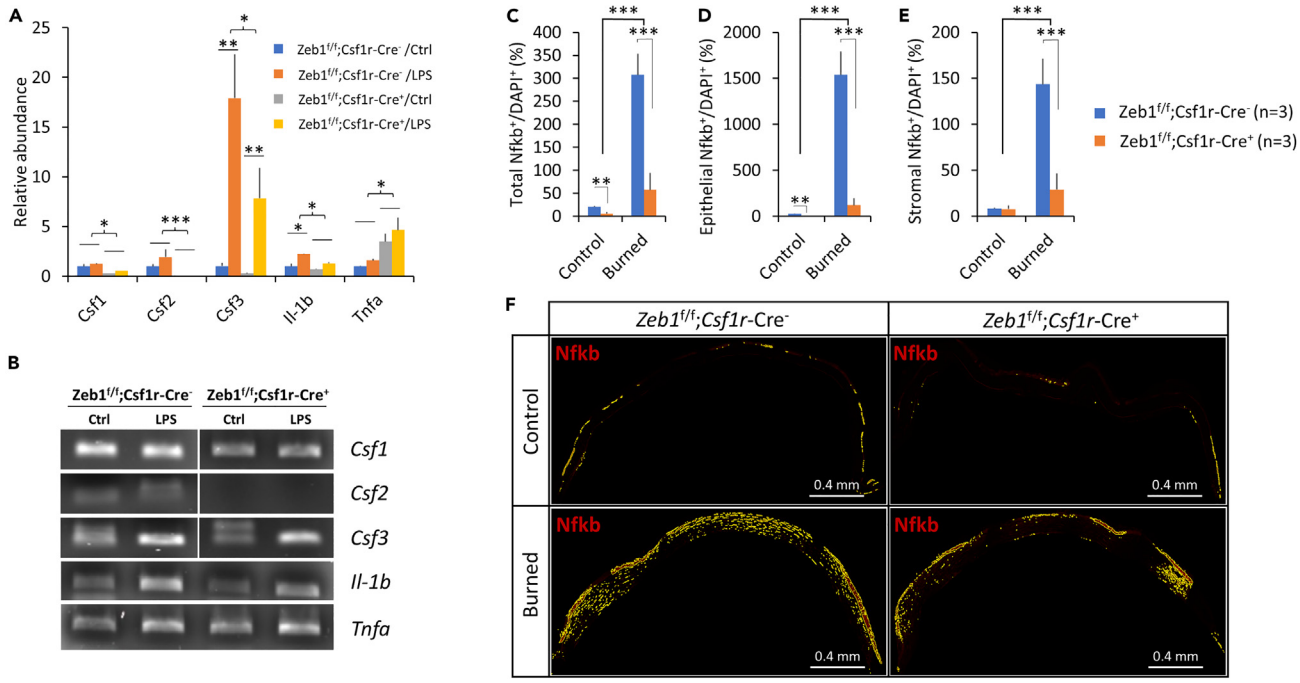

Figure 6. Loss of Zeb1 in Csf1r^{+} cells significantly reduced the expression of the proinflammatory genes in BMCs

(A) detected by qPCR and (B) confirmed by their gel images, (C) in parallel with the reduction of Nfkb in the cornea including (D) the epithelium and (E) the stroma following the alkali burn.

(F) Representative images of Nfkb^{+} cells in both Zeb1 wt and cKO corneas before (Control) and 4days after the alkali burn (Burned). Ctrl, control; LPS, lipopolysaccharides; Ctrl, control; Zeb1$^{f/f}$;Csf1r-Cre^{-}, Zeb wt; Zeb1$^{f/f}$;Csf1r-Cre^{+}, Zeb1 cKO; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Data are represented as mean +SD. two-way ANOVA was used to assign significant difference between corneal samples.

cells (lymphoid cells) whereas <20% are neutrophils and macrophages (myeloid cells) and often with an M1-like phenotype.[21] The myeloid cells are gradually matured to become mostly neutrophils and macrophages during their mobilization from the bone marrow to the circulation and then migration to the affected sites where they produce large amounts of cytokines such as interferon (IFN) and enzymes such as nitric oxide synthase (NOS) thereby nitric oxide (NO) to kill invading microorganism.[40,41] The mature myeloid cells are likely not proliferative and short-lived for a few days and their number in the affected peripheral tissues can only be sustained by continuously circulating new cells from the limbal vasculatur,[42] indicating that the less number of CD11b+ in the Zeb1 cKO BM might also be due to more myelocytes and monocytes were required to mobilize and circulate to the cornea following the alkali burn (Figure 3B).

