BCL6 modulates tissue neutrophil survival and exacerbates pulmonary inflammation following influenza virus infection

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Neutrophils are vital for antimicrobial defense; however, their role during viral infection is less clear. Furthermore, the molecular regulation of neutrophil fate and function at the viral infected sites is largely elusive. Here we report that BCL6 deficiency in myeloid cells exhibited drastically enhanced host resistance to severe influenza A virus (IAV) infection. In contrast to the notion that BCL6 functions to suppress innate inflammation, we find that myeloid BCL6 deficiency diminished lung inflammation without affecting viral loads. Using a series of Cre-transgenic, reporter, and knockout mouse lines, we demonstrate that BCL6 deficiency in neutrophils, but not in monocytes or lung macrophages, attenuated host inflammation and morbidity following IAV infection. Mechanistically, BCL6 bound to the neutrophil gene loci involved in cellular apoptosis in cells specifically at the site of infection. As such, BCL6 disruption resulted in increased expression of apoptotic genes in neutrophils in the respiratory tract, but not in the circulation or bone marrow. Consequently, BCL6 deficiency promoted tissue neutrophil apoptosis. Partial neutrophil depletion led to diminished pulmonary inflammation and decreased host morbidity. Our results reveal a previously unappreciated role of BCL6 in modulating neutrophil apoptosis at the site of infection for the regulation of host disease development following viral infection. Furthermore, our studies indicate that tissue-specific regulation of neutrophil survival modulates host inflammation and tissue immunopathology during acute respiratory viral infection.

neutrophil | influenza | BCL6 | inflammation

Influenza A virus (IAV) infection-associated diseases are largely caused by a combination of the deleterious effects of virus destruction and lung inflammation/injury resulted from host immune response to clear the virus (1, 2). Neutrophils infiltrate into the lungs approximately 2 d post-IAV infection (3). It has been suggested that neutrophils may play important roles in facilitating viral clearance and disease resolution (4). Moreover, evidence indicates that neutrophils facilitate the recruitment of effector $CD8^+$ T cells into the infected lungs to promote viral clearance (5, 6). However, lung neutrophil accumulation has also been correlated with disease severity following IAV infection (7). The elevated numbers of recruited neutrophils may lead to the enrichment of inflammatory/tissue- damaging signaling networks (8, 9). Therefore, the numbers of neutrophils at the infected lungs must be tightly tuned to maximize efficient viral clearance while minimizing immunopathology (10). In such settings, the initiation of neutrophil apoptosis and consequent reduction in neutrophil numbers may be advantageous to limit lung inflammation and bystander tissue injury (11). To this end, however, a direct link between excess neutrophil recruitment and/or survival and the extent of pulmonary inflammation during IAV infection remains unclear. Furthermore, the molecular mechanisms regulating neutrophil fate and function during IAV infection, particularly at the infected site (i.e., lungs), are largely elusive.

The proto-oncogene B cell lymphoma 6 (*Bcl6*) encodes a BTB/POZ-zinc finger transcriptional repressor and is a master transcriptional regulator critical for the development and function of a number of immune cells (12). Beyond its role in T cell and B cell responses (13), BCL6 has also been recognized as an antagonist for NF-κB–mediated gene transcription to repress the production of certain proinflammatory mediators in macrophages (14, 15). A recent report has suggested that BCL6 expression in macrophages may repress the antiviral type I IFN production through its inhibition in IFN-regulatory factor 7 (Irf7) expression (16). However, the physiological function of BCL6 in regulating antiviral innate immune responses is currently unknown, since BCL6 germ line-deficient mice exhibit severe inflammation and autoimmunity (17).

Besides macrophages, the roles of BCL6 in regulating the responses of other myeloid cell populations, including neutrophils, have not been examined before. Here we have addressed the function of BCL6 in regulating innate immune responses and influenza viral pathogenesis using various conditional gene-deleted mice. We demonstrate that BCL6 expression in neutrophils was critical to

Significance

Influenza A virus (IAV) infection poses a significant challenge to public health. The cellular and molecular mechanisms regulating the development of excessive pulmonary inflammation following IAV infection remain largely undefined. We find that the loss of BCL6 in neutrophils mitigates lung inflammation and promotes host resistance to IAV infection. The data provide mechanistic insight into how BCL6 regulates neutrophil-mediated lung inflammation through modulating neutrophil apoptosis at the site of IAV infection. Thus, targeting neutrophil survival in the tissue may be a promising therapeutic option to decrease host inflammation and minimize lung injury for severe IAV infection.

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modulate neutrophil apoptosis in the lungs, but not in the circulation or bone marrow. Our studies suggest that prolonged tissue neutrophil survival, regulated by BCL6, is a critical contributor to lung immunopathology and host morbidity following IAV infection.

