

CNS-targeted autoimmunity leads to increased influenza mortality in mice

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The discovery that central nervous system (CNS)-targeted autoreactive T cells required a process of licensing in the lung revealed an unexpected relationship between these organs. The clinical and immunological significance of this finding is bidirectional in that it showed not only a mechanism by which T cells become pathogenic before entering the CNS, but also the potential for this process to influence lung immunity as well. Epidemiological studies have shown that people with multiple sclerosis (MS) suffer from increased morbidity and mortality from infectious diseases, independent of immunosuppressive therapies. Respiratory infections account for a large percentage of deaths of people with MS. In this study, to investigate the mechanisms responsible for this enhanced susceptibility, we established a comorbid model system in which mice with experimental autoimmune encephalomyelitis (EAE) were administered a sublethal dose of influenza. Whereas mice with either EAE alone or influenza alone survived, 70% of comorbid mice died as a result of uncontrolled viral replication. Immunological analyses revealed that the induction of EAE led to a surprising alteration of the lung milieu, converting an effective stimulatory influenza-reactive environment into a suppressive one. These results provide mechanistic information that may help to explain the unexpected immunological interactions.

INTRODUCTION

The potential for an immune response in one organ to influence activation, reactivation, and suppression of immune responses in other distant organs creates several immunological and clinical complexities. Thus, dissection of the mechanisms behind effects of comorbidities involving the immune system can be difficult. Central nervous system (CNS) immunity is particularly enigmatic in this regard because of its relative isolation via the blood-brain barrier. One CNS-targeted disease that is especially affected by immunological comorbidities is multiple sclerosis (MS). In both MS and the animal model experimental autoimmune encephalomyelitis (EAE), autoreactive activated T cells cross the blood-brain barrier and take up at least temporary residence in the CNS. MS is a heterogeneous disease clinically but is typically characterized by inflammatory demyelination of the CNS and is generally considered to be an autoimmune disease. CD4⁺ T cells migrate to the brain and spinal cord and are reactivated by resident APCs, secrete inflammatory cytokines including IFN- γ and IL-17A, recruit other immune cell types to the CNS to augment inflammation, and cause damage within the CNS. Although the etiology of MS remains undefined,

both genetics and environment likely play a strong role (Stromnes and Goverman, 2006).

People with MS appear to be particularly susceptible to increased morbidity and mortality from additional diseases involving the immune system. Comorbidities of the immune system have been reported in numerous studies in people with MS, with data demonstrating that this patient population suffers an increased incidence and severity of infections compared with people without MS (Sumelahti et al., 2010; Marrie et al., 2014). The overall impact of infections on people with MS is devastating, as they have increased numbers of hospital visits, sick days, higher medical costs, and overall lower quality of life as well as higher death rates from infections (Kang et al., 2010; Montgomery et al., 2013; Marrie et al., 2015). Infections in general account for many of the premature deaths, and respiratory infections such as influenza and pneumonia were reported four times more frequently as a cause of death in people with MS than in their age-, sex-, and ethnicity-matched counterparts (Redelings et al., 2006; Rodríguez-Antigüedad Zarranz et al., 2014). Additional studies supported the finding that respiratory tract infections, in particular, follow a more severe (and more often deadly) course in people with MS (Sumelahti et al., 2010), and numerous studies have shown that infectious disease is a

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Abbreviations used: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cell; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NP, nucleoprotein; PTX, pertussis toxin.

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recurrent MS comorbidity that accounts for excess deaths of MS subjects when compared with non-MS counterparts in carefully controlled retrospective studies (Koch-Henriksen et al., 1998; Lalmohamed et al., 2012; Jick et al., 2014; Capkun et al., 2015; Marrie et al., 2015).

Despite the implications of comorbidity, surprisingly little is known about the interactions between two seemingly disparate diseases such as MS and respiratory infections. A recent interesting and unexpected relationship was demonstrated by Odoardi et al. (2012) whose results revealed that in order for myelin-reactive T cells to become pathogenic, they were required to pass through the lung. This acquisition of pathogenicity or licensure in the lung may provide opportunity for the development of CNS-targeted T cells to influence the milieu of the lung, thereby affecting the generation of additional immune responses. This process may be sustained, as it was also shown that the lung was a repository for myelin-reactive effector cells (Odoardi et al., 2012). In addition, this potential is further enhanced by the finding that the lung itself provides a specialized immunological site, as other studies showed that lung-resident dendritic cells could imprint T cells with a signature profile (Mikhak et al., 2013). Given the apparently active role of the lung in the generation of CNS autoimmunity, we sought to assess the effects of the immunological interactions within the lung resulting from induction of EAE that might contribute to the clinical observation of enhanced respiratory infection susceptibility in people with MS. Toward this end, we developed a comorbid model of EAE and sublethal influenza infection to better understand the cellular and molecular mechanisms by which neuroinflammatory autoimmune disease influences lung immunity.

RESULTS AND DISCUSSION

EAE and influenza-infected comorbid mice exhibit increased morbidity and mortality

To develop a model system to evaluate comorbidity, we immunized C57BL/6 mice for EAE and, 7 d later (before EAE symptom presentation), intranasally inoculated a sublethal dose of influenza A/Puerto Rico/8/34 (PR8). Comorbid mice exhibited a striking increase in mortality, ultimately resulting in death of 70%, whereas no mouse in either the EAE- or influenza-only group suffered mortality (Fig. 1 A). To evaluate morbidity, we compared percentage of weight loss among the three groups, as both EAE and influenza cause weight loss. Each single-affliction group followed a typical course, as EAE mice exhibited a continually variable percentage of body weight loss which peaked at 13% average body weight loss but eventually decreased to 8% (Fig. 1 B). Influenza-infected mice steadily lost weight, dropping to an average of 15% percent body loss, and eventually returned to baseline body weight. In contrast, the comorbid mice continuously lost body weight from initial influenza inoculation and exhibited the highest percent of body weight loss of the three groups at 25% and failed to recover, correlating with progression to death. Two important components of ac-

tive EAE immunization are CFA and pertussis toxin (PTX). To verify that the observed effects were specific to the encephalitogenic peptide myelin oligodendrocyte glycoprotein 35–55 (MOG_{35–55}), we also immunized additional mice with the non-CNS-targeted, foreign control peptide OVA_{323–339} with CFA and PTX and inoculated with influenza. OVA_{323–339} + CFA/PTX mice exhibited minimal mortality at 13% and significantly less morbidity compared with influenza-afflicted mice (Fig. 1, C and D). These findings indicate that CFA and PTX are not sufficient on their own to predispose to exacerbated influenza comorbidity and mortality but, rather, that there is a specificity related to the CNS-targeted MOG_{35–55}.