Zeb1 is highly expressed in embryonic tissues, Zeb1 KO is lethal to the embryo before birth. In most adult tissues, Zeb1 expression is minimal except for stem cell populations including hematopoietic stem cell (HSC[8]).[37] In addition to HSC, Zeb1 is also detected in mature myeloid and lymphoid cell type.[36] Thus, it is not surprising that Zeb1 is not detected in monolayer cultured immature BMCs though the addition of LPS to the culture medium sparked its expression in the LPS-polarized BMCs, implying Zeb1 may not be important for these immune cells under normal conditions, but would be needed for them to function effectively after maturation by an environmental stress such as physical or chemical wounding as we previously reporte[5] to maintain their viability as reported above. What is the molecular mechanism underlying the Zeb1 regulation of corneal inflammation and wound healing induced by the alkali burn? Zeb1 is a transcription factor that either transactivates or represses the expression of genes involved in tissue inflammation and wound healing such as Il-1b and Tnfa that cause tissue inflammation and cell death as we reported previously (Y; [26] We find that the pro-inflammatory cytokine genes *Il-1b*, *Tnfa*, the myeloid activation genes *Csf1/2/3* and their receptors all contain multiple Zeb1-binding consensus sequences (CANNTG) in their putative promoters. Our ChIP assays with human chromatin indicate that ZEB1 binds to *CSF2* and *CSF1R* promoters to possibly transactivate their expression to enhance myeloid cell activation for proliferation, differentiation, chemotaxis, and survival, and to *IL-1βR* and *TNFR1* for signaling pathways leading to inflammation and apoptosis (Figure 7C).[31,43,44] Apparently, alkali burn damages corneal tissues, cytokines are released from the affected tissues and induce TNFα/TNFR1-involved cell apoptosis, IL-1β-involved tissue inflammation and CSF/CSFR-involved myeloid polarization (differentiation), survival, chemotaxis and proliferation (Figure 7C). ZEB1 serves as an important factor in the regulation of the involved genes both directly and indirectly. In culture, however, how does LPS up-regulates ZEB1 as shown in (Figures 1D and 1E)? It is well documented that LPS binds and phosphorylates toll-like receptor 4 (TLR4) to activate inflammation master regulator NF-κB by phosphorylation which translocates to the nucleus to bind multiple inflammatory cytokine genes, including *IL-1β*, to transactivate their expression to induce tissue inflammatio,[45,46] and to repress the expression of E-cadherin (*CDH1*) through the upregulation of the EMT factor ZEB[47] (Figure 7C). It is clear now

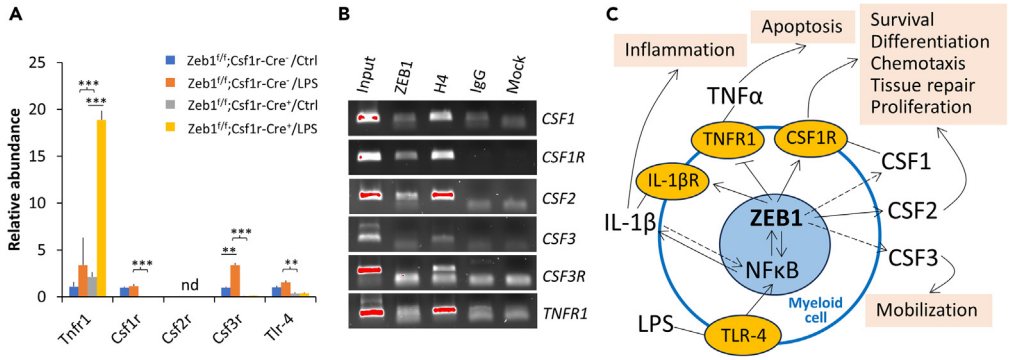

Figure 7. ZEB1 regulates pro-inflammatory and apoptotic cytokines and their receptors in myeloid cells

(A) Expression of pro-inflammatory and apoptotic cytokine receptors detected by qPCR.

(B) Chromatin immunoprecipitation (ChIP) assay to detect ZEB1 binding to putative promoters of the indicated genes.

(C) Schematic diagram depicting Zeb1 functions in the regulation of genes involved in inflammation, apoptosis, and other cellular processes. Data are represented as mean +SD. Input, 1/10 of the original human cell chromatin; ZEB1, ZEB1 antibody to precipitate its bound DNA; H4, pan-histone 4 antibody to precipitate its bound DNA as a positive control; IgG, isotype negative control; Mock, no antibody negative control; LPS, lipopolysaccharides; Ctrl, control; $Zeb1^{f/f}$;Csf1r-Cre^{-}, Zeb wt; $Zeb1^{f/f}$;Csf1r-Cre^{+}, Zeb1 cKO; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

that ZEB1 and NF-κB are the major transcription factors in the regulation of myeloid cell mobilization, proliferation, differentiation, survival, chemotaxis, and apoptosis (Figure 7C), important for tissue inflammation and wound healing.