Results and Discussion

Myeloid BCL6 Deficiency Protects Hosts From IAV Infection Independent of IFNAR1 Signaling. To explore the cell type-specific roles of BCL6 in regulating antiviral responses, we generated myeloid cell-specific BCL6-deficient mice ($Bcl6^{\Delta Lyz2}$). The $Bcl6^{\Delta Lyz2}$ mice and littermate control mice ($Bcl6^{fl/fl}$) were monitored for outcomes after intranasal inoculation with a sublethal dose of IAV (A/PR8/34 strain; PR8). Compared with the $Bcl6^{fl/fl}$ mice, the $Bcl6^{\Delta Lyz2}$ mice exhibited drastically decreased weight loss and enhanced host recovery following sublethal PR8 infection (Fig. 1*A*). We also challenged the mice with a lethal dose of PR8. Approximately 70% of $Bcl6^{fl/fl}$ mice succumbed to infection, whereas all $Bcl6^{\Delta Lyz2}$ mice survived (Fig. 1*B*). The BTB domain of BCL6 is responsible for the recruitment of transcriptional corepressors. Mice engineered to express a BCL6 BTB mutant that cannot bind to corepressors show disruption of certain BCL6 functions in vivo (18). We found that mice expressing BCL6 BTB mutant ($Bcl6^{BTB}$) had diminished host morbidity and increased weight recovery following PR8 infection (Fig. 1*C*).

During viral infection, interferons (IFNs) are suggested to limit viral replication and promote an antiviral state for the host in the days early post-IAV infection (19). A recent report has suggested that BCL6 inhibition in macrophages could lead to enhanced type I IFN production on vesicular stomatitis virus infection (16). We evaluated IFN-α and IFN-λ levels in the lungs and bronchoalveolar lavage (BAL) fluid at 1 and 2 d postinfection (dpi). $Bcl6^{\Delta Lyz2}$ mice showed comparable production of IFN-α and modest reduction of IFN-λ in the lungs and BAL fluid compared with littermate control mice (Fig. 1*D* and *SI Appendix*, Fig. S1*A*). Furthermore, the expression levels of IFN-stimulated genes, including *Isg15*, *Ifitm3*, and *Mx1*, were similar in the lungs of $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice at 1 and 2 dpi (*SI Appendix*, Fig. S1*B*). These data suggest that type I IFNs are probably dispensable for the enhanced protection against IAV infection observed in myeloid BCL6-deficient mice.

To test this hypothesis more critically, we infected $Bcl6^{\Delta Lyz2}$ or $Bcl6^{fl/fl}$ mice lacking expression of type I IFN receptor ($Ifnar^{-/-}$) with PR8 and monitored disease severity. Indeed, loss of *Ifnar1* expression in $Bcl6^{\Delta Lyz2}$ or $Bcl6^{fl/fl}$ mice did not result in significantly increased morbidity following PR8 infection (Fig. 1*E*). To exclude the possibility that abnormalities due to the genetic deficiency of *Ifnar1* could affect the outcomes, we treated $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice with either IFNAR1-blocking or control antibody before and after PR8 infection. Anti-IFNAR1 treatment did not alter the morbidity of $Bcl6^{\Delta Lyz2}$ or $Bcl6^{fl/fl}$ mice compared with control antibody treatment (Fig. 1*F*). Notably, we observed that IFNAR1 deficiency only modestly affected host morbidity following PR8 infection in BCL6 WT mice (Fig. 1*E*). This observation is likely due to the viral evasion of type I IFN responses following PR8 infection (20) and/or the presence of innate antiviral defenses (e.g., type III IFNs) independent of type I IFN signaling (21). Of note, the deficiency in IFNAR1 has been shown to protect severe acute respiratory syndrome coronavirus infection (22). Together, these data suggest that BCL6 expression in myeloid cells is critical for modulating the host response to IAV infection, independent of the IFNAR signaling pathway.

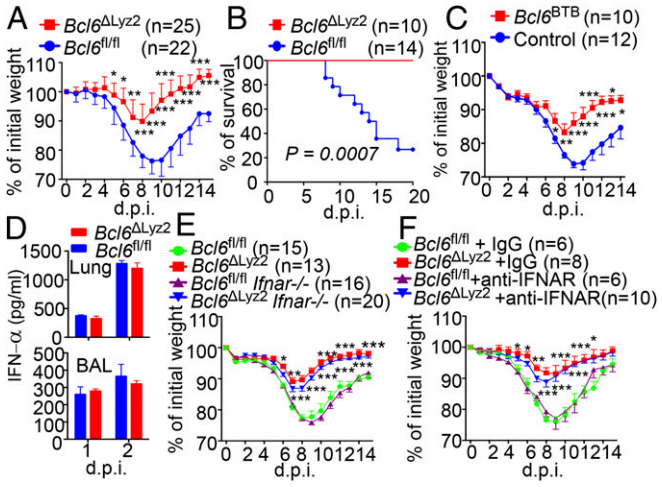

Fig. 1. Myeloid BCL6 deficiency enhances host resistance to severe IAV infection. (*A*) $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice were infected with sublethal PR8, and host morbidity (% of initial weight) was monitored. (*B*) $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice were infected with lethal PR8, and host mortality was monitored. (*C*) $Bcl6^{BTB}$ and C57BL/6 WT (control) mice were infected with sublethal PR8, and host morbidity was monitored. (*D*) IFN-α concentrations were measured in lung homogenate and BAL fluid of PR8-infected $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice at 1 and 2 dpi (n = 4–6). (*E*) Mice with indicated genotypes were infected with sublethal PR8, and host morbidity was monitored. (*F*) Host morbidity of PR8-infected $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice treated with anti-IFNAR or control antibody. In *A*–*C*, *E*, and *F*, data are pooled from at least two independent experiments. In *D*, data are representative of two independent experiments. Statistical differences are indicated. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Myeloid BCL6 Deficiency Diminishes Pulmonary Inflammation. During acute respiratory viral infection, the deleterious effects of viral replication contribute to pathogenesis (1, 23). However, we observed comparable viral titers in $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice and equal rates of viral clearance (Fig. 2*A*). The infectious viruses were completely cleared in both strains of mice at 10 dpi. (Fig. 2*A*). Consistent with the viral clearance data, $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice exhibited similar kinetics and magnitude of IAV-specific T cell responses in the lungs (Fig. 2*B* and *SI Appendix*, Fig. S1 *C* and *D*). These results suggest that the enhanced resistance of $Bcl6^{\Delta Lyz2}$ mice to IAV infection is probably independent of viral burden.

