GABA$_A$ Immunomodulation & Infection

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Statement of Originality

The work included in this thesis is my own. I devised the hypotheses for all the studies included herein. I designed, performed and analyzed all the animal experiments included. For the clinical work, I devised the hypothesis, contributed to experimental design and interpretation of the data. I constructed all the tables and figures unless referenced.

Acknowledgments

Abstract

GABAergic drugs, such as benzodiazepines, are widely used in clinical practice yet their immune side effects are poorly understood. Preliminary studies have suggested that immune cells express GABA$_A$ receptors indicating that they may be controlled by GABA signaling. Herein parallel preclinical, translational and epidemiological approaches are described to help understand the importance of GABA$_A$ immunomodulation. The hypothesis is that GABA signaling acts to reduce responsiveness to a pathogen and thus that GABAergic drugs will increase susceptibility to infection. To inform on the clinical importance of this work, data from a subgroup analysis of the Maximizing Efficacy of Targeted Sedation and Reducing Neurological Dysfunction (MENDS) trial (where the relative effects of lorazepam, and dexmedetomidine were compared) are described in septic and non-septic patients. Consistent with the hypothesis, avoidance of lorazepam sedation decreased mortality by 70% in septic patients but did not affect outcome in non-septic patients. As preclinical data suggests that benzodiazepines increase mortality at subsedative doses we next conducted a population-based cohort and nested case-control design analysis of The Health Improvement Network (THIN), a comprehensive UK general practice database. Benzodiazepines exposure increased the incidence of community acquired pneumonia (CAP) and both 30-day and long-term mortality from CAP. Based on these significant accumulating data of the harm of exposure to benzodiazepines during an infection, animal studies were conducted to understand (i) the biological plausibility of our findings and (ii) the mechanism of the effect. In a series of mouse studies the prototypical benzodiazepine, diazepam, increased

mortality from *Streptococcus pneumoniae* through potentiation of $GABA_A$ signaling. The increased mortality was associated with increased pathogen load and a delayed cytokine response to the infection. However cellular recruitment was not affected, indicating that local mechanisms were perturbed. Immune cell profiling revealed that alveolar macrophage and monocytes abundantly expressed subunits of the $GABA_A$ receptor, compatible with benzodiazepine sensitivity. *Ex vivo* studies showed that $GABA_A$ receptor activation decreased cytokine responses, phagocytosis and bacterial killing by alveolar macrophage likely via inducing an intracellular acidosis. Finally based on the immune cell profile of $GABA_A$ receptors we predicted that benzodiazepines that do not target the α1 $GABA_A$ subunit would lack the immune suppression observed by non-selective drugs. In accordance with this hypothesis we show that these selective benzodiazepines do not provoke intracellular acidosis, affect cytokine release or bacterial killing of macrophage *ex vivo*. *In vivo* the selective benzodiazepine did not increase mortality from infection or increase pathogen load.

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Abbreviations

APACHE acute physiology and chronic health evaluation

BAL bronchoalveolar lavage

CAP community acquired pneumonia

CD cluster of differentiation

CI confidence interval

CAM confusion assessment method

DC dendritic cell

DEX dexmedetomidine

GABA γ-amino-butyric acid

$GABA_A$ γ-amino-butyric acid type A

GAD glutamic acid decarboxylase

GM-CSF granulocyte macrophage colony stimulating factor

HR hazard ratio

ICU intensive care unit

IFN interferon

Ig immunoglobulin

IL interleukin

IP Interferon gamma-induced protein

LPS lipopolysaccharide

LZ lorazepam

MAPK mitogen-activated protein kinase

MARCO macrophage receptor with collagenous structure

MCP monocyte chemoattractant protein

MHC major histocompatibility complex

MIG Monokine Induced by γ interferon

MIP macrophage inflammatory protein

MV mechanical ventilation

NF-kB nuclear factor kappa-light-chain-enhancer of activated B cells

NO nitric oxide

NOD nucleotide binding oligomerization domain

OR odds ratio

PBA phosphate buffered saline with 1% sodium azide

PBS phosphate buffered saline

PRR pattern recognition receptors

RASS Richmond Agitation Sedation Scale

RIG-I-like receptor retinoic acid inducible gene-1

SIRS systemic inflammatory response syndrome

SP surfactant protein

TGF transforming growth factor

THIN The Health Improvement Network

TLR toll-like receptor

TNF Tumour Necrosis Factor

TREM Triggering receptor expressed on myeloid cells

Chapter 1 Introduction

Foreword

Pneumonia is the third highest cause of death (4.3%) in higher income countries but second highest in lower income countries (10.0%) (World Health Organization Annual Report, 2010). However, it is the major cause of death in children worldwide. Given the public health burden of this condition, novel therapeutic strategies are required. Equally it is important to identify ways to prevent iatrogenic harm that may inhibit an appropriate immune response to the infection. One immune regulatory system that has received limited attention in this regard is γ-amino-butyric acid type A (GABA$_A$) immunomodulation. Medications, such as sedatives, anaesthetics, anti-epileptic agents and many other neuropsychiatric drugs, directly affect GABA$_A$ signaling. However the effects of these drugs on immunity, and the endogenous importance of GABA$_A$ signaling on innate immunity in particular, are poorly understood and will be addressed in this thesis. Given that pneumonia is the leading infective cause of death, we will focus on the lung.

1.0 Immune Homeostasis in the Lung

Immunity is site-specific to ensure a balance between immune defence and preservation of the function of the host tissue. For example immune function is highly regulated in the lung to prevent responses to harmless environmental antigens that would interfere with the lung's primary function: to maintain gas exchange[1]. In particular the primary resident immune cells in the lung, alveolar macrophages, exist in an environment of high antigenic load and thus must be restrained to avoid unnecessary inflammatory damage to the lung[1,2]. In the resting state, airway macrophages are restrained by several mechanisms including epithelial secretion of IL-10, nitric oxide and TGF-β explaining their poor phagocytic capabilities relative to peritoneal macrophages (**Figure 1.1**)[1,3]. They also induce a state of reversible T cell inactivation and inhibit antigen presentation by dendritic cells[1]. Furthermore surfactant proteins (SP), such as SP-A and SP-D, reduce airway macrophage toll like receptor (TLR) responsiveness and phagocytic ability[1]. Another important regulatory pathway is the CD200R-CD200 interaction whereby epithelium, endothelium, and lymphocytes expressing CD200 can induce a suppressed state in the alveolar macrophage by activating CD200 receptor (CD200R)[2].
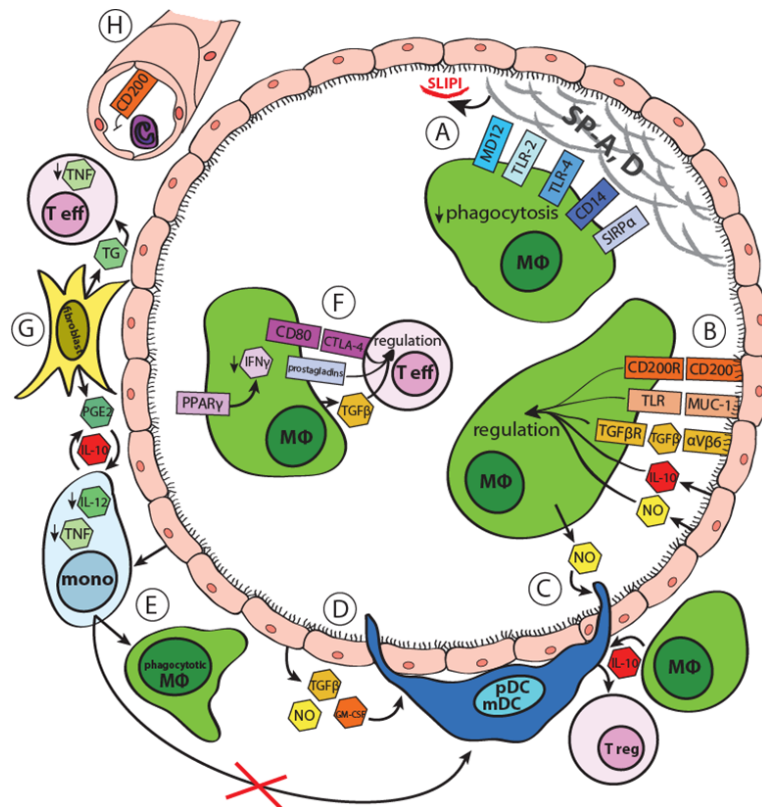
Figure 1.1 Homeostasis of immunology in the lower airways.

(A) Surfactant proteins from type II epithelial cells suppress alveolar macrophage activation through impeding recognition of TLR ligands and through negative regulators of immunity such as SIRP-α. (B) Alveolar macrophage activation is further regulated by epithelial cells via release of anti-inflammatory mediators including TGFβ, nitric oxide (NO) and IL-10 and direct cellular contact leads by CD200 and surface glycoprotein MUC1. (C) Plasmacytoid and myeloid dendritic cells (DCs) promote immunological tolerance to antigen through the release of IL-10 and induction of regulatory T cells. Reciprocal inhibition of DCs is achieved by macrophage-derived NO and IL-10. (D) Airway epithelial cells also limit DC responsiveness through TGFβ, NO and GM-CSF. (E) Airway epithelial cells further act to limit the development of an adaptive immune response by preferentially promoting monocyte development into phagocytic macrophages as opposed to antigen-presenting DCs. (F) Alveolar macrophages have limited antigen presentation capacity and hence poorly stimulate adaptive immunity owing to low co-stimulatory molecule expression or high CD80 expression that binds negative T cell regulator CTLA-4. Alveolar macrophages also directly inhibit T cell responses via the secretion of prostaglandins and TGF-β. Constitutive expression of PPAR-γ in alveolar macrophage negatively regulates the expression of proinflammatory genes. (G) Lung fibroblasts act as a reservoir for triglycerides that subsequently protect the lung from oxygen-radical-imposed injury but also release PGE2 that suppresses TNF and IL-12 production from innate cells and T cell TNF production, activation and proliferation. (H) Endothelia express high levels of CD200 that imparts an inhibitory signal to immune cells as they egress and migrate into the lung. Reproduced from Snelgrove et al.[4].

1.1 Inflammation in the lung

The restraint described above means that airway macrophages are functionally different to their non-epithelial-associated counterparts (e.g. peritoneal macrophage). This is necessary to prevent responses to innocuous antigens and allergens. Nonetheless a robust inflammatory response can be initiated in the lung if stimulation overcomes regulation. Stimulation of innate immunity is via the pattern recognition receptors (PRRs) that recognized conserved structures on pathogens or damaged cells. Such pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are recognized either by membrane bound, cytoplasmic or vesicular PRRs including the Toll-like receptors (TLRs), nucleotide binding oligomerization domain (NOD)-like receptors and retinoic acid like inducible gene-1 (RIG-I)-like receptors. Pathogens stimulate these PRRs activating various intracellular signaling cascades, including mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways, leading to a pro-inflammatory response. However in the resting state, immune regulation in the lung limits responsiveness to pathogens. For example, a bacterium in the lung can be ignored and cleared by non-inflammatory means without inciting an innate immune response that could compromise gaseous exchange. These non-inflammatory approaches include mucociliary clearance, IgA opsonization and by antibacterial peptides[4]. Should the pathogen load exceed a specific threshold however then sufficient TLR activation on the surface of an airway macrophage, or damage to the respiratory epithelium leads to the up-regulation of further

TLRs[5], MHC-II and inhibits IL-10R signaling[6]. Overcoming regulation leads to an increased phagocytic and bactericidal capability of the airway macrophage[1]. Once initiated, innate immunity promotes the release of chemoattractants recruiting neutrophils, monocytes, natural killer cells and/or lymphocytes that perpetuate the inflammatory milieu depending on the nature of the original antigen.

The initial wave of cell recruitment typically involves neutrophils and natural killer cells. Neutrophils clearly play a critical role in controlling pathogens, particularly bacteria, killing by successive enzymatic processes[7]. This initial wave is followed by recruitment of monocytes and then adaptive immune cells such as CD4 and CD8 T cells and B cells. Dendritic cells, that are resident in the lung, play an important role in antigen presentation to lymphocytes following migration to the lymph node. Successive waves of infiltrating cells play an important role in perpetuating the immune response and overcoming this regulation of macrophage in the lung. This is clearly important for pathogen clearance but also represents a "double-edged sword" where increased chance of pathogen clearance increases the risk of inflammatory damage to the pulmonary epithelium (bystander damage).

The threshold for innate immune activation in the lung is adjusted throughout life and depends on the inflammatory or environmental circumstances encountered. For example, severe viral infection may leave a long-lasting imprint

on the lung that in some raises the threshold above which a subsequent antigen can activate innate immunity[3,8]. The evolutionary significance of this effect is that survival of a severe inflammatory event may leave an altered lung that is less able to cope with another immediate inflammatory event. This is likely due to an over-amplification of regulatory pathways that existed in the steady state, a compensatory anti-inflammatory response. It is likely that vulnerability to secondary infections in septic patients occurs via a similar mechanism once the initial inflammation ("cytokine storm") as subsided[9,10]. For example a preceding viral infection leads to desensitization of TLRs to pathogen associated molecular patterns (PAMPs) on pathogens and reduced activation of NF-kB[8]. This changes the threshold for subsequent activation of the immune response hence a bacterial infection during this time may more easily proliferate. A similar finding is apparent in human sepsis where TLR-4 activation with lipopolysaccharide (LPS) provokes reduced cytokine release from splenocytes compared to controls[9]. Through understanding how lung immunology is altered by these differing processes novel therapeutics may be defined. Equally exogenous manipulation of endogenous immune processes, through administration of drugs, may impact on these processes. This has led to the interest in $GABA_A$ immunotransmision, and the effect of benzodiazepines on infection and pneumonia, in this thesis. However despite the description of $GABA_A$ receptors on peritoneal macrophages it is unknown whether alveolar macrophages express $GABA_A$ receptors or if they contribute to immune regulation in the lung.

1.2 The Burden of Pneumonia

As described earlier, community acquired pneumonia (CAP) is a significant cause of morbidity and mortality annually in the United Kingdom and worldwide[11,12]. In the UK the incidence of CAP is between 207 and 233 cases per 100000 of the population per year[11]. The healthcare burden is further increasing as hospital admissions rise; data from hospital episode statistics suggests that between 1997-8 and 2004-5 admissions rose by 34% (from 1.48 to 1.98 per 1000 cases)[12]. Furthermore pneumonia represents the primary cause for intensive care unit admissions with sepsis[13]. Mortality secondary to CAP is 6 to 14% in the United Kingdom[12], however long term mortality is raised for several years after the initial event[11,12]. In 1992-3 CAP cost the United Kingdom £441 million; a more recent analysis from the United States of America set the healthcare burden at $8.4 billion per annum[12].

The term CAP encompasses a wide variety of pathogens including viruses and bacteria that may frequently co-infect in a contemporaneous or sequential fashion[12]. The most common bacterial pathogen is *Streptococcus pneumoniae* (approximately 36% of cases CAP[12]) while influenza A and B are the most common viral pathogens (approximately 13 % of cases CAP[12]). Recent data from the H1N1 pandemic stresses the potential of viral pneumonia and viral-bacterial co-infection to threaten life[14]. In the context of the pandemic, approximately 26-38% of deaths occurred due to viral-bacterial co-infection; the remainder due to viral pneumonia[14]. Accumulating evidence suggests that viral infection itself

increases vulnerability to a secondary bacterial pathogen[1,3]. Indeed evidence from previous influenza pandemics (1918, 1957, 1968 and 1995) suggest that bacterial superinfection occurred in the majority of deaths and/or hospitalizations[1]. Furthermore many host factors also predispose patients to CAP and adverse outcomes from CAP (**Box 1**). Little is known about other factors, including medication that may affect immune responses[15].

Box. 1 Patient risk factors for adverse outcomes in CAP

Risk factor[12, 14]
Age (< 5 or > 65 years old)
Pregnancy (viral pneumonia)
Chronic cardiovascular condition
Chronic lung condition (eg. Chronic Obstructive Pulmonary Disease)
Metabolic disorder (eg. Diabetes Mellitus)
Neurological disorder (eg. Stroke)
Obesity
Immunosuppression
Chronic renal disease
Chronic hepatic disease
Chronic connective tissue disorder (eg. Rheumatoid Arthitis)
Long history of smoking
Malignancy (eg. Lung Cancer)

The immune response to the primary pathogen for CAP, *Streptococcus pneumoniae*, is increasingly well characterized. *Streptococcus pneumoniae* is a gram positive diplococcus pathogen that is widely carried in the population

(approximately 10% of healthy adults and up to 60% of infants)[16]. It has developed various virulence factors to hinder an appropriate immune response such as the invasion genes, iron and other heavy-metal transporters, oxidative stress protection, adherence factors, host-defence evasion, pneumolysin and bacteriocin production, and biofilm formation[16]. However it is the capsule that is the most important virulence factor, inhibiting mucociliary clearance from the respiratory tract and phagocytosis by macrophage, monocytes and neutrophils[16]. Pneumolysin acts to kill host cells as well as to impair respiratory burst in phagocytic cells. *Streptococcus pneumoniae* is detected by PRRs such as TLR-2, -4 and -9 as well as MARCO and NOD-2 (that detects muramyl dipeptide component of pneumococcal peptidoglycan in the cell cytoplasm)[16]. Furthermore the acute phase protein, C-reactive protein (CRP) acts as a PRR binding to the *Streptococcus pneumoniae* cell wall and activating complement. In particular the classic complement cascade aids clearance of *Streptococcus* pneumoniae. Epithelial responses to the pathogen include release of cytokines, chemokines and anti-bacterial peptides such as defensins and SP-D. Alveolar macrophages phagocytose and kill low numbers of *Streptococcus pneumoniae* in the airways[17]. Unfortunately *Streptococcus pneumoniae* is only slowly killed in phagolysosomes especially if poorly opsonized. Alveolar macrophages may then resort to apoptosis to kill the pathogen. Higher numbers of bacteria induce a neutrophilia to clear bacteria, recruited both by the host response but also by pneumolysin. CD4 and γδ T cells as well as natural killer cells also play crucial roles in potentiating the host response to infection, partly through affecting neutrophil recruitment[16].

Increased understanding of the pathogen and immune response to pneumonia has led to the development of vaccines that have reduced the incidence of pneumococcal disease by up to 75%[16]. However, beyond the refinement of antibiotic therapy, the treatment of CAP has not been similarly informed. Current treatment focuses on provision of antibiotics and supportive therapy including supplemental oxygen, ventilation (both non-invasive and invasive ventilation), haemodynamics (use of inotropes and vasoconstrictors) and support of disparate end organ insults including treatment for renal failure and the acute confusional state of delirium. When a systemic inflammatory response (**Table 1.1**) to an infection is noted, the syndrome is referred to as sepsis.

Table 1.1 Variables required for the diagnosis of a systemic inflammatory response

Variable	Value
Temperature	<36°C or >38°C
Heart Rate	> 90 beats per minute
Respiratory	Rate >20 or PaCO2 <4.3KPa
White Blood Cells	$<4x10^9$/L or $>12x10^9$/L or 10% bands

1.3 The Burden of Sepsis

Sepsis is a systemic inflammatory response syndrome associated with infection, septic shock is evidence of sepsis combined with hemodynamic compromise (**Table 1.1**). Many of the features of the systemic inflammatory response syndrome can be attributed to an exuberant immune response by the host, the so

called "cytokine storm"[10]. Here excessive cytokine production (such as TNF-α, IL-1β, IL-6, IL-12, IL-8, and IL-18) and release of DAMPs such as high mobility group B1, leads to septic shock. Inflammation also impairs organ function producing acute brain dysfunction including delirium, acute respiratory distress syndrome, dysfunction of coagulation, renal failure, liver failure, and circulatory failure[10]. However patients with sepsis also exhibit profound immunosuppression and are unable to mount an appropriate inflammatory response to a pathogen[9,10]. Typically patients will enter a compensatory anti-inflammatory phase where impairment of innate immunity leads to secondary infections and, all to often, to patient death[10]. The mechanisms of this immunosuppression are unclear though apoptosis ("programmed cell death") of immune effectors including lymphocytes and dendritic cells appears one important mechanism[10,18]. Immune dysfunction results from both loss of the immune cells and phagocytic clearance of the apoptotic cells that leads to the release of immunosuppressive cytokines TGF-β and IL-10.

Sepsis is a significant public health burden, killing more than 750,000 patients per year in the USA[10] and incurring a mortality of up to 45% in the UK[13]. As such strategies are urgently required to improve both supportive care (such as fluids, inotropes, ventilation and sedatives) and therapeutic care (that currently focuses on appropriate antibiotics). However, recent randomized controlled trials of therapies designed to improve outcomes have failed[19]. This has led to abandonment of research avenues, including the TLR-4 antagonist, eritoran tetrasodium, and the withdrawal of Xigris (Activated Protein C) after the findings

of the initial randomized controlled trial could not be repeated[19]. This latter finding is crucial as Xigris was the only licensed therapeutic for sepsis. The CORTICUS study showed that hydrocortisone therapy could reduce the duration of shock[20] and improved organ dysfunction[21] but did not improve mortality[20], suggesting that reducing inflammation may help but is not a cure for sepsis. The evolution of pathogens resistant to current therapies including antibiotic resistant strains of bacteria[22], stress the importance of supporting innate immune defence in combating the pathogen. While anti-apoptotic therapies have been trialed in laboratory studies[10] they have yet to reach clinical development. Furthermore it is unlikely a syndrome as heterogeneous as sepsis may have a single cure, it is possible that outcomes may be improved by reducing iatrogenic injury. Therefore the search for modifiable risk factors such as sedatives was proposed[23].

1.4 The impact of sedatives on infection

Sedatives exert profound effects on the central nervous system yet their effects on other organ systems, including their immunological impact, are potentially under appreciated. Secondary infections in critically ill patients (i.e. infections that are the not present on admission), such as ventilator-associated pneumonia, are one type of potentially common complication of sedation and mechanical ventilation in the intensive care unit. The most commonly used sedatives target γ-amino-butyric-acid (GABA) receptors in the central nervous system to produce sedation. A secondary analysis of the SEDCOM trial, recently demonstrated that

avoidance of midazolam sedation (a GABAergic drug), by substitution with the α2 adrenoceptor agonist dexmedetomidine, halved the number of secondary infections[24] though two recent randomized controlled trials comparing dexmedetomidine to either propofol or midazolam did not repeat this finding[25]. Therefore there is some evidence that sedative doses of GABAergic drugs may have important effects on immunity but further data are clearly required.

