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Benzodiazepine Augmented γ -Amino-Butyric Acid Signaling Increases Mortality From Pneumonia in Mice*

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Abstract

Objectives—Benzodiazepines are used for treating anxiety, epilepsy, muscle spasm, alcohol withdrawal, palliation, insomnia, and sedation as they allosterically modulate γ -amino-butyric acid type A (GABA_A) receptors. Despite widespread use, the importance and mechanism of their immune side-effects are poorly understood. Herein we sought to elucidate the impact and mechanism of benzodiazepine-induced susceptibility to infection at anxiolytic doses in mice.

Design—Animal randomized controlled trial.

Setting—Laboratory.

Subjects—Adult female C57BL/6 and BALB/c mice.

Interventions—The effect of a sub-sedative, anxiolytic dose of diazepam (2 mg kg⁻¹ intraperitoneal) was investigated in a murine *Streptococcus pneumoniae* pneumonia model.

Measurement and Main Results—Mortality, bacterial and cytokine load, cell recruitment, and intracellular pH were measured. Diazepam treatment did not affect immune homeostasis in the lung. However, diazepam increased mortality and bacterial load from *S. pneumoniae* pneumonia. The increases in mortality and bacterial load were reversed by a GABA_A antagonist, bicuculline, indicating dependence on GABA_A receptor signaling. While cell recruitment was unaltered by

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Dr. Sanders developed the hypothesis with Drs. Maze and Hussell. Drs. Sanders, Godlee, Fujimori, Goulding, Xin, Salek-Ardakani, Snelgrove, Ma, Maze, and Hussell designed and/or conducted the animal experiments. Dr. Sanders wrote the manuscript with input from all authors.

diazepam, the cytokine response to infection was affected, suggesting that local responses to the pathogen were perturbed. Macrophage and monocytes expressed benzodiazepine sensitive ($\alpha 1$ - $\gamma 2$) GABA_A receptors. Interestingly macrophage GABA_A receptor expression was regulated by bacterial toll-like receptor agonists and cytokines indicating an endogenous role in the immune response. Functionally diazepam appeared to counteract the endogenous down-regulation of GABA_A signaling during infection. Consistent with augmented GABA_A signaling, diazepam provoked intracellular acidosis in macrophage, leading to impaired cytokine production, bacterial phagocytosis and killing. In contrast, selective benzodiazepines that do not target the $\alpha 1$ GABA_A subunit did not affect macrophage function ex vivo or increase susceptibility to pneumonia in vivo.

Conclusions—Our data highlight the regulation of macrophage function by GABA_A receptor signaling and the potential harm of benzodiazepine exposure during pneumonia. Therapeutically, selective drugs may improve the safety profile of benzodiazepines.

Keywords

γ -amino-butyric acid type A receptor; benzodiazepine; pneumonia

Benzodiazepines are World Health Organization essential medicines, used for treating anxiety, epilepsy, muscle spasm, alcohol withdrawal, palliation, insomnia, and sedation. In the United States and the United Kingdom, approximately 2% of the general population has taken benzodiazepines for 12 months or more (1), and their use is even more prevalent in elderly patients (up to 10%) (2). Furthermore, they are the most common sedative used in critically ill patients (3). To produce their clinical effects, benzodiazepines allosterically modulate γ -amino-butyric acid type A (GABA_A) receptors sensitizing them to GABA (4–8). GABA_A receptors are pentameric, ligand-gated channels that conduct chloride and bicarbonate anions (9). The most prevalent GABA_A receptor in the brain is the benzodiazepine-sensitive $\alpha 1\beta 2\gamma 2$ receptor with the benzodiazepine recognition site formed by the α - $\gamma 2$ subunit interface (4, 8).

GABA_A receptors are also expressed on immune cells. Macrophage and monocytes express α -subunits (10, 11) and β -subunits (10, 12, 13) but studies of lymphocytes are contradictory (10, 12, 14). Currently, it is unclear whether any immune cell expresses the $\gamma 2$ subunit and thus whether their GABA_A receptors are benzodiazepine sensitive. Macrophages synthesize GABA (12, 15), upregulate the expression of the synthetic enzyme of GABA, glutamic acid decarboxylase, on immune challenge (12), and serum GABA becomes detectable in experimental sepsis (16). Therefore, it is conceivable that GABA signaling acts to regulate immune responses. Consistent with an immune regulatory role, preliminary evidence suggests that GABA_A receptor expression is also influenced by inflammatory stimuli as a form of biological feedback (11, 17). Therefore we investigated the immune-regulated expression of the GABA_A $\gamma 2$ subunit on immune cells.

Activation of GABA_A receptors leads to a measurable electrophysiological response (12) and suppression of cytokine release from macrophage ex vivo (11, 12) and improves experimental autoimmune encephalitis in vivo (12), implying immunomodulatory activity. Of course, perturbing appropriate inflammation can be detrimental, and in human sepsis this has been correlated with increased mortality (3, 18). Consistent with this premise, avoiding

benzodiazepine sedation in septic critically ill patients reduced mortality by 70% (19, 20). In the Safety and Efficacy of Dexmedetomidine Compared with Midazolam study, sedation with midazolam appeared to double the risk of secondary infections in critically ill patients compared with sedation with dexmedetomidine (21). However, a more recent study did not observe the difference in infection rates (22). Several studies have shown that benzodiazepines increase mortality from infection including those from *Klebsiella pneumoniae* (23), *Mycobacterium bovis* (24), *Salmonella typhimurium* (25), and Vaccinia (26). In particular, the prototypical benzodiazepine, diazepam, increases mortality from intraperitoneal *K. pneumoniae* at subsedative doses (23). However, the mechanism of this effect and the relevance to clinical routes of infection, such as pneumonia, are unknown. Our objective was to clarify the mechanism of this effect using a pneumonia infection model with exposure to anxiolytic doses of diazepam. Indeed, we have recently observed in a cohort of 4,964 patients that benzodiazepine administration was associated with increased 30-day (adjusted hazard ratio [HR], 1.22; 95% confidence interval [CI], 1.06–1.39) and long-term mortality (adjusted HR, 1.32; 95% CI, 1.19–1.47) from community-acquired pneumonia (27). Using a case-control design, comparing the 4,964 cases with 29,697 controls, we have also recently shown that benzodiazepines increase the odds of developing pneumonia (adjusted odds ratio, 1.54; 95% CI, 1.42–1.67) (27). In these analyses, we adjusted for age, social deprivation, previous pneumonia, pulmonary disease, ischemic heart disease or other comorbidity, psychiatric disease, smoking, and alcohol use. Thus, despite accounting for multiple confounders a significant signal was still noted from benzodiazepine exposure. Further, understanding of the biological plausibility for this effect is still required, hence our interest in further preclinical mechanistic work.