Zeb1 was previously reported to be involved in alkali burn-induced corneal neovascularization (NV), a serious condition in the clinic, by activating vascular endothelial cell proliferation; and the inhibition of Zeb1 activation by the ZEB1-CtBP inhibitor NSC95397 could significantly reduce the corneal N.[4] NV goes with tissue inflammation and mouse corneal alkali burn is a well-documented animal model to study corneal N.[48] We also found previously that the alkali burn-induced corneal inflammation was divided into two phases – the early phase before NV appearance and the later phase when NV was established in the cornea. Although monoallelic Zeb1-KO significantly reduced the alkali burn-induced corneal inflammation over the entire course following the alkali burn, the levels of corneal Zeb1 expression had no significant difference between wt and monoallelic Zeb1 KO until the later phase (day 14) after the alkali bur.[4] Intriguingly, in this study, we found that Zeb1 cKO in the $Csf1r^{+}$ myeloid cells did not affect both total BMC and $CD11b^{+}$ myeloid cell reproduction in the BM until day 14 (Figure 3) when NV should be established in wt cornea.[4] This correlation indicates that the Zeb1-regulation of corneal NV is not only through the regulation of vessel endothelial cell proliferation[4] but also through the regulation of immune cell mobilization and viability as we reported herein. Zeb1-regulation of corneal inflammation in the cornea following the alkali burn appeared not directly through regulating the expression of pro-inflammatory cytokine genes such as *Il-1b* and *Tnf*.[5] In this study we have clarified that Zeb1 could significantly enhance BMC viability by directly regulating the expression of *CSF2* and their receptors and *TNFa/TNFR1* (Figure 7). In our opinion, to effectively control corneal NV, the suppression of severe inflammation following the alkali burn is critical either by blocking and/or neutralizing pro-inflammatory cytokine production and/or activities. Alternatively, it is also applicable to utilize inhibitors that repress the activity of the major inflammation transcription factors NF-κB and ZEB1 such as NSC95397.

Limitations of the study

Using the conditional knockout of Zeb1 mouse model, we provide convincing evidence that the deletion of Zeb1 in Csf1r-positive immune cells significantly reduces corneal inflammation after an alkali burn. We clearly demonstrated that the alkali-burn significantly upregulated Zeb1 expression in corneal cells, mostly those infiltrated immune cells into the cornea. The underlying cellular mechanism is Zeb1 maintenance of Csf1r-positive myeloid cell viability in the affected tissue. However, we do not know what proportion of the infiltrated immune cells is not affected by the Zeb1 cKO and what are these cell types. Further investigation is needed to clarify these issues.

STAR★METHODS

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● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109694.