1.5 The impact of community use of GABAergic drugs on infection

GABAergic medication is widely prescribed in the community, typically for neuropsychiatric disorders[26]. One of the commonest used, benzodiazepines are World Health Organization essential medicines used for treating anxiety, epilepsy, muscle spasm, alcohol withdrawal, palliative care, insomnia, and sedation. Therefore it is clear that given the abundant use of GABAergic drugs a thorough understanding of their immune side effects is required.

A systematic review was conducted to identify whether benzodiazepines effect mortality from infection (**Figure 1.2**). Two studies were identified. Hak et al, 2005 did a retrospective cohort analysis to determine prognostic factors for severe complicated lower respiratory tract infection in patients aged over 60 in primary care and found that complications from pneumonia, hospitalisation or all cause mortality were independently associated with use of antidepressants or benzodiazepines (OR 1.89; 95% CI 1.02-3.52)[27]. However the same group, Van de

Nadort et al, performed a similar cohort study in over 80 year olds which showed a conflicting result; benzodiazepine or antidepressant use was not associated with hospital admission or death in this cohort with pneumonia after multivariate analysis (OR 1.2; 95% CI 0.2-1.2)[28]. This may be attributable to studying an older, potentially "higher risk" population with more comorbidities, leading to loss of statistical power to identify the benzodiazepine effect ("drowning the benzodiazepine signal"). Also from these studies the differential effect between antidepressants and benzodiazepines on mortality cannot be ascertained, nor can any effect on mortality from benzodiazepines alone be derived.

INITIAL SEARCH
Pubmed database
"benzodiazepine and mortality"
(limits: human and English)
894 articles

↓

INCLUDED FROM TITLES
13 articles

↓

All abstracts found and scrutinised

↓

8 abstracts identified

↓

2 ARTICLES IDENTIFIED
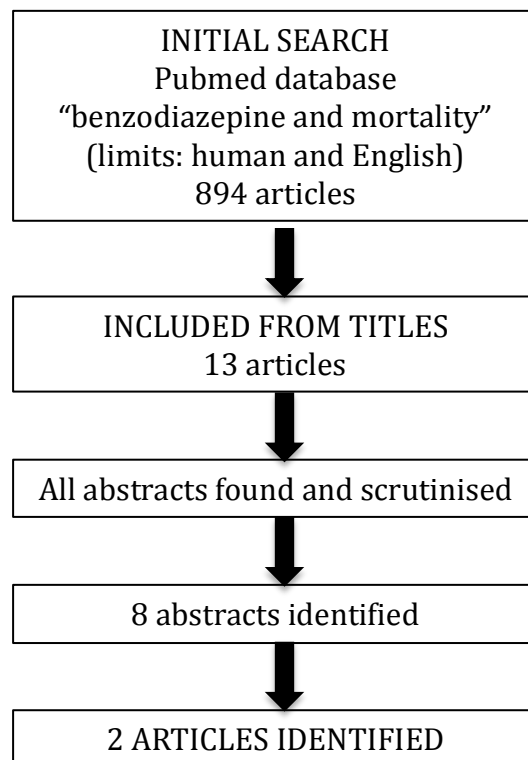
Figure 1.2 Search strategy for systematic review to identify whether benzodiazepines increase mortality from infection in the community.

Therefore limited clinical data support the hypothesis that augmenting GABAergic signaling with drugs such as benzodiazepines may increase mortality from infection; in particular pneumonia and sepsis. Next the mechanisms of GABAergic signaling and the immune effects are reviewed in the context of the published preclinical work.

1.6 GABA Signaling

γ-amino-butyric-acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. It is synthesized from glutamate by glutamic acid decarboxylase (GAD) and activates $GABA_A$, $GABA_B$ and $GABA_C$ receptors to transduce its effect. $GABA_A$ and $GABA_C$ receptors are ionotropic chloride and bicarbonate channels while $GABA_B$ receptors are G-protein coupled metabotropic receptors. Through chloride flux, $GABA_A$ receptor activation hyperpolarizes the neuron decreasing responsiveness to stimuli. The focus of the following section will be on $GABA_A$ receptors that perform the majority of the sedative, anticonvulsant and anxiolytic function of benzodiazepines (the most commonly prescribed class of GABAergic drug) as well as many other anesthetic, anticonvulsant and sedative medications.

There are two genes for GAD, *Gad1* and *Gad2* encoding the isoforms GAD_{67} and GAD_{65} (molecular weights of 67 and 65 KDa respectively). Both GAD isoforms are expressed in the brain and lung. GAD_{65} is expressed in the pancreas and

dendritic cells, CD4 lymphocytes and macrophages[29]. GAD67 is expressed in thymic epithelial cells[30,31]. The isoforms equally synthesize GABA however, in neurons, they function differently; GABA produced by GAD_{67} acts as a neurotrophic factor during synaptogenesis, a regulator of redox potential during oxidative stress and also provides energy via the GABA shunt (producing succinate for entry into the Krebs cycle)[32]. While GAD_{67} appears evenly spread through the cytoplasm, GAD_{65} is concentrated in nerve terminals where it acts to synthesize GABA for neurotransmission[32]. The catalytic reaction of the decarboxylation of glutamate to produce GABA is shown below:

$$HOOC\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \rightarrow CO_2 + HOOC\text{-}CH_2\text{-}CH_2\text{-}CH_2NH_2$$
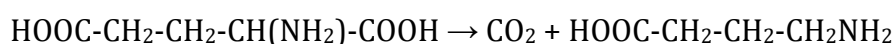
The $GABA_A$ receptor subunits are encoded for by a total of 19 genes, with 8 subunit classes: α1–α6, β1–β3, γ1–γ3, δ, ε, θ, π, ρ1–ρ3 [33]. $GABA_A$ receptors are heteropentameric chloride channels that typically consist of 2 α subunits, 2 β subunits and one γ or δ subunit (**Figure 1.3**). γ2 subunit containing receptors are the most prevalent in the CNS (most typically α1β2γ2) and typically constitute the synaptic pool of $GABA_A$ receptors that show relatively low affinity for GABA[33,34]. The receptor becomes activated with the binding of two GABA molecules between the α and β subunits. The presence of the γ2 subunit i important as benzodiazepines bind at the interface of the α and γ2 subunits[35]. Benzodiazepine refers to a specific chemical structure consisting of a fusion of a benzene ring and a diazepine ring. Via action at the α and γ2 interface benzodiazepines allosterically modulate the $GABA_A$ channel to the increase probability the channel will open with the binding of only one GABA molecule[36].

The δ subunit may commonly substitute for the γ2 subunit rendering the GABA_A

receptor insensitive to benzodiazepines. δ subunit containing receptors are often

referred to as "extrasynaptic" channels that help regulate tonic inhibitory tone in

the brain[37]. They have relatively high affinity to GABA, which is important as the

extrasynaptic ambient GABA concentration is relatively low.
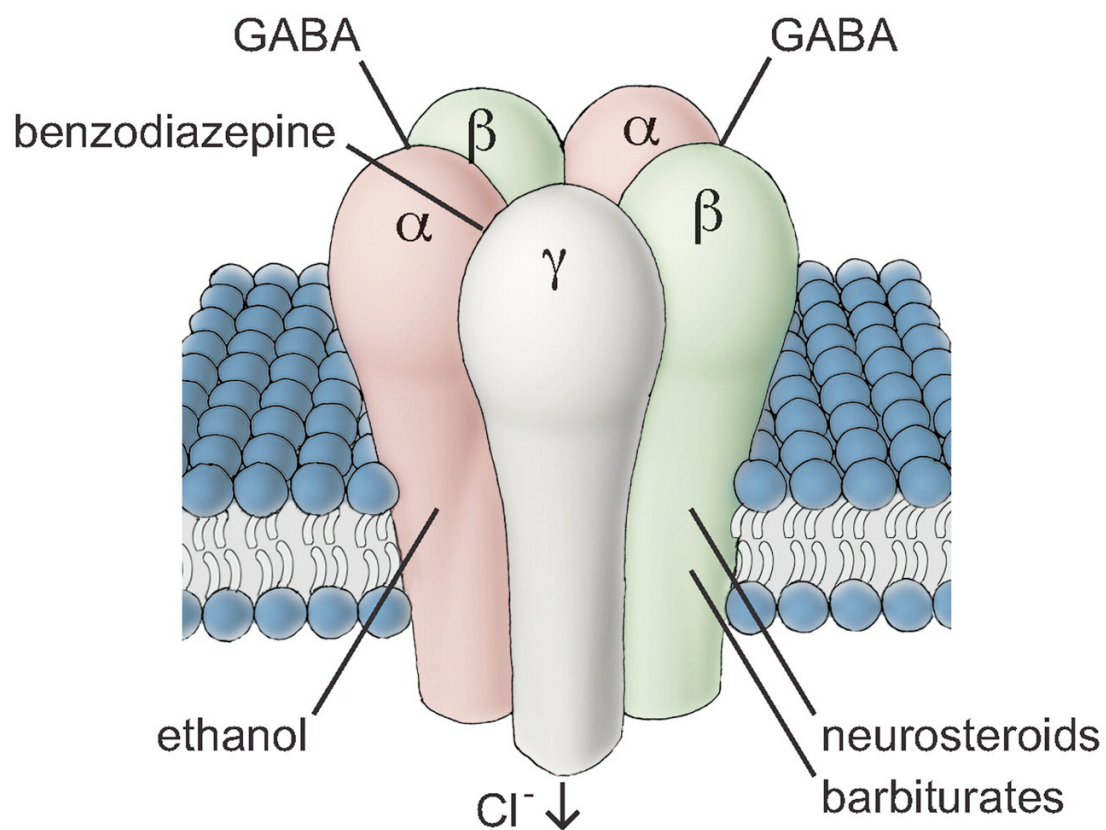
Figure 1.3 GABA_A receptor structure.

The GABA_A receptor is a heteropentameric structure typically composed of two α and β subunits and one other subunit (most often the γ subunit). GABA binds at the junction of the α and β subunits, benzodiazpines at the junction of the α and γ subunits. Receptor function is in part dictated by the chloride ion gradient across the membrane. In mature neurons and macrophages chloride influx typically follows GABA_A receptor activation [29,34,38] however in lung epithelial cells efflux of chloride has been noted [39]. Reproduced from Olsen & Sieghart [38].

1.7 GABA Signaling in the lung

While the central nervous system effects of GABA are relatively well characterized, recently a GABAergic signaling system has been described within the lung[39] and on immune cells[23,29,40,41]. Lung epithelial cells possess GAD and functional $GABA_A$ receptors and increase the expression of both following inflammatory challenge (with OVA or IL-13 treatment) leading to chloride anion (Cl^-) efflux and increased mucus production[39,42]. Activation of epithelial $GABA_A$ receptors also inhibits both basal and stimulated alveolar fluid clearance through active secretion of Cl^-[42]. Consistent with a role in regulation of mucus production and fluid clearance, type 2 alveolar epithelial cells express $GABA_A$ receptor subunits more abundantly than type 1 cells. Both cell types express α1, α3, α4, α6 β2, γ3, ε, π and ρ1-3. Type 2 cells were seen to express γ2 at low levels but this was absent from type 1 cells.

1.8 GABA Signaling in immune cells

Splenic lymphocytes, dendritic cells and peritoneal macrophages express GAD[29]. Immune cells, including macrophages, dendritic cells and CD4 lymphocytes also express GABA receptor mRNA and protein[41] and functional $GABA_A$ receptors are present on lymphocytes[40] and macrophages[29]. Human peripheral blood monocytic cells express mRNA for α1, α3, α4, β2, β3, δ and ε, however it is unclear whether they express the γ2 subunit[41].

In addition to the changes observed with epithelial cells[39], evidence suggests that the expression of GABA receptor subunits is modulated by lipopolysaccharide (LPS) treatment of peritoneal macrophages[43] and IL-1β treatment of hippocampal neurons *in vitro*[44]. Influenza treatment of hippocampal cultures also increases the frequency of GABA mediated inhibitory post-synaptic potentials[45], an effect mimicked by interferon-γ treatment[46]. It is unknown whether inflammation drives changes in GABA receptor expression following pathogen challenge *in vivo* although serum GABA levels are raised in septic animals[47]. At present we know little about the regulation of GAD and GABA$_A$ receptor expression within the lung; in particular we remain ignorant of whether pulmonary immune cells may be regulated in a similar manner to lung epithelium[39].

1.9 Immune Function of GABA$_A$ receptor activation

GABA$_A$ receptor activation on mouse peritoneal macrophage[29] and THP-1 cells (an immortalized human monocytic cell line)[48] is associated with Cl$^-$ influx (similar to mature neurons) and cellular hyperpolarization. This is associated with reduced p38 MAPK activity and inflammatory cytokine production *ex vivo*[29]. Consistent with this, application of GABA reduces stimulated intracellular calcium concentrations in human peripheral blood monocytic cells[41]. GABAergic agents also appear to suppress the activity of critical intracellular signaling cascades such as the p38 MAPK pathway reducing the production of

inflammatory cytokines such as IL-6[49-51]. However dependence on GABAergic signaling, tested by using $GABA_A$ antagonists, has only been explored for the anti-epileptic agents vigabatrin and topiramate *ex vivo*[29]. So while review of the evidence suggests that disparate $GABA_A$ modulators such as benzodiazepines and propofol exert similar effects[23], further experiments testing the dependence on $GABA_A$ receptors are required. In particular, no study has shown that $GABA_A$ antagonists can reverse the effects these drugs *in vivo*.

In addition to its effects on p38 MAPK, midazolam also inhibits degradation of the NF-κB negative regulator, IκB-α, as well as nuclear translocation of the p65 subunit of NF-κB inhibiting transcriptional activity. These actions further reduce the inflammatory potential of the cell. This mimics changes seen during post-viral pneumonia (in animals) where desensitization of toll like receptor signaling with reduced NF-κB activity is associated with increased susceptibility to bacterial infection[8,51,52].

As a class, drugs that activate $GABA_A$ receptors exhibit anti-inflammatory effects[23,29] that have shown benefit in animal models with toxicity associated with extreme inflammation such as endotoxic shock (propofol)[53], acute severe sepsis induced by cecal ligation and puncture (midazolam)[54] and also in an animal model of multiple sclerosis (topiramate and vigabatrin)[29]. In these settings the GABAergic drugs *decrease* mortality. Conversely *in vivo* studies demonstrate that benzodiazepines increases mortality in live bacterial infection

with intraperitonal *Klebsiella pneumoniae*[55], *Mycobacterium bovis*[56], *Salmonella typhimurium*[57] and *Vaccinia*[58] infection in animals. Thus, in animal models with active pathogen replication, *increased* mortality has been noted. Another GABAergic anesthetic propofol impairs bacterial clearance from the lung and spleen in rabbits injected with *Escherichia coli in vivo* (compared to saline with baseline ketamine/xylazine anaesthesia)[59] and *Escherichia coli* and *Staphylococcus aureus in vitro*[23]. Stimulation of GABA$_A$ receptors on THP-1 cells is associated with impaired phagocytosis; however it is unclear whether this effect also obtains in non-immortalized cells too.

GABA$_A$ receptor activation remains a plausible mechanism that would explain the common effects of benzodiazepines, propofol, vigabatrin and topiramate. However benzodiazepines may also modulate immune function by targeting the peripheral benzodiazepine receptor[23]. Indeed studies using the peripheral benzodiazepine antagonist, PK11195, suggest that benzodiazepines may alter neutrophil functions by targeting this receptor[60]. However these studies are difficult to interpret as PK11195 has mixed agonist/antagonist properties[61], the function of the peripheral benzodiazepine receptor is poorly understood[61] and its immune effects are variably reported[60,62,63]. Furthermore propofol, topiramate and vigabatrin do not influence this receptor, although they exert similar effects to benzodiazepines on immunity. Rather all these compounds share GABA$_A$ modulation as a common property. Based on review of the current evidence for GABA$_A$ modulators[23], it seems plausible that benzodiazepines may act through GABA$_A$ receptors to transduce their immune effects.

In contrast to the detrimental effects of GABA$_A$ agonists, the GABA$_A$ antagonist[64], bicuculline, improves acute mortality in septic rats[65] and securinine, also a GABA$_A$ antagonist, enhances killing of *Coxiella burnetii in vitro* by alveolar macrophages[66]. This latter *in vitro* effect suggests that GABA produced by alveolar macrophages plays a role in autoregulating macrophage function. Securinine activates macrophages *in vitro*, resulting in increased cytokine production, increased CD11b and MHC-II antigen expression, down-regulation of L-selectin, and increased cathepsin D expression. Furthermore the findings *in vitro* are supported by increased clearance of *Coxiella burnetii in vivo* by securinine[66].

Therefore structurally diverse GABA$_A$ ligands, agonists and antagonists, affect the immune response, though in opposite directions. However insufficient work has been conducted to characterize the type of GABA$_A$ receptors expressed on immune cells and to confirm the functional role of GABA$_A$ receptor signaling in the effects of drugs such as benzodiazepines. The animal models used so far have focused on intraperitoneal infection and so it is important to confirm the immune effects of benzodiazepines on outcomes from pneumonia. Furthermore limited work has been conducted to identify whether GABAergic drugs, such as benzodiazepines, exert a clinically meaningful affect on infection. In this thesis parallel preclinical and clinical approaches to this problem are described to identify the potential clinical impact and mechanisms of GABA$_A$ immunomodulation on infection.

1.10 Hypothesis

GABA$_A$ receptor signaling plays a role in the response to infection and that augmentation of GABA$_A$ signaling, with drugs such as benzodiazepines, may compromise the immune responses to infection.

1.11 Objectives

The chapters that follow describe preliminary data and the experimental approach to following questions:

1. Does GABAergic sedation worsen mortality in sepsis relative to an alternate non-GABAergic sedative dexmedetomidine?

2. Are sub-sedative doses of benzodiazepines and zopiclone associated with an increased incidence of, and mortality from, pneumonia?

3. Does diazepam impair the immune response to *Streptococcus pneumoniae* pneumonia in a GABA$_A$ receptor dependent manner?

4. Does diazepam impair the immune response to influenza?

5. Do parenteral fluids during influenza affect the immune response?

6. Which immune cells express GABA$_A$ receptors?

7. What are the functional effects of GABA$_A$ receptor signaling in alveolar macrophage?

Chapter 2 Methods

2.1. Methods for Chapter 3: Effect of dexmedetomidine versus lorazepam on outcome in patients with sepsis: an a priori-designed analysis of the MENDS randomized controlled trial

2.1.1 Clinical Trial Study Design

The Maximizing Efficacy of Targeted Sedation and Reducing Neurological Dysfunction (MENDS) study (Trial Registration Identifier: NCT00095251), conducted between August 2004 and May 2006, was approved by the institutional review boards (IRBs) at Vanderbilt University Medical Center and Washington Hospital Center[67]. Patients were randomized after obtaining informed consent, from either the patient or approved surrogate, in a double-blind fashion, to receive dexmedetomidine (DEX)-based (maximum 1.5 mcg/kg/hr) or lorazepam (LZ)-based (maximum 10 mg/hr) sedation for up to 5 days, titrated to target Richmond Agitation-Sedation Scale scores determined by the managing ICU team each day. Patients were monitored daily for delirium with the Confusion Assessment Method for the ICU[68]. In this subgroup analysis, we compared the effects of dexmedetomidine *vs* lorazepam in patients with sepsis, diagnosed within 48 hours of admission to the clinical trial, to the effects of these sedatives in patients without sepsis (now published as Pandharipande et al., 2010[69]). Patients were classified as being septic if they had at least two systemic inflammatory response syndrome (SIRS) criteria and a known or

suspected infection within 48 hours of enrollment. A patient was "suspected" to have an infection if the treating physicians stated this in the medical record or started antibiotics or drotrecogin alfa (activated protein C). SIRS criteria and known/suspected infection were recorded by study personnel prospectively, and one author (Dr Timothy Girard), blinded to study group assignment, also confirmed each case of sepsis by retrospectively examining electronic medical records. Apart from sedation, all other aspects of medical management were according to standardized ventilator management protocols and sepsis treatment algorithms, provided by the critical care team, blinded to the sedative intervention.

2.1.2 Clinical Trial Primary and Secondary Outcomes

The primary outcome of interest was delirium/coma-free days, defined as the days alive without delirium or coma during the 12-day study period[67,69]. Secondary outcomes of the study included delirium-free days, daily prevalence of delirium while patients received study drug, coma-free days, lengths of stay on the ventilator and in the ICU, and 28-day mortality. Ventilator-free days were calculated as the number of days alive and off mechanical ventilation over a 28-day period.

Delirium was measured daily until hospital discharge or for 12 days using the Confusion Assessment Method for the ICU (CAM-ICU)[68]. Efficacy of the study

drug was defined as the ability to achieve a sedation score within 1 point of the desired goal sedation level determined by the managing ICU team each day. Sedation level was assessed using the Richmond Agitation Sedation Scale (RASS), a highly reliable and well-validated sedation scale for use within patients over time in the ICU[68]. Both the RASS and the CAM-ICU instruments are described in more depth at www.icudelirium.org. For other outcomes, patients were followed in the hospital from enrollment for 28 days, or until discharge or death if earlier.

2.1.3 Clinical Trial Statistical Analysis

Data were analyzed using an intention-to-treat approach. Continuous data were described using medians and interquartile ranges or means and standard deviations, and categorical data using frequencies and proportions. We used Pearson chi-square tests for categorical variables and Wilcoxon rank-sum tests for continuous variables to test for baseline differences between the two interventional (treatment) cohorts, stratifying by the presence or absence of sepsis.

We used multivariable regression to examine associations between treatment group and outcomes, assessing for interactions between sepsis and the effect of treatment group on each outcome (i.e., testing for homogeneity of treatment effect according to presence or absence of sepsis). All regression models

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included sepsis, treatment group, and a treatment group by sepsis interaction term as independent variables, in addition to the following covariates: age, severity of illness according to the acute physiology component of the APACHE II score at enrollment, and use of drotrecogin alfa (activated) within 48 hours of enrollment. Because the trial was not powered to detect interactions, we considered an interaction term p value <0.15 to be significant, indicating that the treatment group affected the outcome in question differently among septic than among non-septic patients.