Comorbidity leads to failure to control viral replication in the lung but does not affect EAE severity

The profound increase in morbidity and mortality of the comorbid group led us to assess each individual aspect of the two afflictions. EAE, Flu, and comorbid disease groups were thus established and either evaluated for EAE progression for the duration of the study or sacrificed to analyze intensity of viral disease as measured by viral titers and lung pathology. In the analysis of EAE progression, we determined both disease severity and immunological parameters, including expansion and cytokine secretion of myelin-reactive 2D2 CD4⁺ T cells. No significant differences between the severities of the EAE scores were noted between the EAE and comorbid groups (Fig. 2 A). Further, similar numbers and percentages of myelin-reactive 2D2 CD4⁺ T cells and T helper 17 cells (Th17 cells) were noted in the lungs of EAE and comorbid groups (Fig. 2, B and C), together indicating that the increased mortality was not likely caused by increased EAE severity. In contrast, evidence of viral-mediated pathology was notably different between the influenza-only group and the comorbid group. Mice treated with influenza alone exhibited a typical viral course of early weight loss, followed by recovery, with weight returning to normal and viral clearance achieved. Control of viral replication requires the mobilization and expansion of both innate immune cells and ultimately virus-reactive CD8⁺ effector T cells. Activated CD8⁺ T cells provide protective immunity against acute influenza infection and are required for efficient viral clearance (Bender et al., 1992; Thomas et al., 2006). One marker of antiviral CD8⁺ T cell activation is production of the proinflammatory, antiviral cytokine IFN- γ . As comorbid mice failed to resolve lung cell infiltration and influenza virus replication, we sought to determine whether comorbid mice exhibit defective influenza-specific CD8⁺ T cell responses. Therefore, we analyzed and compared the immune responses of mice that were infected with influenza alone or in conjunction with EAE. After infection, the singly infected group mobilized both innate (NK cells) and later tetramer-positive influenza-specific CD8⁺ T cells that secreted IFN- γ . In contrast, the comorbid mice had significantly fewer effector cells, either innate or acquired, suggesting the lack of immune stimulation in response to infection.

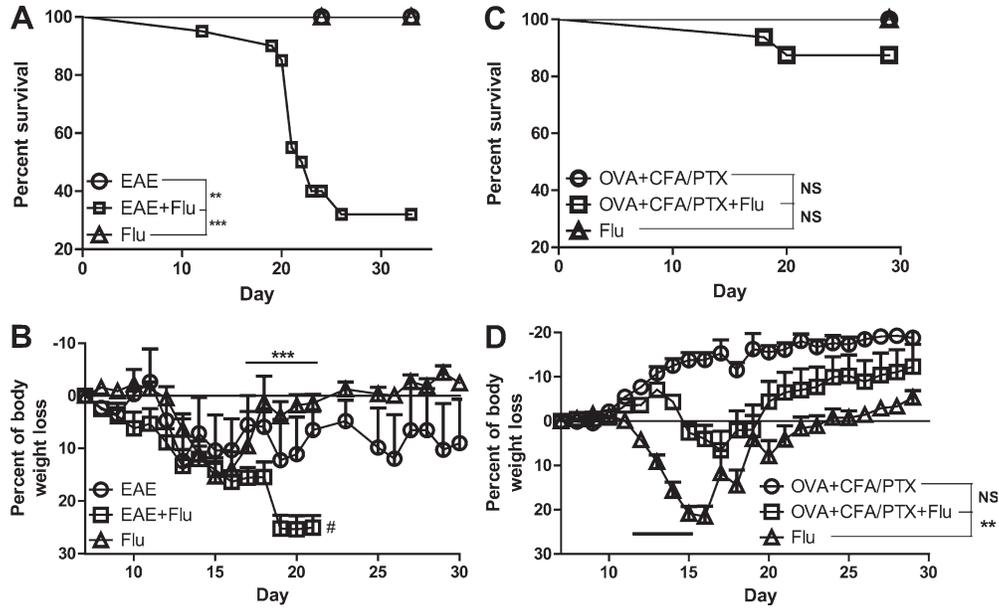


Figure 1. **EAE predisposes mice to increased morbidity and mortality from influenza infection.** (A–D) C57BL/6 mice were immunized with MOG_{35–55} EAE (A and B) or OVA_{323–339} + CFA/PTX (C and D) on day 0. On day 7, naive mice and a subset of EAE or OVA-administered mice were inoculated with a sub-lethal dose of influenza. Mice were monitored daily for survival (A and C) and body weight loss (B and D). (B) Comparison of comorbid mouse body weight loss measurement to EAE and Flu mouse groups was ceased at day 22 because of significant loss of comorbid mice for meaningful analysis, denoted by the symbol #. Each graph is a combination of two independent experiments. (A and B) EAE, *n* = 7; Flu, *n* = 13; EAE + Flu, *n* = 20. (C and D) OVA + CFA/PTX, *n* = 8; Flu, *n* = 10; OVA + CFA/PTX + Flu, *n* = 16. To evaluate the significance of observed differences, the log-rank (Mantel-Cox) test (A and C) and one-way ANOVA (Tukey's multiple comparison test; B and D) were used. **, *P* < 0.01; ***, *P* < 0.001. The mean and standard error of means are represented.

In acute infection at day 3 after influenza infection, NK and CD8⁺ T cells likely control early influenza infection, and indeed, they up-regulated IFN- γ production in influenza-

only-infected mice but failed to do so in the comorbid condition (Fig. 3). During the virus resolution phase, influenza-infected mice effectively cleared the virus, and their lungs