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AUTHOR CONTRIBUTIONS

K.K.D. and F.W.: performed most of the experiments, collected and analyzed the data, wrote the article; Y.Z.: performed an additional experiment for validation; W.L., J.Y.L., D.Y.J., and X.L.: assisted in cell culture, RNA extraction, and Western blot analysis; L.Z.: contributed critical resources and helped analyzing the data; W.W.: shared a critical idea; D.C.D. and Y.L.: designed experiments, analyzed the data, and wrote the article. All authors read and approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Feizi, S. (2018). Corneal Blindness Caused By Mustard Gas (Sine Loco: IntechOpen).
2. Takagi, T., Moribe, H., Kondoh, H., and Higashi, Y. (1998). DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. Development *125*, 21–31.
3. Sanchez-Tillo, E., Liu, Y., de Barrios, O., Siles, L., Fanlo, L., Cuatrecasas, M., and Postigo, A. (2012). EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. Cell. Mol. Life Sci. *69*, 3429–3456. https://doi.org/10.1007/s00018-012-1122-2.
4. Jin, L., Zhang, Y., Liang, W., Lu, X., Piri, N., Wang, W., and Liu, Y. (2020). Zeb1 promotes corneal neovascularization by regulation of vascular endothelial cell proliferation. Commun. Biol. *3*, 349. https://doi.org/10.1038/s42003-020-1069-z.
5. Liang, W., Zhang, Y., Zhou, L., Lu, X., Finn, M.E., Wang, W., and Liu, Y. (2022). Zeb1 regulation of wound-healing-induced inflammation in alkali-damaged corneas. iScience *25*, 104038. https://doi.org/10.1016/j.isci.2022.104038.
6. Higashi, Y., Moribe, H., Takagi, T., Sekido, R., Kawakami, K., Kikutani, H., and Kondoh, H. (1997). Impairment of T cell development in deltaEF1 mutant mice. J. Exp. Med. *185*, 1467–1479. https://doi.org/10.1084/jem.185.8.1467.
7. Dean, K.C., Huang, L., Chen, Y., Lu, X., and Liu, Y. (2015). An Rb1-dependent amplification loop between Ets1 and Zeb1 is evident in thymocyte differentiation and invasive lung adenocarcinoma. BMC Mol. Biol. *16*, 8. https://doi.org/10.1186/s12867-015-0038-4.
8. Almotiri, A., Alzahrani, H., Menendez-Gonzalez, J.B., Abdelfattah, A., Alotaibi, B., Saleh, L., and Rodrigues, N.P. (2021). Zeb1 modulates hematopoietic stem cell fates required for suppressing acute myeloid leukemia. J. Clin. Invest. *131*. https://doi.org/10.1172/JCI129115.
9. Guan, T., Dominguez, C.X., Amezquita, R.A., Laidlaw, B.J., Cheng, J., Henao-Mejia, J., and Kaech, S.M. (2018). ZEB1, ZEB2, and the miR-200 family form a counterregulatory network to regulate CD8(+) T cell fates. J. Exp. Med. *215*, 1153–1168. https://doi.org/10.1084/jem.20171352.
10. Loschko, J., Rieke, G.J., Schreiber, H.A., Meredith, M.M., Yao, K.H., Guermonprez, P., and Nussenzweig, M.C. (2016). Inducible targeting of cDCs and their subsets in vivo. J. Immunol. Methods *434*, 32–38. https://doi.org/10.1016/j.jim.2016.04.004.
11. Shi, J., Hua, L., Harmer, D., Li, P., and Ren, G. (2018). Cre Driver Mice Targeting Macrophages. Methods Mol. Biol. *1784*, 263–275. https://doi.org/10.1007/978-1-4939-7837-3_24.
12. Hill, L., Browne, G., and Tulchinsky, E. (2013). ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. Int. J. Cancer *132*, 745–754. https://doi.org/10.1002/ijc.27708.
13. Sanchez-Tillo, E., Siles, L., de Barrios, O., Cuatrecasas, M., Vaquero, E.C., Castells, A., and Postigo, A. (2011). Expanding roles of ZEB factors in tumorigenesis and tumor progression. Am. J. Cancer Res. *1*, 897–912.
14. Ghafouri-Fard, S., Askari, A., Behzad Moghadam, K., Hussen, B.M., Taheri, M., and Samadian, M. (2023). A review on the role of ZEB1-AS1 in human disorders. Pathol. Res. Pract. *245*, 154486. https://doi.org/10.1016/j.prp.2023.154486.
15. Liu, Y., El-Naggar, S., Darling, D.S., Higashi, Y., and Dean, D.C. (2008). Zeb1 links epithelial-mesenchymal transition and cellular senescence. Development *135*, 579–588. https://doi.org/10.1242/dev.007047.
16. Takizawa, H., Boettcher, S., and Manz, M.G. (2012). Demand-adapted regulation of early

hematopoiesis in infection and inflammation. Blood *119*, 2991–3002. https://doi.org/10.1182/blood-2011-12-380113.