For the primary outcome, we used bootstrap multiple linear regression to calculate a non-parametric 95% confidence interval (CI) for the adjusted difference in mean delirium/coma-free days between the two treatment groups, because of this outcome variable's skewed distribution. Specifically, we fitted a multiple linear regression model (which included the independent variables described above) in each of 2,000 datasets randomly generated from the original data using the bootstrap method (i.e., resampling with replacement) and determined the 95% CI of the adjusted difference in mean delirium/coma-free days using the 2.5 and 97.5 percentiles of the 2,000 regression coefficients of these models. The same approach was used to analyze delirium-free days, coma-free days, and ventilator-free days.

For time-to-event outcomes (time to ICU discharge and death), Cox proportional hazards models were used. Kaplan-Meier survival curves were created for

graphical representation of these time-to-event outcomes. When examining 28-day mortality, patients were censored at the time of last contact alive or at 28 days from enrollment, whichever was first. Censoring for ICU or hospital discharge analyses occurred at time of death or, rarely, at study withdrawal.

To examine the effect of treatment group on the probability of being delirious each day during the study drug period (*vs* having a normal mental status), we used Markov logistic regression. These models, with an outcome of daily mental status, adjust for the previous day's mental status as well as the relevant covariates described above. Due to the multiple assessments included for each patient, generalized estimating equations (GEE) were applied to this regression model to account for the correlation of these observations within each patient. For all results except for interaction terms, two-sided p-values of 0.05 or less were considered to indicate statistical significance. We used R (version 2.10) for all statistical analyses.

2.2 Methods for Chapter 3: The impact of benzodiazepines and zopiclone on pneumonia incidence and mortality: A population-based cohort and nested case-control study

2.2.1 Data source

The Health Improvement Network (THIN) database is a large, longitudinal collection of records of patients registered to various primary care facilities all over the United Kingdom. Approximately 479 general practices are registered to supply data to the research databank of THIN[70]. This contains about 9.1 million registered patients, of which 3.4 million are categorized as 'active patients'[70]. Data are entered using Read codes that map onto the International Classification of Disease (ICD)-9 codes. All patient data contained in THIN are depersonalized and anonymised. THIN was approved by the NHS South-East Multi-centre Research Ethics Committee (MREC) in 2002 for studies using pre-collected, anonymised data with the only requirement being to undergo a scientific review by an Independent Scientific Review Committee (SRC). Approval for this study was obtained from the SRC (application reference number: 11-010R).

2.2.2 Study design and population

We conducted two studies within the THIN database: (i) a population-based matched case-control study nested within THIN to address whether the

incidence of pneumonia was altered by benzodiazepine exposure; and (ii) a cohort study using Cox regression analysis within the cases to understand the impact of benzodiazepine exposure on mortality from pneumonia.

Cases were patients of all ages in the database with a diagnosis of pneumonia occurring between 1 July 2001 and 1 July 2002. We used only the first recorded pneumonia diagnosis within this period for each case. This ensured that only incident cases were picked. Furthermore, our analyses were limited to a single year because of the vast number of cases in the database. Identification of cases was done using specific medical Read codes corresponding to a pneumonia diagnosis (available on request). The date of pneumonia diagnosis was designated the index date. For each case, six controls were matched by practice, sex and age at index date (within three years). Controls were constantly contributing data to THIN at the time the cases were diagnosed. We did not exclude controls or cases if they had a recorded pneumonia diagnosis before our selected study period and included previous pneumonia episodes as a covariate in our analyses. Cox regression was then used to address the impact of drug exposure on mortality in pneumonia cases.

2.2.3 Exposure definition

All recorded prescriptions of benzodiazepines were used. Exposure to drug was classified as 'current' when the most recent prescription was within 30 days

before the pneumonia index date. Prescriptions within 31 to 90 days before the index were treated as 'recent' exposures, while prescriptions of 90 days or more before the index date, were treated as 'past' exposure. An additional category was created for 'no use' where subjects had never been prescribed any benzodiazepine. Zopiclone, a non-benzodiazepine that acts on $GABA_A$ receptors, was used as a test drug in our study to investigate the hypothesis that any observed association between benzodiazepines and pneumonia risk is linked to action on benzodiazepine-sensitive $GABA_A$ receptors.

2.2.4 Potential confounders

Both cases and controls required at least a year's worth of prior data to be included in the study to allow for adequate capture of confounding variables. We evaluated the following comorbidities as potential confounders: ischemic heart disease (IHD), pulmonary disease (including chronic obstructive pulmonary disease (COPD) and asthma) and previous pneumonia episodes. We also used a combined weighted comorbidity index, the Charlson index adapted for use with ICD-9 codes[71]. This takes account of comorbidities such as congestive heart failure, peripheral vascular disease, cerebrovascular disease, myocardial infarction, dementia, chronic pulmonary disease, connective tissue disease diabetes, hemiplegia, renal disease, liver disease, neoplasm and AIDS. Other potential confounders considered include alcohol consumption (categorized as above or at/below recommended weekly units for both males and females), diagnosis of depression and psychosis, current smoking (the most recent record

of smoking status was used) and socioeconomic status measured using the Townsend deprivation score quintiles (the first quintile being the least deprived, while the fifth quintile being the most deprived). The Townsend index measures multiple deprivations by output area (approximately 150 households) and was derived from Census 2001 data. It is calculated using data on house and car ownership, overcrowding of accommodation and employment status [72].

2.2.5 Statistical analysis

Conditional logistic regression was used to assess the strength of the association between exposure with the different drugs and risk of pneumonia. Cox regression was used to assess the association between drug exposure and mortality. Results have been expressed as odds ratios (OR) or hazard ratios (HR) with 95% confidence intervals (CI). The multivariate model included all variables that were either a risk factor for pneumonia in the univariate analysis or were found to modify the OR for drug association by at least 10% when included in a bivariate model with the main exposure variable. Given that age (and perhaps comorbidity)[27,28] and gender[73], may influence the benzodiazepine effect, we tested for interactions between any benzodiazepine effect and age, gender or Charlson comorbidity status. The numbers needed to harm was calculated as 1/absolute risk increase. All analyses were carried out in Stata11[74].

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2.3 Methods for Chapter 4: Effect of diazepam on in vivo pneumonia

infection models

2.3.1 In vivo animal models of infection

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 to 19g) unless stated.

In the bacterial infection animal model, mice were infected intranasally with 1 x 10^6 colony forming units of *Streptococcus pneumoniae* (serotype 2), strain D39 (NCTC 7466, London, UK) under isoflurane anesthesia. For the influenza infection model, as a single infection or preceding *Streptococcus pneumoniae* seven days later, mice were infected with 50 Haemagglutination units (HA) of A/X31 (H3N2) influenza. In some experiments 67 HA infection was used to give a more severe infection. For the influenza-*Streptococcus pneumoniae* coinfection model, mice were infected with influenza (50 HA) seven days before 1 x 10^4 (lethal) or 2 x 10^3 (sublethal) colony forming units of *Streptococcus pneumoniae*. Allergic pulmonary disease was induced by intranasal 15mg house dust mite extract (*Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, United States of America) in BALB/c mice on alternate days for 3 weeks. C57BL/6 mice were not used for this latter experiment as they do not produce a robust allergic response.
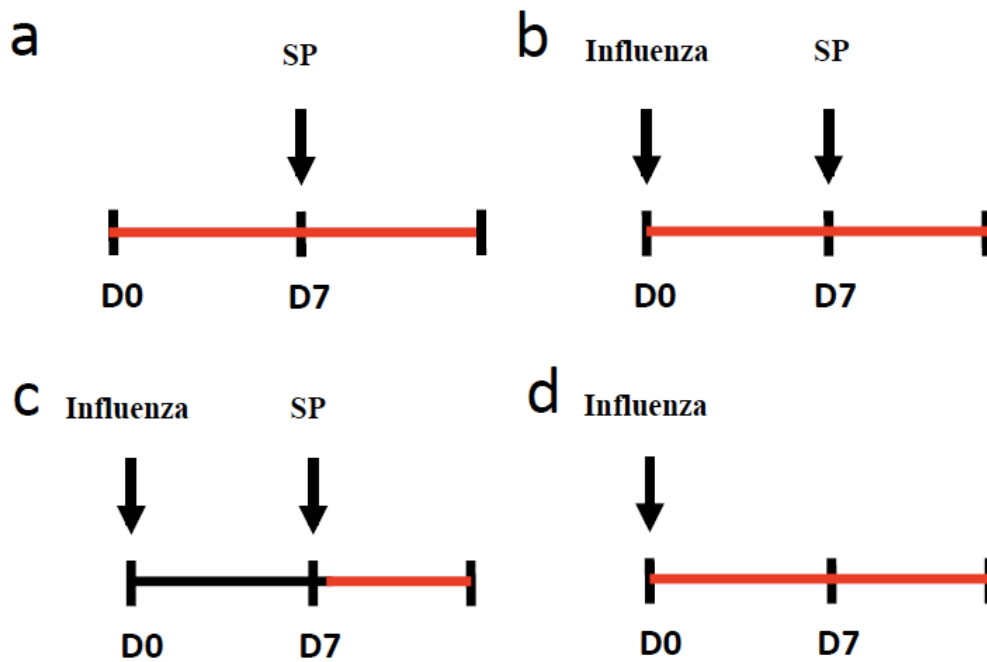
Figure 2.1 Timelines for preclinical bacterial infection models involving diazepam.

Drug administration is denoted by the red line. D refers to day. (**a**) Drugs were given twice daily, from day 0 (D0), for seven days prior to intranasal *Streptococcus pneumoniae* infection (1 x 10^6 CFU) on day 7 (D7). The drugs were also continued twice daily post infection. (**b**) Drugs were given immediately following influenza infection (on D0) and continued throughout the experiment. 1 x 10^4 CFU *Streptococcus pneumoniae* were given intranasally on day seven of influenza infection. (**c**) Drug treatment was commenced four hours after bacterial superinfection of influenza pneumonia. Two doses of *Streptococcus pneumoniae* were used in separate experiments: high dose (1 x 10^4 CFU) and low dose (2 x 10^3 CFU). (**d**) Diazepam and vehicle were also compared during influenza infection. Drug administration was started four hours after influenza infection and continued throughout the experiment.

2.3.2 Drugs

For *in vivo* work, clinical grade diazepam (Hameln Pharmaceuticals, Gloucester, United Kingdom) was diluted in PBS; the vehicle control was 4% ethanol in PBS. Diazepam was given at 2 mg kg^{-1} as this dose provides anxiolysis but not

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sedation[75-78]. The α2/3 GABA$_A$ subunit selective benzodiazepine, NS11394 was obtained by Material Transfer Agreement 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 similar anxiolytic efficacy[75]. The GABA$_A$ antagonist, bicuculline methiodide (Tocris, Bristol, United Kingdom) was diluted in PBS and also given at given at 2 mg kg^{-1} based on previous work[79]. All drugs were given twice daily in a volume of 200 μL by intraperitoneal injection. For the fluid treatment experiments compound sodium lactate (CSL) or 0.9% sodium chloride (NaCl) were administered in a volume of 400 μL by intraperitoneal injection.

2.3.3 Study Endpoints

Several endpoints were examined in the *in vivo* animal work. Survival, weight loss in response to influenza infection, pathogen load, cytokine response and cell recruitment to infection were all assessed. 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. Mice were sacrificed by administration of pentobarbitone and exsanguinated from the femoral artery.

2.3.4 Recovery of Samples

In separate cohorts, blood was typically taken for bacterial load at sacrifice. Bronchoalveolar lavage fluid was then obtained by inflation of the lung four times with 1.5 ml 5 mM EDTA in HBSS via an intratracheal cannula. 100 μL was used for bacterial CFU counts and the remainder centrifuged. The supernatant was then stored at – 80 °C and the cells resuspended for counting and flow cytometric analysis. Lung tissue was divided into cells for rapid freezing in liquid nitrogen (right upper lobe) stored for viral and cytokine load, left lower lobe for histology and the right lung was disrupted through a 100 μM sieve (BD labware, New Jersey, USA) with 100 μL then set aside for bacterial CFU counts, the balance was used for cell counts and flow cytometric analysis. This remainder was then spun and red blood cells lysed by adding ACK 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 and 2 mM L-glutamine. BAL and lung cell viability was assessed by trypan blue exclusion of cells re-suspended in RPMI containing 10% fetal calf serum and 2 mM L-glutamine at 1×10^6 cells ml^{-1}.

2.3.5 Bacterial load

Colony forming unit counts were used to assess bacterial load. Serial dilutions of samples were made in PBS and plated onto Columbia agar supplemented with

5% defibrinated horse blood and counted after incubation at 37°C in air supplemented with 5% carbon dioxide for 16 hours.

2.3.6 Viral load

Lung tissue was freeze-thawed three times, then centrifuged at 4,000g, and supernatants 'titrated' in two-fold dilutions on Madine-Darby canine kidney cell monolayers. After incubation for four hours at 37°C, samples were overlaid with 1% methycellulose and were incubated for 72 h at 37°C. Cell monolayers were washed and incubated with anti-influenza (Serotec, Kidlington, United Kingdom) followed by horseradish peroxidase–conjugated anti-mouse (Dako, Ely, United Kingdom). Infected cells were detected with 3-amino-9-ethylcarbazole substrate. Infectious units were counted by light microscopy and total plaque-forming units per lung were quantified (plaques x dilution factor x lung homogenate volume).

2.3.7 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/peritoneal macrophage (F480$^+$ CD11b$^+$), monocyte (CD11c$^-$ CD11b$^+$ F480$^-$ Ly6G$^-$), neutrophil (CD11c$^-$ CD11b$^+$ Ly6G$^+$), CD4 positive lymphocyte (CD4$^+$ CD3$^+$ CD8$^-$), B cell (CD19$^+$) and CD8 positive lymphocyte (CD4$^-$ CD3$^+$ CD8$^+$).

2.3.8 Cytokine quantification

Cytokine quantification was performed by enzyme linked immunosorbant assay for Interleukin-6 and Tumour Necrosis Factor-α or by Luminex according to the instructions (R&D systems, Minneapolis, United States of America).

2.3.9. Statistical analysis of animal experiments

Data are presented as mean ± standard deviation in the text. Data in the figures are presented as mean ± standard error of the mean. Survival data were analyzed by Log-Rank test. Bacterial load was analyzed by Kruskal-Wallis or Mann-Whitney. Cell counts and cytokine levels were analyzed by Mann-Whitney or analysis of variance and post-hoc Tukey. Data were analyzed on Prism software. Significance was set at $p<0.05$.

2.4 Methods for Chapter 5: Immune regulation and function of

Benzodiazepine sensitive GABA$_A$ receptor expression

2.4.1 Additional Antibodies

Cells were identified as in **2.3.7** above. The antibodies for GABA$_A$ subunits (α1-4, β2, γ2 and δ) came from Abcam (Cambridge, United Kingdom) and were used at 1:100 dilution. The glutamic acid decarboxylase 65/67 antibody (1:100 dilution) came from Millipore (Billerica, United States of America). The secondary Allophycocyanin conjugated antibody was used at a 1:200 dilution (ebioscience, Hatfield, United Kingdom). The isotype antibody was purchased from Santa Cruz Biotechnology (California, United States of America). Positive staining was defined as from 1% of the upper limit of the isotype control but was not considered significant unless (i) it exceeded a minimum value of 3% and (ii) the standard deviations did not overlap with a minimum value of 1%. Cells were washed in PBS supplemented with 1% sodium azide (PBA) and data acquired on a BD FACS LSR II and 30,000 lymphocyte or myeloid events analysed with the FlowJo analysis program.

2.4.2 Ex-vivo TLR and cytokine stimulation of alveolar macrophage

Cells were obtained by bronchoalveolar lavage, and underwent red blood cell lysis, to give a 95% pure alveolar macrophage population. Alveolar macrophage were then plated on plastic for 2 hours at 37°C in 5% CO$_2$ before being washed to

ensure a pure cell population[2]. Alveolar macrophage were then treated for 16 hours with different TLR agonists (TLR 1/2 [PamCSK4] 100 ng ml^{-1}, TLR 2 [HKLM] 10^8 cells ml^{-1}, TLR 3 [PolyIC] 100 ng ml^{-1}, TLR 4 (LPS) 100 ng ml^{-1}, TLR 5 [STFLA] 100 ng ml^{-1}, TLR 6/2 [FSL-1] 100 ng ml^{-1}, TLR 7 [ssRNA40] 100 ng ml^{-1}, or TLR9 (ODN1826] 5μM) from Invivogen, San Diego, United States of America. In further experiments alveolar macrophage were treated for 16 hours with different 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-Ɣ 100 ng ml^{-1}, TNF-α 100 ng ml^{-1}, IL-6 100 ng ml^{-1}, IL-1β 1 ng ml^{-1}) from ebioscience Hatfield, United Kingdom. LPS stimulations conducted in parallel with cytokine experiments were also done at 100 ng ml^{-1}. Cells were then harvested and stained as above.

2.4.3 Ex-vivo phagocytosis and bacterial killing assays

Alveolar macrophage phagocytosis was assessed by incubation for 1 hour with phrodo-labelled *Staphylococcus aureus* (Invitrogen, Paisely, United Kingdom). Phrodo-labelled *Staphylococcus aureus* fluoresces at 600 nm when acidified in a vacuole within a cell and can be detected by flow cytometry.

While neutrophils and RAW cells (an immortalized macrophage cell line) will kill bacteria spontaneously, preliminary studies showed that bacterial killing by alveolar macrophage required pretreatment of cells for 16 hours with IFN-Ɣ (100 ng ml^{-1}) at 37^{0}C to stimulate bacteriocidal capacity. *Streptococcus*

pneumonia were pre-opsonized 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 in water and the supernatant plated in serial dilutions for bacterial load.

Neutrophil assays were conducted with human neutrophils. *Staphylococcus aureus* (MM85T, Oxford strain, NCTC 6571/ATCC 8144, TCS Bioscience) were pre-opsonized with human IgG for 20 minutes and then mixed with neutrophils at a 1:1 ratio for 20 minutes (at 37°C). Cells were the lysed, plated and counted after incubation overnight. Neutrophil respiratory burst was measured by Amplex assay (Invitrogen, Paisely, United Kingdom) following stimulation with Phorbol 12-myristate 13-acetate.

2.4.4 Assessment of Alveolar macrophage intracellular pH

pH was assessed by loading cells with a pH sensitive probe, 2′,7′–bis–(2–carboxyethyl)–5–(and–6)–carboxyfluorescein (BCECF) (Invitrogen, Paisely, United Kingdom), in 10mM HEPES, 8mM MES, 140mM NaCL, 5mM KCL and 5 mM glucose. Cells were left to equilibrate for 10 minutes at 37°C before fluorescence was measured on a Omega Fluorostar plate reader.

2.4.5 Statistical analysis of ex vivo assays

Ex-vivo assays were analyzed by Mann-Whitney or analysis of variance and post-hoc Tukey. Data were analyzed on Prism software. Significance was set at $p < 0.05$.

Chapter 3 Clinical Effects of Benzodiazepines on Sepsis and Pneumonia

3.1 Introduction

3.1.1 Choice of Sedative in Sepsis

Whereas sedative and analgesic medications, routinely administered to mechanically ventilated (MV) patients[80], to improve patient comfort and synchrony with the ventilator, these drugs may contribute to increased time on mechanical ventilation (MV), acute brain dysfunction (delirium and coma) and intensive care unit (ICU) length of stay[81]. Additionally, preliminary evidence suggests that GABAergic sedatives may be particularly deleterious in the setting of infection[23].

Recent advances in critical care medicine have identified acute brain dysfunction as a highly prevalent manifestation of organ failure in the critically ill that is associated with increased morbidity and mortality[67-69,82-86]. Accumulating evidence also shows that the degree and duration[86] of acute brain dysfunction are important risk factors for adverse clinical outcomes. The presence of delirium and coma can potentially worsen outcomes in septic patients; this may be linked to septic perturbation of inflammatory, coagulopathic and neurochemical mechanisms that can contribute to the pathogenesis of acute brain dysfunction. Benzodiazepines, in particular, enhance the risk of developing

acute brain dysfunction, i.e., delirium and coma[67,83]. Other studies have demonstrated that benzodiazepines are associated with worse clinical outcomes when compared with either propofol or with opioid-based sedation regimens[87,88], although these studies did not evaluate the role of changing sedation paradigms on acute brain dysfunction

The MENDS trial[67] demonstrated that dexmedetomidine, an alpha$_2$ (α_2) adrenoceptor agonist[89], provided safe and efficacious sedation in critically ill MV patients, with significant improvement in brain dysfunction (delirium and coma) compared with the benzodiazepine, lorazepam. The principal findings from the MENDS trial were recently corroborated by the SEDCOM trial of 366 critically ill patients, which showed a reduction in the prevalence of delirium in patients sedated with dexmedetomidine vs midazolam; patients on dexmedetomidine also showed a reduction in the duration of mechanical ventilation[24] though Jakob et al. did not see such impressive benefits[25]. In the absence of knowledge of the mechanisms whereby choice of dexmedetomidine may improve patient outcome, it will be necessary to postulate testable hypotheses; hypothesis-testing data can provide the basis for designing future comparative efficacy trials for sedation for the wide-range of ICU patients.

The α_2 adrenoceptor agonists and benzodiazepines have different molecular targets (α_2 adrenoceptors *versus* GABA$_A$ receptors) and neural substrates for their hypnotic effects that may play a critical role in maintaining sleep

architecture in critically ill patients[90,91]; improved sleep may potentially improve delirium outcomes and immune function[23,92].

Furthermore benzodiazepines and α_2 adrenoceptor agonists exert opposing effects on innate immunity, apoptotic injury and mortality in preclinical models of infection[23]. Benzodiazepines increase mortality in animal models of bacterial infection[55-57], perhaps by impairment of macrophage function[49] while $GABA_A$ receptor antagonists are under investigation as anti-infective agents[66]. Contrastingly, α_2 adrenoceptor agonists enhance phagocytosis and bacterial clearance[93-95], while exerting minimal effect on neutrophil function[96], and are associated with improved outcomes in animal models of bacterial sepsis[54,97]. Dexmedetomidine per se exerts superior anti-inflammatory and organ-protective properties compared to other sedatives[54,98] and is neuroprotective in models of hypoxia-ischemia[99] and apoptosis[100], and thus may prevent sepsis-induced brain and other organ injury. The anti-apoptotic effects of dexmedetomidine are greater than midazolam[54] and may be useful, given that sepsis-related mortality has been associated with apoptotic injury[10]. Sympatholysis has also been shown to improve outcome in sepsis[101]; in line with previous evidence[89], presumptive evidence for the more profound sympatholytic actions of dexmedetomidine over its benzodiazepine comparators was suggested by the higher incidence of bradycardia and reduced tachycardia in both the MENDS[67] and SEDCOM[24] studies. Finally the SEDCOM trial showed that midazolam sedation doubled the risk of secondary infections in critically ill patients compared to dexmedetomidine[24].