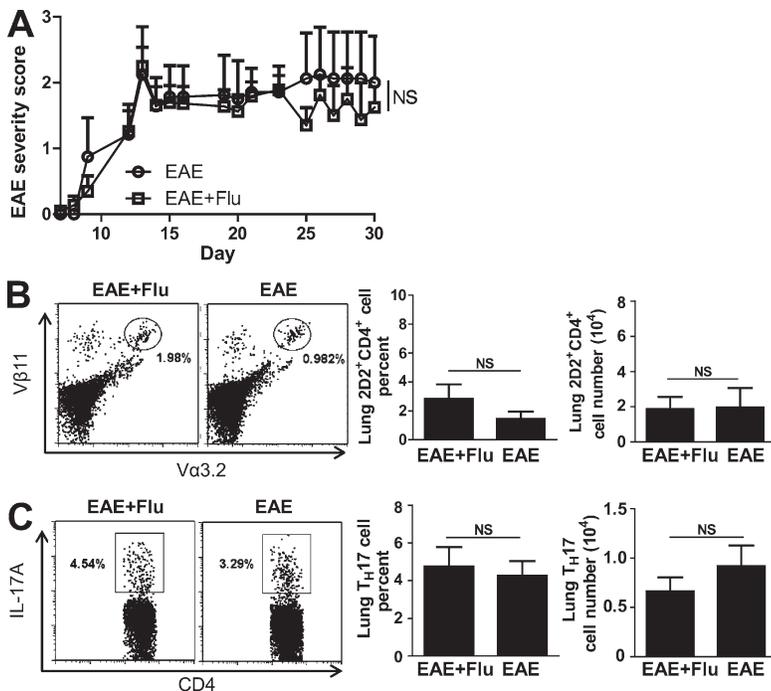


Figure 2. **Comorbid mice do not exhibit exacerbated EAE.** (A–C) Mice were immunized for EAE and then inoculated with influenza during the EAE priming phase as described in Fig. 1. (B) Mice were immunized for EAE, injected with 2×10^7 lymphocytes from 2D2 mice 2 d after EAE, and then inoculated with influenza. (A) Mice were monitored for EAE severity. The graph shown is a combination of two independent experiments. EAE, *n* = 7; EAE + Flu, *n* = 20. The Mann-Whitney test was used to evaluate significance. (B and C) During acute influenza infection (d13), single-cell suspensions were prepared for flow cytometry from lungs and analyzed. Shown are representative flow cytometric plots of 2D2 (V β 11⁺V α 3.2⁺) CD4⁺ T cells (B) and IL-17A-producing CD4⁺ T cells (Th17 cells; C) from singly EAE and comorbid mouse lungs. Bar graphs of compiled cell percentages are shown. Numbers of two independent experiments with at least four mice per group are shown. Student's *t* tests were conducted for statistical analysis. The mean and standard error of means are represented.

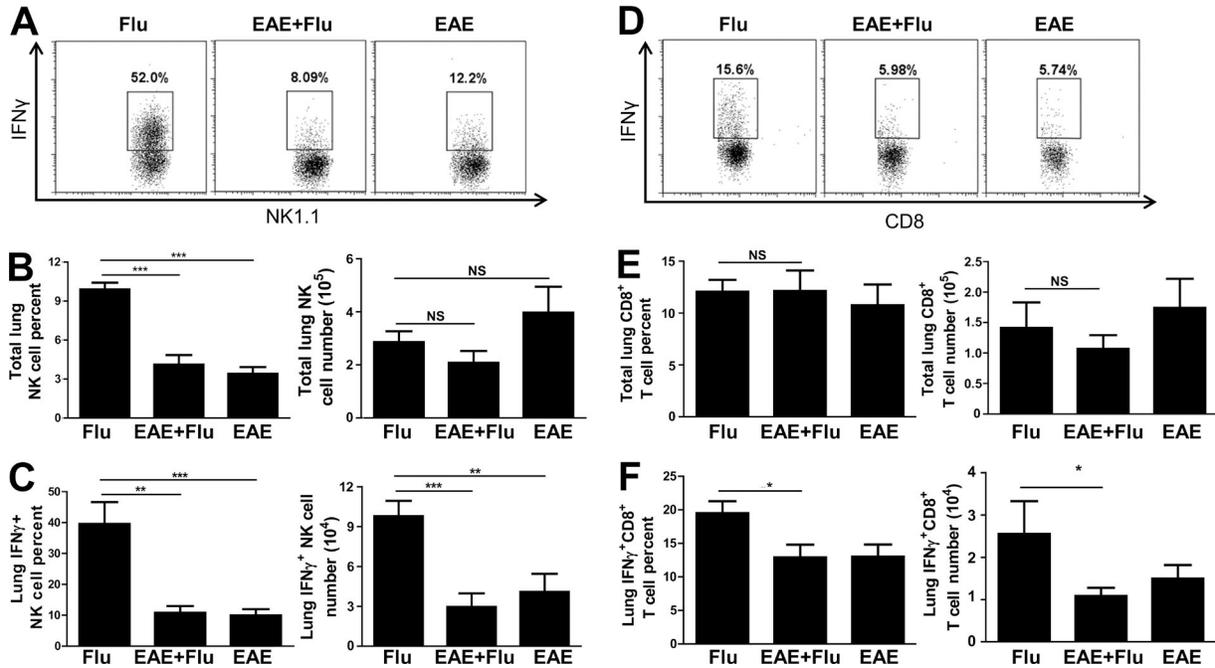


Figure 3. Comorbid mice exhibit suppressed NK cell and CD8⁺ T cell activation during early influenza infection. (A and D) Mice were immunized and then inoculated as described in Fig. 1. Cellular composition of the lungs was analyzed 3 d after influenza inoculation. Shown are representative flow cytometric plots of IFN- γ production from NK cells obtained from either singly influenza or comorbid (A) and total CD8⁺ T cells (D). (B–F) Summary bar graphs of compiled cell percentages and numbers for total NK cells (B), IFN- γ -producing NK cells (C), compiled cell percentages and numbers for total CD8⁺ T cells (E), and total IFN- γ -producing CD8⁺ T cells (F) are from two independent experiments with at least three mice per group. Student's *t* tests were conducted for statistical analysis. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. The mean and standard error of means are represented.

contained reduced cellular infiltrates. The comorbid mice demonstrated notable differences in their responses to influenza. Both groups began with similar lung viral titers early in infection at day 6, but comorbid mice had an eightfold higher viral titer at day 11 after influenza infection compared with singly influenza mice and maintained massive cellular infiltration in the lungs (Fig. 4, A and B). Notable differences in the numbers of lung NK cells, CD8⁺ T cells, inflammatory monocytes, and myeloid-derived suppressor cells (MDSCs) were also observed between the two groups at these viral titer time points (Fig. 4, C–F). To determine mechanisms of reduced viral clearance in the comorbid mice, we first compared the efficacy of the antiviral immune responses by analyzing IFN- γ production in virus-specific lung CD8⁺ T cells. During the resolution phase of viral infection, influenza viral nucleoprotein (NP) 366–374-reactive tetramer-positive CD8⁺ T cells were recruited to the lungs of both singly flu and comorbid mice, but comorbid lungs harbored a significantly lower percentage and total number of IFN- γ ⁺ NP tetramer-positive CD8⁺ T cells (Fig. 4 G). Thus, the presence of EAE exacerbated influenza-induced morbidity and mortality and led to increased lung cell infiltration and pathology, elevated viral titer, and decreased antiviral CD8⁺ T cell function.