As a class, GABA_A modulators including benzodiazepines, appear to exert similar effects on immune responses *ex vivo* (3). However, dependence on GABA_A receptor signaling *ex vivo* or *in vivo* has not been tested using pharmacological antagonists. We hypothesized that the mechanism of benzodiazepine-induced susceptibility to pneumonia would involve GABA_A receptor activation.

Materials and Methods

Drugs

For the *in vivo* work, clinical grade diazepam (Hamel Pharmaceuticals, Gloucester, UK) was diluted in phosphate buffered saline (PBS); the vehicle control was 4% ethanol in PBS. Diazepam was given at 2 mg kg⁻¹ as this dose provides anxiolysis but not sedation in mice (6, 7, 28, 29); 2 mg kg⁻¹ is a large dose of diazepam for humans, but in mice this produces the behavioral response we were attempting to model: anxiolysis but not sedation in mice (6, 7, 28, 29); 2 mg kg⁻¹ is a large dose of diazepam for humans, but in mice this produces the behavioral response we were attempting to model: anxiolysis at subsedative levels. The α 2/3 GABA_A subunit selective benzodiazepine NS11394 was obtained from Neurosearch, Ballerup, Denmark. NS11394 was diluted in 4% ethanol in PBS similar to diazepam and given at 2 mg kg⁻¹ as it has comparable anxiolytic efficacy (28). The GABA_A antagonist, bicuculline methiodide (Tocris, Bristol, UK), was diluted in PBS and also given at 2 mg kg⁻¹ based on previous work (30). All drugs were given twice daily in a volume of 200 μ L by

intraperitoneal injection. Additionally, for the ex vivo work diazepam, the $\alpha 2/3$ GABA_A subunit selective benzodiazepine, L-818-437, and the nonselective non-benzodiazepine GABA_A agonists, GABA and muscimol, were obtained from Tocris.

Animal Models

All protocols were approved by the Home Office (UK), conforming to the United Kingdom Animals (Scientific Procedures) Act of 1986. All animals used were C57BL/6 mice (weighing 17–19 g) unless stated. In the single infection animal model, mice were infected intranasally with 1×10^6 colony forming units (CFUs) of *Streptococcus pneumoniae* (serotype 2), strain D39 (NCTC 7466, London, UK). In the coinfection model, mice were infected with 50 hemagglutination units of A/X31 (H3N2) influenza seven days before 1×10^4 (lethal) or 2×10^3 (sublethal) CFUs of *S. pneumoniae*. Allergic pulmonary disease was induced by intranasal 15 μ g house dust mite extract (*Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, NC) in BALB/c mice on alternate days for 3 weeks. Survival was assessed according to Home Office rules limiting the severity of animal illness. If three or more of the following criteria were achieved, the animal was culled: piloerection, increased docility or aggression, immobility, hunched posture, sunken eyes, respiratory distress, dehydration, and loss of more than 25% of body weight.

Recovery of Samples

Mice were sacrificed by administration of pentobarbitone and exsanguination. In separate cohorts, bronchoalveolar lavage (BAL) fluid was obtained by inflation of the lung four times with 1.5 mL of 5 mM EDTA in Hank's Balanced Salt Solution (HBSS) via an intratracheal cannula; 100 μ L was used for bacterial CFU counts, the remainder was centrifuged, and the supernatant was stored at -80°C . Lung tissue was disrupted through a 100- μ m sieve (BD Labware, Franklin Lakes, NJ) with 100 μ L then set aside for bacterial CFU counts. The remainder was then spun and red blood cells lysed by adding ammonium-chloride-potassium buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate and 0.01 mM EDTA, pH 7.2) and washed with RPMI containing 10% fetal calf serum. BAL and lung cell viability was assessed by trypan blue exclusion and cells resuspended in RPMI containing 10% fetal calf serum and 2 mM L-glutamine at 1×10^6 cells mL^{-1} .

Bacterial Load

CFU counts were used to assess bacterial load in the airway (BAL) and lung and for the ex vivo killing assay. Serial dilutions were made in PBS and plated onto Columbia agar supplemented with 5% defibrinated horse blood and counted after incubation at 37°C in 5% carbon dioxide for 16 hours.

Cell Staining

Cells were identified by antibody purchased from BD Pharmingen, (Heidelberg, Germany). For receptor staining of mouse cells, cells were selected by forward/side scatter and the following surface markers: alveolar macrophage (CD11c⁺ F480⁺ CD11b⁺), splenic and peritoneal macrophage (F480⁺ CD11b⁺), monocyte (CD11c⁻ CD11b⁺ F480⁻ Ly6G⁻), neutrophil (CD11c⁻ CD11b⁺ Ly6G⁺), CD4 positive lymphocyte (CD4⁺ CD3⁺ CD8⁻), CD8

positive lymphocyte (CD4⁻ CD3⁺ CD8⁺) and B cell (CD19⁺ CD3⁻). The antibodies for GABA_A subunits (α 1–4, β 2, γ 2, and δ) came from Abcam (Cambridge, UK). The glutamic acid decarboxylase 65/67 antibody came from Millipore (Billerica, MA), and the secondary allophycocyanin-conjugated antibody was from ebioscience (Hatfield, UK). The isotype antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Positive staining was defined as within 1% of the upper limit of the isotype control. Cells were washed in phosphate-buffered azide and data were acquired on a BD FACS LSR II; 30,000 lymphocyte or myeloid events were analyzed with the FlowJo analysis program.