Multiple levels of evidence thus converge to support the hypothesis that sedation with dexmedetomidine may lead to better outcomes for patients with sepsis than benzodiazepine sedation. We therefore conducted an *a priori*-planned subgroup analysis among MENDS patients to determine if sedation with dexmedetomidine compared to lorazepam in septic *vs* non-septic patients, affected clinical outcomes, including duration and prevalence of acute brain dysfunction and 28-day mortality.

3.1.2 Effect of benzodiazepines on outcomes in community acquired pneumonia

While we have presented a hypothesis that sedative doses of GABAergic benzodiazepines may be inferior to α_2 adrenoceptor agonists, it is unclear whether subsedative dosing of benzodiazepines increases susceptibility to infection. Benzodiazepines are World Health Organization essential medicines used in the community for treating anxiety, epilepsy, muscle spasm, alcohol withdrawal, palliative care, and insomnia. A previous study showed that diazepam (2 mg kg^{-1}) increased mortality from intraperitoneal *Klebsiella pneumoniae* infection. This dose of diazepam was subsequently found to be anxiolytic and subsedative in mice[76,78].

The influence of sub-sedative doses of benzodiazepines on infectious outcomes is incompletely evaluated in humans. One group reported that benzodiazepines and/or anti-depressants increase mortality from pneumonia in over 60 year olds[27] but not over 80 year olds[28]. In contrast, another group has suggested that benzodiazepines may be associated with a decreased incidence of pneumonia however this study used questionnaires to determine drug exposure and thus is prone to recall and reporting bias[102]. Furthermore each of these studies were small (<500 patients) and the outcomes varied and limited, for example currently it is unclear whether there is any long term effect on mortality. We therefore decided to conduct a more rigorous investigation of our dataset based on our hypothesis that predicts that benzodiazepines should exert a "class effect" on outcomes from pneumonia (i.e. all individual drugs should act in a similar manner), as all drugs of this class activate the responsible $GABA_A$ receptors. To further analyze this hypothesis we included the non-benzodiazepine, zopiclone. This hypothesis has clear ramifications for clinical practice as all benzodiazepines, and other $GABA_A$ modulators, would be implicated similarly. We focus here on the incidence of pneumonia and 30-day and long term mortality from pneumonia.

3.2 Results: Choice of sedative in sepsis

3.2.1 Effect of dexmedetomidine versus lorazepam on outcome in patients with sepsis: an *a priori*-designed analysis of the MENDS randomized controlled trial

Sixty-three patients in the MENDS study[67] met the consensus criteria definition of sepsis within 48 hours of enrollment, with 31 randomized to DEX and 32 randomized to LZ. Forty patients were enrolled without sepsis, of which 21 were randomized to the DEX group and 19 to the LZ group. Baseline demographics and clinical characteristics according to treatment group and sepsis are shown in **Table 3.1**. Among non-septic patients, many were admitted with pulmonary diseases, including: pulmonary embolus, pulmonary hypertension, and pulmonary fibrosis (n=13); acute respiratory distress syndrome without infections (n=3); and chronic obstructive pulmonary disease (n=2). Other admission diagnoses among non-septic patients included cardiac surgery (n=6); malignancies (n=3), airway obstruction (n=2); hemorrhagic shock (n=2); gastrointestinal surgery (n=2); neuromuscular disease (n=1); coagulopathy (n=1) and other surgeries (n=5). Sepsis management was similar between septic patients receiving DEX and LZ with regard to number of antibiotics [2 (1, 3) *vs* 2 (1, 3), p=0.37], percentage of patients receiving antibiotics on study day 1 (81% *vs* 81%, p=0.94), and percentage treated with corticosteroids (61% *vs* 59%, p=0.90). Though not statistically significant, activated protein C administration may have been less common among DEX septic patients than LZ septic patients

(21% *vs* 35%, p=0.20) despite a similar severity of illness according to APACHE II scores (**Table 3.1**).

3.2.2 Major clinical outcomes and mortality

Septic patients sedated with DEX had a mean (95% CI) of 3.2 (1.1, 4.9) more delirium/coma-free days, 1.5 (-0.1, 2.8) more delirium-free days, and 6 (0.3, 11.0) more ventilator-free days than patients receiving LZ, after adjusting for relevant covariates. However, no substantial difference was seen in these outcomes between non-septic patients treated with DEX and LZ (**Figure 3.1** and **Table 3.2**). Sedation with DEX had a greater impact on patients with sepsis *versus* without sepsis for delirium/coma-free days (p for interaction = 0.09) and for ventilator-free days (p for interaction = 0.02) (**Figure 3.1**). Alternatively, the effect of DEX *vs* LZ sedation on the probability of being delirious was the same for septic and for non-septic patients (p for interaction = 0.94); among all patients (regardless of sepsis), DEX-treated patients had 70% lower odds, compared with LZ-treated patients, of being delirious on any given day [odds ratio (CI), 0.3 (0.1, 0.7)] (**Figure 3.2**). Amongst the four CAM-ICU features, the beneficial effects of DEX (*vs* LZ) on delirium outcomes were driven by lower odds of development of inattention (CAM-ICU Feature 2) [OR (CI) 0.3 (0.1, 0.7), p= 0.005] and disorganized thinking (CAM-ICU Feature 3) [OR (CI) 0.2 (0.1, 0.5), p<0.001] (i.e. features associated with content of arousal), and not as much by level of arousal.

Table 3.1 Baseline characteristics of patients with and without sepsis*

	Patients with sepsis		Patients without sepsis	
Variable	DEX (N=31)	LZ (N=32)	DEX (N=20)	LZ (N=19)
Age	60 (46, 65)	58 (44, 66)	61 (50, 68)	60 (52, 67)
Males	58%	41%	57%	53%
APACHE II	30 (26, 34)	29 (24, 32)	27 (20, 31)	25 (20, 30)
SOFA score	10 (9,13)	9 (8, 12)	9 (8, 12)	8 (7, 9)
IQCODE at enrollment	3 (3, 3)	3 (3, 3)	3 (3, 3)	3 (3, 3)
Medical ICU	77%	81%	62%	47%
Surgical ICU	23%	19%	38%	53%
Pre-enrollment lorazepam (mg)	1.5 (0, 5)	0 (0, 4)	0 (0, 4)	0 (0, 2)
Enrollment RASS	-3 (-4, -2)	-4 (-4, -3)	-3 (-4, 0)	-3 (-4, -1)
SIRS criteria				
Temperature (Celcius)	37.5 (37, 38.3)	38 (37.2, 38.6)	36.7 (35.8, 37.8)	37.2 (36.2, 38.3)
White blood count ($10^3/\mu L$)	12.5 (6.6, 21.7)	12.5 (7.7, 18.8)	14.6 (8.9,17.9)	10 (7.5,14)
Systolic BP (mm Hg)	88 (78, 100)	83 (79, 100)	92 (90, 100)	90 (80,110)
Heart rate (per minute)	113 (100, 134)	119 (96, 130)	80 (65,123)	107 (99, 126)
Respiratory rate	26 (20, 33)	33 (27, 39)	20 (15 ,24)	24 (20,28)
Organ dysfunction at enrollment				
PaO2/FiO2 ratio	128 (105, 209)	126 (94, 198)	127 (72, 211)	145 (81, 223)
Creatinine (mg/dL)	1.7 (0.8, 2.9)	1.0 (0.8, 1.8)	1.2 (1.0, 1.7)	0.9 (0.8, 1.4)
Vasopressors	32%	56%	19%	5%
Bilirubin (mg/dL)	0.5 (0.4, 0.8)	0.9 (0.4, 1.8)	0.6 (0.5, 1.6)	0.6 (0.4, 1.1)
Platelets ($10^3/\mu L$)	176 (61, 304)	183 (107, 266)	186 (101,242)	145 (114, 242)

Median (interquartile range) unless otherwise noted

*Abbreviations: DEX, dexmedetomidine; LZ, lorazepam; APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential Organ Failure Assessment; IQCODE, Informant Questionnaire on Cognitive Decline in the Elderly; ICU, intensive care unit; SIRS, Systemic Inflammatory Response Syndrome; BP, Blood pressure.

Outcome	Diff. in Means (95% CI)	P–Value for Interaction
Delirium/Coma–Free Days	3.2 (1.1, 4.9)	0.09
0.0 (−3.2, 2.9)
Delirium–Free Days	1.5 (−0.1, 2.8)	0.39
0.3 (−2.2, 2.5)
Coma–Free Days	3.3 (1.3, 5.2)	0.01
−0.9 (−3.5, 1.6)
Ventilator–Free Days	6.0 (0.3, 11.0)	0.02
−5.8 (−13.7, 2.6)

● Septic Patients
▲ Non–Septic Patients

−15 −10 −5 0 5 10 15

Favors Lorazepam **Favors Dexmedetomidine**
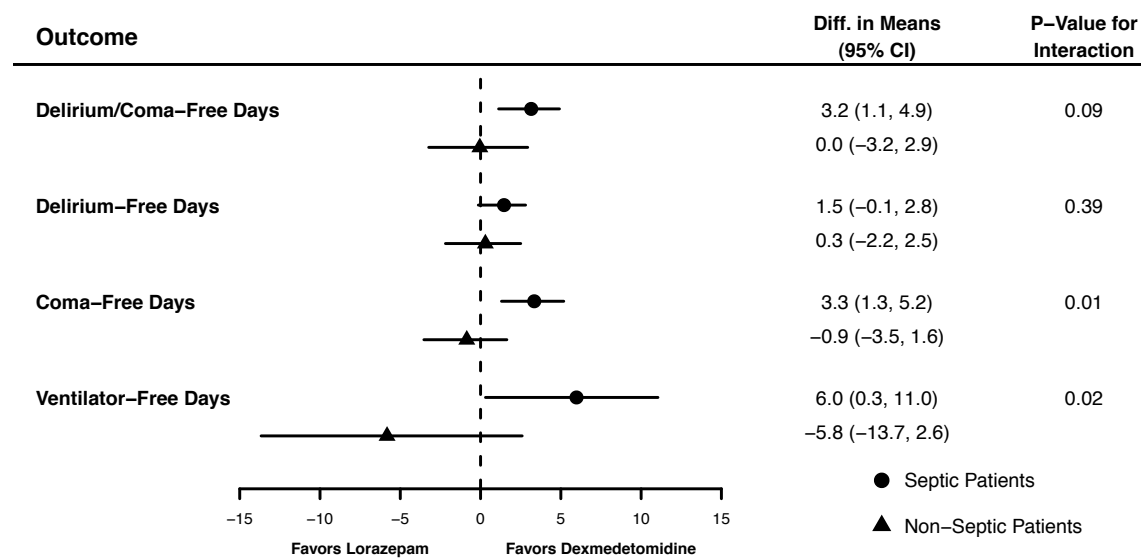
Figure 3.1 Forest plot demonstrating interactions between sepsis and the effect of sedative group on delirium/coma-free days, delirium-free days, coma-free days and ventilator-free days.

For each outcome, the adjusted difference in the means between the dexmedetomidine group and lorazepam group is presented, first for the septic patients (heavy circle) and then for the non-septic patients (heavy triangle), along with 95% confidence intervals (CI) for the difference. Differences, CIs and p-values were calculated using bootstrap multiple linear regression, adjusting for age, the acute physiology component of the APACHE II score at enrollment, administration of drotrecogin alfa (activated), treatment group, sepsis, and treatment group by sepsis interaction. If the difference in means is greater than 0, it reflects an improved outcome with dexmedetomidine; if less than 0, then patients on lorazepam had a better outcome. We considered a p-value for interaction < 0.15 to indicate that the effect of sedative group on the outcome in question was different for septic patients than for non-septic patients. A p value for interaction ≥ 0.15, alternatively, indicated that the effect of sedation group on outcomes was the same for all patients, regardless of sepsis.

Table 3.2 Outcomes of patients with and without sepsis*

Outcome variable	Patients with sepsis			Patients without sepsis		
	DEX (N=31)	LZ (N=32)	Adj. P value**	DEX (N=21)	LZ (N=19)	Adj. P value**
Delirium/coma-free days**	6.1 (4.3)	2.9 (3.2)	0.005	6 (4.7)	5.5 (3.6)	0.97
Delirium-free days†	8.1 (3.1)	6.7(2.9)	0.06	8.1 (3.5)	7.9 (2.8)	0.80
Coma-free days⁜	9.4 (2.9)	5.9 (4.2)	<0.001	8.9 (4)	8.8 (2.6)	1
MV-free days‡	15.2 (10.6)	10.1 (10.3)	0.03	12.8 (11.5)	17.2 (10)	0.15
ICU days	13.4 (15.1)	12.2 (9.8)	0.81	14.9 (16.5)	10.4 (8.9)	0.28
28-day mortality	5 deaths (16%)	13 deaths (41%)	0.03	4 deaths (19%)	1 death (5%)	0.21

Mean (standard deviation) unless otherwise noted

Abbreviations: DEX, dexmedetomidine; LZ, lorazepam; ICU, intensive care unit; MV, mechanical ventilation

* Adjusted (Adj.) P value obtained from the bootstrap multiple linear regression that calculated a difference in mean for each outcome between the two treatment groups, adjusting for age, severity of illness, use of drotrecogin alfa (activated) within 48 hours of enrollment, sepsis, treatment group, and a treatment group by sepsis interaction.

**Indicates the number of days alive without delirium or coma from study day 1 to 12.

†Indicates the number of days alive without delirium from study day 1 to 12.

⁜Indicates the number of days alive without coma from study day 1 to 12.

‡Indicates the number of days alive breathing without assistance of the ventilator from study day 1 to 28.

All Patients

P for treatment = 0.004

Legend: □ Dexmedetomidine, ■ Lorazepam

Y-axis: % Delirious; X-axis: Study Day

Number Assessed for Delirium*

	1	2	3	4	5	6
DEX	29	33	41	42	42	41
LZ	19	17	27	29	25	27

Septic Patients

Y-axis: % Delirious; X-axis: Study Day

Number Assessed for Delirium*

	1	2	3	4	5	6
DEX	17	21	23	25	28	25
LZ	11	8	14	14	13	16
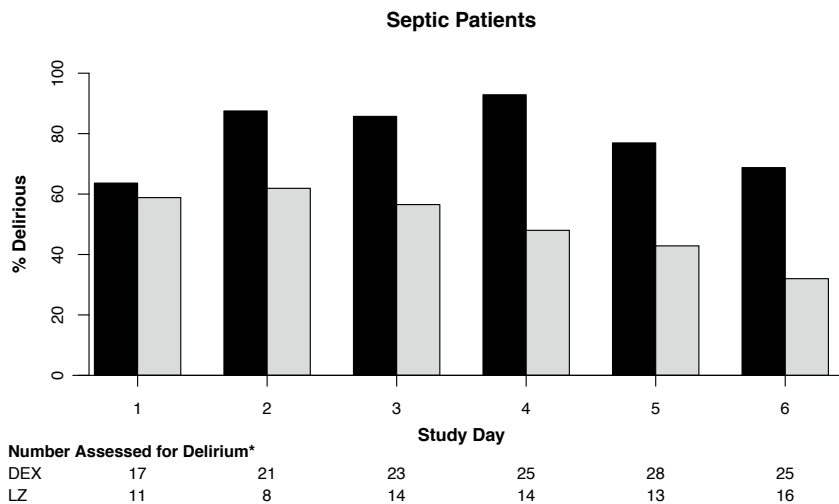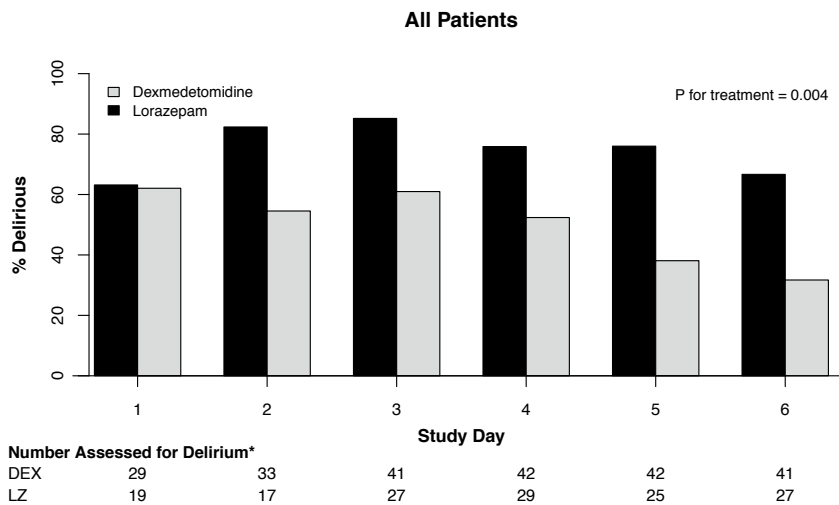
Figure 3.2 Prevalence of delirium while on study drug.

The top panel demonstrates that, among all patients, those sedated with dexmedetomidine had a 70% lower likelihood of having delirium on any given day compared with patients sedated with lorazepam. Sepsis did not modify this relationship (adjusted p for interaction = 0.94), meaning that dexmedetomidine reduced the risk of developing delirium whether patients had sepsis (lower panel) or not.

*Number of patients assessed denotes the number of patients who were alive, in the ICU, and not comatose (RASS -3 or lighter) and are therefore assessable for delirium. Percentages of patients alive and without coma, but with delirium, are represented with black bars if on lorazepam and gray bars if on dexmedetomidine.

Septic patients sedated with DEX additionally had a lower risk of death at 28 days as compared with those sedated with LZ [hazard ratio, HR (CI)= 0.3 (0.1, 0.9)] (**Figure 3.3**); however, this beneficial effect was not seen in non-septic patients [HR (CI) = 4.0 (0.4, 35.5), p for interaction = 0.11]. The proportional hazards assumption for time to death within 28 days was validated graphically and *via* examining model residuals[69].
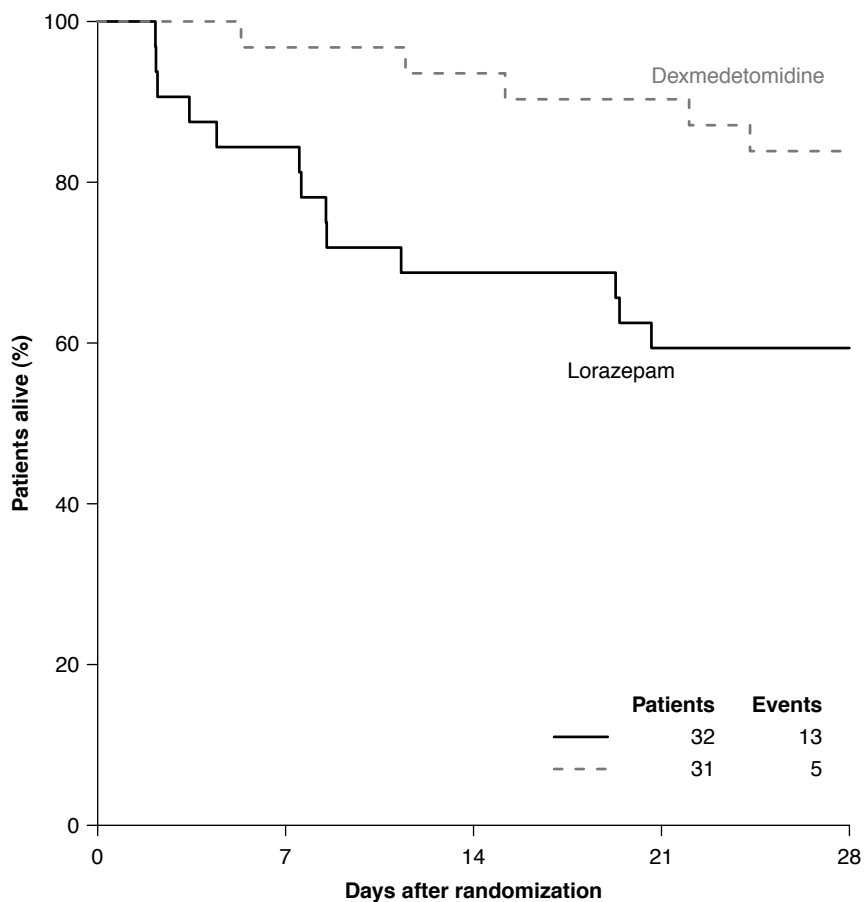
Figure 3.3 Kaplan-Meier curve showing probability of survival during the first 28 days according to treatment group, among patients with sepsis.

Dexmedetomidine decreased the probability of dying within 28 days by 70%; this beneficial effect was not seen in patients who were not septic (p value for interaction=0.11 implying an interaction between sepsis and the treatment groups).

3.2.3 Efficacy of sedation

Among the septic patients, those sedated with DEX achieved sedation within 1 point of their ordered RASS target more often than those sedated with LZ [accurately sedated on 67% of days (50%, 83%) *vs* 52% of days (0%, 67%), p=0.01]; however, efficacy of sedation among the non-septic patients was similar for both treatment groups [67% of days (50%, 86%) *vs* 60% of days (27%, 75%), p=0.27]. Median (interquartile range) DEX dose was 0.8 mcg/kg/hour (0.3, 1.1) and LZ dose was 3.6 mg/hr (2.2, 7.1) in the septic patients. In the non-septic group, median infusion rate were 0.6 mcg/kg/hr for DEX and 2.7 mg/hr for LZ. Septic patients sedated with DEX received more fentanyl per day [1114 mcg/day (212, 2997) *vs* 117 (0, 1460), p=0.01] than septic patients sedated with LZ, while fentanyl use was similar in the non-septic DEX and LZ groups [520 mcg/day (133, 1778) *vs* 262 (10, 775), p=0.20].