Odoardi et al. recently reported that myelin-reactive T cells must transit through the lungs to gain access to the

CNS, and gene expression–profiling studies showed that these migratory T cells down-regulated their activation and proliferation programs, including that of proinflammatory cytokine production (Odoardi et al., 2012). This study suggests regulation of T cell inflammatory properties in the lungs during EAE. Consistent with the results of this study, we also found the myelin-specific 2D2 T cell populations in the lungs of both the single-affliction EAE and comorbid groups during disease induction. The process of EAE induction was already established at the time point of influenza infection, and no significant differences were noted in EAE disease severity, 2D2 CD4⁺ T cell number, or expansion of potentially lung-damaging Th17 cells. As the comorbid mice began to die within a relatively short period after influenza infection, long-term effects on EAE progression could not be assessed. The lack of difference in EAE progression along with the increased viral titers and presence of lung pathology in the comorbid group suggested that uncontrolled viral infection might be the cause of increased morbidity and mortality.

EAE induction robustly recruits MDSCs to the lungs

To discern the mechanism behind the ineffective antiviral T cell responses in the comorbid mice, we sought to characterize the local immune environment within the lung and determine the effects of EAE on lung immunity. Toward this end, we conducted a comprehensive assessment of the cellular

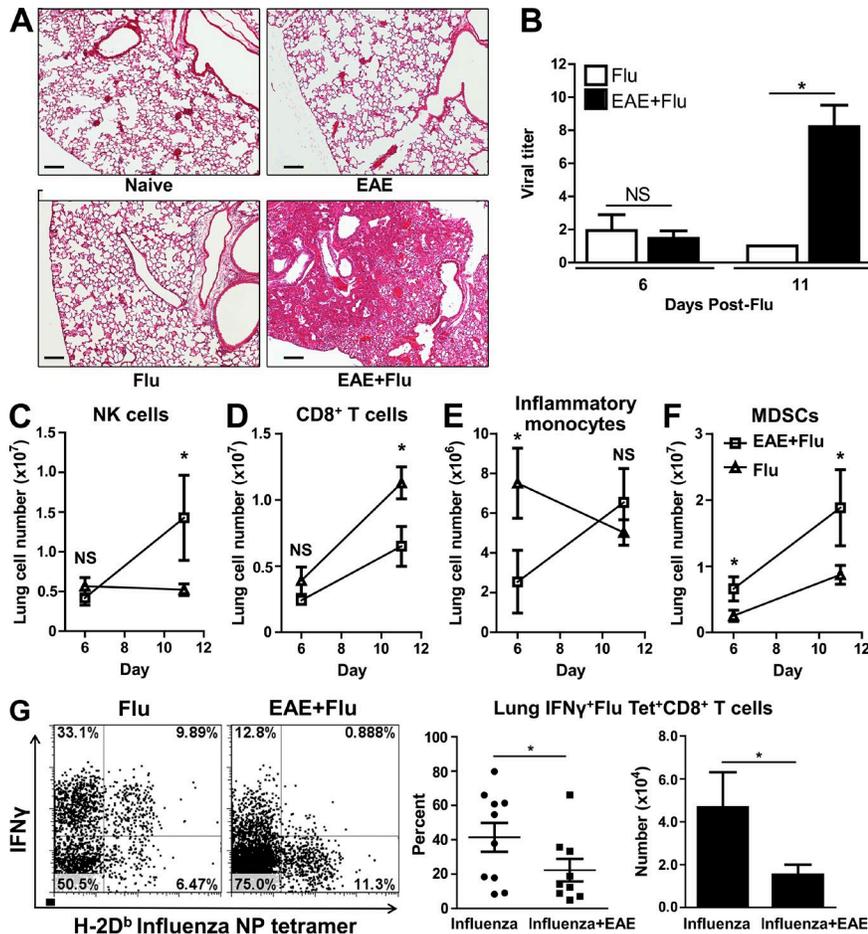


Figure 4. Comorbid mice fail to resolve influenza infection and exhibit deficient lung antiviral CD8⁺ T cell function. (A–G) Mice were immunized and inoculated with influenza for analysis of viral replication, immunity, and pathology 11–16 d after influenza infection. (A) Left lungs were perfused, harvested, and sectioned for immune infiltration evaluation via hematoxylin and eosin tissue staining 14 d after influenza infection. Bar, 100 μ m. (B) Lung influenza viral titer was determined in influenza and comorbid mice by qPCR analysis of influenza matrix protein, normalized to β -actin. Data are compiled from two independent experiments per time point with $n = 8$ –10 mice per group. (C–F) Lung cell suspensions were prepared from influenza and comorbid mice at the specified time points, and flow cytometry was used to identify lung cell type. Shown are kinetic analyses of lung NK cells (C), CD8⁺ T cells (D), inflammatory monocytes (E), and MDSC abundance (F). Each time point contains 8–10 mice per group. (G) Cytokine production by influenza-specific CD8⁺ T cell function in influenza-infected and comorbid mice was analyzed by conducting intracellular cytokine staining on lung cell suspensions 11–16 d after influenza infection. Shown are representative flow cytometric plots of IFN- γ production from influenza NP tetramer⁺ CD8⁺ T cells harvested from either singly influenza-infected or comorbid mice, with bar graphs of compiled cell percentages and numbers of two independent experiments, with 9–10 mice per group. Student's t tests were conducted for statistical analysis. *, $P < 0.05$. The mean and standard error of means are represented.