17. Minutti, C.M., Knipper, J.A., Allen, J.E., and Zaiss, D.M.W. (2017). Tissue-specific contribution of macrophages to wound healing. Semin. Cell Dev. Biol. *61*, 3–11. https://doi.org/10.1016/j.semcdb.2016.08.006.
18. Shahriary, A.P., Sabzevari, M.M., Jadidi, K.M., Yazdani, F.M., and Aghamollaei, H.P. (2021). The Role of Inflammatory Cytokines in Neovascularization of Chemical Ocular Injury. Ocul. Immunol. Inflamm. 1–13. https://doi.org/10.1080/09273948.2020.1870148.
19. Stoikou, M., van Breda, S.V., Schafer, G., Vokalova, L., Giaglis, S., Plattner, A., and Buser, A. (2020). G-CSF Infusion for Stem Cell Mobilization Transiently Increases Serum Cell-Free DNA and Protease Concentrations. Front. Med. *7*, 155. https://doi.org/10.3389/fmed.2020.00155.
20. Liu, T., Zhang, L., Joo, D., and Sun, S.C. (2017). NF-kappaB signaling in inflammation. Signal Transduct. Targeted Ther. *2*. https://doi.org/10.1038/sigtrans.2017.23.
21. Fortingo, N., Melnyk, S., Sutton, S.H., Watsky, M.A., and Bollag, W.B. (2022). Innate Immune System Activation, Inflammation and Corneal Wound Healing. Int. J. Mol. Sci. *23*. https://doi.org/10.3390/ijms232314933.
22. Arnold, C.N., Pirie, E., Dosenovic, P., McInerney, G.M., Xia, Y., Wang, N., and Beutler, B. (2012). A forward genetic screen reveals roles for Nfkbid, Zeb1, and Ruvbl2 in humoral immunity. Proc. Natl. Acad. Sci. USA *109*, 12286–12293. https://doi.org/10.1073/pnas.1209134109.
23. Szerenyi, K.D., Wang, X., Gabrielian, K., and McDonnell, P.J. (1994). Keratocyte loss and repopulation of anterior corneal stroma after de-epithelialization. Arch. Ophthalmol. *112*, 973–976. https://doi.org/10.1001/archopht.1994.01090190121031.
24. Wilson, S.E., Chaurasia, S.S., and Medeiros, F.W. (2007). Apoptosis in the initiation, modulation and termination of the corneal wound healing response. Exp. Eye Res. *85*, 305–311. https://doi.org/10.1016/j.exer.2007.06.009.
25. Ljubimov, A.V., and Saghizadeh, M. (2015). Progress in corneal wound healing. Prog. Retin. Eye Res. *49*, 17–45. https://doi.org/10.1016/j.preteyeres.2015.07.002.
26. Zhang, Y., Do, K.K., Wang, F., Lu, X., Liu, J.Y., Li, C., and Liu, Y. (2023). Zeb1 facilitates corneal epithelial wound healing by maintaining corneal epithelial cell viability and mobility. Commun. Biol. *6*, 434. https://doi.org/10.1038/s42003-023-04831-0.
27. Mohan, R.R., Mohan, R.R., Kim, W.J., and Wilson, S.E. (2000). Modulation of TNF-alpha-induced apoptosis in corneal fibroblasts by transcription factor NF-kappaB. Invest. Ophthalmol. Vis. Sci. *41*, 1327–1336.
28. Xiao, T., Yan, Z., Xiao, S., and Xia, Y. (2020). Proinflammatory cytokines regulate epidermal stem cells in wound epithelialization. Stem Cell Res. Ther. *11*, 232. https://doi.org/10.1186/s13287-020-01755-y.
29. van Loo, G., and Bertrand, M.J.M. (2023). Death by TNF: a road to inflammation. Nat. Rev. Immunol. *23*, 289–303. https://doi.org/10.1038/s41577-022-00792-3.
30. Chen, Y., Lu, X., Montoya-Durango, D.E., Liu, Y.H., Dean, K.C., Darling, D.S., and Liu, Y. (2017). ZEB1 Regulates Multiple Oncogenic Components Involved in Uveal Melanoma Progression. Sci. Rep. *7*, 45. https://doi.org/10.1038/s41598-017-00079-x.
31. Stanley, E.R., and Chitu, V. (2014). CSF-1 receptor signaling in myeloid cells. Cold Spring Harbor Perspect. Biol. *6*. https://doi.org/10.1101/cshperspect.a021857.
32. Wajant, H., and Siegmund, D. (2019). TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages. Front. Cell Dev. Biol. *7*, 91. https://doi.org/10.3389/fcell.2019.00091.
33. Terstappen, L.W.M.M., and Levin, J. (1992). Bone-Marrow Cell Differential Counts Obtained by Multidimensional Flow-Cytometry. Blood Cell *18*, 311–330.
34. Sen, P., Kemppainen, E., and Oresic, M. (2017). Perspectives on Systems Modeling of Human Peripheral Blood Mononuclear Cells. Front. Mol. Biosci. *4*, 96. https://doi.org/10.3389/fmolb.2017.00096.
35. Verhoeckx, K., Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., and Lea, T. (2015). SpringerLink. The Impact of Food Bioactives on Health : In Vitro and Ex Vivo Models, 1st 2015 (Springer International Publishing: Imprint: Springer).
36. Radhakrishnan, K., Truong, L., and Carmichael, C.L. (2023). An "unexpected" role for EMT transcription factors in hematological development and malignancy. Front. Immunol. *14*, 1207360. https://doi.org/10.3389/fimmu.2023.1207360.
37. Zhang, K., Zhao, H., Sheng, Y., Chen, X., Xu, P., Wang, J., and Zhu, H.H. (2022). Zeb1 sustains hematopoietic stem cell functions by suppressing mitofusin-2-mediated mitochondrial fusion. Cell Death Dis. *13*, 735. https://doi.org/10.1038/s41419-022-05194-w.
38. Wang, J.Q., Farkas, C., Benyoucef, A., Carmichael, C., Haigh, K., Wong, N., and Haigh, J.J. (2021). Interplay between the EMT transcription factors ZEB1 and ZEB2 regulates hematopoietic stem and progenitor cell differentiation and hematopoietic lineage fidelity. PLoS Biol. *19*. https://doi.org/10.1371/journal.pbio.3001394.
39. Liu, J., and Li, Z. (2021). Resident Innate Immune Cells in the Cornea. Front. Immunol. *12*, 620284. https://doi.org/10.3389/fimmu.2021.620284.