Since the lung inflammation and injury resulting from the host's excessive immune response to IAV contribute to host disease development (22–24), we next evaluated whether myeloid BCL6 regulates inflammatory responses during PR8 infection. We analyzed the levels of cytokines and chemokines in the BAL fluid of $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice at 2, 4, or 10 dpi by multiplex assay. A number of inflammatory cytokines and chemokines were significantly decreased in the BAL fluid of $Bcl6^{\Delta Lyz2}$ mice, particularly at 4 and 10 dpi (Fig. 2*C* and *SI Appendix*, Fig. S1*E*). These data suggest that myeloid BCL6 deficiency diminishes pulmonary inflammation following IAV infection in vivo. $Bcl6^{\Delta Lyz2}$ mice also exhibited diminished accumulation of inflammatory immune cells, including neutrophils and inflammatory monocytes, in the respiratory tract (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S1*F*), which has been shown to contribute to pulmonary immune pathology and mortality following IAV infection (1, 22, 23, 25). Notably, no differences in the number of neutrophils and inflammatory monocytes were detected in the respiratory tract of $Bcl6^{\Delta Lyz2}$ and $Bcl6^{fl/fl}$ mice at the baseline (Fig. 2 *D* and *E*), but those cells in the respiratory tract of $Bcl6^{\Delta Lyz2}$ mice were significantly reduced starting at 4 dpi. (Fig. 2 *D* and *E*). These results suggest that the decreased morbidity observed in $Bcl6^{\Delta Lyz2}$ mice to IAV infection is possibly due to decreased host inflammation rather than to viral burden following IAV infection.

Neutrophil BCL6 Deficiency Results in Diminished Host Inflammation and Morbidity. Lyz2-cre–mediated gene recombination occurs mainly in myeloid cells, including macrophages, neutrophils, and, to a lesser extent, $CD11b^+$ monocyte populations, following IAV infection (26) (*SI Appendix*, Fig. S2*A*). Thus, we sought to identify the cell types that expressed BCL6 and modulated host inflammation during IAV infection. We first crossed $Bcl6^{fl/fl}$ mice to CD11c-cre

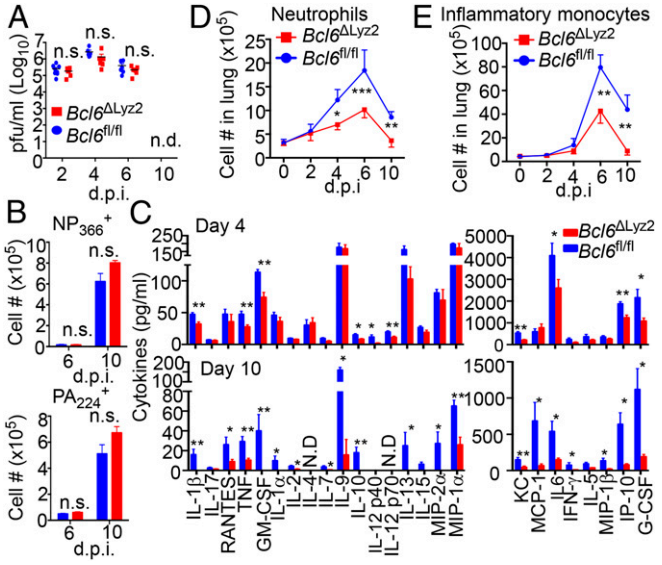

Fig. 2. Myeloid BCL6 promotes pulmonary inflammation following IAV infection. (*A–E*) *Bcl6*$^{\Delta Lyz2}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8. (*A*) Viral titers in BAL fluid were monitored over time. Each point represents one animal. (*B*) Lung IAV-specific NP_{366} and PA_{224} tetramer$^+$ CD8$^+$ T cells at 6 and 10 dpi. (*C*) Cytokine and chemokine concentrations were quantified by multiplex assay at 4 and 10 dpi ($n = 6$). (*D* and *E*) Flow cytometry quantification of neutrophils (CD11b$^+$Ly6G$^+$) (*D*) and inflammatory monocytes (CD11b$^+$Ly6G$^-$Ly6c$^+$) (*E*) in the lungs. In *A* and *C*, data are pooled from two independent experiments. In *B*, *D*, and *E*, data are representative of at least two independent experiments (n = 4–6 mice/group/experiment). Statistical differences are indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.s., not significant. n.d., not detected.

transgenic mice to generate *Bcl6*$^{\Delta CD11c}$ mice, in which BCL6 was disrupted in CD11c-expressing alveolar macrophages and dendritic cells. We also crossed *Bcl6*$^{fl/fl}$ mice to CX3CR1-cre transgenic mice to generate *Bcl6*$^{\Delta CX3CR1}$ mice, in which BCL6 was disrupted in both alveolar macrophages, CD11b$^+$Ly6C$^-$ monocytes and macrophages, and partially in Ly6C$^+$ monocytes (27, 28). We confirmed the reported gene recombination in CD11c-cre and CX3CR1-cre transgenic mice (*SI Appendix*, Fig. S2 *B* and *C*). Interestingly, *Bcl6*$^{\Delta CD11c}$ and *Bcl6*$^{\Delta CX3CR1}$ mice lost comparable amounts of weight compared with their WT littermate controls following PR8 infection (Fig. 3 *A* and *B*), suggesting that BCL6 deficiency in macrophages or Ly6C$^-$ monocytes does not alter mouse morbidity during IAV infection.