infiltrates and found most remarkably a disparity in the populations of myeloid cells (Fig. 4, E and F). Not surprisingly, in the generation of an effective immune response, the mice infected with influenza recruited a large population of inflammatory CD11b⁺ cells bearing a CD11b^{lo}Ly6-C^{hi} phenotype during acute infection, and upon resolution, these cells disappeared. In contrast, mice administered EAE alone or in conjunction with influenza exhibited a notable absence of these inflammatory cells and, instead, had a predominant population of CD11b^{hi}Ly6-C^{lo} cells, suggestive of an MDSC subtype. These cells were prominent during both acute and resolution phases (Fig. 5, A–D). The myeloid cells present in lungs of singly influenza mice successfully up-regulated cell-surface molecules important for CD8⁺ T cell activation and restimulation, such as CD40, CD86, and H-2k^b (Fig. 5 E). In contrast, recruited lung myeloid cells of singly EAE or comorbid mice failed to express CD40 and CD86 and exhibited ~50% of the level of H-2k^b expression than singly influenza mice. Next, we sought to investigate the underlying mechanism behind this early suppressive response. EAE induction has been shown to expand a population of CD11b⁺ suppressive monocytes in the bone marrow, blood, spleen, and CNS (Zhu et al., 2007; Ioannou et al., 2012). Thus, we asked whether a similar pop-

ulation of CD11b⁺ myeloid cells might also be recruited to the lungs during EAE pathogenesis, which could account for the suppression of the proinflammatory, antiinfluenza CD8⁺ T cell response. Our results showed that EAE pathogenesis did, in fact, mobilize a large population of CD11b⁺ MDSCs into the lungs during early disease induction, which is a previously undocumented observation. Interestingly, whereas influenza infection alone led to an expansion of inflammatory CD11b⁺ cells in the lung, the CD11b⁺ cells in EAE were comprised of a distinctly different population, both phenotypically and functionally. This distinction is of critical importance because CD11b^{lo}Ly6-C^{hi} inflammatory myeloid cells have been shown to promote local protective CD8⁺ T cell responses in the lung against influenza infection (Aldridge et al., 2009), whereas MDSCs potently suppress T cells.

EAE lung MDSCs directly suppress CD8⁺ T cell activation

To determine the potential for the MDSCs recruited by EAE to the lungs to functionally inhibit a CD8⁺ T cell response, we compared their effects on activation of CD8⁺ T cells in vitro. Mice were again immunized for EAE and infected with influenza or coinfected, and during the acute viral infection stage, CD11b⁺ myeloid cells were harvested and puri-

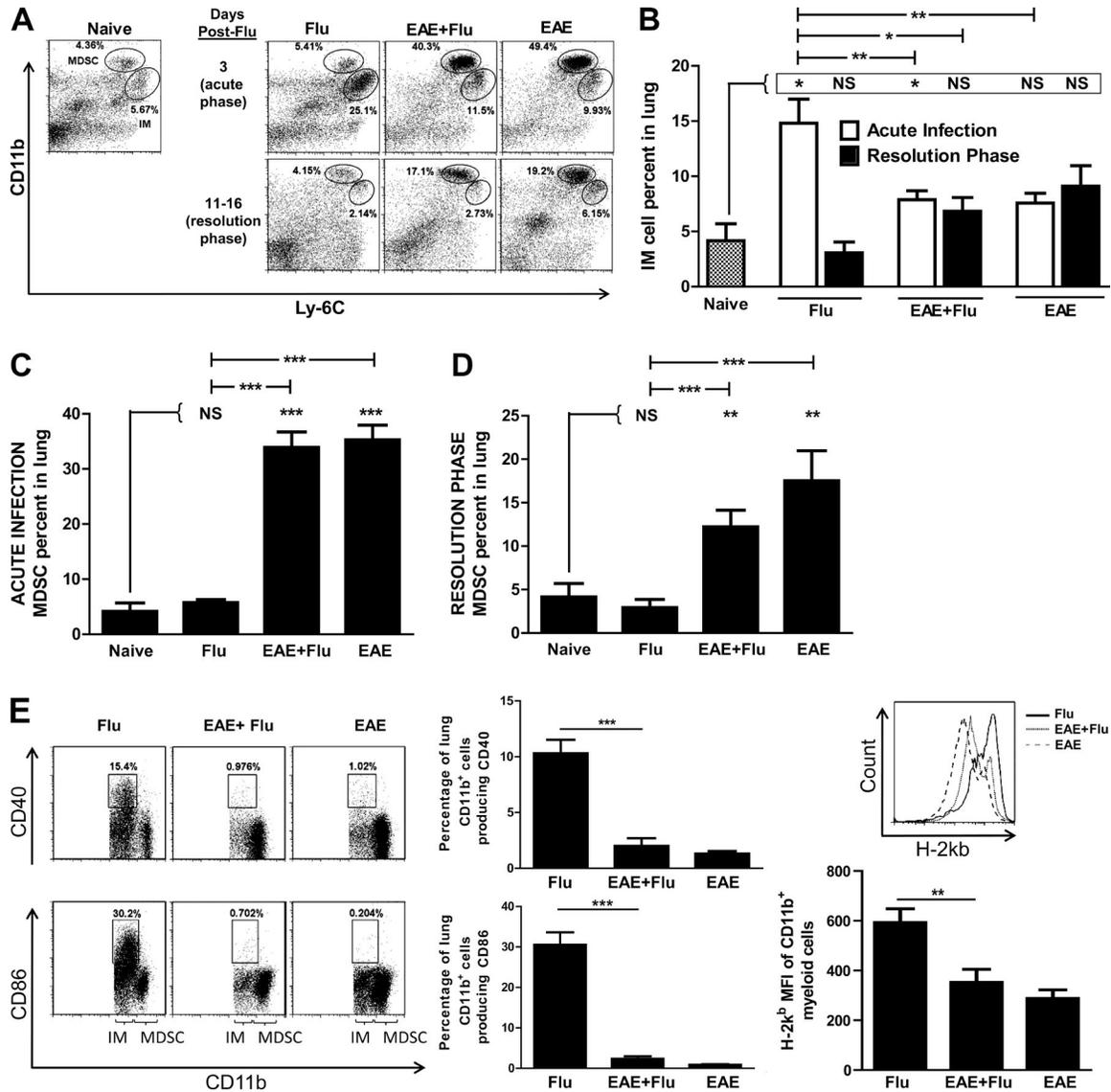


Figure 5. EAE recruits a phenotypically nonstimulatory population of CD11b⁺ myeloid cells to the lungs that is also retained in comorbid mice. (A–D) C57BL/6 mice were immunized and then inoculated as described in Fig. 1. (A) At 3 d (acute infection phase) and 11–16 d (influenza resolution phase) after influenza infection, whole lungs were perfused and harvested, and cellular composition was analyzed. Shown are representative flow cytometric plots of live-gated CD11b^{hi} Ly-6C^{lo} MDSCs and CD11b^{lo} Ly-6C^{hi} inflammatory monocytes (IMs). Compiled cellular percentages of inflammatory monocytes in both phases (B) and MDSCs in acute infection (C) and resolution phase (D) are shown from two to four independent experiments, with at least three mice per group. (E) Stimulatory phenotypes of inflammatory monocytes and MDSCs were also determined from mice in acute influenza infection phase. Shown are representative flow cytometric and histogram plots of live-gated cells and compiled cellular percentages of two to four independent experiments with at least three mice per group. MFI, mean fluorescence intensity. Student's *t* tests were conducted for statistical analysis. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. The mean and standard error of means are represented.

fied from the lungs for evaluation. A standard proliferation assay for CD8⁺ T cells was conducted, and CD11b⁺ cells from mice were added to the culture. Whereas activated CD8⁺ T cells cultured with CD11b⁺ myeloid cells derived from lungs of influenza-infected mice vigorously divided and produced IFN- γ , CD8⁺ T cells cultured with CD11b⁺ cells derived from either EAE-only or comorbid lungs exhibited significantly suppressed proliferation and IFN- γ production (Fig. 6, A and B).