40. Stegelmeier, A.A., van Vloten, J.P., Mould, R.C., Klafuric, E.M., Minott, J.A., Wootton, S.K., and Karimi, K. (2019). Myeloid Cells during Viral Infections and Inflammation. Viruses *11*. https://doi.org/10.3390/v11020168.
41. Xue, Q., Yan, Y., Zhang, R., and Xiong, H. (2018). Regulation of iNOS on Immune Cells and Its Role in Diseases. Int. J. Mol. Sci. *19*. https://doi.org/10.3390/ijms19123805.
42. Janssen, W.J., Bratton, D.L., Jakubzick, C.V., and Henson, P.M. (2016). Myeloid Cell Turnover and Clearance. Microbiol. Spectr. *4*. https://doi.org/10.1128/microbiolspec.MCHD-0005-2015.
43. Dinarello, C.A. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol. Rev. *281*, 8–27. https://doi.org/10.1111/imr.12621.
44. Jang, D.I., Lee, A.H., Shin, H.Y., Song, H.R., Park, J.H., Kang, T.B., and Yang, S.H. (2021). The Role of Tumor Necrosis Factor Alpha (TNF-alpha) in Autoimmune Disease and Current TNF-alpha Inhibitors in Therapeutics. Int. J. Mol. Sci. *22*. https://doi.org/10.3390/ijms22052719.
45. Ohto, U., Fukase, K., Miyake, K., and Shimizu, T. (2012). Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. Proc. Natl. Acad. Sci. USA *109*, 7421–7426. https://doi.org/10.1073/pnas.1201193109.
46. Swanson, L., Katkar, G.D., Tam, J., Pranadinata, R.F., Chareddy, Y., Coates, J., and Ghosh, P. (2020). TLR4 signaling and macrophage inflammatory responses are dampened by GIV/Girdin. Proc. Natl. Acad. Sci. USA *117*, 26895–26906. https://doi.org/10.1073/pnas.2011667117.
47. Chua, H.L., Bhat-Nakshatri, P., Clare, S.E., Morimiya, A., Badve, S., and Nakshatri, H. (2007). NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene *26*, 711–724. https://doi.org/10.1038/sj.onc.1209808.
48. Anderson, C., Zhou, Q., and Wang, S. (2014). An alkali-burn injury model of corneal neovascularization in the mouse. J. Vis. Exp. *86*. https://doi.org/10.3791/51159.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal Zeb1 anti-serum	Dr. Douglas Darling	N/A
Rabbit pre-immune serum	Dr. Douglas Darling	N/A
Rabbit polyclonal iNOS antibody	Novus Biologicals	Cat.# NB300-605; RRID: AB_10002794
Mouse monoclonal Ki67 antibody	BD Pharmingen	Cat.# 550609; RRID: AB_393778
Rat monoclonal CD45 antibody	eBioscience	Cat.# 14-0451-81; RRID:AB_467250
Rabbit polyclonal NF-κB (p65) antibody	Cell Signaling	Cat.# 8242; RRID:AB_10859369
Mouse monoclonal β-Actin antibody	Sigma	Cat.# A1978; RRID:AB_476692
Mouse monoclonal CD11b antibody	eBioscience	Cat.# 53-0112-82; RRID:AB_469901
Mouse monoclonal H4 antibody	Qiagen	Cat.# GAH-2206; RRID:AB_10405876
Chemicals, peptides, and recombinant proteins		
Lipopolysaccharide (LPS)	Sigma-Aldrich	Cat.#L6529
Trypan blue solution	Sigma-Aldrich	Cat.# 93595
Invitrogen SYBR Green	ThermoFisher	Cat.#S7563
Critical commercial assays		
Red blood cell (RBC) lysis buffer	eBioscience	Cat.# 00-4333-57
Enhanced chemiluminescence (ECL) detection reagents	Cytiva	Cat.# RPN2134
Invitrogen SuperScript III reverse transcriptase	ThermoFisher	Cat.# 18080044
Nuclear dye Hoechst 33342	ThermoFisher	Cat.# 62249
Experimental models: Cell lines		
Mouse bone marrow-derived cells (BMC) of both Zeb1$^{f/f}$;Csf1r-Cre^{-} (Zeb1 wt) and Zeb1$^{f/f}$; Csf1r-Cre^{+} (Zeb1 cKO)	This paper	N/A
Experimental models: Organisms/strains		
Csfr1^{Cre} *(C57BL/6-Tg(Csf1r-cre)1Mnz/J Mus musculus* ***Genetic Background:*** *C57BL/6*	The Jackson Laboratory	Stock # 029206
Zeb1$^{f/f}$ *Mus musculus* ***Genetic Background:*** *C57BL/6*	Dr. Susan Kaech	N/A
Zeb1 wt: *Zeb1$^{f/f}$;Csf1r-Cre^{-} Mus musculus* ***Zeb1 cKO:*** *Zeb1$^{f/f}$;Csf1r-Cre^{+} Mus musculus* ***Genetic Background:*** *C57BL/6*	This paper	N/A
Oligonucleotides		
Primers, see Tables S1 and S2	This paper	N/A
Software and algorithms		
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
FlowJo	Tree Star Inc.	http://www.treestar.com/
Prism - GraphPad	Dotmatics	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for data should be directed to and will be fulfilled by the lead contact Yongqing Liu (y0liu016@louisville.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Csf1r-Cre mice[10] (C57BL/6-Tg(Csf1r-cre)1Mnz/J, Stock #: 029206) were purchased from the Jackson Laboratory while the Zeb1-floxed mice were kindly provided by Dr. Susan Kaech at Yale University School of Medicine. The Zeb1$^{f/f}$;Csf1r-Cre^{+} (Zeb1 conditional knockout or cKO in Csf1r^{+} cells) and their Cre^{-} control (Ctrl) mice were created by breeding Csf1-Cre mice with and backcrossing to the Zeb1-floxed mice. Tail tips of 1–2 weeks old pups are collected for genomic DNA isolation. PCR genotyping for *Zeb1 loxP* site is performed with the pair of primers: Zeb1-loxP F 5′- CCTGTAACCATAACTGGGTTAGAA and Zeb1-loxP R 5′- CAAACAGTGTGAAGCCAGAGG to identify the wt locus of 206-bp and the *loxP* locus of 240-bp. The floxed *Zeb1* exon 6 was excised by the Cre recombinase activity and was detected by PCR genotyping with cultured Zeb1$^{f/f}$;Csf1r-Cre^{+} bone marrow-derived cells (BMCs) using the primers: Zeb1 exon 6 F 5′- CCGCTGGATGGAGTTAAAAA and Zeb1 exon 6 R 5′- TGATGTGCAGCTTCTGGAAC. The *Cre* gene is identified by the primer pair of Cre F 5′- GCACTGATTTCGACCAGGTT and Cre R 5′- GCTAACCAGCGTTTTCGTTC.