Interleukin-6 and Tumor Necrosis Factor-alpha Enzyme-Linked Immunosorbant Assay

Cytokine quantification was performed by enzyme-linked immunosorbant assay or luminex according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Ex Vivo Toll-Like Receptor and Cytokine Stimulation

Alveolar macrophages were treated for 16 hours with different Toll-like receptor (TLR) agonists (Invivogen, San Diego, CA) or cytokines (IL-4, 100 ng mL⁻¹; IL-10, 100 ng mL⁻¹; IL-13, 100 ng mL⁻¹; IL-33, 30 ng mL⁻¹; IFN- γ , 100 ng mL⁻¹; TNF- α , 100 ng mL⁻¹; IL-6, 100 ng mL⁻¹; IL-1 β , 1 ng mL⁻¹). Lipopolysaccharide (LPS) stimulations were also done at 100 ng mL⁻¹. Cells were then harvested and stained as previously described. For the ex vivo experiments, diazepam and L838-417 were obtained from Tocris.

Ex Vivo Phagocytosis and Bacterial Killing Assays

Alveolar macrophage phagocytosis was assessed by incubation for 1 hour with phrodo-labeled *Staphylococcus aureus* (Invitrogen, Paisely, UK). Bacterial killing by alveolar macrophage was assessed following pretreatment of cells for 16 hours with IFN- γ (100 ng mL⁻¹) at 37°C. *S. pneumoniae* were preopsonized with mouse serum at 37°C for 20 minutes and then mixed in a 1:1 ratio with cells. After incubation for 60 minutes at 37°C, cells were lysed, and the supernatant was plated in serial dilutions for bacterial load. Neutrophil assays were conducted with human neutrophils. *S. aureus* strains (MM85T, Oxford strain, NCTC 6571/ATCC 8144, TCS Bioscience Buckingham, UK) were preopsonized with human IgG for 20 minutes and then mixed with neutrophils at a 1:1 ratio for 20 minutes (at 37°C). Cells were then lysed, plated, and counted after incubation overnight. Neutrophil respiratory burst was measured by Amplex assay (Invitrogen) following stimulation with Phorbol 12-myristate 13-acetate. Alveolar macrophage pH was assessed using a 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) probe (Invitrogen), in 10 mM HEPES, 8 mM MES, 140 mM NaCL, 5 mM KCL, and 5 mM glucose, measured on an Omega Fluorostar plate reader (Buckingham, UK).

Statistical Analysis

Data are presented as mean \pm SD in the text. Data in the figures are presented as mean \pm SEM. Survival data were analyzed by log-rank test. Bacterial load were analyzed by Kruskal-Wallis and post hoc Dunn's multiple comparison test for multiple comparisons or Mann-Whitney when there were only two groups to compare. Cell counts, cytokine levels, and ex

vivo assays were analyzed by Mann-Whitney *U* test or analysis of variance and post-hoc Tukey test. Significance was set at a *p* value of less than 0.05.

Results

We hypothesized that that diazepam would increase susceptibility to pneumonia through inappropriate augmentation of GABA_A immune regulation, and this was tested by investigating the immune effects of an anxiolytic, sub-sedative dose of diazepam (6) that has previously been shown to increase susceptibility to infection in mice (23). We initially established that pretreatment of C57BL/6 mice for seven days with twice daily diazepam did not affect immune homeostasis in the healthy lung. Cell populations in the lung and surface receptors on the resident immune cells and alveolar macrophages were unchanged (Supplementary Fig. S1, Supplemental Digital Content 1, <http://links.lww.com/CCM/A604>).

When given on day 7 of the drug-treatment protocol, intranasal *S. pneumonia* (1×10^6 CFUs) produced an aggressive pneumonia in C57BL/6 mice. Despite the lack of changes to the baseline state, treatment with diazepam increased mortality from *S. pneumoniae* pneumonia (HR, 2.52 [1.08–5.90]; *p* = 0.033)—an effect attenuated by the GABA_A receptor antagonist, bicuculline, indicating a critical role of GABA_A receptor activation (Fig. 1A). Forty-eight hours into the infection, diazepam also increased airway bacterial counts. This was again dependent on GABA_A receptor activation (Fig. 1B). To ascertain whether diazepam exerted effects before 48 hours, we harvested animals that were treated with vehicle or diazepam at 24 and 48 hours. Unlike at 48 hours into the infection, bacterial counts were unaffected by diazepam at 24 hours (Fig. 1, C and D). Diazepam also did not affect cell recruitment at either 24 or 48 hours (Supplementary Figs. S2 and S3, Supplemental Digital Content 1, <http://links.lww.com/CCM/A604>), suggesting that it perturbs local responses to the infection. Consistent with this, diazepam inhibited the TNF- α , IL-6, and MCP-1 airway inflammatory response at 24 hours postinfection (Fig. 1E–h). This was followed by an exacerbated cytokine response at 48 hours with high levels of proinflammatory and anti-inflammatory mediators (Fig. 1e–h; Supplementary Fig. S4, Supplemental Digital Content 1, <http://links.lww.com/CCM/A604>), likely attributable to the increased bacterial load (Fig. 1, c and D).

Bacterial superinfection contributed to 26% to 38% of mortality in the recent H1N1 influenza pandemic (31) and occurred in 20% to 24% of critically ill influenza-infected patients (31) where benzodiazepines are typically administered (19). Therefore, we tested whether diazepam still increased mortality when animals were made vulnerable to *S. pneumoniae* by influenza infection seven days earlier. Diazepam treatment throughout the influenza and bacterial superinfection phases increased the risk of death HR, 22.4 [4.3–117.5]; *p* = 0.001; Fig. 1I). Furthermore diazepam, even when started after the bacterial superinfection of influenza, increased mortality rate from 1×10^4 CFU (HR, 3.1 [1.16–8.35]; *p* = 0.020; Fig. 1j) and 2×10^3 CFU (HR, 34.8 [6.32–191.7]; *p* = 0.001; Fig. 1k) of *S. pneumoniae*.

Next we profiled GABA_A receptor expression on immune cells to identify which local responses may be affected by diazepam. We initially screened immune cells from naïve

C57BL/6 mice for $\gamma 2$ expression (as a marker of benzodiazepine-sensitive GABA_A receptors). Alveolar macrophages abundantly expressed $\gamma 2$ ($64\% \pm 17\%$ of cells) with evidence of $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2A) (Table 1). Lower levels of $\gamma 2$ were present on splenic macrophages ($36\% \pm 5\%$; $p < 0.05$) and monocytes ($20\% \pm 7\%$; $p < 0.05$) with very low levels on CD4 cells ($6\% \pm 1\%$; $p < 0.05$). $\gamma 2$ subunits were not detected on other cell types. As benzodiazepines that are selective for specific α subunits are under development (7, 28), we investigated the expression of other α subunits. While $\alpha 1$ subunits were highly expressed on macrophages and monocytes, $\alpha 2-4$ subunits were rarely detected or absent (Table 1). Greater than 80% of all immune cells expressed the synthetic enzyme for GABA (Table 1), consistent with data from macrophages (15).