To study the contribution of inflammatory monocytes (Ly6C$^+$ monocytes) in regulating influenza-associated diseases in *Bcl6*$^{\Delta Lyz2}$ mice, we crossed *Bcl6*$^{\Delta Lyz2}$ and *Bcl6*$^{fl/fl}$ mice to *Ccr2*$^{-/-}$ mice to block inflammatory monocytes trafficking to infected lungs (25, 29). Consistent with a previous report (30), CCR2 deficiency did not affect host weight loss in WT mice. CCR2 deficiency also did not alter host morbidity in *Bcl6*$^{\Delta Lyz2}$ mice (Fig. 3*C*), suggesting that lung inflammatory monocyte infiltration is not required for the phenotypes observed in *Bcl6*$^{\Delta Lyz2}$ mice following IAV infection.

We next sought to determine whether BCL6 expression in neutrophils is responsible for the modulation of host disease during infection. To this end, we observed that neutrophils expressed detectable BCL6 following staining with BCL6 Ab or using BCL6-YFP reporter mice (Fig. 3*D*). We then crossed *Bcl6*$^{fl/fl}$ mice with MRP8-cre mice to generate *Bcl6*$^{\Delta MRP8}$ mice to delete BCL6 in neutrophils. We confirmed that MRP8-cre targeted mainly neutrophils but not other myeloid cells (28) (*SI Appendix*, Fig. S2*D*). *Bcl6*$^{\Delta MRP8}$ mice showed significantly lower host morbidity than control mice following a sublethal dose of PR8 infection (Fig. 3*E*). We also challenged the mice with a lethal dose of PR8. Strikingly, 80% of *Bcl6*$^{fl/fl}$ mice succumbed to lethal PR8 infection, whereas approximately 80% of *Bcl6*$^{\Delta MRP8}$ mice survived (Fig. 3*F*). *Bcl6*$^{\Delta MRP8}$ mice exhibited similar kinetics of infectious virus clearance as control mice (Fig. 3*G*). Consistent with the viral clearance data, *Bcl6*$^{\Delta MRP8}$ mice had comparable numbers of T cell responses in the lungs as control mice (Fig. 3*H* and *SI Appendix*, Fig. S2 *E–G*).

We next examined the effects of neutrophil BCL6 deficiency on the accumulation of inflammatory mediators in the airway during IAV infection. Loss of BCL6 in neutrophils resulted in diminished levels of inflammatory cytokines and chemokines in *Bcl6*$^{\Delta MRP8}$ mice compared with *Bcl6*$^{fl/fl}$ mice at 3 and 9 dpi (Fig. 3*I*). Collectively, the loss of BCL6 in neutrophils rather than in dendritic cells, macrophages, or monocytes significantly protected the mice from severe IAV infection. Of note, *Bcl6*$^{\Delta MRP8}$ mice appeared to develop modestly more severe disease during sublethal IAV infection and showed relatively less protection against lethal IAV infection compared with *Bcl6*$^{\Delta Lyz2}$ mice. Thus, the deficiency of BCL6 in