METHODS DETAILS

Mouse model of alkali burn-induced corneal inflammation

Zeb1 cKO and their wildtype littermates (Zeb1 wt) of both sexes were anesthetized by an intraperitoneal (IP) injection of 100 mg/kg ketamine and 5 mg/kg xylazine and the corneal alkali burn was created as previously reporte.[4] Briefly, a 2-mm diameter filter disc with 2.5 μL of 1N NaOH was applied on the central cornea of one eye followed by a thorough rinse with PBS whereas the other eye was treated with PBS in the same way as a sham control. Three or four Zeb1 cKO and wt mice per time point for corneal sectioning were collected on day 0, 1, 4, 7 and 14 following alkali burn. One cornea was alkali-burned for each animal according to the Association for Research in Vision and Ophthalmology (ARVO)'s regulation. Animal studies were conducted according to the policies and guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) and approved by the University of Louisville, Kentucky, USA.

Bone marrow-derived cell (BMC) isolation and culture

For mouse bone marrow-derived cell (BMC) preparation, 3–4 above Zeb1 wt and Zeb1 cKO mice for each time point before and after the alkali burn were euthanized by CO_2 in a sealed container and their femur and tibia bones of rear legs were cut at both ends under sterile conditions as previously describe.[5] The BMCs were collected and counted directly for flow cytometric analysis. The BMCs were collected and cultured in 30% L929 cell culture-conditioned DMEM with 10% FBS as previously reporte.[5] The confluent BMCs were treated with 1 μg/mL of lipopolysaccharide (LPS, Sigma Cat. #L6529) for 1 h to mimic cell inflammatory response. These cultured BMCs were utilized for Western blot (WB) analysis.