While alveolar and splenic macrophages abundantly expressed the $\gamma 2$ subunit, peritoneal macrophages did not (Table 1). Given this evidence for GABA_A receptor regulation, we investigated what factors modulated alveolar macrophage $\gamma 2$ subunit expression. Ex vivo stimulation with bacterial TLRs (TLR-4, -5, -6/2, and -9), but not viral TLRs (TLR-3 and -7), reduced $\gamma 2$ subunit expression (Fig. 2B). In vivo, 48 hours following *S. pneumoniae* infection, $\gamma 2$ subunit expression decreased by 46% (Fig. 2C). Endogenous reduction of GABA_A receptor expression during infection explains why bicuculline did not act antithetically to diazepam (Fig. 1, A and B) and protect against *S. pneumoniae*. Indeed bicuculline alone exerted no effect on the response to infection, perhaps due to the endogenous (physiological) antagonism of GABA_A receptor signaling induced by receptor down-regulation. This could be expected to reduce the effect of the pharmacological antagonist. Nonetheless, bicuculline was able to attenuate the effect of diazepam confirming that the benzodiazepine augmented GABA_A receptor activity was responsible for diazepam's immune effects. We also investigated the effects of cytokine signaling on alveolar macrophage GABA_A $\gamma 2$ subunit expression ex vivo. IL-1 β reduced, while IL-4 increased, expression (Fig. 2D). In vivo, treatment of BALB/c mice with the allergen house dust mite, promoting a Th2 cytokine environment (including IL-4), also caused an increase in $\gamma 2$ expression (Fig. 2E).

To further enhance the plausibility of our findings, we investigated the ex vivo effects of augmenting GABA_A receptor signaling. GABA_A receptor activation inhibited LPS-induced alveolar macrophage TNF- α (Fig. 3A) and IL-6 release (Supplementary Fig. S5, Supplemental Digital Content 1, <http://links.lww.com/CCM/A604>). To functionally confirm our finding that alveolar macrophages express $\alpha 1$ containing GABA_A receptors, we used a benzodiazepine that is selective for $\alpha 2/3$, but lacks activity at $\alpha 1$, containing GABA_A receptors (7): L-838-417. L-838-417 did not affect cytokine release, suggesting a critical role for $\alpha 1$ containing GABA_A receptors (Fig. 3A). GABA_A receptor activation, with diazepam or GABA, also reduced phagocytosis of phrodo-labeled *S. aureus* (Fig. 3B). Again L-838-417 lacked effect. IFN- γ -stimulated killing of *S. pneumoniae* was inhibited by diazepam by 28%, a defect antagonized by bicuculline and not mimicked by L-838-417 (Fig. 3C). Consistent with a lack of GABA_A receptor expression (10), GABAergic drugs did not modify neutrophil bacterial killing or respiratory burst ex vivo (Supplementary Fig. S6, Supplemental Digital Content 1, <http://links.lww.com/CCM/A604>).

Activation of neuronal GABA_A receptors leads to cytoplasmic acidification through bicarbonate efflux (9). As acidification of the cytoplasm is known to reduce TNF- α production (32) and bacterial phagocytosis and killing (33) by alveolar macrophage, we hypothesized that GABA_A receptor stimulation perturbed immune responses by altering intracellular pH. Augmenting GABA_A signaling with diazepam (or another GABA_A agonist muscimol [100 μ M, unpublished observations]) increased the intracellular H⁺ concentration by 10% (baseline pH 7.13 \pm 0.03 vs. diazepam pH 7.08 \pm 0.01; p = 0.004; Fig. 3D). α 1 containing GABA_A receptors were again implicated as L-838-417 and did not alter pH (pH 7.12 \pm 0.02; Fig. 3D). Increasing GABA_A receptor expression with IL-4 pretreatment increased H⁺ concentration by 167% (pH 6.91 \pm 0.01; p < 0.001 vs. baseline pH; Fig. 3E); this was further augmented by diazepam (Fig. 3E). In contrast, reducing GABA_A receptor expression with LPS led to cytoplasmic alkalosis (pH 7.16 \pm 0.01; p < 0.001 vs. baseline; Fig. 3F). Despite reduced receptor expression by LPS, diazepam still increased the cytoplasmic H⁺ concentration (pH 7.11 \pm 0.01; p < 0.001), an effect antagonized by bicuculline (pH 7.13 \pm 0.01; p < 0.001; Fig. 3F). In contrast, L-838-417 (pH 7.17 \pm 0.01) and bicuculline (pH 7.16 \pm 0.02) exerted no effect alone. Furthermore, H⁺ concentration correlated with changes in GABA_A subunit expression (r^2 = 0.80; p < 0.001; Fig. 3G). To summarize, augmenting GABA_A receptor activity acidifies the cytoplasm of alveolar macrophage through α 1- γ 2 containing GABA_A receptors.

To show whether α 1 subunit containing GABA_A receptors were responsible for the benzodiazepine-induced increase in susceptibility to infection, we compared diazepam and a subunit selective benzodiazepine in vivo. We did not choose to use L-838-417 in vivo due to its extremely short half-life (7). Rather, we selected NS11394, as its pharmacokinetics are compatible with twice daily dosing in mice (28), and it produces anxiolysis at comparable doses to diazepam (6, 28). This is possible as anxiolysis is mediated by α 2/3-, not α 1-, containing GABA_A receptors (5, 6, 34). Unlike diazepam, NS11394 did not increase mortality from infection (HR, 1.23 [0.56–2.67]; p = 0.6; Fig. 4A) nor were bacterial counts higher than vehicle-treated controls (Fig. 4B); again cell recruitment was unaffected (data not shown).