3.2.4 Safety evaluation

Incidence of hypotension, vasopressor use and cardiac arrhythmias monitored during the study are shown in **Table 3.3**. There were no differences in cardiac, hepatic, renal and endocrine functional and injury parameters between the DEX and LZ groups, regardless of sepsis at enrollment (all $p > 0.10$). Development of new secondary infections beyond the first 48 hours after enrollment was similar in the originally non-septic group in the DEX and LZ study arms (17% *vs* 15%).

Table 3.3 Hemodynamic parameters in patients with and without sepsis*

	Patients with sepsis			Patients without sepsis		
Hemodynamic variable**	**DEX** (N=31)	**LZ** (N=31)	**P value**	**DEX** (N=21)	**LZ** (N=19)	**P value**
Number of days on vasoactive drugs	1 (1)	2 (2)	0.08	1.5 (2.2)	0.3 (0.9)	0.08
Average daily number of vasoactive drugs	1.1 (0.2)	1.6 (0.5)	0.004	1.6 (0.9)	1 (0)	0.2
Ever vasoactive drugs increased	26%	47%	0.08	33%	16%	0.2
Sinus bradycardia (< 60 beats/min)	13%	6%	0.4	24%	0%	0.02
Sinus tachycardia (> 100 beats/min)	81%	84%	0.7	52%	53%	1

Mean (standard deviation) unless otherwise noted *Abbreviations: DEX, dexmedetomidine; LZ, lorazepam ** Measured during 120-hour study drug protocol, except for sinus bradycardia & sinus tachycardia, which are measured during entire study

3.3 Results: Effect of benzodiazepines and zopiclone on the incidence of and mortality from community acquired pneumonia

3.3.1 Demographic information of controls and cases

The sample used in this research had a total of 34,661 patients, of which there were 29,697 controls and 4,964 cases. The characteristics of cases and controls are summarized in **Table 3.4**. Cases were more likely to have had previous

episodes of pneumonia, myocardial infarction, a history of depression, a history of psychosis related diagnosis, be current smokers, and have a higher Charlson's comorbidity index score as compared with controls.

Table 3.4 Characteristics of Cases and Controls (n=34,661)

Characteristics	Controls (n=29,697)	Cases (n=4,964)	Unadjusted OR (95%CI)
Age (years)			
<25	4,598 (15.48%)	766 (15.43%)	
25-50	5,585 (18.81%)	932 (18.78%)	*matching variable
51-75	10,082 (33.95%)	1,675 (33.74%)	
>75	9,432 (31.7%)	1,591 (32.05%)	
Sex			
Male	13,760 (46.33%)	2,304 (46.41%)	*matching variable
Female	15,937 (53.67%)	2,660 (53.59%)	
Current smokers			
No	24,591 (82.81%)	3,745 (75.44%)	1.00
Yes	5,106 (17.19%)	1,219 (24.56%)	**1.69 (1.57-1.83)‡**
Previous pneumonia			
No	28,935 (97.43%)	4,518 (91.02%)	1.00
Yes	762 (2.57%)	446 (8.98%)	**4.04 (3.56-4.59)‡**
Townsend score deprivation quintile			
1(least deprived)	6,546 (22.04%)	983 (19.80%)	1.00
2	6,408 (21.58%)	973 (19.60%)	1.02(0.93-1.13)
3	5,541 (18.66%)	947 (19.08%)	**1.18(1.07-1.31)**
4	5,132 (17.28%)	904 (18.21%)	**1.24(1.12-1.38)**
5(most deprived)	3,971 (13.37%)	781 (15.73%)	**1.42(1.27-1.60)**
Missing	2,099 (7.07%)	376 (7.57%)	-
			P trend <0.001
Myocardial infarction			
No	26,757 (90.10%)	4,334 (87.31%)	1.00
Yes	2,940 (9.90%)	630 (12.69%)	**1.36(1.23-1.50)‡**
Charlson's comorbidity index score			
0	17,303 (58.27%)	1,939 (39.06%)	1.00
1-2	9,173 (30.89%)	1,964 (39.56%)	**2.27(2.11-2.44)**
3-5	2,997 (10.09%)	929 (18.71%)	**3.74(3.38-4.13)**
>5	224 (0.75%)	132 (2.66%)	**7.43(5.91-9.34)**
			P trend <0.001
Depression			
No	26,380 (88.83%)	4,123 (83.06%)	1.00
Yes	3,317 (11.17%)	841 (16.94%)	**1.71(1.57-1.87)‡**
Psychosis			
No	29,565 (99.56%)	4,930 (99.32%)	1.00
Yes	132 (0.44%)	34 (0.68%)	**1.54(1.06-2.25)**
Alcohol			
Below limit	18,308 (61.65%)	3,137 (63.20%)	1.00
Above limit	11,389 (38.35%)	1,827 (36.80%)	**0.90(0.83-0.98)**
Lung diseases			
No	26,493 (89.21%)	3,763 (75.81%)	1.00
Yes	3,204 (12.44%)	1,201 (24.19%)	**2.74(2.54-2.96)‡**

Note: Statistically significant (p≤0.05) results are in bold; †p<0.001

3.3.2 Effect of benzodiazepines and zopiclone on the incidence of community acquired pneumonia

Table 3.5 shows the adjusted and unadjusted odds ratios for benzodiazepine as a class, and as individual benzodiazepines. After adjusting for current smoking, presence of lung disease, Townsend deprivation index, diagnosis of depression or psychosis, Charlson's comorbidity index, myocardial infarction and previous episode of pneumonia a significant association was seen between benzodiazepine use and increase in pneumonia risk (adj. OR 1.54, 95% CI 1.42-1.67). Prescriptions of diazepam, lorazepam and temazepam were associated with an increased risk of pneumonia (**Table 3.5**). However we did not find a statistically significant association between current use of chlordiazepoxide and pneumonia risk (**Table 3.5**). Furthermore, use of zopiclone, a non-benzodiazepine drug acting on $GABA_A$ receptors, similarly showed an increase in risk of pneumonia (**Table 3.5**). Refinement of the analysis to look at current prescriptions showed that diazepam, temazepam and zopiclone all increased the odds of pneumonia. A test for interaction was carried out with age, gender and Charlson's comorbidity index score for the main drug exposure, benzodiazepine use. No interactions were found with age or gender for drug exposure, however, a statistically significant interaction was found by Charlson's comorbidity index score (p <0.001). **Table 3.6** shows the stratified analysis results. The association between benzodiazepine use and pneumonia incidence was stronger in people with less comorbidity.

Table 3.5 Association between drug exposure and the incidence of CAP

Exposure variable	Cases (n=4,964)	Controls (n=29,697)	Unadjusted OR (95% CI)	P value	Adjusted OR (95%CI)¥	P value
Benzodiazepines						
No	3,695(74.4%)	25,071(84.4%)	1.00		1.00	
Yes	1,269(25.6%)	4,626(15.6%)	**2.00 (1.85-2.16)**	<0.001	**1.54 (1.42-1.67)**	<0.001
Benzodiazepines						
Current	328 (6.61%)	976 (3.29%)	**2.53 (2.21-2.89)**		**1.89 (1.64-2.18)**	
Recent	145 (2.92%)	433 (1.46%)	**2.49 (2.05-3.03)**	*P trend*	**1.95 (1.58-2.39)**	*P trend*
Past	796 (16.04%)	3,217(10.83%)	**1.79 (1.64-1.96)**	<0.001	**1.39 (1.27-1.53)**	<0.001
Diazepam						
No	4,321 (87.1%)	27,505(92.6%)	1.00		1.00	
Yes	643 (12.95%)	2,192(7.38%)	**1.93 (1.76-2.13)**	<0.001	**1.49 (1.34-1.65)**	<0.001
Diazepam						
Current	103 (2.07%)	243 (0.82%)	**2.81 (2.22-3.55)**		**2.11 (1.64-2.71)**	
Recent	49 (0.99%)	151 (0.51%)	**2.12 (1.53-2.93)**	*P trend*	**1.60 (1.14-2.25)**	*P trend*
Past	491 (9.89%)	1,798 (6.05%)	**1.80 (1.62-2.01)**	<0.001	**1.39 (1.24-1.56)**	<0.001
Lorazepam						
No	4,891(98.53%)	29,495(99.3%)	1.00		1.00	
Yes	73 (1.47%)	202 (0.68%)	**2.20 (1.68-2.89)**	<0.001	**1.65 (1.24-2.20)**	0.001
Lorazepam						
Current	21 (0.42%)	63 (0.21%)	**2.01 (1.22-3.31)**		1.66 (0.98-2.81)	
Recent	8 (0.16%)	21 (0.07%)	**2.36 (1.04-5.37)**	*P trend*	1.76 (0.74-4.18)	*P trend*
Past	44 (0.89)	118 (0.40%)	**2.28 (1.61-3.23)**	<0.001	**1.63 (1.13-2.35)**	0.002
Chlordiazepoxide						
No	4,901(98.7%)	29,460(99.2%)	1.00		1.00	
Yes	63 (1.27%)	237 (0.80%)	**1.62 (1.22-2.15)**	0.001	1.19 (0.88-1.62)	0.248
Chlordiazepoxide						
Current	10 (0.20%)	31 (0.10%)	1.94 (0.95-3.95)		1.51 (0.71-3.23)	
Recent	7 (0.14%)	14 (0.05%)	**3.01 (1.22-7.46)**	*P trend*	**2.65 (1.03-6.77)**	*P trend*
Past	46 (0.93%)	192 (0.65%)	**1.46 (1.05-2.03)**	0.004	1.04 (0.74-1.48)	0.445
Temazepam						
No	4,310(86.8%)	27,391(92.2%)	1.00		1.00	
Yes	654 (13.17%)	2,306 (7.77%)	**1.87 (1.70-2.06)**	<0.001	**1.43 (1.29-1.59)**	<0.001
Temazepam						
Current	149 (3.00%)	459 (1.55%)	**2.20 (1.81-2.66)**		**1.69 (1.38-2.06)**	
Recent	66 (1.33%)	185 (0.62%)	**2.40 (1.79-3.20)**	*P trend*	**1.90 (1.41-2.57)**	*P trend*
Past	439 (8.84%)	1,662 (5.60%)	**1.74 (1.55-1.94)**	<0.001	**1.32 (1.17-1.49)**	<0.001
Zopiclone						
No	4,883(98.4%)	29,512(99.4%)	1.00		1.00	
Yes	81 (1.63%)	185 (0.62%)	**2.68 (2.05-3.49)**	<0.001	**1.98 (1.49-2.64)**	<0.001
Zopiclone						
Current	18(0.36%)	36 (0.12%)	**3.10 (1.75-5.48)**		**2.07 (1.13-3.81)**	
Recent	7 (0.14%)	16 (0.05%)	2.38 (0.97-5.89)	*P trend*	1.62 (0.61-4.31)	*P trend*
Past	56 (1.13%)	133 (0.45%)	**2.60 (1.89-3.57)**	<0.001	**2.01 (1.43-2.81)**	<0.001

Table 3.6 Association between benzodiazepines and incidence of pneumonia stratified by Charlson comorbidity index score

Exposure variable	Adjusted[1] OR (95% CI) stratified by Charlson Comorbidity Index score			
	CCI score 0	CCI score 1-2	CCI score >3	CCI score >5
Benzodiazepine use	**1.98 (1.68-2.35)**	**1.46 (1.26-1.69)**	1.09 (0.86-1.40)	-*
Benzodiazepine use				
Current	**2.81 (2.02-3.90)**	**2.01 (1.54-2.62)**	0.77 (0.52-1.15)	
Recent	**1.93 (1.20-3.11)**	**1.91 (1.29-2.83)**	**2.31 (1.31-4.09)**	-*
Past	**1.80 (1.48-2.19)**	**1.26 (1.07-1.50)**	1.09 (0.81-1.46)	

Note: Reference category is 'no use'
[1]Adjusted for Charlson's index score, Townsend Score, Depression, myocardial infarction, previous pneumonia, current smoke and lung disease ; Statistically significant results (p<0.05) in bold
*Could not be calculated to insufficient data

3.3.3 Effect of benzodiazepines and zopiclone on the mortality from community acquired pneumonia

We next analyzed whether benzodiazepine exposure increased 30-day or long term mortality (median follow up 2.8 years) from pneumonia and that current and recent prescriptions exerted the greatest effect (**Table 3.7**). After adjusting for potential confounders (age, sex, Townsend's social deprivation score, current smoking, Charlson Comorbidity Index score, alcohol use, depression and psychosis), any benzodiazepine exposure prior to CAP increased the likelihood of pneumonia-related mortality (adjusted Hazard Ratio (HR): 1.22, [95% Confidence Interval: 1.06-1.39]; p=0.004). The number needed to harm was 6.9 [(95% CI: 5.8-8.5]; thus for every six[§] patients with CAP taking a benzodiazepine, one additional death can be expected within 30 days. Benzodiazepines similarly increased the risk of long term mortality (adjusted Hazard Ratio (HR): 1.32, [95% Confidence Interval: 1.19-1.47]; p<0.001). The numbers needed to harm for long term mortality for any exposure to a benzodiazepine prior to pneumonia

equates to one additional death for every 4 patients taking benzodiazepines with CAP (NNH: 4.4 [3.9, 5.1]).

Individually all drugs tested, except zopiclone, increased all cause mortality. However thirty-day mortality was only affected by diazepam and lorazepam (**Table 3.7**). When individual benzodiazepine use was divided by the timing of prescription, only current diazepam prescription appeared to influence mortality (**Table 3.7**). We also investigated whether the mortality effect of the class of benzodiazepines was affected by age, gender or comorbidity; similar to the effect on the incidence of pneumonia, we found that increasing comorbidity reduced the impact of benzodiazepines on mortality (**Table 3.8**) however age and gender did not impact upon the outcome.

Table 3.7 Association between 30-day and all-cause mortality following pneumonia and benzodiazepine use, all ages (n=4964)

Drug	Numbers dead at 30 days (%) (n=947)	30-day Adjusted Hazard Ratio [1] (95% CI)	P value	Numbers dead over study period (%) (n=1547)	All cause mortality Adjusted Hazard Ratio[1] (95% CI)	P value
Benzodiazepine						
No	568 (15.4)	1.00		938 (25.4)	1.00	
Yes	379 (29.9)	**1.22 (1.06- 1.39)***	0.004	609 (48.0)	**1.32 (1.19-1.47)**	<0.001
Benzodiazepine						
Current	123 (37.5)	**1.35 (1.10- 1.64)**	*P*	185 (56.4)	**1.42 (1.21- 1.67)**	
Recent	61 (42.1)	**1.36 (1.04- 1.79)**	*trend*	93 (64.1)	**1.49 (1.19- 1.85)**	*P trend*
Past	195 (24.5)	1.12 (0.95- 1.32)	0.081	331 (41.6)	**1.24 (1.09- 1.41)**	<0.001
Diazepam						
No	781 (18.1)	1.00		1279 (29.6)	1.00	
Yes	166 (25.8)	**1.24 (1.04- 1.47)**	0.014	268 (41.7)	**1.27 (1.11-1.46)**	0.001
Diazepam						
Current	40 (38.8)	**2.00 (1.45- 2.75)**	*P*	49 (47.6)	**1.71 (1.28- 2.28)**	
Recent	16 (32.7)	1.48 (0.90- 2.43)	*trend*	27 (55.1)	**1.86 (1.27- 2.73)**	*P trend*
Past	110 (22.4)	1.06 (0.87- 1.30)	0.225	192 (39.1)	1.14 (0.98- 1.34)	<0.001
Chlordiazepoxide						
No	930 (19.0)	1.00		1519 (31.0)	1.00	
Yes	17 (27.0)	1.58 (0.98- 2.57)	0.063	28 (44.4)	**1.49 (1.02- 2.17)**	0.038
Chlordiazepoxide						
Current	2 (20.0)	0.92 (0.23- 3.69)	*P*	4 (40.0)	1.01 (0.38-2.70)	
Recent	4 (57.1)	1.98 (0.74- 5.34)	*trend*	6 (85.7)	1.95 (0.87-4.37)	*P trend*
Past	11 (23.9)	1.68 (0.93- 3.06)	0.045	18 (39.1)	1.53 (0.96-2.45)	0.030
Lorazepam						
No	913 (18.7)	1.00		1502 (30.7)	1.00	
Yes	34 (46.9)	**1.61 (1.14-2.28)**	0.007	45 (61.6)	**1.48 (1.10- 2.00)**	0.010
Lorazepam						
Current	10 (47.6)	1.54 (0.82- 2.88)	*P*	13 (61.9)	1.29 (0.75- 2.24)	
Recent	7 (87.5)	**2.57 (1.21- 5.47)**	*trend*	8 (100.0)	**2.79 (1.38- 5.64)**	*P trend*
Past	17 (38.6)	1.44 (0.89- 2.33)	0.018	24 (54.6)	1.38 (0.92- 2.06)	0.017
Temazepam						
No	738 (17.1)	1.00		1209 (28.1)	1.00	
Yes	209 (32.0)	1.11 (0.95- 1.29)	0.208	338 (51.7)	**1.20 (1.06- 1.36)**	0.003
Temazepam						
Current	46 (30.9)	0.91 (0.67- 1.22)	*P*	85 (57.1)	1.12 (0.90- 1.40)	
Recent	29 (43.9)	1.12 (0.77- 1.63)	*trend*	40 (60.6)	1.02 (0.74- 1.40)	*P trend*
Past	134 (30.5)	1.19 (0.99- 1.44)	0.071	213 (48.5)	**1.28 (1.11- 1.49)**	0.001
Zopiclone						
No	932 (19.1)	1.00		1517 (31.1)	1.00	
Yes	15 (18.5)	0.92 (0.55- 1.53)	0.738	30 (37.0)	1.11 (0.77- 1.60)	0.564
Zopiclone						
Current	5 (27.8)	1.43 (0.59- 3.44)	*P*	10 (55.6)	**2.17 (1.17- 4.06)**	
Recent	1 (14.3)	0.30 (0.04- 2.12)	*trend*	5 (71.4)	0.85 (0.35- 2.04)	*P trend*
Past	9 (16.1)	0.95 (0.49- 1.83)	0.641	15 (26.8)	0.91 (0.55- 1.52)	0.943

[1] Adjusted for age, sex, townsend's deprivation score, current smoking, Charlson Comorbidity Index score, alcohol use, depression and psychosis

Note: All comparisons are in reference to 'no use'; percentages shown represent 'row' percentages i.e. the proportion of cases who died within each exposure category.

Table 3.8 Association between benzodiazepines and all-cause mortality following a pneumonia diagnosis stratified by Charlson comorbidity index score (reference category is never)

Exposure variable	Adjusted[1] HR (95% CI) stratified by Charlson Comorbidity Index score			
	CCI score 0	CCI score 1-2	CCI score >3	CCI score >5
Benzodiazepine use	**1.77 (1.34-2.33)**	**1.28 (1.09-1.50)**	**1.25 (1.04-1.50)**	1.13 (0.73-1.73)
Benzodiazepine use				
Current	**1.96 (1.34-2.85)**	**1.30 (1.01-1.68)**	**1.34 (1.00-1.79)**	1.26 (0.70-2.26)
Recent	**2.78 (1.61-4.79)**	1.33 (0.93-1.90)	1.29 (0.90-1.85)	2.15 (0.99-4.67)
Past	**1.45 (1.00-2.08)**	**1.26 (1.04-1.52)**	1.21 (0.98-1.50)	0.89 (0.51-1.53)

Note: Reference category is 'no use'

[1]Adjusted for Charlson's index score, Townsend Score, Depression, myocardial infarction, previous pneumonia, current smoke and lung disease ; Statistically significant results (p<0.05) in bold

3.4 Discussion

We have used two approaches to determine the potential impact of benzodiazepine exposure in the setting of infection. In our first approach we looked at the subgroup of patients who had sepsis in the MENDS trial, finding that lorazepam exposure increased the risk of 28-day mortality relative to dexmedetomidine. Septic patients treated with dexmedetomidine also had shorter duration of acute brain dysfunction (delirium and coma), lower daily probability of delirium and shorter time on the ventilator compared with septic patients treated with lorazepam. Our results further suggest that sedation regimens avoiding lorazepam have a greater impact on these important outcomes in patients with sepsis than in patients without sepsis. These findings suggest that choice of sedative is important in the vulnerable septic patient population and, along with other strategies[103], needs to be addressed at the time sedative regimens are initiated for mechanical ventilation.

Our second approach, using a population-based nested case control design and cohort study, demonstrated an approximately 50 percent increase in risk of pneumonia with users of benzodiazepines with a similar increase in mortality from pneumonia. However a statistically significant association was not observed between chlordiazepoxide use and pneumonia risk. Interestingly the non-benzodiazepine zopiclone was associated with an increased in risk of pneumonia but not pneumonia related mortality. We also investigated whether age, gender or comorbidity influenced the benzodiazepine effect, finding that

increasing comorbidity reduced the impact of the drug on risk of pneumonia and mortality from pneumonia.

3.4.1 Sedation and Immunomodulation

The findings from the patients with sepsis could be the result of either a beneficial effect of dexmedetomidine in the setting of sepsis, a deleterious effect of lorazepam in this setting, or both[23]. Benzodiazepines inhibit macrophage function (although the mechanism is unclear)[49], whereas α_2 adrenoceptor agonists appear to promote macrophage phagocytosis and bactericidal killing[93-95]. Given the crucial role of macrophages in mucosal immunity and clearance of bacteria, the opposing effects of these sedatives on macrophage function may explain our findings herein. These alternate effects are also consistent with the reduced number of secondary infections experienced in dexmedetomidine-sedated (*vs* midazolam-sedated) patients in a secondary analysis from the SEDCOM trial[24].

Therefore the mortality benefit that was provided by dexmedetomidine over lorazepam in our patients with sepsis may be due to several factors. These include differences in the effects of these sedative regimens on both innate immunity and inflammation and also on the anti-apoptotic role of dexmedetomidine[54,100] that may mitigate the deleterious effect of apoptosis in the pathogenesis of sepsis[54]. Indeed, we have recently observed that dexmedetomidine reduces the burden of apoptosis from severe sepsis to a

greater degree than midazolam in the cecal ligation and puncture model[54]. Furthermore, the anti-inflammatory effects of dexmedetomidine may have also contributed to both the reduction in the risk of delirium and the shorter duration of brain dysfunction since inflammation likely plays an important role in the pathophysiology of ICU delirium[104]. The benefits provided by dexmedetomidine may also be attributed to consequences of the quality of sedation. Dexmedetomidine sedation is more akin to non-rapid eye movement (NREM) sleep, than is sedation with GABAergic drugs[90,91,105]; thus, it is possible that improved sleep in critically ill patients could have contributed to improved outcomes given the relationship between sleep with immunity and delirium[85]. Sleep deprivation has been associated with higher levels of inflammatory cytokines, decreased glucose tolerance and increased insulin resistance and activation of the hypotalmic-pituitary axis[85]; all of which could contribute to worse clinical outcomes[85]. Previous polysomnographic studies have revealed that intensive care patients sleep for less than 2 hours in a 24 hours period; thus, prolonged stays in intensive care may result in a huge sleep debt with all the attendant complications of sleep deprivation[85]. The putative contribution of the more natural sleep-enhancing properties of dexmedetomidine[90,91,105] to the observed outcome benefits in septic patients requires further investigation.