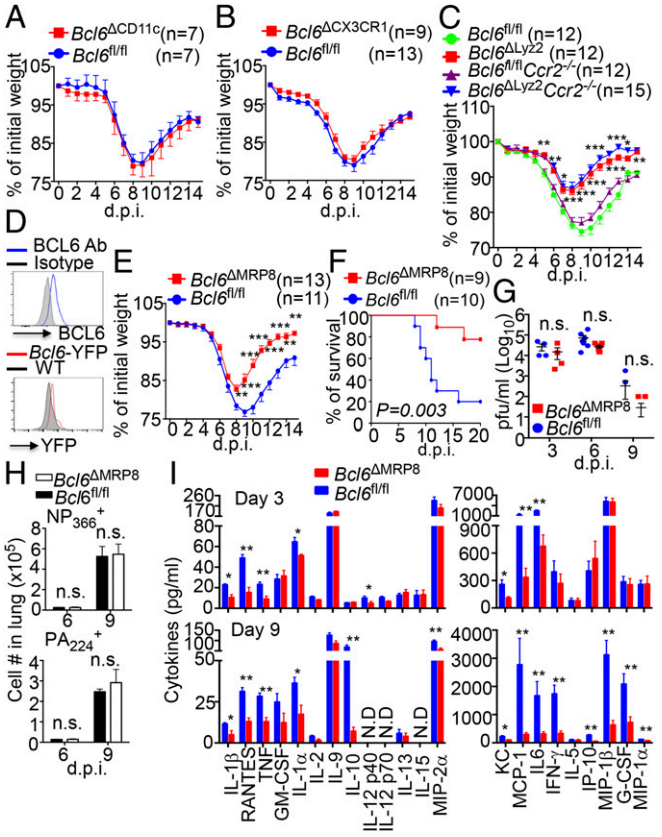

Fig. 3. Neutrophil BCL6 deficiency decreases host inflammation and morbidity. (*A*) *Bcl6*$^{\Delta CD11c}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8, and host morbidity (% of initial weight) was monitored. (*B*) *Bcl6*$^{\Delta CX3CR1}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8, and host morbidity was monitored. (*C*) Mice with indicated genotypes were infected with sublethal PR8, and host morbidity was monitored. (*D*) Flow cytometry analysis of BCL6 expression, with BCL6 staining in neutrophils (*Upper*) and YFP expression in neutrophils in WT and BCL6-YFP reporter mice (*Lower*). (*E*) *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8, and host morbidity was monitored. (*F*) *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice were infected with lethal PR8, and host mortality was monitored. (*G–I*) *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8. (*G*) Viral titers in the BAL fluid over time (n = 4–7). (*H*) Lung IAV-specific NP_{366} and PA_{224} tetramer$^+$ CD8$^+$ T cells at 6 and 9 dpi. (*I*) Cytokine and chemokine concentrations were quantified by multiplex assay at 3 and 9 dpi ($n = 6$). In *A–C* and *E*, data are pooled from at least two independent experiments. In *D* and *H*, data are representative of two independent experiments (n = 4–6 mice/group/experiment). Statistical differences are indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. n.s., not significant. n.d., not detected.

other myeloid cells may "aid" neutrophil BCL6 deficiency to maximally protect the host from IAV-induced inflammation and diseases.

BCL6 Represses Apoptotic Gene Expression in Neutrophils Specifically at the Site of Infection. To gain insight into why loss of BCL6 expression in neutrophils resulted in decreased inflammatory responses and enhanced host resistance to IAV infection, RNA sequencing was performed to profile the transcriptional signatures in neutrophils from lungs of PR8-infected *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice. Approximately 133 genes were down-regulated and 294 genes were up-regulated in BCL6-deficient neutrophils vs. WT neutrophils (Fig. 4*A*).

To further characterize these differences, we performed gene set enrichment analysis on the gene expression profiles using the hallmark collection of the Molecular Signatures Database. The loss of BCL6 expression in neutrophils resulted in significant enrichment of both the inflammatory response and TNF signaling via NF-κB gene sets compared with WT neutrophils (Fig. 4*B* and *SI Appendix*, Fig. S3*A*), suggesting that BCL6 suppresses inflammatory cytokine expression in neutrophils following IAV infection. These data are consistent with previous reports that BCL6 suppresses proinflammatory mediators in macrophages, possibly through inhibition of NF-κB transcriptional activity (14, 15).

Although we observed increased enrichment of mRNA for the inflammatory response in neutrophils from BCL6-deficient mice (Fig. 4*B* and *SI Appendix*, Fig. S3*A*), the overall protein levels of proinflammatory cytokines and chemokines were significantly lower in the lungs of BCL6-deficient mice (Fig. 3*I*). Thus, we postulated that the diminished pulmonary inflammation observed in *Bcl6*$^{\Delta MRP8}$ mice may be related to the decreased neutrophil numbers but not to the diminished expression of inflammatory cytokines on a per cell basis. Consistent with this hypothesis, genes associated with apoptosis signature were significantly up-regulated in neutrophils from *Bcl6*$^{\Delta MRP8}$ mice compared with *Bcl6*$^{fl/fl}$ mice (Fig. 4*C*). In germinal center B cells, BCL6 has been reported to suppress expression of the P53 tumor-suppressor gene and modulate the P53-dependent apoptotic response (31). Consistently, BCL6 repressed gene signatures in P53 pathway in neutrophils following IAV infection (Fig. 4*D*). Indeed, the P53 pathway has been shown to regulate neutrophil apoptosis in the tissue (32). Analysis of the core genes from apoptosis gene sets indicated up-regulation of certain apoptosis-related genes in BCL6-deficient neutrophils, including *Pmaip1* (encodes Noxa), *Casp3*, and *Pdcd4* (Fig. 4*E*, genes marked in red). We confirmed that BCL6-deficient neutrophils in the BAL fluid exhibited enhanced *Pmaip1*, *Casp3*, and *Pdcd4* expression compared with their WT counterparts through quantitative RT-PCR (Fig. 4*F*). Interestingly, BCL6-deficient neutrophils isolated from the blood or bone marrow showed comparable *Pmaip1*, *Casp3*, and *Pdcd4* expression as their WT counterparts (Fig. 4*F*), suggesting that BCL6 controls apoptotic gene expression specifically at the site of infection.

We next sought to determine the underlying mechanisms by which BCL6 controls apoptotic gene expression in neutrophils at the infection site, but not in the circulation or in the bone marrow. We first examined BCL6 expression levels in the bone marrow, blood, and infected lungs and found that neutrophils in the bone marrow and blood had comparable BCL6 expression to that of their counterparts in the lungs, kidney, and liver before and after IAV infection (Fig. 4*G* and *SI Appendix*, Fig. S3*B*), suggesting that the tissue-specific effects of BCL6 in controlling neutrophil apoptotic gene expression are not due to differential BCL6 expression levels. BCL6 acts mainly as a transcriptional repressor to modulate gene expression (33).