Discussion

In summary, we provide evidence that benzodiazepines increase mortality from pneumonia in mice. Mechanistically, our results suggest that benzodiazepines increase susceptibility to *S. pneumoniae* by augmenting GABA_A receptor activity. These in-vivo changes were subsequently correlated with ex vivo changes in macrophage function. Indeed GABA_A receptor activation leads to a fundamental change in macrophage physiology via cytoplasmic acidification. To fine tune alveolar macrophage responses, GABA_A signaling is tightly controlled through immune regulation. During infection, administration of benzodiazepines opposes the endogenous drive to reduce GABA_A signaling, increasing susceptibility to pneumonia. In contrast, treatment with IL-4 ex vivo or house dust mite in vivo increased GABA_A subunit expression, and this led to cytoplasmic acidification. This intracellular pH change is consistent with the “alternatively activated” macrophage phenotype induced by IL-4: reduced TNF- α production and bacterial phagocytosis and killing (35).

The site-specific regulation of macrophage is also of interest. Macrophage GABA_A receptor expression was highest in the lung (a mucosal site) and was lower in the nonmucosal sites (spleen and peritoneal cavity). Future studies should address the source of GABA, possibly from epithelial (36) or immune cell (12) sources. Another possibility is that GABA is released from bacterial pathogens (37, 38), especially as serum GABA becomes detectable in sepsis (16). In this scenario, pathogen-produced GABA may act to disable the host immune response. In contrast, down-regulation of host GABA_A receptors on macrophages may reveal an attempt to block this pathogen–host interaction. Finally, macrophage GABA_A channels may have tonic activity that does not require GABA for activation (39).

Macrophages and monocytes expressed $\alpha 1$ subunit containing GABA_A receptors. Consistent with this, benzodiazepines that lack activity at $\alpha 1$ subunits lack the immunosuppression of nonselective drugs; thus enhanced benzodiazepine selectivity may improve the safety profile of this widely used class of drug. This is achievable for endpoints such as anxiolysis, which are mediated by $\alpha 2/3$ subunit containing GABA_A receptors (34). Despite studying a sub-sedative, anxiolytic dose of a benzodiazepine, our results may also have relevance to sedative doses of the drugs. The sedative actions of GABAergic drugs are mediated by $\alpha 1\beta 2\gamma 2$ GABA_A receptors (5, 34), and the same receptors are expressed by macrophages and monocytes. Therefore, it is unclear whether the sedative actions of GABAergic drugs such as benzodiazepines can be separated from their immunological effects. Further studies are required to compare the immune effects of GABAergic and non-GABAergic sedatives (19), to further build on investigations into their central nervous system side-effects (19, 20).

We are confident that the immune effects of diazepam are mediated by the GABA_A receptor rather than other targets such as the mitochondrial translocator protein (also known as the peripheral benzodiazepine receptor [3]), as the diazepam effect is reversed by the GABA_A antagonist bicuculline, diazepam does not effect neutrophil function (where GABA_A receptor expression is absent but the translocator protein is present), and the selective benzodiazepine, NS11394 (that activates the translocator protein but not $\alpha 1$ containing GABA_A receptors) did not cause immune suppression. Furthermore, activation of translocator protein is thought to stimulate chemotaxis in macrophage and monocytes *ex vivo*; we, and others (40), found no effect on chemotaxis *in vivo*. Finally, the immune function of the translocator protein is variably reported *ex vivo* (41, 42) with a lack of *in vivo* studies conducted, making any immune function obscure.

An important caveat to this work is that we are unable to definitively demonstrate that the benzodiazepine effect is mediated by a specific cell type. We suggest that our findings may be explained by perturbed function of alveolar macrophage based on their abundant expression of GABA_A receptors, the immune regulation of their expression, and our *ex vivo* functional studies. Studies with genetically modified mice are complex due to the critical role of the $\gamma 2$ subunit in neurodevelopment (8, 43). Future studies should genetically manipulate $\gamma 2$ subunit expression in individual immune cell populations to definitively identify the cell type responsible for the benzodiazepine effect. However, from a drug development perspective, the exact immune cellular target may not be critical given that the drugs need to be able to penetrate the blood-brain barrier to have the clinically desired effect

and so will be able to access the lung and that therapeutically, selective benzodiazepines that do not target the $\alpha 1$ GABA_A subunit did not increase susceptibility to infection.

Conclusions

In our studies, diazepam appeared to oppose the endogenous regulation of GABA_A receptor activity to increase susceptibility to infection via activation of $\alpha 1$ -subunit-containing GABA_A receptors. Activation of GABA_A receptors on macrophages leads to cytoplasmic acidification and impaired antipathogen responses. Our data highlight the regulation of macrophage function by GABA_A receptor signaling and the potential harm of benzodiazepine exposure during pneumonia. These data are supported by our parallel epidemiological study, suggesting that benzodiazepines increase susceptibility to pneumonia in humans (27). Nonetheless, it would be imprudent to immediately extrapolate these findings to clinical care, and further prospective cohort studies and randomized controlled trials are required to understand the impact of benzodiazepines on outcomes from infection. Our data also suggest that selective benzodiazepines may have an improved safety profile compared with their nonselective counterparts. Further preclinical study is required to develop these drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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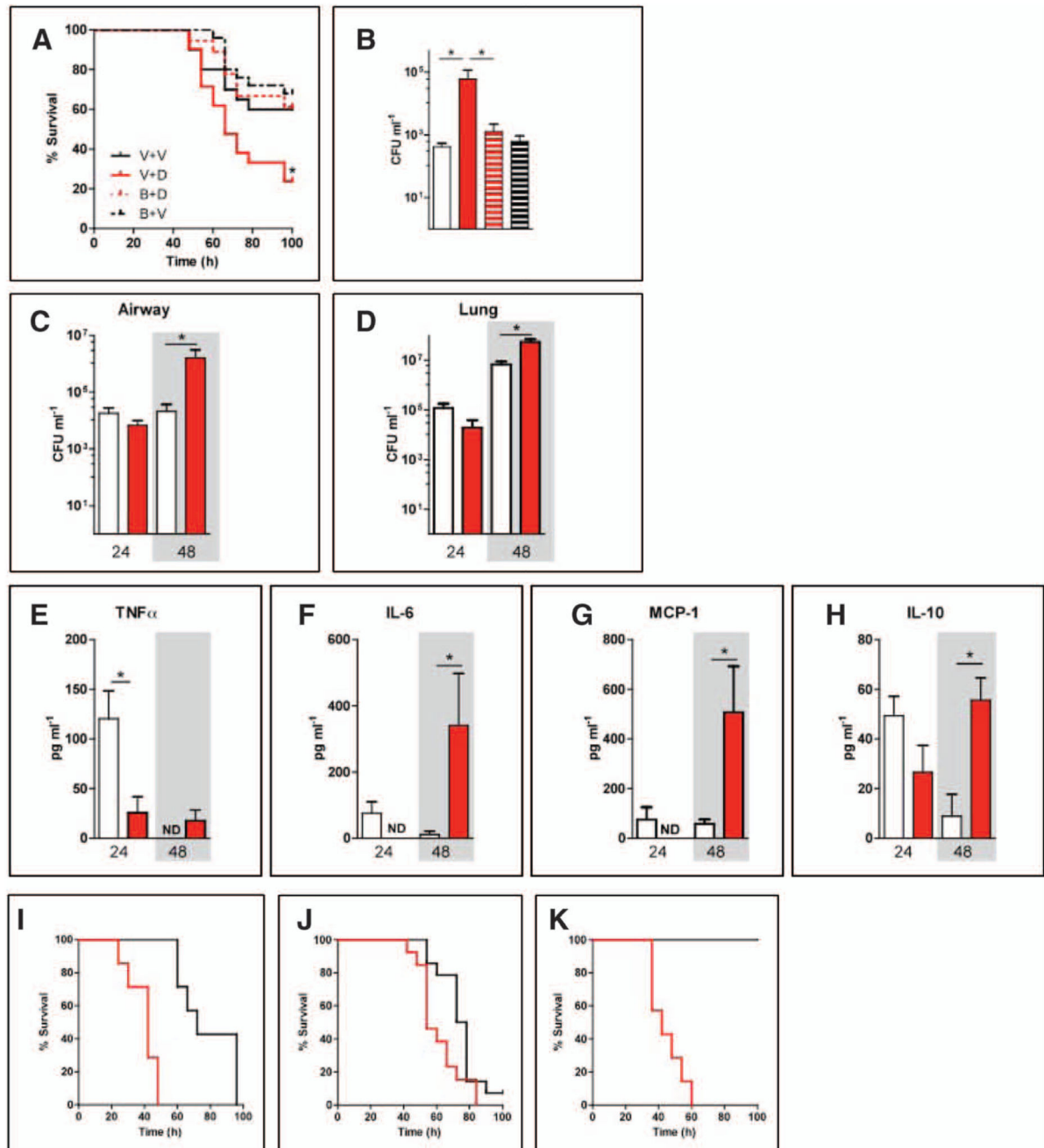