3.4.2 Safety of the two sedative regimens

We did not observe any adverse events in the septic dexmedetomidine group (with the possible exception of bradycardia), and there were no differences in

liver, renal, cardiac or endocrine safety outcomes (e.g., cortisol levels) in septic patients treated with dexmedetomidine *vs* lorazepam, attesting to its safety in critically ill septic patients. Dexmedetomidine has been reported to cause hypotension and bradycardia in patients, due to inhibition of central sympathetic outflow, peripheral vasodilation and a vagomimetic action[89]. While this may be concerning in septic patients who are at risk for the development of shock, we observed no difference in the incidence of hypotension between treatment groups. In fact, dexmedetomidine treated patients required fewer daily vasopressors and had trends towards shorter duration of hypotension that may reflect improvement in sepsis severity due to the putative effects of dexmedetomidine on inflammation and immunity. This reduction in vasopressor use in the septic patients is corroborated by a decrease in hypotension seen in animals receiving dexmedetomidine during septic shock[106,107] and reduced epinephrine requirements in dexmedetomidine treated patients following cardiac surgery[108]. In the animal studies, the improved hemodynamic stability correlated with reduced inflammation following dexmedetomidine administration[106,107]. Indeed in two recent studies dexmedetomidine sedation has been associated with a reduction in pro-inflammatory cytokines in patients with sepsis relative to midazolam[109] and propofol[110]. It is plausible that hemodynamic-stabilizing and anti-inflammatory effects of dexmedetomidine are linked by central sympatholysis[54,106,107]; though appearing counter-intuitive, we consider that a reduction in pro-inflammatory cytokines would outweigh any direct hypotensive effect of dexmedetomidine, the net effect being improved hemodynamic stability.

While fentanyl doses were significantly greater in septic dexmedetomidine treated patients vs lorazepam treated patients—likely because supplemental analgosedation may be needed to achieve heavy sedation for a dexmedetomidine-treated patient—it is unlikely that the benefits observed in the dexmedetomidine group were attributable to the use of fentanyl. Indeed, available evidence indicates that opioids have immunosuppressive effects and are capable of increasing mortality in animal models of infection[111-113]. Additionally, fentanyl may contribute to delirium[114]. Thus, we would expect the increased opioid use in the dexmedetomidine group to have reduced rather than promoted the observed benefits.

3.4.3 Effect in non-septic patients

Interestingly, while we observed significant benefits of α_2 adrenoceptor agonist based sedation compared to GABAergic sedation in septic patients, we did not observe all these benefits in the non-septic group. Dexmedetomidine treated patients did have lower odds of development of delirium, whether septic or non septic, however, the improvements in duration of brain dysfunction were predominantly seen in the septic patients on dexmedetomidine. This may be because the non-septic group was smaller than the septic group and thus had limited statistical power to identify any beneficial or detrimental effect of either treatment. Additionally differences in pathogenesis of delirium may account for the greater benefit seen in septic patients, for example increased inflammation. Furthermore septic shock is associated with cellular apoptosis of both immune

and neuronal cells (including the locus ceruleus[115], where there is an abundance of α_2 adrenoceptors). Given that dexmedetomidine prevents central neuroapoptosis via activation of α_2 adrenoceptors[100], these neuroprotective effects may have contributed to the benefits observed in the septic group to a greater extent than in the non-septic group. Finally any detrimental effect of lorazepam may be greater in septic patients, for examples impairments in innate immunity pathogen clearance[23].

3.4.4 Limitations of the clinical trial sub group analysis

There are several limitations to this investigation. First, we categorized patients as septic *vs* non-septic based on the presence of at least two SIRS criteria and suspected infection, in accordance with the consensus definition[116]. As in clinical practice, these determinations were not always supported by microbiological evidence. However, a certified critical care physician confirmed all suspected cases of sepsis to ensure that postoperative patients on prophylactic antibiotics were not misclassified as septic. Future prospective studies should include referral to a clinical evaluation committee to confirm the diagnosis of sepsis and appropriateness of other therapeutic interventions designed to survive sepsis. However alternate analysis of these data, where patients were classified by admission diagnosis of sepsis rather than sepsis diagnosed within 48 hours of admission, found similar results to those presented herein strengthening our findings[117]. Second, this is a subgroup analysis of a larger study, and the study was not powered to specifically examine interactions. Our data are therefore

vulnerable to Type II error, and we advise cautious interpretation of these preliminary findings[118,119]. Interestingly differences in the *magnitude* of a treatment effect based on subgroup analyses are commonplace however as further evidence accumulates qualitative differences (differences in the *direction* of treatment effect) are rarely found[120,121]. However it is important to highlight that for the interaction analysis an *a priori* decision was made to set the threshold for significance at p < 0.15 due to the anticipated limited statistical power for the analysis. Therefore the findings should be treated as preliminary data. Third, the subset population of septic individuals in the MENDS trial may not be generalizable to the entire septic population because of certain exclusion criteria, including severe liver failure, alcohol abuse and ongoing cardiac ischemia. Fourth, randomization was not specifically applied to the septic and non-septic cohort and hence demographic imbalances, common in subgroup analyses, could have occurred. Fortunately, the dexmedetomidine and lorazepam groups were balanced for several important criteria, including severity of illness and organ failure scores (**Table 3.1**). However, some imbalances did exist; for example more non-septic patients randomized to dexmedetomidine were admitted in the medical ICU, which often have higher mortality than surgical ICUs due to associated comorbidities. We were unable to assess whether this difference had a role in the non-significant trends towards lower survival in the dexmedetomidine non-septic group as compared to the lorazepam non-septic patients. We did, however, try to account for potential confounding by including important clinical covariates in our model (including age, severity of illness according to the acute physiology component of the APACHE II score at enrollment, and use of drotrecogin alfa (activated) within 48 hours of

enrollment). Finally, the MENDS study was designed to compare dexmedetomidine to the current recommended sedative, lorazepam. Further studies are required to understand whether dexmedetomidine is similarly superior to other benzodiazepine and non-benzodiazepine agents, such as propofol, that also act via the $GABA_A$ receptor. Indeed lorazepam is a significant risk factor for delirium[83] and may have exaggerated any perceived benefit from dexmedetomidine; it is therefore important that future studies concentrate on alternate agents. These studies should also focus on long-term outcomes such as 90 day mortality to ensure a persistent survival benefit. Thus, these results must be confirmed in a adequately powered prospective randomized controlled studies before widespread changes are made to clinical practice. To this end we have submitted a RO1 application (10786444) to the National Institutes of Health for a multicenter randomized controlled trial of dexmedetomidine versus propofol sedation in 530 septic patients. Propofol is another GABAergic sedative that was chosen in preference to lorazepam due to the reducing use of lorazepam sedation in clinical practice.

In this *a priori*-identified subgroup analysis, sedation with dexmedetomidine reduced the duration of brain organ dysfunction, lowered the probability of delirium, increased time off mechanical ventilation, and reduced 28-day mortality as compared with lorazepam in septic patients; the benefit of dexmedetomidine sedation was greater for septic patients than for non-septic patients in terms of duration of acute brain dysfunction (delirium or coma), time on mechanical ventilation and mortality. Prospective multi-center, randomized controlled trials are needed to confirm these results and examine the

mechanisms underlying the effect of dexmedetomidine and GABAergic sedation on outcomes, including mortality, in sepsis.

3.4.5 Effect of benzodiazepine exposure on the incidence of and mortality from community acquired pneumonia

Benzodiazepine exposure increased the incidence of pneumonia and 30-day and long-term mortality from pneumonia in the community. Consistent with a previous animal study[55], even at subsedative doses benzodiazepines increase vulnerability to infection. Interestingly this effect was less impressive with increasing Charlson comorbidity score.

We included zopiclone in our analysis as it is a non-benzodiazepine that acts on $GABA_A$ receptors. Our findings that zopiclone and benzodiazepines act in a similar manner to increase vulnerability to catching a pneumonia suggest there may be a common mechanism of action (likely augmenting $GABA_A$ receptor signaling). While zopiclone increased risk of pneumonia, the low numbers of patients taking this drug in the cases of pneumonia preclude definitive conclusions on mortality.

We suspect that chlordiazepoxide did not affect the incidence of pneumonia as it is used in the treatment of alcohol dependence and we adjusted for this factor, hence the impact of chlordiazepoxide was dwarfed by the underlying disease. When current prescriptions (those issued within 30 days of the pneumonia)

were analyzed diazepam, temazepam and zopiclone all increased the risk of pneumonia however lorazepam did not. Nonetheless the point estimate for the effect of a current prescription of lorazepam on the incidence of pneumonia remained above 1.

Analysis of the long term mortality data showed a similar pattern of increased mortality for each benzodiazepine. However zopiclone also did not affect mortality, although we suspect that this relates to the low usage of zopiclone in the population of "cases". Further analysis of the mortality data showed that when current prescriptions were considered, only diazepam increased mortality from pneumonia. This may indicate that diazepam produced the biggest effect on mortality of the individual drugs or loss of statistical power in these subgroups (by subdividing the cohort further). The lack of clear signal regarding the relationship between the timing of the prescription and mortality may also be because benzodiazepines are often used on an "as needed" [*pro re nata* (prn)] basis and so a prescription may last greater than one month.

Our finding that the benzodiazepine effect is bigger in patients with minimal comorbidity may be explained by the influence of cumulative disease processes outweighing the effects of the drug. Another explanation is that inflammation from comorbid diseases influences the expression of the $GABA_A$ receptor something we will probe in future preclincial studies (see **Chapter 4**).

While our findings are novel they are complemented by some previous studies. The increase in incidence of pneumonia is similar to a previous study by Knol *et*

al (2008) which recorded a 60% increased risk during the first week of exposure to "anti-psychotic" medication[122]. However, we must also highlight the research carried out by Almirall *et al.* (1999) that found a decreased risk of pneumonia with current use of benzodiazepines (OR 0.46, 95% CI 0.23-0.94)[123]. One potential reason for the variation in results might be from the mode of collection of exposure variables that was done using questionnaires and thus is susceptible to both reporting and recall bias. Vergis et al (2001) showed that the use of "tranquilisers" increased mortality from pneumonia in patients in long term care[124]. Using a retrospective cohort study design, Hak *et al.* (2006) followed up 229 people over 60 years of age with pneumonia over a period of two years and found that the use of benzodiazepines and/or antidepressants increased the risk of pneumonia by 89% (OR 1.89; 95% 1.02-3.52)[125]. The same group performed a similar study in over 80 year olds which showed a conflicting result; benzodiazepine and/or antidepressant use was not associated with hospital admission or death in this cohort with pneumonia after multivariate analysis[28]. These two findings may be consistent with our observations as the more elderly cohort likely had greater comorbidity, reducing the impact of benzodiazepines on mortality.

3.4.6 Strengths and limitations of the population based study

This is a large population-based case control and cohort study with 29,697 controls and 4,946 cases. We have used THIN database which holds longitudinal data on over 9.1 million people registered at 479 general practices throughout the UK[70]. The database is representative of the UK population and results from

research using THIN were in keeping with research from other large databases[126]. This makes our findings applicable to the general population and ensures no recall bias as exposures were recorded prospectively before the diagnosis of pneumonia. The mode of collection of data (i.e. routinely, non interventional and in a prospectively manner prior to the diagnosis of pneumonia) also ensures that reporting bias is eliminated at the point of data collection. THIN has proven to be a credible database for research as results generated from the use of THIN are also consistent with other large databases like the General Practice Research Database (GPRD)[126]. Nesting of this case-control study within a prospective cohort ensures the avoidance of temporal bias as relates to the timing of the exposure to benzodiazepines. Cases and controls were individually matched by age, sex and location of practice, therefore reducing the confounding effects of these variables. In particular, due to the prescribing policies and variations in different regions, matching by practice harmonizes this factor. Findings were adjusted for presence of lung disease in cases and controls, previous pneumonia, depression, myocardial infarction, socio-economic factors, comorbidity, and smoking status.

One possible limitation is the error of misclassification of pneumonia that may arise from differences in coding practices. However, if this happens, results would be biased towards unity because the misclassification is independent of exposure status. The absence of a chest x-ray as a confirmation for the diagnosis of pneumonia brings to the fore a question of the validity of diagnosis in the study. However, from past records, it had been reported that general practitioners diagnose pneumonia and other chest infections with a reasonable

degree of accuracy[127-129]. Codes for 'acute lower respiratory tract infection' (ALRTI) have been included in the case definition as some cases of pneumonia may be coded as ALRTI in the absence of a confirmatory radiograph. Nonetheless our findings suggest that the clinical diagnosis of pneumonia and the associated mortality are both affected by the drugs.

Prescription data were used as a proxy for exposure to the drugs therefore we cannot be certain that the drugs were taken as recommended by the general practitioner. This might lead to an overestimation of exposure to these drugs in the analysis. This limitation however, would be non-differential and would again bias the results towards unity. In order to avoid counting a single case multiple times (for example if the patient re-consults for the same episode of illness), only the first ever record of pneumonia diagnosis occurring within the study period was considered. Differences in coding practices of some of the confounding variables (lung disease and Charlson's cormobidity index) might also bring in some form of bias in the study. However, this misclassification should be independent of exposure and outcome status, thus bias will be non-differential. The Charlson's Comorbidity index score is generated by calculating weights of disease burdens in comorbid patients, with mortality being the outcome [71,130,131]. Therefore, critics have argued that this index is inappropriate when the outcome is morbidity. However, the index had previously been validated in literature and accepted for use in morbidity studies[130,131].

Controlling for socio-economic status using the Townsend index score may have introduced some misclassification errors. This is because data on deprivation is

not personalized but recorded as an area output of 150 households[72]. This assumes that all individuals within a particular geographical area are homogeneous in character that is prone to error. However, from previous evidence, this measure of socio-economic status has proven to be a credible and effective marker of social deprivation[72].

3.4.7 Future directions for the epidemiology study

Our data indicate that benzodiazepines may affect the incidence of, and mortality from, pneumonia. However, given the limitations of the study, further work is required to confirm our findings. In particular prospective cohort studies to are warranted to understand the impact of benzodiazepines on outcomes from infection. This next step is especially important because randomized controlled trials are potentially difficult given the lack of therapeutic alternatives at present. Further studies should also investigate the safety of other drugs, such as the anti-epileptic medication topiramate, that targets $GABA_A$ receptors[29].
.

3.5 Conclusions

In this chapter, two approaches are described to ascertain the importance of benzodiazepine exposure during infection. In critically ill patients with sepsis, lorazepam sedation increased the risk of 28-day mortality. While these data are vulnerable to Type I error, we believe there is sufficient biological plausibility to

warrant investigation in a large randomized controlled trial. The population based nested case control and cohort studies further widens the public health impact of our findings, implicating subsedative doses of benzodiazepines as risk factors for the increased incidence of, and mortality from, CAP. This is of significant concern given the abundance of benzodiazepine use in the community. However neither of these clinical investigations are definitive, they indicate further clinical investigation is required. Likewise mechanistic studies are required to define the biological plausibility of the findings.

Chapter 4 Diazepam increases susceptibility to infection in vivo via augmenting GABA$_A$ signaling

4.1 Introduction

In the previous chapter we have provided evidence that benzodiazepine exposure may increase mortality from sepsis when provided at sedative doses (compared to a non-GABAergic sedative) and from community acquired pneumonia when given at sub-sedative doses. However residual confounding limits definite conclusions based on these studies as neither study was randomized. Further data are needed to (i) support the biological plausibility of the findings and (ii) provide evidence from a randomized controlled trial regarding the impact of augmented GABAergic signaling on immune outcomes. Next we focus on the mechanism of diazepam-induced susceptibility to pneumonia to inform us regarding the biological plausibility of our findings.

Diazepam is known to increase susceptibility to infection *in vivo* from intraperitoneal *Klebsiella pneumoniae*[55], *Salmonella typhimurium*[57], and *Mycobacterium bovis*[132] and *Vaccinia* and cowpox viral infection[58]. However the *in vivo* mechanism for the increase in vulnerability is unknown. *Ex vivo*, drugs that activate the GABA$_A$ receptor exert similar effects on phagocytosis, bacterial clearance and cytokine production[23]. Given the homogeneity in these responses for GABA$_A$ modulating drugs[23], we hypothesize that the benzodiazepine affect will be mediated through GABAergic mechanisms. Indeed propofol[53,59],

thiopentone[133] and benzodiazepines[133] all exert similar effects on phagocytosis, bacterial killing and inflammation[23]. To test whether the benzodiazepine effect was mediated through augmented $GABA_A$ signaling, we first confirmed that we could simulate the benzodiazepine effect on human immunity in mice before investigating whether the effect was reversible with a $GABA_A$ antagonist, bicuculline. To do this we used three animal models of infection: *Streptococcus pneumoniae* pneumonia, *Streptococcus pneumoniae* superinfection of influenza (H2N3 A/X31) pneumonia and thirdly influenza infection alone.

4.2 Results: Benzodiazepines increase susceptibility to infection

4.2.1 Benzodiazepines increase mortality from infection in mice in a $GABA_A$ receptor dependent manner

We hypothesized that this increase in susceptibility to pneumonia was mediated by inappropriate augmentation of GABAergic immunomodulation and tested this by investigating the immune effects of an anxiolytic, subsedative dose of diazepam[76] (2 mg kg^{-1} I.P.) that has previously been shown to increase susceptibility to intraperitoneal infection in mice[55]. We initially established that pretreatment of C57BL/6 mice for seven days with twice daily diazepam, to simulate community use of the drug, did not affect immune homeostasis in the healthy lung. Cell populations in the lung and surface receptors on the resident immune cells, alveolar macrophages were unchanged (**Figure 4.1**).
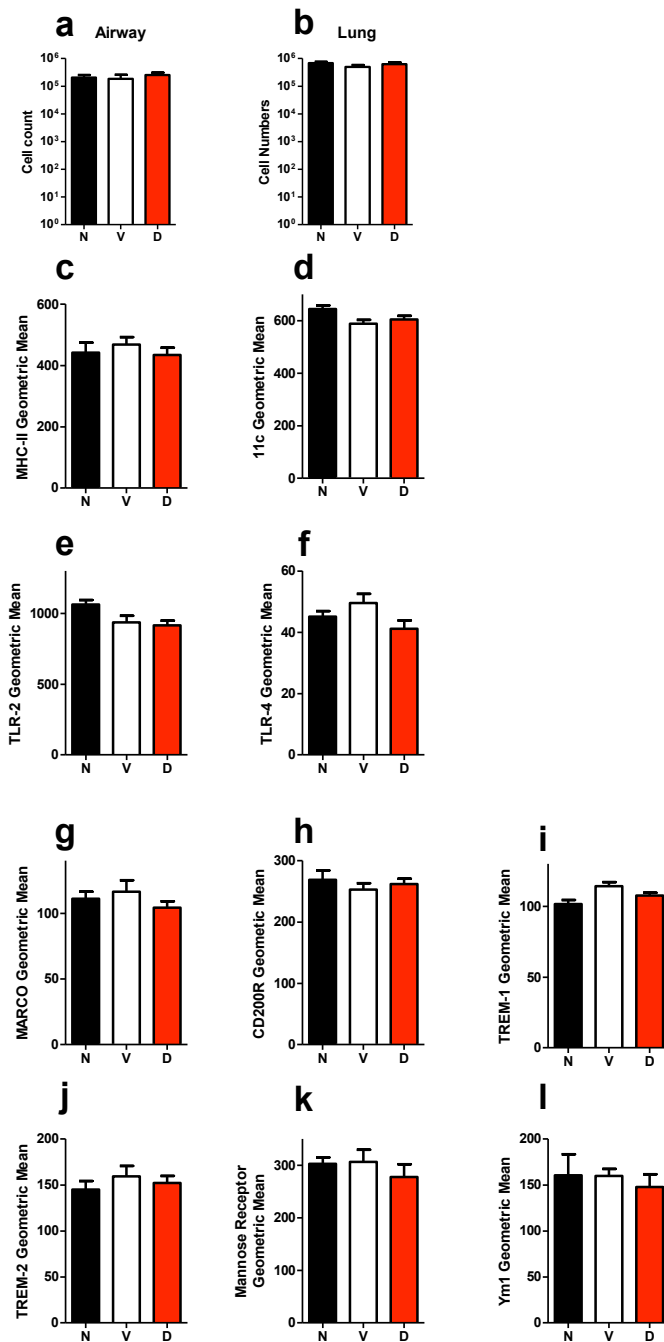
Figure 4.1 Diazepam does not affect immune homeostasis in the lung.

Mice were pre-treated with diazepam (D, 2 mg kg^{-1}), vehicle (V, 4% ethanol in PBS) or left untreated (N, naïve) and 7 days later total airway (**a**) and lung (**b**) cellularity assessed by trypan blue exclusion. Airway alveolar macrophages were also assessed for (**c**) MHC-II, (**d**) CD11c, (**e**) TLR-2, (**f**) TLR-4, (**g**) MARCO, (**h**) CD200R, (**i**) TREM-1, (**j**) TREM-2, (**k**) mannose receptor, and (**l**) YM-1 expression by flow cytometry. Results presented as the geometric mean of fluorescence (± s.e.m) of n=5 mice/group.