We next asked whether BCL6 was recruited to the loci of apoptotic genes in neutrophils, and whether the recruitment was subject to tissue-specific regulation. We performed a chromatin immunoprecipitation (ChIP) assay for BCL6 in neutrophils purified from the infected lungs or the bone marrow. Due to the large numbers of cells required for the ChIP assay, we did not perform BCL6 ChIP analysis in blood neutrophils. Strikingly, BCL6 robustly bound to the *Casp3* and *Pmaip3* loci, and to a lesser extent to the *Pdcd4* locus, in neutrophils isolated from the infected lungs (Fig. 4*H*). However, BCL6 showed limited binding to these gene loci in neutrophils isolated from the bone marrow, even though bone marrow neutrophils expressed robust BCL6 (Fig. 4 *G* and *H*). Thus, BCL6 appears to control apoptotic gene transcription in a tissue type-specific fashion in neutrophils, which explains the specific increase of apoptotic gene expression in tissue neutrophils but not in the circulation or the bone marrow following BCL6 deficiency.

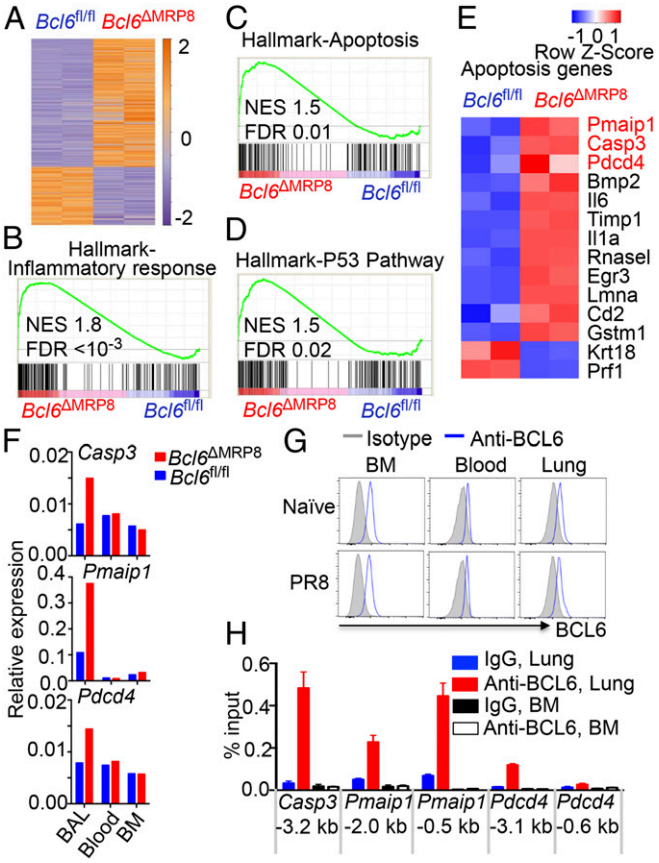

Fig. 4. BCL6 represses apoptotic gene expression in neutrophils of lungs following IAV infection. (*A–E*) Transcriptomic data for lung neutrophils sorted from *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice infected with sublethal PR8 at 6 dpi. (*A*) Heat map of 427 out of 11,523 RNA transcripts differentially expressed in neutrophils. (*B–D*) Enrichment plots from gene set enrichment analysis of BCL6-deficient neutrophils compared with WT neutrophils using the hallmark gene set collection from the Molecular Signatures Database. (*E*) mRNA expression (normalized log2) of core genes from apoptosis gene sets in lung neutrophils from *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice. Red and blue represent overexpressed and underexpressed genes, respectively. (*F*) *Casp3*, *Pmaip1*, and *Pdcd4* transcript levels in neutrophils of BAL fluid, blood, and bone marrow from *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice infected with PR8 at 6 dpi were analyzed by RT-PCR. Neutrophils were harvested from four mice per genotype and pooled for gene expression studies. (*G*) Representative flow cytometry plots of BCL6 expression showing BCL6 staining in neutrophils of bone marrow, blood, and lung from naïve mice and PR8-infected mice at 6 dpi ($n = 4$). (*H*) ChIP analysis of BCL6 enrichment relative to input chromatin in lung and bone marrow neutrophils of PR8-infected WT mice at 6 dpi. The annotated gene names and positions of the BCL6-binding sites relative to the transcription start sites are listed. Neutrophils were harvested from 10 mice and pooled for ChIP studies.