Figure 1. Benzodiazepines increase mortality from pneumonia in C57BL/6 mice through GABA_A signaling.

A, Mice were treated with the vehicle for bicuculline (phosphate-buffered saline [PBS]) and then the vehicle for diazepam (V+V; *black solid line*) or diazepam (2 mg kg⁻¹) (V+D; *red solid line*). Two other groups received bicuculline followed by diazepam (B+D; *red dotted line*) or the vehicle for diazepam (4% ethanol in PBS; *black dotted line*) (B+V). Survival ($n = 20$ per group) after intranasal *Streptococcus pneumoniae* was assessed. **B**, Bacterial load in the airway at 48 hr ($n = 8$ per group) was also assessed. The groups are vehicle for

bicuculline (PBS) and then the vehicle for diazepam (*white bar*) or diazepam (*red bar*); two other groups received bicuculline followed by diazepam (*red striped bar*) or the vehicle for diazepam (*black striped bar*). In a separate experiment, bacterial load in the airway (**C**) and lung (**D**) and airway lavage, tumor necrosis factor (TNF)- α (**E**), interleukin (IL)-6 (**F**), monocyte chemoattractant protein (MCP)-1 (**G**), and IL-10 (**H**) were assessed at 24 and 48 hr after *S. pneumoniae* infection ($n = 5$ mice per group). The two treatment groups for these experiments (**C–H**) were the vehicle for diazepam (*white bar*) or diazepam (2 mg kg^{-1} ; *red bar*). Survival was also analyzed in mice given *S. pneumoniae* 7 days after influenza (H3N2 X31/A) infection. **I**, Diazepam was administered prior to and during bacterial superinfection of influenza ($n = 8$ per group; *red* = diazepam; *black* = vehicle). In separate experiments, diazepam was administered twice daily following (**J**) 1×10^4 colony-forming units ($n = 13$ per group) or (**K**) 2×10^3 CFU ($n = 7$ per group) bacterial superinfection of influenza. $*p < 0.05$. Graphs show mean \pm SEM. ND = not detected..