EAE-mobilized lung MDSCs suppress CD8⁺ T cell function via inducible nitric oxide synthase (iNOS)

In our studies, MDSCs isolated from lungs of comorbid mice potently suppressed CD8⁺ T cell proliferation and inflammatory cytokine production, thus providing direct evidence of comorbid lung MDSC suppression of CD8⁺ T cell activity. MDSCs have been extensively reported to use diverse mechanisms to achieve immunosuppression, including

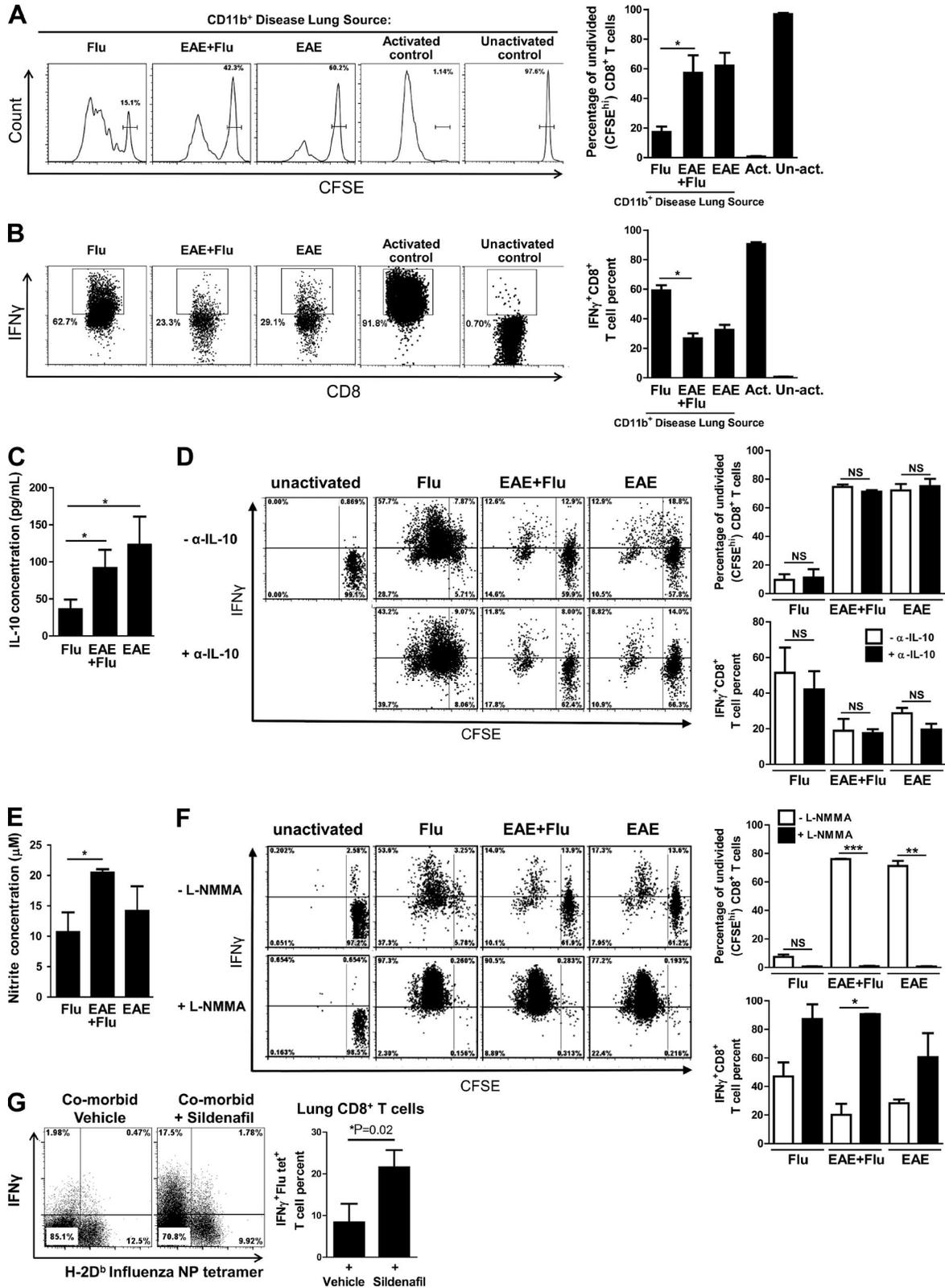


Figure 6. EAE-induced lung CD11b⁺ cells effectively suppress CD8⁺ T cell activation by iNOS. (A–F) CD11b⁺ cells were isolated from lungs obtained from each of the groups of mice (EAE only [10 d after EAE], influenza only [3 d after flu], and comorbid [10 d after EAE/3 d after flu]) and added to cultures of CFSE-labeled CD8⁺ T cells, which were activated with plate-bound α -CD3 and soluble α -CD28 at a CD11b⁺ myeloid cell/CD8⁺ T cell ratio of 10:1. Then,

production of the immunosuppressive cytokine IL-10 and NO synthesis via iNOS (Sinha et al., 2007; Zhu et al., 2007; Bunt et al., 2009; Gabrilovich and Nagaraj, 2009; Ioannou et al., 2012). To measure production of these molecules in our system, we harvested supernatants from co-cultures and compared production of IL-10 and nitrite (oxidized NO). Supernatants from co-cultured cells obtained from both comorbid and EAE-only mice contained significantly higher concentrations of IL-10 and nitrite than supernatants from co-cultures of CD11b⁺ and CD8⁺ cells obtained from influenza-only mice (Fig. 6, C and E). Next, we assessed relative contributions of these molecules and found that whereas neutralization of IL-10 had no effect on MDSC suppression of CD8⁺ T cell proliferation and IFN- γ production, the iNOS inhibitor L-NMMA efficiently reversed the inhibitory effect, generating robust CD8⁺ T cell activation and IFN- γ production (Fig. 6, D and F). To determine whether MDSC suppression could rescue influenza-specific CD8⁺ T cell function in vivo, we pharmacologically suppressed iNOS by administering sildenafil to comorbid mice. Sildenafil is a 5-phosphodiesterase inhibitor that has been demonstrated to down-regulate MDSC function, including iNOS expression, which leads to restoration of cytotoxic T cell function (Serafini et al., 2006, 2008; Meyer et al., 2011). Our results show that sildenafil administration led to a notable reversal of suppression with significant increases in the influenza-targeted T cell response, as shown by IFN- γ production in Flu tetramer-specific CD8⁺ T cells in the lungs of comorbid mice (Fig. 6 G). This finding suggests a functional role for the MDSCs in suppressing influenza responses. Collectively, these results indicate that the induction of EAE ultimately results in a diversion of lung immunity that leads to an inhibition of an effective viral response.