BCL6 Regulates Tissue Neutrophil Cellular Apoptosis. Previous studies have shown that IAV infection induces neutrophil apoptosis (34). Our RNA-sequencing data also indicate that gene signatures implicated in apoptosis process were up-regulated in neutrophils from *Bcl6*$^{\Delta MRP8}$ mice, suggesting that BCL6-deficient neutrophils may undergo enhanced cell death compared with control WT neutrophils. To examine this idea, we measured neutrophil apoptosis in *Bcl6*$^{\Delta Lyz2}$, *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice by FACS analysis using propidium iodide (PI) or Annexin V staining on PR8 infection. The percentage of apoptotic neutrophils (Annexin V$^+$/PI$^-$) was higher in BAL fluid and lungs from *Bcl6*$^{\Delta Lyz2}$ and *Bcl6*$^{\Delta MRP8}$ mice compared with *Bcl6*$^{fl/fl}$ mice, whereas inflammatory monocyte apoptosis remained unchanged (Fig. 5*A* and *SI Appendix*, Fig. S3 *C* and *D*). Notably, lung neutrophil apoptosis was comparable in *Bcl6*$^{fl/fl}$ and *Bcl6*$^{\Delta MRP8}$ mice without PR8 infection (*SI Appendix*, Fig. S3*E*). These data suggest that BCL6 is critical to the control of neutrophil apoptosis in the lungs following IAV infection. To further confirm this notion, we also assessed active caspase 3/7 activity in neutrophils following PR8 infection. BCL6 deficiency resulted in increased caspase 3/7 activity in the respiratory tract but not in the bone marrow (*SI Appendix*, Fig. S3*F*), further suggesting that BCL6 deficiency promotes neutrophil apoptosis at the site of infection.

We next determined whether neutrophils in bone marrow and blood were similarly affected in *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice. Strikingly, no significant differences in the percentage of apoptotic neutrophils in bone marrow or blood were observed between *Bcl6*$^{\Delta MRP8}$ or *Bcl6*$^{fl/fl}$ mice (Fig. 5 *B* and *C* and *SI Appendix*, Fig. S4*A*). Thus, BCL6 modulates neutrophil survival specifically in the lungs following IAV infection.

To determine whether the increased lung neutrophil apoptosis observed in neutrophil-specific BCL6-deficient mice is influenza-strain specific, we infected *Bcl6*$^{\Delta MRP8}$ or *Bcl6*$^{fl/fl}$ mice with influenza A/X31 (X31, H3N2) strain and assessed neutrophil apoptosis in the BAL fluid, lung, blood, and bone marrow. Loss of BCL6 on X31 infection increased neutrophil apoptosis in the BAL fluid and lung but not in bone marrow or blood (*SI Appendix*, Fig. S3*G*). These results demonstrate that BCL6 deficiency-promoted lung neutrophil apoptosis is not viral strain-specific. The frequencies of mature neutrophils in bone marrow and blood were similar in *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice (*SI Appendix*, Fig. S4*B*).

Next, we sought to determine whether a difference in neutrophil survival contributes to neutrophil accumulation in the lungs of *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice. To do so, we quantified neutrophil numbers in the lungs and BAL fluid following PR8 infection. The *Bcl6*$^{\Delta MRP8}$ mice had lower numbers of neutrophils in the lung and BAL fluid, particularly at 4 and/or 6 dpi (Fig. 5*D* and *SI Appendix*, Fig. S4*C*), which marks the peak neutrophil levels in both groups, ruling out a delayed neutrophil response in *Bcl6*$^{\Delta MRP8}$ mice. Therefore, increased apoptosis of neutrophils in the lungs of BCL6-deficient mice resulted in the decrease of those cells in the respiratory tract following IAV infection.

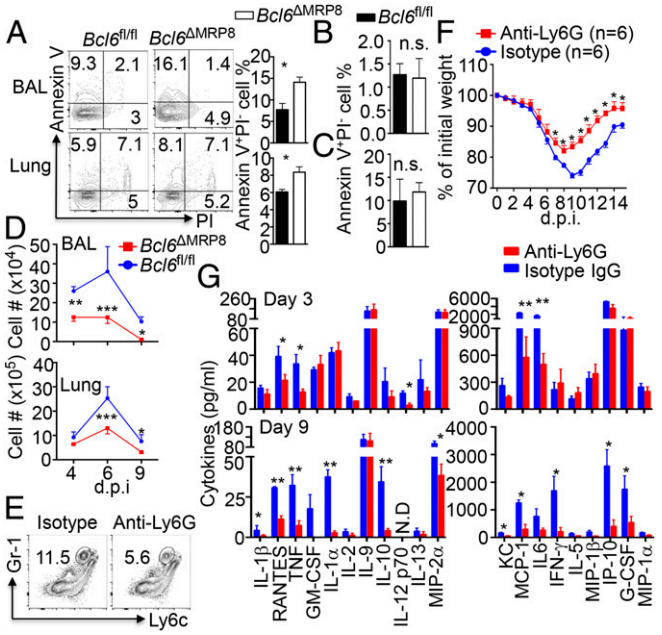

Fig. 5. Attenuation of neutrophils ameliorates host inflammation. (*A–C*) *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice were infected with sublethal PR8. (*A*) Neutrophil apoptosis stained with Annexin V and PI in the BAL fluid and lung at 2 dpi. Representative flow cytometry plots (*Left*) and the percentages of apoptotic neutrophils (Annexin V$^+$ PI$^-$) (*Right*) are shown. (*B* and *C*) Neutrophil apoptosis stained with Annexin V and PI in the bone marrow (*B*) and blood (*C*) at 2 dpi. The percentages of apoptotic neutrophils (Annexin V$^+$ PI$^-$) are shown. (*D*) Absolute numbers of neutrophils in the BAL fluid and lung from *Bcl6*$^{\Delta MRP8}$ and *Bcl6*$^{fl/fl}$ mice infected with PR8 over time. Data are representative of three independent experiments (n = 4–6 mice/group/experiment). (*E–G*) C57BL/6 mice treated with anti-Ly6G antibodies or isotype antibodies were infected with sublethal PR8. (*E*) Representative flow cytometry plots showing the percentage of lung neutrophils at 3 dpi (n = 3–4). (*F*) Host morbidity (% of initial weight) was monitored. (*G*) Cytokine and chemokine concentrations were quantified by multiplex assay at 3 and 9 dpi (n = 3–4). Statistical differences are indicated. *P < 0.05; **P < 0.01; ***P < 0.001. n.d., not detected. n.s., not significant.