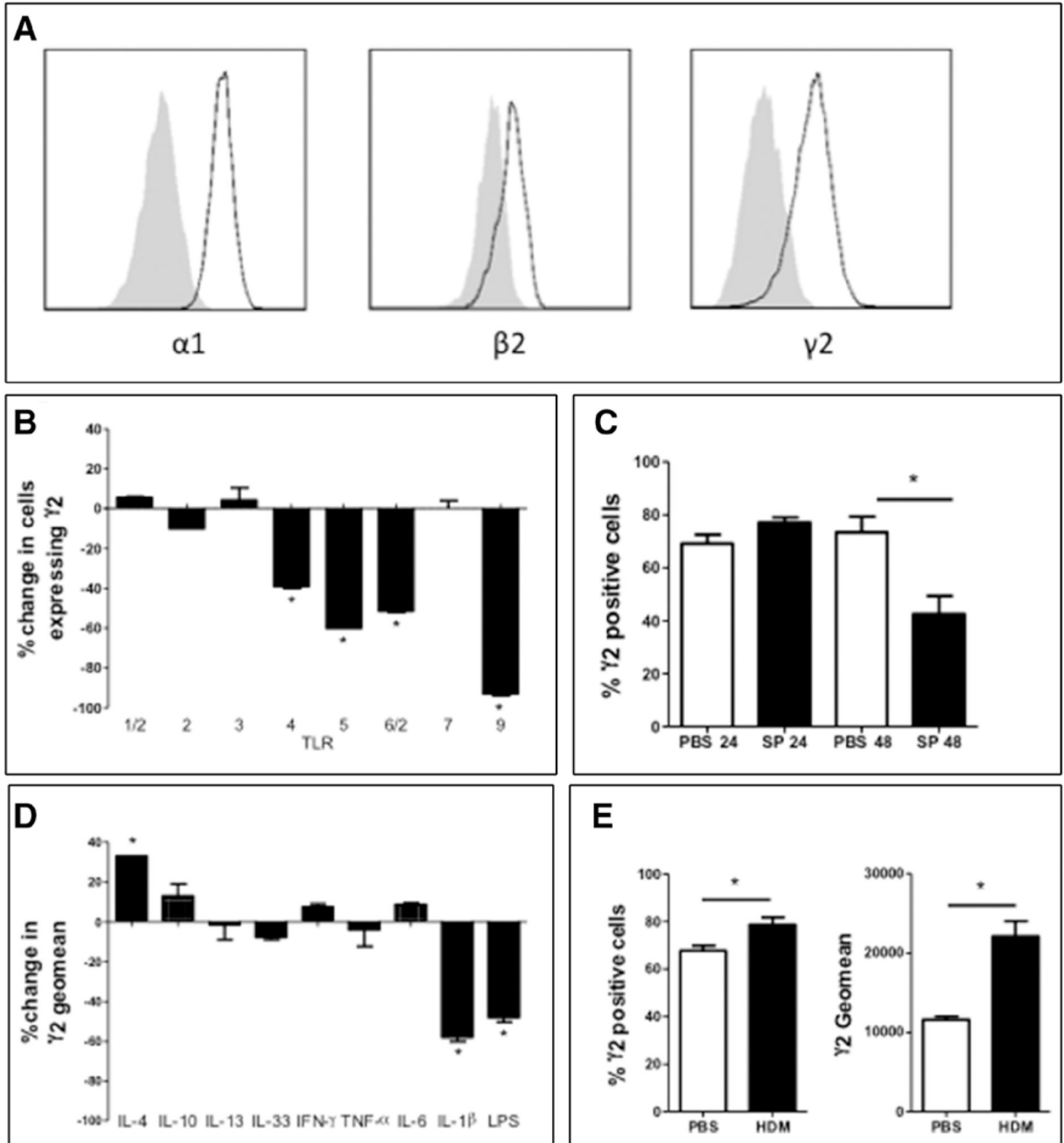


Figure 2. Alveolar macrophage express benzodiazepine-sensitive, $\alpha 1$ - $\gamma 2$ subunit containing $GABA_A$ receptors.

A, Alveolar macrophage, purified by lavage of C57BL/6 mice, were assessed for $\alpha 1$, $\beta 2$, and $\gamma 2$ $GABA_A$ subunit expression by flow cytometry. *Gray shading* = isotype. *Black outline* = $GABA_A$ subunit. Alveolar macrophage from C57BL/6 mice were incubated with **(B)** toll-like receptor (TLR) agonists or **(D)** cytokines (and lipopolysaccharide [LPS]) and $\gamma 2$ subunit expression determined by flow cytometry ($n = 3$ wells of 100,000 cells/treatment) at 16 hr. **C**, Mice were given *Streptococcus pneumoniae* (SP) or phosphate-

buffered saline (PBS) intranasally, alveolar macrophage $\gamma 2$ subunit expression was assessed at 24 and 48 hr ($n = 5C57BL/6$ mice/group). **E**, Alveolar macrophage $\gamma 2$ subunit expression in response to 3 wk of house dust mite (HDM) treatment ($n = 5$ BALB/c mice/group) assessed by flow cytometry. $*p < 0.05$ versus control or vehicle. Graphs show mean \pm SEM. IL = interleukin; TNF- α = tumor necrosis factor- α ; IFN = interferon.

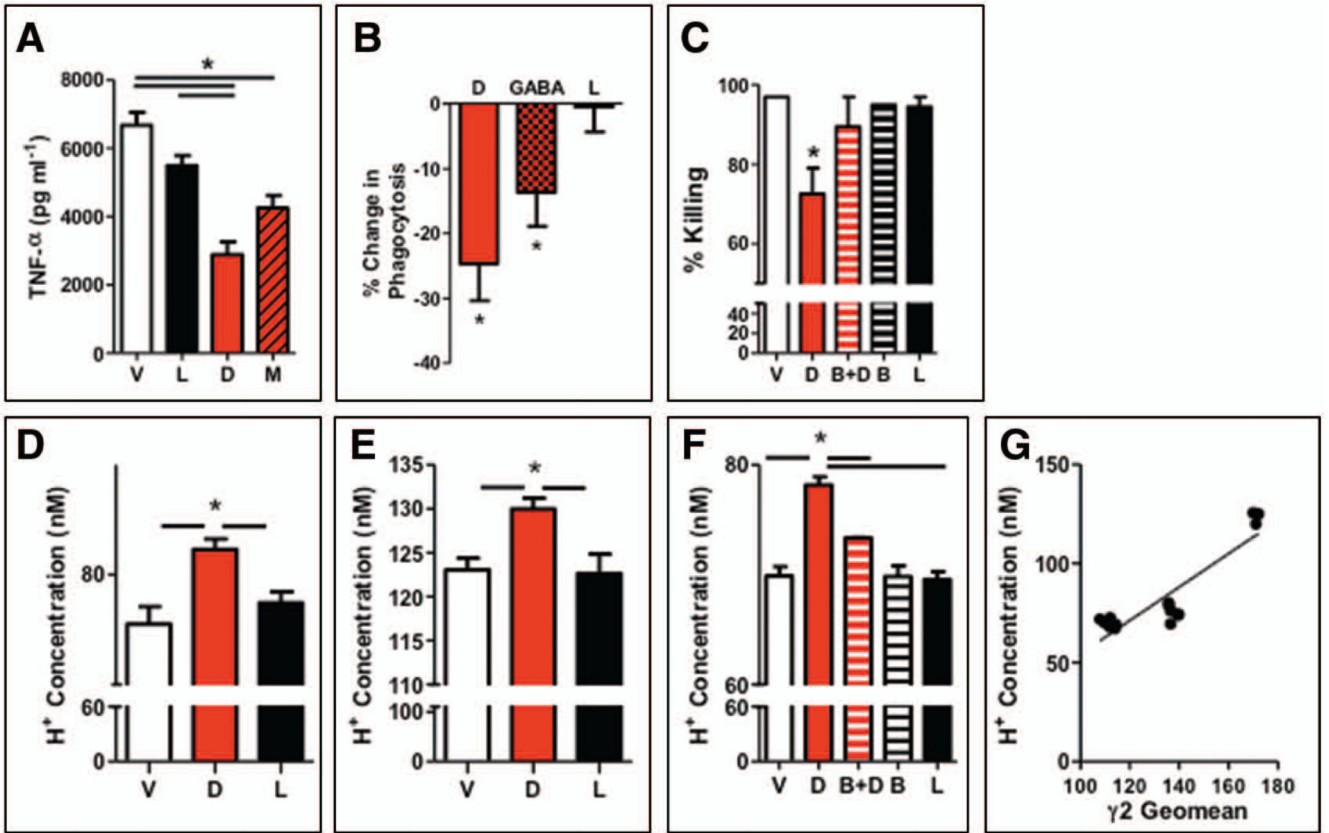


Figure 3. Only benzodiazepines that activate $\alpha 1$ subunit containing GABA_A receptors affect macrophage function.