Flow cytometry

Aliquots of 1x10^{6} BMCs were first blocked with CD32 and then stained with Alexa Fluor 488-conjugated CD11b (eBioscience Cat. # RM2820) or mouse IgG2a control (Biolegend Cat. # 400233) for 30 min at room temperature. Cells were passed through a flow cytometer (FACScaliber, BD Biosciences) to collect the flow data which was further analyzed by FlowJo (Ashland, OR).

Immunohistochemistry (IHC)

The eyeballs were enucleated and fixed for paraffin-section as previously describe.[4] All sections are sagittal cuts to ensure that the anterior central cornea and the posterior optical nerve bundle are in the same plane. Paraffin sections were used for IHC to identify Nfkb^{+} (Cell Signaling Cat. # 8242T), Ki67^{+} (BD Pharmingen Cat. # 550609), Zeb1^{+} (kindly provided by Dr. Douglas Darling) and TUNEL^{+} (Promega Cat. #G3250) cells. The respective primary antibody only and the secondary antibody only were served as negative background controls. For corneal wholemount immunostaining against CD45 (eBioscience Cat. # 14-0451-81) and iNOS (Novus Biologicals Cat. # NB300-605), the cornea with the limbus was isolated using a pair of surgical scissors and transferred to a 96-well plate. The stained corneas were thereafter placed on a glass slide with the epithelium layer facing up and cut quarterly using surgical spring scissors and forceps under a binocular dissecting microscope to make a butterfly-tie shape. All images were captured using the 20x lens by a Nikon confocal microscope with a fixed exposure setting. The staining areas were measured by ImageJ after setting up a fixed threshold, normalized to the DAPI staining areas in the same selected location, i.e., a marker intensity vs. DAPI intensity as a percentage (%) and statistically analyzed by Prism - GraphPad.

Real-time quantitative PCR (qPCR)

Total RNA was extracted using Trizol solution (ThermoFisher Cat. # 15596026) according to the manufacturer's instruction and the RNA quality and content were estimated by an 1% ethidium bromide-stained agarose gel. The first strand cDNA was synthesized using the Invitrogen SuperScript III reverse transcriptase (ThermoFisher Cat. # 18080044) according to the manufacturer's instruction. Real-time qPCR was performed with the Strategene Mx3000P system to collect the threshold cycle (Ct) values using SYBR green (ThermoFisher Cat. #S7563). The double delta formula was used to calculate the expression values of each desired gene after normalized to the expression of the house keeping gene *Rpl9*. Three independent tests were completed for each gene. Primer sequences for a particular gene were selected by the online software "Primer3" at the default settings and then synthesized by Integrated DNA Technology (IDT) (Table S1). All PCR products were verified by their size on an 1% agarose gel.

Total protein extraction and western blot (WB)

Total soluble proteins were extracted by lysing the cultured BMCs treated with 1 μg/mL of LPS or PBS control in the culture plates and separated by a 4–21% gradient SDS-PAGE gel as previously describe.[5] The proteins in the gel were transferred to a PVDC membrane at 4°C overnight. The membrane was incubated in a 5% milk blocking solution and then with the rabbit polyclonal Zeb1 anti-serum provided by Dr. Douglas Darling at the University of Louisville in the blocking solution at 4°C overnight. The amounts of Zeb1 protein on the membrane was visualized with the Cytiva ECL kit (Cat. # RPN 2134) and detected by an X-ray film. Beta actin (Actb, Sigma Cat. # A1978) was served as an internal control.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The TUNEL assays were performed using the DeadEnd Fluorometric TUNEL System (Promega Cat. #G3250) according to the manufacturer's instruction.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously using human uveal melanoma C918 cell genomic DN.[30] Rabbit polyclonal antiserum for Zeb1 as WB was used for immunoprecipitation whereas equal amount of pre-immune serum was used as a background control (IgG). The pan-histon 4 antibody (H4) from EpiTect ChIP antibody kit (Qiagen Cat. # GAH-2206) was used as a positive control. ChIP-PCR primer sequences and the expected PCR amplicon size are shown in Table S2.

QUANTIFICATION AND STATISTICAL ANALYSIS

Student's t-tests were conducted for two independent animal groups or cell samples comparison after an F-test confirmation that the comparable samples have an equal level of variance. two-way ANOVA was utilized to compare Zeb1 wt vs. Zeb1 cKO, and the corneas with vs. without the alkali burn. The animal sample size for all corneal section immunostaining is $n = 3$. All values in the graphs are presented as means ± standard deviations. '***' indicates p-value ≤ 0.001, '**' indicates p-value ≤ 0.01, whereas '*' indicates p-value ≤ 0.05. For qPCR, results were obtained from at least 3 independent experiments of three technical replicates.