When given on day seven of the drug treatment protocol, intranasal *Streptococcus pneumoniae* (1×10^6 colony forming units (CFU) D39 serotype 2) produced an aggressive pneumonia in C57BL/6 mice. Despite the lack of changes to the baseline state, treatment with diazepam increased mortality from *Streptococcus pneumoniae* pneumonia (HR 2.52 [1.08-5.90]; p=0.033); this effect was attenuated by the GABA$_A$ receptor antagonist, bicuculline, indicating a critical role of GABA$_A$ receptor activation (**Figure 4.2a**). The diazepam effect was confirmed in a second experiment (HR 1.71 [1.18 to 2.25]; p=0.004) although there was a more severe infection evidenced by the increased mortality in the control group (p=0.017 by Fischer's exact test vs. prior experiment) (**Figure 4.2b**). As a lack of fluid resuscitation may confound our model we conducted further experiments to test whether administration of fluids would affect mortality in this model. Four intraperitoneal fluid boluses of 20ml kg^{-1} 0.9% normal saline and 10% glucose per day (administered six hourly) did not affect survival in our model (HR 2.38 [0.61-3.29]; p=0.21) though the point estimate was towards increased harm with fluid treatment. In contrast, treatment with ampicillin (200 mg kg^{-1} day^{-1} QDS IP) started four hours after infection completely attenuated mortality (100% vs. 14%; p < 0.001 by Fischer's exact test). Given that (a) fluid resuscitation did not affect mortality in our model, and (b) the antibiotic regimen produced such a significant change (perhaps due to too early administration of the antibiotics to simulate clinical practice) and (c) that the model without fluids or antibiotics reproduced our clinical findings, we pursued this latter model to understand the mechanism of the benzodiazepine effect. Nonetheless we acknowledge the limited clinical parallels of this model (lack of fluid resuscitation and antibiotic therapy).
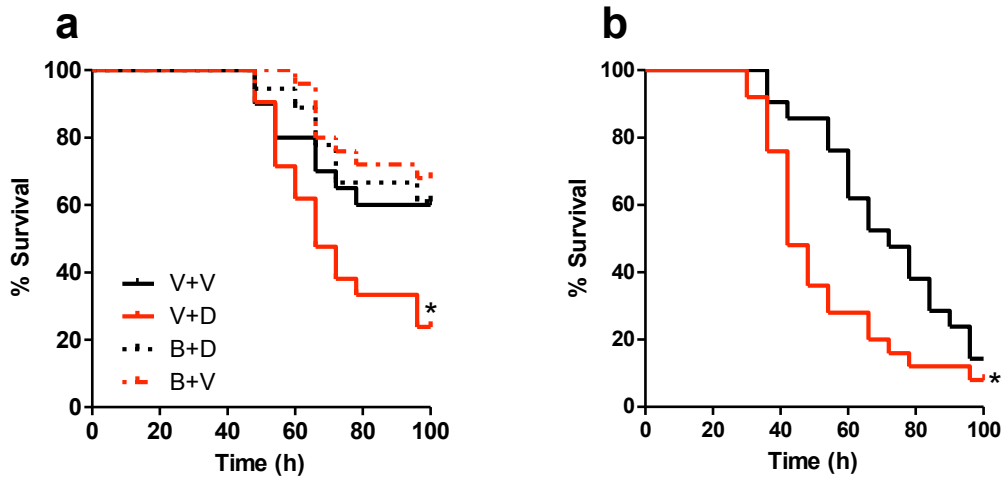
Figure 4.2 Benzodiazepines increase mortality from pneumonia in C57BL/6 mice through GABA$_A$ signaling.

The timeline for this experiment can be seen in **Figure 2.1a**. (**a**) Mice were treated with the vehicle for bicuculline (PBS) and then the vehicle for diazepam (V+V) or diazepam (2 mg kg^{-1}) (V+D). Two other groups received bicuculline followed by diazepam (B+D) or the vehicle for diazepam (4% ethanol in PBS) (B+V). Survival was monitored following *Streptococcus pneumoniae* infection (n = 20/group). (**b**) A second experiment directly compared diazepam (red; n = 13) with vehicle (black; n = 10) following *Streptococcus pneumoniae* infection.

Consistent with the effects on survival, 48 hours into the *Streptococcus pneumoniae* infection, diazepam increased bacterial counts in the airway (**Figure 4.3**). Again this affect was antagonized by the administration of bicuculline indicating dependence on GABA$_A$ receptor activation (**Figure 4.3**). However at this time point cell recruitment was unaffected at 48 hours (**Figure 4.4**) suggesting that either a defect in cell recruitment occurred prior to 48 hours or local responses were perturbed by diazepam.

Figure 4.3 Benzodiazepines increase bacterial load from pneumonia in C57BL/6 mice through GABA$_A$ signaling.

a, Mice were treated with the vehicle for bicuculline (PBS) and then the vehicle for diazepam (V+V) or diazepam (2 mg kg^{-1}) (V+D). Two other groups received bicuculline followed by diazepam (B+D) or the vehicle for diazepam (4% ethanol in PBS) (B+V). Bacterial load in the airway at 48 hours (n = 8/group) after intranasal *Streptococcus pneumoniae* was assessed.

Time course analysis showed that bacterial counts were unaffected at 24 hours but that bacterial load was increased in the airway and lung at 48 hours (**Figure 4.5**). Cell recruitment was unaffected at either time point (**Figure 4.6**), suggesting that diazepam perturbs local responses that would otherwise control the pathogen. Consistent with this, diazepam inhibited the TNF-α, IL-6 and MCP-1 inflammatory response at 24 hours post-infection (**Figure 4.7**). This was followed by an exacerbated cytokine response at 48 hours with high levels of pro-, and anti-inflammatory, mediators (**Figure 4.7**). We consider this exacerbation at 48 hours is likely attributable to the increased bacterial load at this time point (**Figure 4.3 & 4.5**).
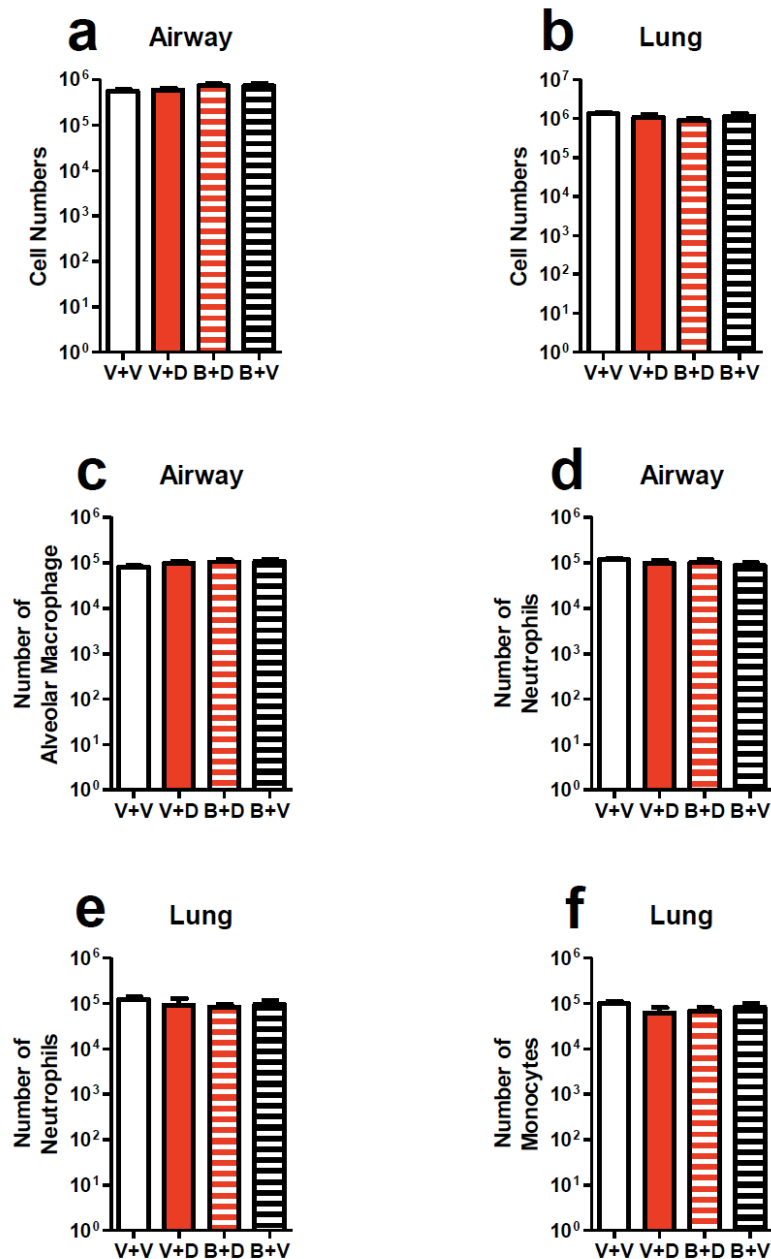
Figure 4.4 Modulation of GABA$_A$ receptors does not affect cell recruitment during infection.

Mice were treated with the vehicle for bicuculline and 30 minutes later given the vehicle for diazepam (PBS) (V+V) or diazepam (2 mg kg^{-1}) (V+D). Two other groups received bicuculline followed by diazepam (B+D) or the vehicle for diazepam (4% ethanol in PBS) (B+V). Total cell counts in the (a) airway or (b) lung were assessed 48 hours after intranasal *Streptococcus pneumoniae* infection by trypan blue exclusion and the number of airway macrophages (c) and neutrophils (d) or lung neutrophils (e) and monocytes (f) assessed by flow cytometry. n=12-14 mice/group. All graphs show the mean ± s.e.m (n=8/group).
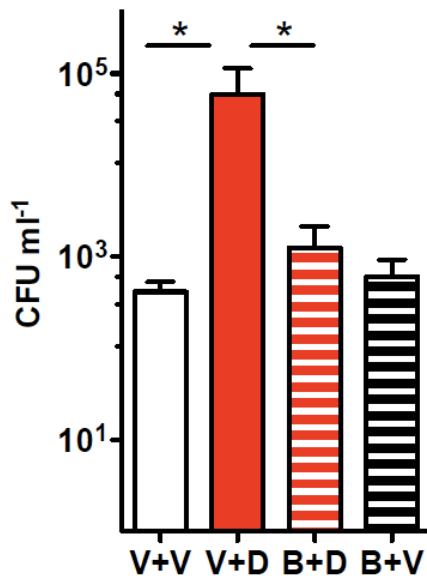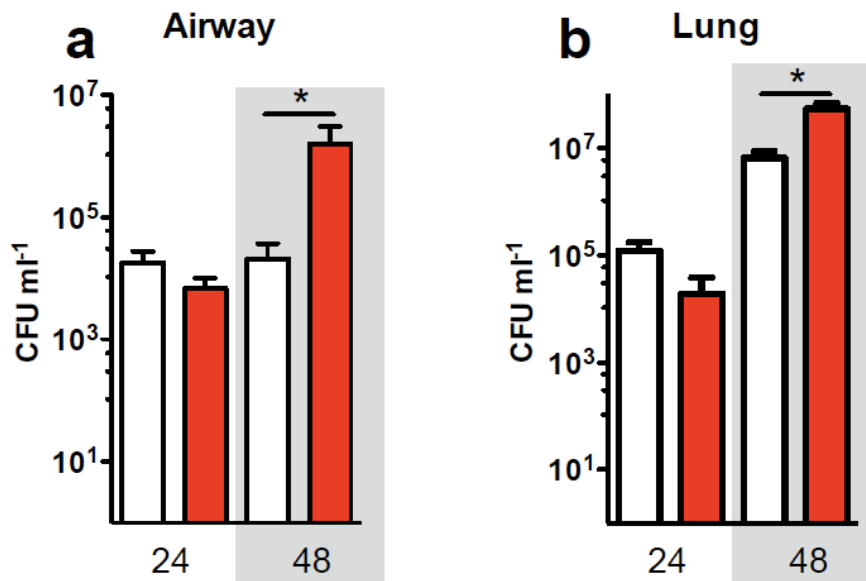
Figure 4.5 Benzodiazepines increase bacterial load from pneumonia in C57BL/6 mice in a time-dependent manner.

Mice were treated with the vehicle for diazepam (4% ethanol in PBS) (white) or diazepam (2 mg kg^{-1}) (red). Bacterial load in the airway (**a**) and lung (**b**) was assessed after intranasal *Streptococcus pneumoniae* at 24 and 48 hours post-infection (n=5/group).
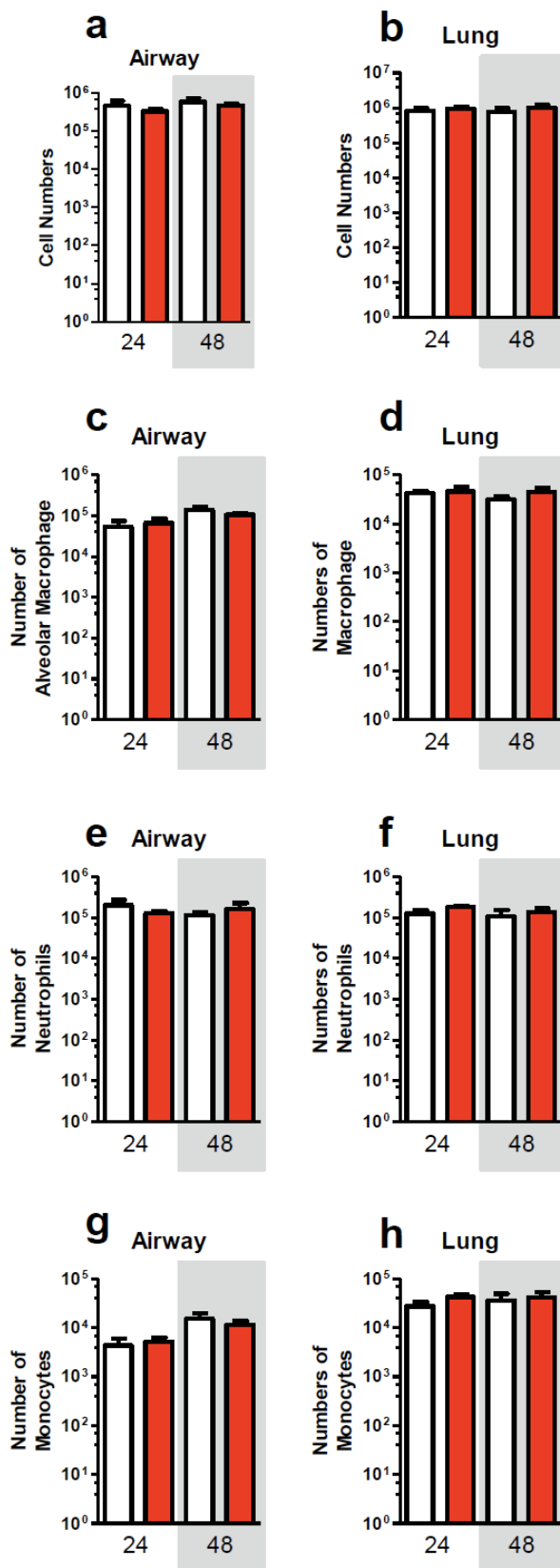
Figure 4.6 Diazepam pre-treatment does not affect cell recruitment post-infection.

Mice were administered diazepam (2 mg kg^{-1}) (red bar) or vehicle (4% ethanol in PBS) (white bar) twice daily for seven days prior to and during infection with *Streptococcus pneumoniae*. Total viable cell counts in the airway (**a**) or lung (**b**) were enumerated by trypan blue exclusion at 24 and 48 hours after the infection. The number of airway macrophages (**c**), neutrophils (**e**) and monocytes (**g**) were assessed by flow cytometry, as were macrophages (**d**), neutrophils (**f**) and monocytes (**h**) in homogenised lung. All graphs show mean ± s.e.m. n = 5/group.
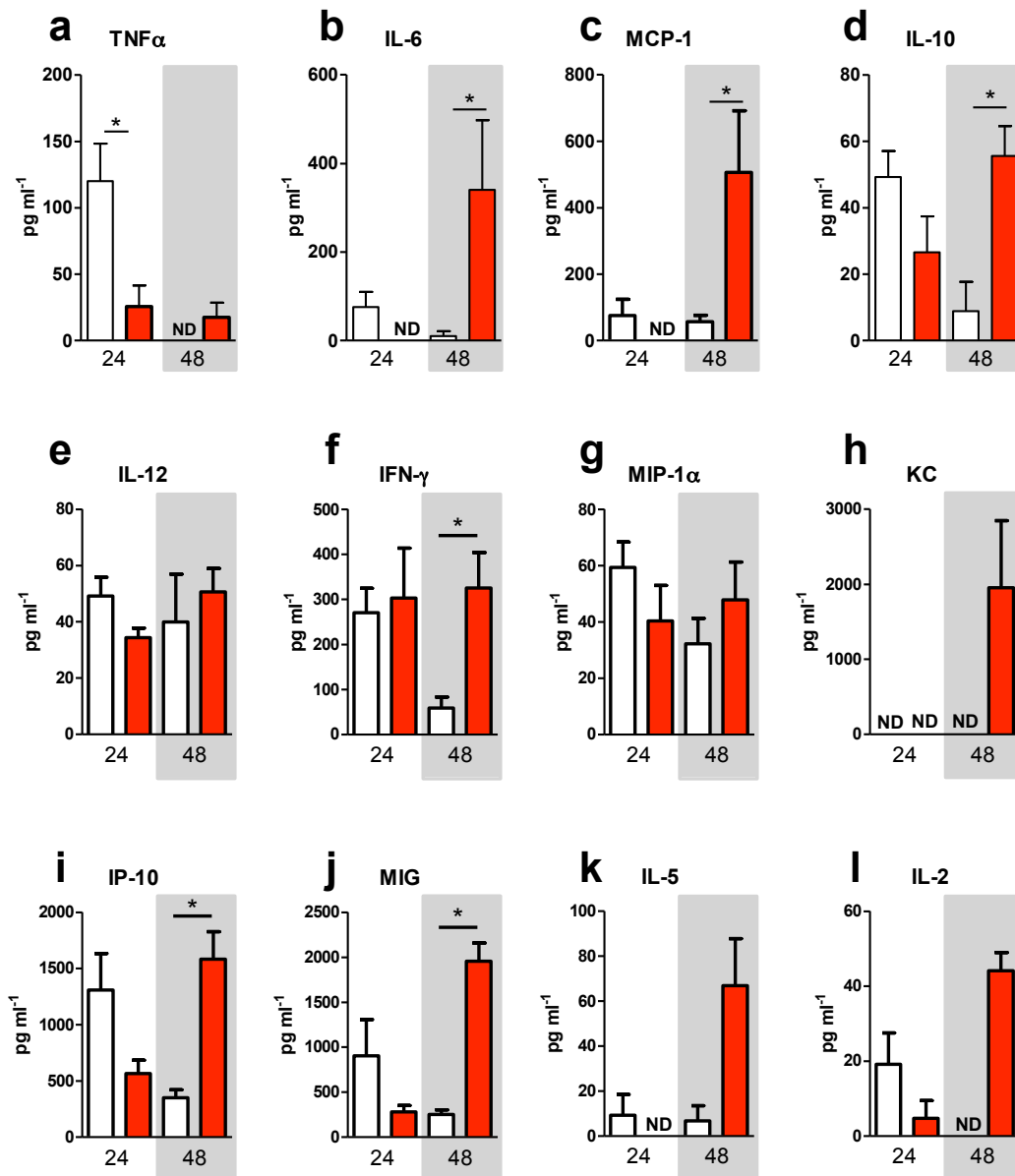
Figure 4.7 Diazepam delays the cytokine response to pneumonia in C57BL/6 mice at 24 hours leading to exacerbation at 48 hours.

Mice were administered diazepam (2 mg kg^{-1}) (red bar) or vehicle (4% ethanol in PBS) (white bar) twice daily for 7 days prior to and during infection with *Streptococcus pneumoniae*. TNF-α (**a**), IL-6 (**b**), MCP-1 (**c**) and IL-10 (**d**), IL-12 (**e**), IFN-γ (**f**) MIP-1α (**g**) KC (**h**), IP-10 (**i**), MIG (**j**), IL-5 (**k**) and IL-2 (**l**) were assessed by Luminex on airway lavage. *p<0.05 vs. vehicle. All graphs show the mean ± s.e.m (n = 5/group).

Given that a local response was likely perturbed by diazepam treatment we investigated whether epithelial mucus production was affected. Epithelial cells express both GABA$_A$ receptors and the synthetic enzyme for GABA (GAD 65/67)[39]. GABA signaling controls epithelial cell proliferation and mucus production, for example over production of mucus in an animal model of asthma is associated with increased expression of GAD and GABA$_A$ receptor subunits and is blocked with GABA$_A$ antagonists[39]. However the γ2 subunit of the GABA$_A$ receptor, that is critical for benzodiazepine binding[38], is only expressed at very low levels by type II alveolar epithelial cells[42] but not airway epithelial cells[134]. Nonetheless given that increased mucus production has been implicated in impairment of pathogen clearance[135], we investigated whether diazepam may affect mucus production using Periodic Acidic Schiff staining. Consistent with limited γ2 subunit expression on epithelial cells, treatment with diazepam did not affect mucus levels in the absence or presence of infection (**Figure 4.8**).