A, Alveolar macrophages, purified from C57BL/6 mice, were incubated with vehicle (V), diazepam (D) (10 μ M), muscimol (M) (100 μ M) or L-838-417 (L) (567nM; an equivalent Ki multiple to diazepam6), and lipopolysaccharide (LPS) (100 ng ml⁻¹), tumor necrosis factor (TNF)- α release was measured by ELISA ($n = 3-7$ per group). **B**, Change in phagocytosis of phrodo-labeled *Staphylococcus aureus* ($n = 3-6$ per group) assessed at 1 hr with diazepam, GABA (100 μ M), or L. **C**, Alveolar macrophages were incubated with V, D, bicuculline (B), and B and D (B+D), or L, and killing of *Streptococcus pneumoniae* was assessed by counting bacterial colonies ($n = 3$ wells/group). Cytoplasmic pH was measured by BCECF probe in untreated (**D**), interleukin-4-treated (**E**), and LPS-treated alveolar macrophage (**F**) ($n = 10$ wells per treatment). **G**, H⁺ concentration correlates with expression of $\gamma 2$ subunit expression as assessed by geomean.

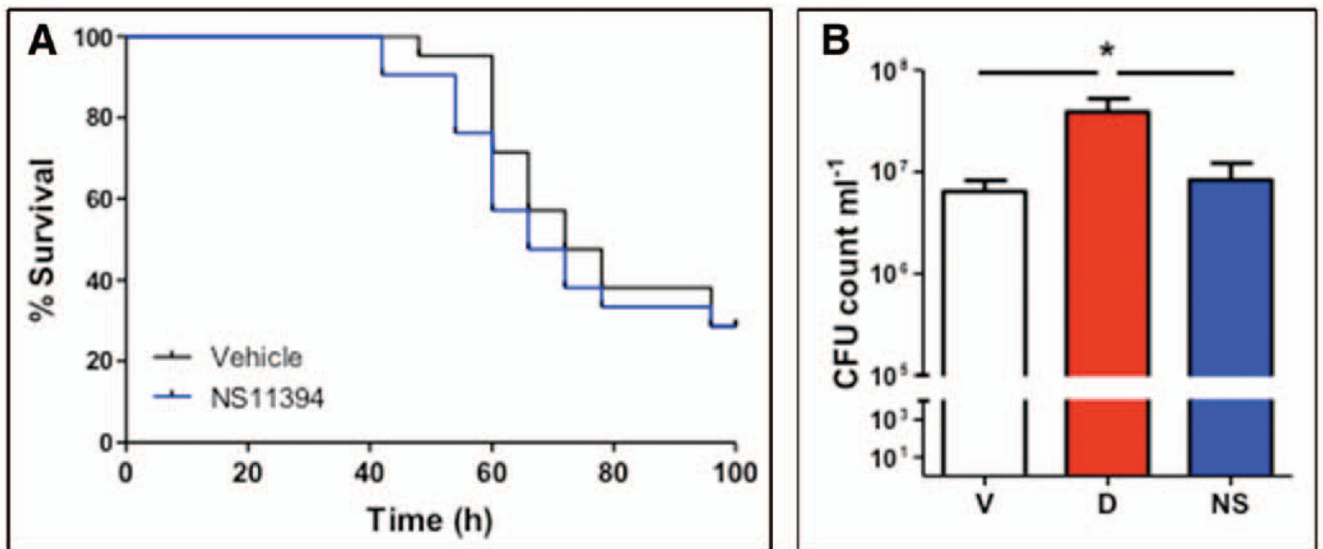


Figure 4. A selective benzodiazepine that does not activate $\alpha 1$ subunit containing GABA_A receptors does not affect outcomes from pneumonia.

A, Mice were treated with NS11394 (NS) (2 mg·kg⁻¹; blue line) or vehicle (black line) for seven days prior to and during *Streptococcus pneumoniae* infection and survival assessed ($n = 20$ /group). **B**, Bacterial load in lung homogenates at 48 hr was monitored comparing treatments of vehicle (V) (black bar), NS11394 (NS) 2 mg·kg⁻¹; blue bar) or diazepam (D) (2 mg·kg⁻¹; red bar) ($n = 16$ per group). * $p < 0.05$ versus control unless otherwise specified. Graphs show mean \pm SEM.

Table 1
**Percentage Expression of GABA_A Subunits and the Synthetic Enzyme for GABA,
 Glutamic Acid Decarboxylase, on Cells From C57BL/6 Mice**

	GABA _A Signaling Proteins							Glutamic Acid Decarboxylase
	α1	α2	α3	α4	β2	γ2	σ	
Alveolar macrophage	99 (1)	4 (1)	4 (2)	ND	42 (19)	71 (11)	ND	93 (5)
Splenic macrophage	37 (7) ^a	5 (2)	7 (3)	NT	NT	36 (5) ^a	NT	NT
Peritoneal macrophage	21 (8) ^a	NT	NT	NT	NT	13 (4) ^a	NT	NT
Monocyte	34 (12) ^a	6 (2)	8 (3)	ND	NT	20 (7) ^a	ND	90 (5)
Neutrophil	5 (1) ^a	ND	ND	ND	NT	ND	ND	94 (3)
CD4	10 (2) ^a	NT	NT	ND	NT	6 (1) ^a	ND	93 (3)
CD8	4 (1) ^a	NT	NT	ND	NT	ND	ND	91 (1)
B Cell	8 (3) ^a	NT	NT	ND	NT	ND	ND	84 (4)
NK cell	9 (2) ^a	NT	NT	ND	NT	ND	ND	89 (3)

ND = not detected; NT = not tested.

Data are presented as mean (SD).

^a $p < 0.05$ versus alveolar macrophage.