In summary, the results of our present studies showed that mice afflicted with EAE were highly susceptible to minor influenza infection, with fatal outcomes, mirroring epidemiological studies in people. Influenza viral infection has consistently been found to be a contributor to MS-related morbidity, and MS survivors are twice as likely to be admitted for hospitalization because of influenza infection and are at greater risk of developing MS relapses after infection (De Keyser et al., 1998; Oikonen et al., 2011; Montgomery et al., 2013; Marrie et al., 2014). The results of our studies may

help to at least partially elucidate some of the mechanisms behind this finding as we show that EAE pathogenesis, which involves T cell passage through the lung, surprisingly left in its wake a converted and suppressive immune milieu. The surprising finding that EAE recruits a population of suppressive myeloid cells into the lungs that are phenotypically distinct from those recruited by influenza infection provides a potential mechanism to link the immune responses in these distinct organs and the failure to control viral infection.

MDSCs have been extensively reported to use diverse mechanisms to achieve immunosuppression, including production of the immunosuppressive cytokine IL-10 and NO synthesis via iNOS (Sinha et al., 2007; Zhu et al., 2007; Bunt et al., 2009; Gabrilovich and Nagaraj, 2009; Ioannou et al., 2012). In our studies, MDSCs isolated from lungs of comorbid mice potently suppressed CD8⁺ T cell proliferation and inflammatory cytokine production, thus providing direct evidence of comorbid lung MDSC suppression of CD8⁺ T cell activity, which may account for the uncontrolled viral replication. Further, blockade of iNOS in these cells led to at least a partial reversal of inhibition, with improved T cell function, both in vitro and in vivo. The reasons these cells become recruited are unknown, but it is possible that they serve to limit collateral damage that could be induced by encephalitogenic T cells migrating to the CNS through the lungs or to provide a regulatory response to control the autoreactive process.

Collectively, these results suggest that the process of generating EAE and the passage of cells through the lung leads to a conversion of local immunity from stimulatory to suppressive, which may in part account for the heightened susceptibility to a comorbid condition. These findings may help to explain mechanisms by which neuroinflammatory autoimmune disease may exacerbate influenza infection.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice 6–7 wk of age were purchased from the National Cancer Institute; females were used for all experiments. 2D2 mice (T cell receptor specific for MOG on a C57BL/6 background) were purchased from The Jackson Laboratory and bred in house. All studies were approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee.

cells were analyzed by flow cytometry. (A) CD8⁺ T cell proliferation is shown by representative flow histograms of CFSE dilution and compiled percentages of undivided CD8⁺ T cells gated on live cells from three independent experiments. (B) IFN- γ production from CD8⁺ T cell activated under the same conditions is shown by representative flow cytometric plots and compiled percentages of two independent experiments. After 72 h, supernatants were harvested and assayed for IL-10 secretion (C) and nitrite production (E). IL-10 or NO-producing iNOS was blocked by addition of anti-IL-10 neutralizing antibody or L-NMMA, respectively, at the start of culture. After 72 h, cells were analyzed by flow cytometry. Shown are representative flow cytometric plots and bar graphs of compiled experiments of CFSE dilution indicating division of CD8⁺ T cells and IFN- γ production with IL-10 blockade (D) and L-NMMA-mediated iNOS blockade (F). Data are combined from two independent experiments. (G) Cytokine production by influenza-specific CD8⁺ T cell function in comorbid mice given no treatment or 60 mg/kg/d sildenafil citrate was analyzed by conducting intracellular cytokine staining on lung cell suspensions 9 d after influenza infection. Shown are representative flow cytometric plots of live cells gated on CD8⁺ T cells, which were harvested from comorbid mice given no treatment or sildenafil citrate, with bar graphs of compiled cell percentages from two independent experiments, with four to five mice per group. Un-act., unactivated. Student's *t* tests were conducted for statistical analysis. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. The mean and standard error of means are represented.

EAE induction and influenza inoculation

For induction of EAE, each mouse was injected with 100 μg of pure MOG_{35–55} in complete CFA (8 mg/ml *Mycobacterium tuberculosis*) in the abdomen subcutaneously. When done, OVA_{323–339} immunization followed the same preparation as MOG_{35–55} preparation. At the time of immunization and again 2 d later, 250 ng PTX was administered intraperitoneally. For inoculation with mouse-adapted H1N1 influenza (PR8/A), mice were briefly anesthetized with isoflurane and intranasally administered $2.6\text{--}3.5 \times 10^3$ PFU of virus in 40 μl PBS. To generate comorbid mice, mice immunized with EAE were inoculated with influenza during the priming phase of EAE (before the onset of EAE symptoms), 7 d after EAE immunization. Mice were monitored daily for changes in weight, clinical score, and mortality. For in vivo analysis of 2D2 expansion in the lungs, spleens and lymph nodes were harvested from 6-wk-old female 2D2 mice, and single-cell suspensions were prepared, combined, and injected intravenously via the tail vein into recipient mice (2×10^7 cells per recipient) 2 d after EAE immunization.

Lung histopathology

Lungs of either naive, EAE-immunized, influenza-inoculated, or comorbid mice were harvested 9–14 d after influenza inoculation (viral resolution phase). The left lung of each mouse was formalin fixed for 24 h, washed three times with deionized water, and stored in 70% ethanol. Then, mouse lungs were sliced into 2- μm sections and stained with hematoxylin and eosin. Then, sections were visualized by light microscopy with an upright microscope (80i; Nikon) and photographed using a DS Camera Control unit (DS-L2; version 4.0).