Excess Neutrophils Drive Pulmonary Inflammation. Although exuberant neutrophil recruitment has been associated with enhanced host morbidity and detrimental inflammation (7, 8), the direct link between excessive neutrophil recruitment and pulmonary inflammation following IAV infection has been missing. To explore this, we partially depleted pulmonary neutrophils during the early phase of IAV infection using a low dose of a neutrophil-depleting antibody (anti-Ly6G, 25 μg/mouse). Here the limited anti-Ly6G–based neutrophil depletion caused roughly 50% decrease in the number of lung neutrophils without affecting inflammatory monocytes during infection (Fig. 5*E* and *SI Appendix*, Fig. S4*D*). Mice with reduced neutrophil numbers showed diminished morbidity to PR8 infection compared with mice with normal neutrophil levels (Fig. 5*F*), a phenotype similar to that of *Bcl6*$^{\Delta MRP8}$ mice (Fig. 3*E*).

We also measured cytokine and chemokine levels in the BAL fluid of mice treated with or without anti-Ly6G at 3 and 9 dpi (Fig. 5*G*). A number of inflammatory cytokines and chemokines were decreased in the BAL fluid of anti-Ly6G–treated mice (Fig. 5*G*), similar to observations in *Bcl6*$^{\Delta MRP8}$ mice (Fig. 3*I*). These data suggest that excessive accumulation of neutrophils in the lungs drives an exaggerated inflammatory response and increases disease severity following IAV infection. Consistently, dysregulated neutrophil responses in the lungs led to the accumulation of inflammatory damaging signaling networks, which act as an important driver of H1N1 IAV-induced lethality (8). Of note, neutrophils may contribute to viral clearance and adaptive immunity (3, 6, 35). Indeed, previous data show that total ablation of neutrophils by the injection of a higher dose of anti-Ly6G promotes host mortality (4, 36), although it is possible that high-dose anti-Ly6G antibody may cause bystander deletion of Ly6G^{low} non-neutrophil cells that are important in antiviral responses (8). Nevertheless, our data are consistent with previously reported findings that the attenuation of deleterious neutrophil responses with low doses of anti-Ly6G provides protection against lethal IAV infection (8). Our data and the findings reported by Brandes et al. (8) support the view that early engagement of neutrophils instigates a damaging feedforward innate inflammatory circuit responsible for severe disease development following IAV infection.

Neutrophils are the first responders to infiltrate the sites of infection to clear bacterial infections (37, 38). In this regard, enhanced tissue neutrophil apoptosis caused by BCL6 deficiency may increase pulmonary bacterial burden and worsen outcome during certain respiratory bacterial infections, such as *Staphylococcus pneumonia* infection (37, 38). Conversely, excessive neutrophil recruitment is a hallmark of acute susceptibility to *Mycobacterium tuberculosis* and is associated with overexuberant inflammatory response and disease progression (39). In this case, loss of neutrophil BCL6 may provide benefits to decrease tissue damage during *M. tuberculosis* infection. Thus, the precise roles of neutrophil BCL6 in modulating differential disease outcomes following respiratory infections may ultimately depend on the type of pathogen.

In conclusion, we have identified a role for BCL6 in modulating neutrophil survival in the tissue, thereby regulating the severity of diseases following IAV infection. Furthermore, our studies indicate that neutrophil survival is tightly regulated specifically at the sites of infection, thereby influencing host inflammation and tissue immunopathology during acute respiratory viral infection. An important unanswered question is why the enhanced neutrophil death following BCL6 deficiency is restricted to the lungs and does not occur in the blood or bone marrow. To this end, our data show that BCL6 could bind to *Pmaip1* (encoding Noxa) promoter, and that BCL6 deficiency resulted in increased levels of *Pmaip1* in lung neutrophils. Noxa is a BH3-only protein that can bind and trigger proteasome-mediated degradation of the prosurvival protein Mcl-1 (myeloid cell factor 1) (40), which plays essential roles in neutrophil survival (38, 41). Notably, lung neutrophils express higher Noxa levels than blood and bone marrow neutrophils in WT mice, whereas BCL6 deficiency further increases Noxa expression specifically in the lungs. Thus, it is possible that enhanced Noxa expression promotes lung neutrophil apoptosis through antagonizing Mcl-1 expression and/or function in BCL6-deficient neutrophils. Further studies are needed to test this possibility. Further studies are also warranted to elucidate the exact downstream mechanisms by which live neutrophils contribute to host inflammation and injury following IAV infection. The development of pharmacologic agents that target neutrophil survival and function in the tissue may be promising to decrease host inflammation and minimize lung injury for the treatment of severe IAV infection.

Materials and Methods

All animal experiments were approved by the Institutional Animal Care and Use Committees of Indiana University School of Medicine and the Mayo Clinic. The animals, virus infection, experimental setup, sample collection, and statistical analysis are described in detail in *SI Appendix, Materials and Methods*.

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