Influenza viral titer quantitation

The right lung lobes of influenza-inoculated and comorbid mice were harvested 9–14 d after influenza inoculation (viral resolution phase). Lung lobes were finely sliced, and 30 mg of tissue per mouse was homogenized in 600 μl of RNeasy mini kit buffer RLT (QIAGEN) plus 100-fold-diluted β -mercaptoethanol using a pellet mixer (VWR). After centrifugation, the supernatant was taken and RNA was harvested with the RNeasy mini kit (QIAGEN). Then, RNA was reversed transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories). Influenza viral quantitation was achieved by quantitative PCR analysis. cDNA of influenza viral A matrix protein and β -actin (normalization control) was assayed with iQ SYBR green supermix (Bio-Rad Laboratories) with the indicated primers: influenza A matrix protein: forward, 5'-GACCAATCCTGTACCTCTGAC-3' and reverse, 5'-AGGGCATTGTTGGACAAAGCGTCTA-3'; mouse β -actin: forward, 5'-TACAGCTTCACCACCACAGC-3' and reverse, 5'-TCTCCAGGGAGGAAGAGGAT-3'. Samples were processed on an iCycler (Bio-Rad Laboratories), and cycle threshold (Ct) values were calculated with the iCycler iQ multicolor real-time detection system (version 3.1). Data

were analyzed with the $2^{-\Delta\Delta\text{Ct}}$ method, and values were normalized to β -actin transcription.

Flow cytometry for ex vivo cell analysis

For ex vivo cell analyses, mice were EAE immunized and influenza inoculated as described in the EAE induction and influenza inoculation section. Mice were sacrificed either 3 d after influenza inoculation (10 d after EAE immunization [early EAE/acute viral infection phase]) or 9–14 d after influenza inoculation (16–21 d after EAE immunization), which is during the viral resolution phase, and perfused with cold $1 \times$ HBSS (without cations) introduction into heart right atrium, and whole lungs were harvested. Lungs were dissociated with $1 \times$ HBSS (with cations) containing 200 U collagenase II (Thermo Fisher Scientific) for 45 min at 37°C. Then, single-cell suspensions were prepared by passing lung preparations through a 100- μm filter, followed by red blood cell lysis with ACK lysis buffer. Single-cell suspensions were immediately restimulated with 2 $\mu\text{l}/\text{ml}$ Cell Stimulation cocktail (eBioscience) for 4 h at 37°C and stained. Antibodies used from BioLegend were Ly-6C-FITC and CD11b-PerCp; those from BD were V β 11-PE, V α 3.2-FITC, CD4-PE, Ly-6C-APC, CD11b-APC, CD8a-PerCp, CD8a-APC, and NK1.1-PE; those used from eBioscience were IL-17A-APC, CD40-PE, CD86-PE, H-2k^b-APC, IFN- γ -FITC, and IFN- γ -PerCp; and that of influenza viral NP (Remes et al., 2015) 66–374-reactive tetramer-APC were from the National Institutes of Health tetramer facility. For intracellular cytokine staining, the Foxp3 transcription factor staining buffer set (eBioscience) was used for cell permeabilization and fixation. Then, cells were interrogated by flow cytometry on a cell analyzer (FacsCalibur; BD) and analyzed with FlowJo software (Tree Star).

Co-culture analysis of CD11b⁺ myeloid cells and CD8⁺ T cells

Disease groups were prepared as described in the EAE induction and influenza inoculation section. 10 d after EAE immunization (3 d after influenza inoculation), mice were perfused and lungs were harvested from EAE, comorbid, and influenza mice ($n = 3\text{--}5/\text{group}$). Lung CD11b⁺ cells were isolated with a Mouse CD11b Positive Selection kit (STEMCELL Technologies) and cultured with CFSE (Thermo Fisher Scientific)-labeled CD8⁺ T cells isolated from naive mouse spleen with the CD8 Positive T cell Negative Selection kit (STEMCELL Technologies) at a 10:1 ratio. CD8⁺ T cells were polyclonally activated for 3 d with 3 $\mu\text{g}/\text{ml}$ plate-bound α -CD3 and 2 $\mu\text{g}/\text{ml}$ of soluble α -CD28. Cells were restimulated, stained, flow cytometrically interrogated, and analyzed as described in the Flow cytometry for ex vivo cell analysis section. For IL-10 neutralization and iNOS inhibition studies, activated CD8⁺ T cells \pm CD11b⁺ myeloid cells were given 50 $\mu\text{g}/\text{ml}$ anti-mouse IL-10 functional-grade purified antibody (clone JES5-2A5; eBioscience) or 0.5 mM L-NMMA citrate (Cayman Chemical), respectively, at the start of co-culture.

ELISA

For supernatants from MDSCs, CD8⁺ T cell co-cultures were harvested after 3 d of culture and passed through a 0.22- μ m filter. IL-10 secretion was interrogated using the Mouse IL-10 ELISA Ready-SET-Go! kit (second generation; eBioscience) according to the manufacturer's protocol.

Nitrite quantitation

For supernatants from MDSCs, CD8⁺ T cell co-cultures were harvested after 3 d of culture and passed through a 0.22- μ m filter. Nitrite was quantified with the Griess Reagent kit for Nitrite Determination (Thermo Fisher Scientific) according to the manufacturer's protocol.

Sildenafil administration

Sildenafil citrate (Teva Pharmaceuticals) was administered to mice by dissolution in drinking water acidified to pH 3.0 at 60 mg/kg/d at 5 d after EAE immunization (2 d before influenza inoculation). Mice untreated with sildenafil received acidified water, pH 3.0, only. Mice were allowed to drink ad libitum.

Statistical analysis

All statistics shown were conducted using Student's *t* test (unless specified) on the Prism program (GraphPad Software), where *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001. For all figures, the mean and standard error of means are represented.

ACKNOWLEDGMENTS

This work was supported by the Maryland Stem Cell Research Fund, a National Multiple Sclerosis Society Research Grant (RG1501027770) and Collaborative Center Grant, and the Silverman Foundation and the Johns Hopkins School of Public Health Histology Core for tissue sectioning and staining.

The authors declare no competing financial interests.

Author contributions: K.A. Whartenby, P.A. Calabresi, and J.D. Glenn conceived and designed the experiments. J.D. Glenn, K.A. Whartenby, P. Xue, M.D. Smith, Y. Chan-Li, and S. Collins performed the experiments. J.D. Glenn, M.R. Horton, and K.A. Whartenby analyzed the data. J.D. Glenn and K.A. Whartenby wrote the paper.

Submitted: 11 April 2016

Revised: 12 September 2016

Accepted: 9 December 2016

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