4.2.2 Diazepam increases mortality from bacterial superinfection of influenza in mice

Bacterial superinfection contributed to 26-38% of mortality in the recent H1N1 influenza pandemic[14] and occurred in 20-24% of critically ill influenza-infected patients[14] where benzodiazepines are typically administered[69]. Therefore we tested whether diazepam still increased mortality when animals were made vulnerable to *Streptococcus pneumoniae* by influenza infection seven days earlier. Diazepam treatment started four hours after influenza infection and continued
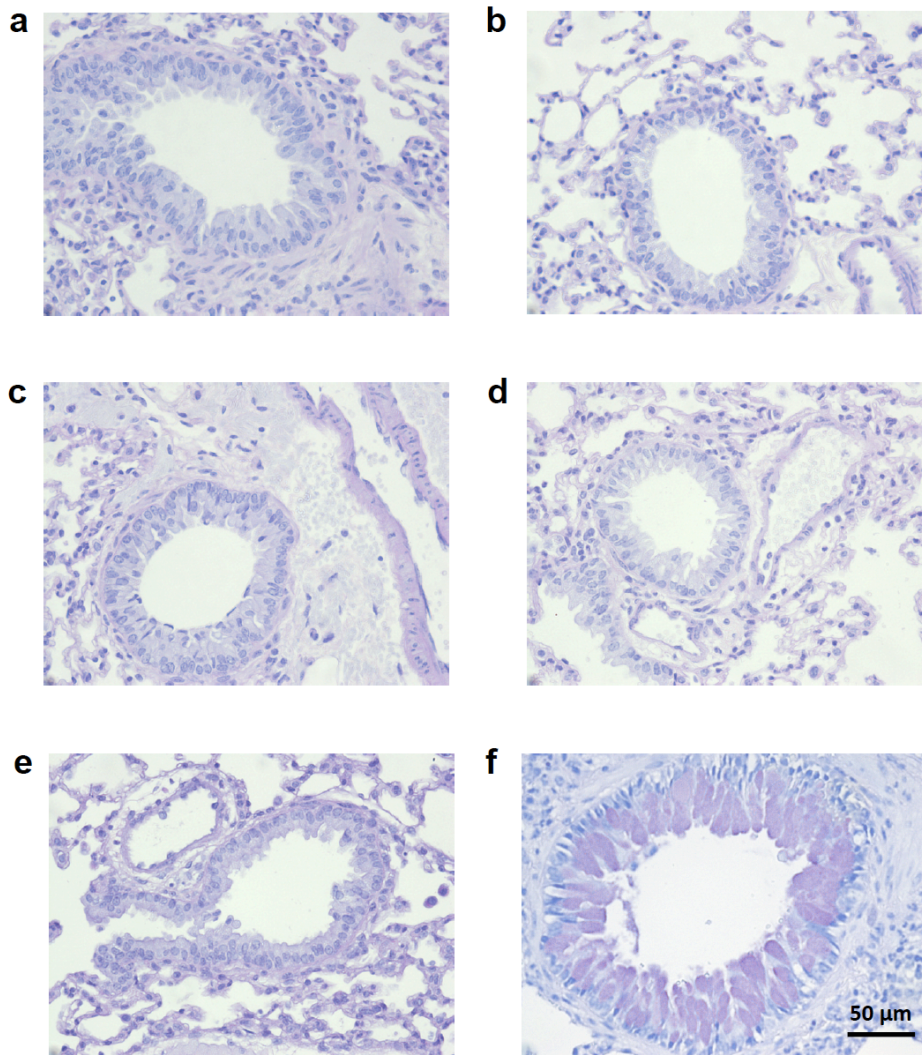
Figure 4.8 Diazepam does not modify epithelial mucus production.

Mucus production was assessed in de-waxed paraffin-embedded lung sections from naïve mice (**a**) and those additionally treated with vehicle (4% ethanol in PBS) (**b**) or diazepam (2 mg kg^{-1}) (**c**). Periodic-acid Schiff staining was also assessed 48 hours after *Streptococcus pneumoniae* infection in C57BL/6 mice treated with vehicle (**d**) or diazepam (**e**). The positive control for PAS staining is the house dust mite sensitized airways, evidenced by pink staining (**f**) (data representative of n=5 mice/group).
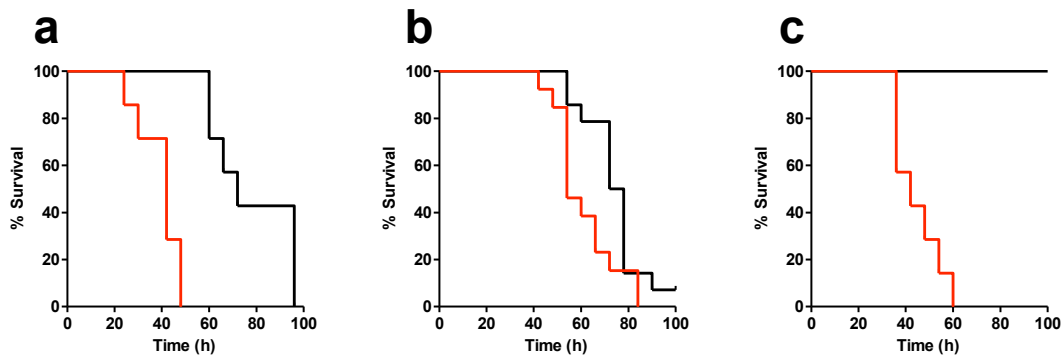
Figure 4.9 Diazepam reduces survival from secondary bacterial superinfection compared to vehicle treatment.

Survival was also analyzed in mice given *Streptococcus pneumoniae* seven days after influenza (H3N2 X31/A) infection. (**a**) Diazepam (2 mg kg^{-1} IP; red line) or vehicle (black line) was administered twice daily from four hours following influenza infection and continued during bacterial infection (1 x 10^4 CFU of *Streptococcus* pneumoniae; n = 8/group). The timeline for this experiment is available in **Figure 2.1b**. In two further cohorts (**b, c**), diazepam (2 mg kg^{-1} IP; red line) or vehicle (black line) were started four hours after *Streptococcus pneumoniae* superinfection of influenza infection. Two doses of bacteria were studied: (**b**) high dose (1 x 10^4 CFU of *Streptococcus pneumoniae* (n = 13/group)) and (**c**) low dose (2 x 10^3 CFU of *Streptococcus pneumoniae* (n = 7/group)). The timeline for (**b**) and (**c**) can be see in **Figure 2.1c**.

throughout the influenza and bacterial superinfection phases increased the risk of death (HR 22.4 [4.3-117.5]; p=0.001; **Figure 4.9a**). Furthermore diazepam increased mortality rate from high dose (HR 3.1 [1.16-8.35]; p=0.02; **Figure 4.9b**) and low dose (HR 34.8 [6.32-191.7]; p=0.001; **Figure 4.9c**) *Streptococcus pneumoniae* superinfection of influenza even when diazepam was started after the bacterial superinfection.

4.2.4 Diazepam increases the severity of influenza infection in mice

Given that diazepam increases mortality from bacterial pneumonia and bacterial superinfections of influenza, we analyzed whether diazepam affected responses to influenza infections alone (timeline is available in **Figure 2.1d**). Diazepam treatment started four hours after influenza infection delayed weight loss on day 3 but exacerbated weight loss between day 7 and 9 (**Figure 4.10a**). Two out of 10 diazepam treated mice died on day 8 and no mice died in the group not treated with diazepam (p=0.47 by Fisher's exact test). Despite seeing a mortality difference in the bacterial experiments, mortality was not expected in the influenza experiments and hence the experiments were not prospectively powered to observe any difference in mortality. Subsequent power analysis (α = 0.05; β = 0.8) suggests 35 mice would be required per group to test for a difference in mortality. Blood sugars were checked in animals on day 8 to understand whether significant hypoglycaemia occurs coincident with weight loss. Hypoglycaemia is a potential confound of animal experimental models of infection if not corrected (as it would be clinically)[136]. However there was no difference in glycaemic load between the groups on day 8 (**Figure 4.10b**).

Interestingly unlike during the bacterial pneumonia, cell recruitment was altered on day 10 of the influenza infection with increased total cell numbers, as well as monocyte, CD4 and CD8 subsets, noted in the airway (**Figure 4.11**). However cell recruitment was not altered in the lung at any time point (**Figure 4.12**). While airway TNF-α levels were unchanged by diazepam during influenza, IL-6

levels were significantly higher on day 7 when increased weight loss was first noted. Airway albumin levels, a marker of increased lung damage, were not affected by diazepam treatment (**Figure 4.13**) though on day 14 our sample size was limited to three mice as two had died earlier in the experiment. Hence we may be underpowered to address the effect on lung damage. The increase in IL-6 (**Figure 4.13b**) appeared similar to the findings at 48 hours in bacterial pneumonia when cytokine levels were raised with diazepam treatment.
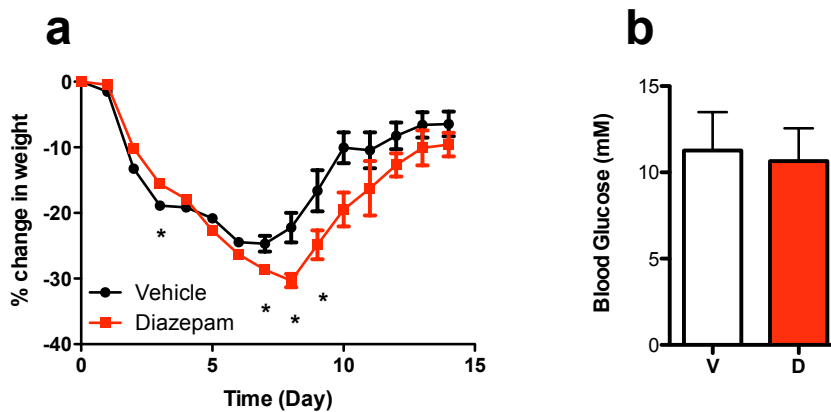
Figure 4.10 Diazepam increases weight loss from influenza but does not alter glycaemic load.

C57BL/6 mice were infected with influenza (H3N2 X31/A) and monitored for weight loss (**a**) and blood glucose (**b**) on day 8. Diazepam (2 mg kg^{-1} IP) or vehicle control (4% ethanol in PBS) was administered twice daily starting four hours after influenza infection. Blood glucose was assessed using a tail prick and measured on an Optium XL glucometer. *p<0.05. Graphs show mean±s.e.m. Each data point reflects 5 animals per group except on day 14 when there are 3 animals in the diazepam group (due to animal deaths on day 8).
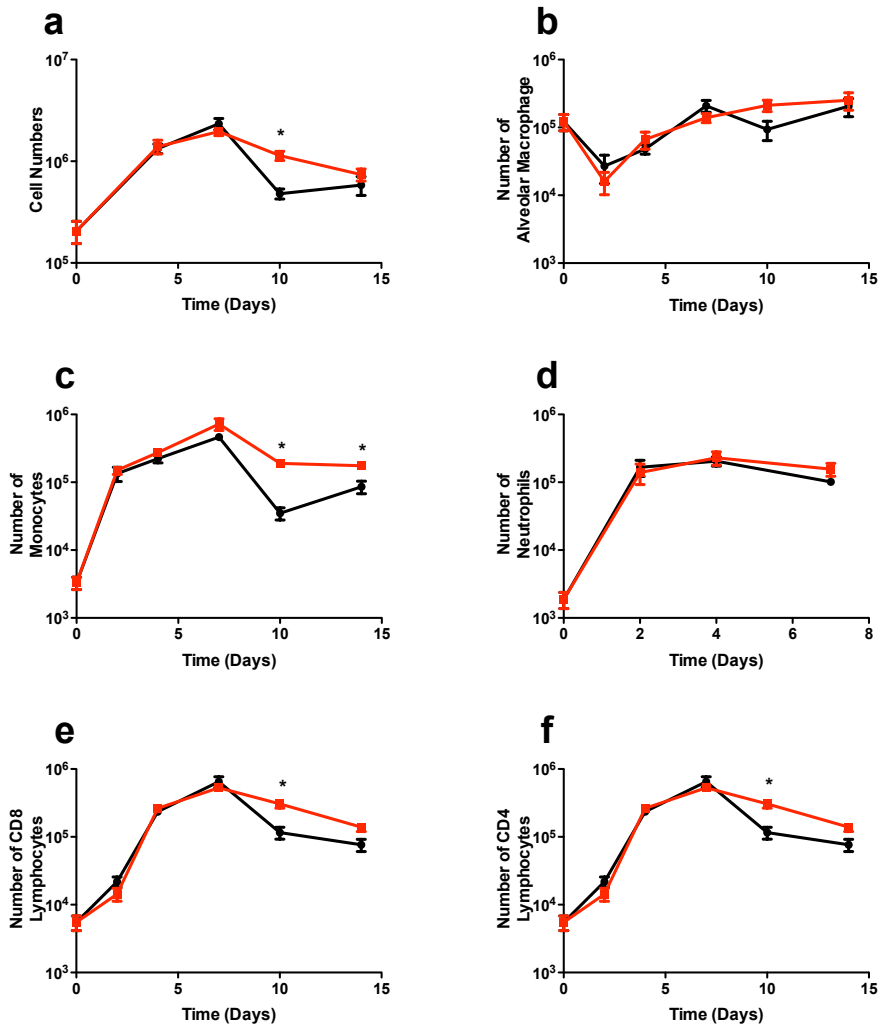
Figure 4.11 Cell recruitment to the airway is increased on day 10 following influenza infection.

Bronchoalveolar lavage was conducted on designated days during influenza infection and cells counted by typan blue exclusion. Treatment groups were diazepam (red) and vehicle (black). Subsets were identified by flow cytometry. All cells were measured until day 14 except neutrophils that were followed until day 7. (**a**) total cell count, (**b**) alveolar macrophages, (**c**) monocytes, (**d**) neutrophils, (**e**) CD4 cells and (**f**) CD8 cells. *$p<0.05$. Graphs show mean±s.e.m. Each data point reflects 5 animals per group except on day 14 when there are 3 animals in the diazepam group (due to animal deaths on day 8).
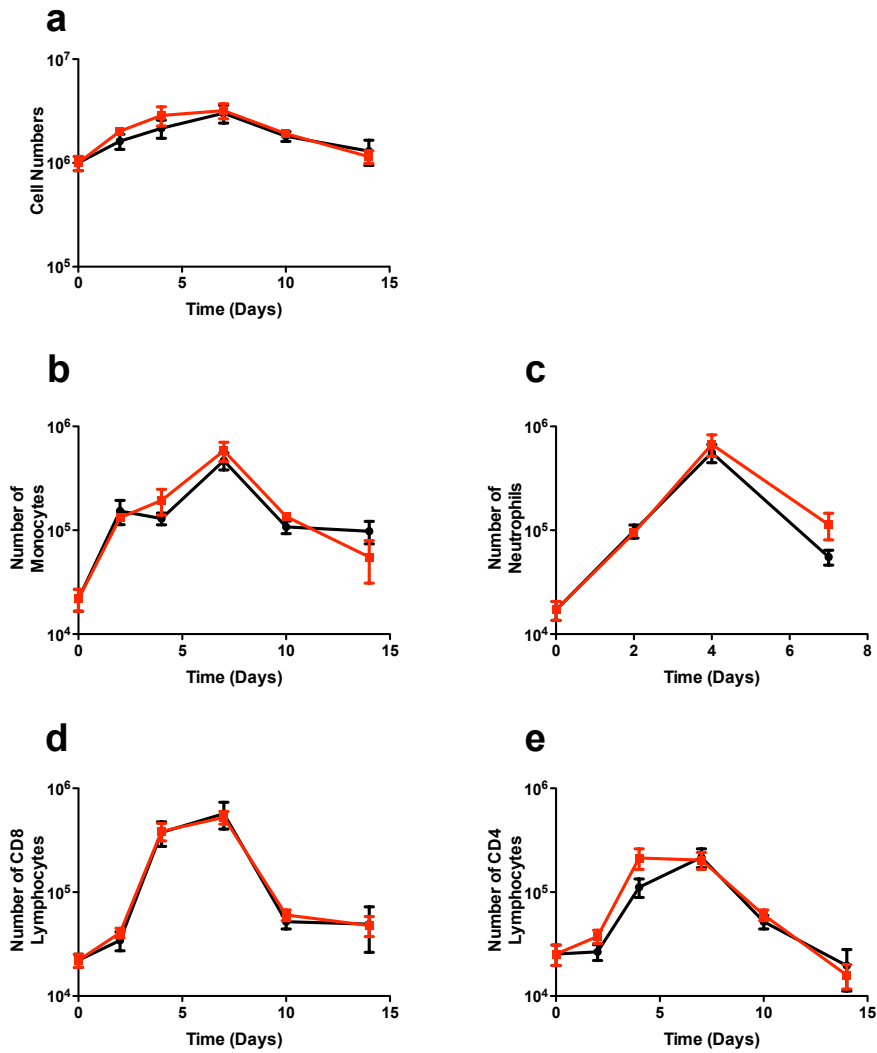
Figure 4.12 Cell recruitment to the lung is unaffected following influenza infection.

Bronchoalveolar lavage was conducted on designated days during influenza infection and cells counted by typan blue exclusion. Treatment groups were diazepam (red) and vehicle (black). Subsets were identified by flow cytometry. All cells were measured until day 14 except neutrophils that were followed until day 7. (**a**) total cell count, (**b**) alveolar macrophages, (**c**) monocytes, (**d**) neutrophils, (**e**) CD4 cells and (**f**) CD8 cells. *p<0.05. Graphs show mean±s.e.m. Each data point reflects 5 animals per group except on day 14 when there are 3 animals in the diazepam group (due to animal deaths on day 8).
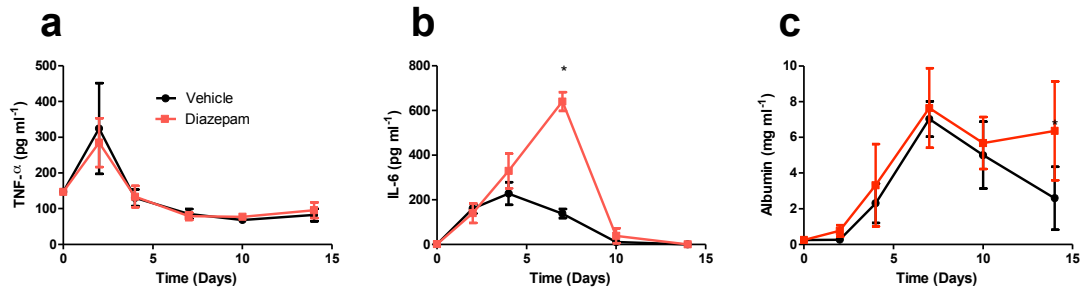
Figure 4.13 Airway IL-6 is increased on day 7 following influenza infection.

Bronchoalveolar lavage was conducted on designated days during influenza infection and the supernatant stored for cytokine analysis by ELISA. TNF-α (**a**), IL-6 (**b**) and albumin (**c**) were assessed. Each data point reflects 5 animals per group except on day 14 when there are 3 animals in the diazepam group (due to animal deaths on day 8).*$p<0.05$. Graphs show mean±s.e.m.

In the *Streptococcus pneumoniae* experiments the increase in pro-inflammatory cytokines was attributed to increased bacterial load. In order to understand whether pathogen load was changed by diazepam treatment we assayed viral load until day 7 and bacterial load throughout the infection. Viral load was statistically unaffected at any time point though it appeared to be increased at each time point (**Figure 4.14a**). On day 2 there was a trend to an increase in viral load ($p=0.111$). At day 7, influenza virus persisted in the diazepam group in 3 out of 5 samples but was absent in all of the vehicle treated animals ($p=0.167$ by Fischer's exact test). At day 10 and 14, significant spontaneous bacterial infections (presumably from the commensal flora) were noted in the airway of influenza-infected mice treated with diazepam (**Figure 4.14b**).
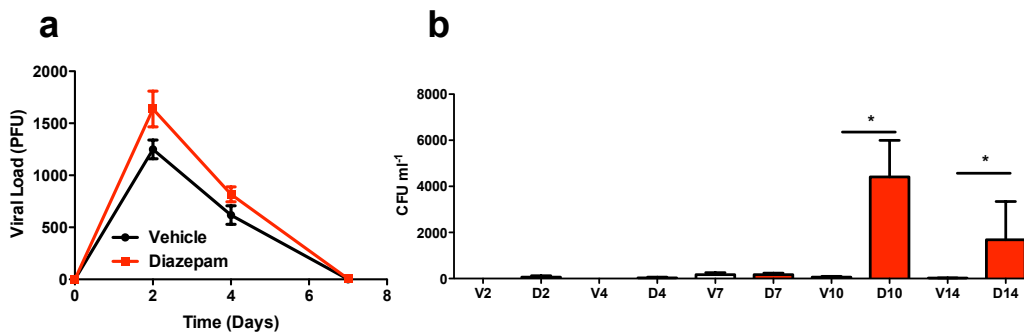
Figure 4.14 Diazepam treatment throughout influenza does not affect viral load but leads to increased bacterial superinfection.

Lung was frozen in liquid nitrogen on day of harvest and viral load was subsequently assessed by plaque assay. Viral load was measured up to day 7 (**a**). Bronchoalveolar lavage fluid was serially diluted and plated on blood agar. Bacterial load was measured by counting colony forming units (CFU) (**b**). V=vehicle (day). D=diazepam (day). Each data point reflects 5 animals per group except on day 14 when there are 3 animals in the diazepam group (due to animal deaths on day 8).

4.2.5 Parenteral Fluids do not affect Pulmonary Immune Responses to Influenza or Susceptibility to Secondary Bacterial Pneumonia in Mice

Weight loss from influenza may be driven by inflammatory mechanisms[137]; indeed diazepam exacerbated weight loss and increased airway IL-6 prior to the beginning of this weight loss. However we wished to confirm that the mice infected with influenza were not losing weight from dehydration. We also wished to reconfirm that hypoglycaemia did not complicate the influenza infection model. This was achieved by treating the mice with intraperitoneal

fluid boluses while monitoring weight loss, glycaemic load and immune parameters.

Table 4.1 Influenza infection induces weight loss but not hypoglycemia.

The data are presented as mean (standard deviation). Weight loss and glycaemic load are unaffected by fluid treatment. (* p < 0.05 versus naïve). NT = not tested.

		Naive	Influenza	Control	CSL	NS
BALB/c	Starting weight (g)	19.4 (0.4)	19.6 (0.7)	19.9 (1.1)	19.9 (0.8)	19.8 (0.6)
	Weight loss on Day 6 (g)	+0.2 (0.4)	-2.5 (0.8)*	-2.5 (0.9)*	-3.3 (1.0)*	-2.8 (0.7)*
	Glucose on Day 3 (mmol L^{-1})	7.9 (1.0)	9.2 (5.2)	9.8 (2.0)	8.8 (2.6)	NT
	Glucose on Day 6 (mmol L^{-1})	7.2 (0.4)	6.8 (1.7)	7.3 (0.8)	6.9 (0.5)	7.2 (0.6)
C57BL/6	Starting weight (g)	18.4 (0.8)	19.5 (0.5)	19.0 (0.5)	19.1 (0.7)	18.5 (1.3)
	Weight change on Day 6 (g)	+0.6 (0.5)	-4.3 (0.6)*	-4.0 (0.8)*	-3.5 (1.1)*	-4.2 (0.5)*
	Glucose on Day 6 (mmol L^{-1})	9.8 (2.0)	7.0 (1.7)	8.8 (1.3)	10.0 (1.5)	7.9 (1.3)

Animals were treated with 20 ml kg^{-1} once daily (OD) compound sodium lactate (CSL) or normal saline (NS) by intraperitoneal injection during days 3 to 7 of influenza infection (**Table 4.1**). In this experiment we confirmed a lack of hypoglycaemia on day 6 of influenza infection in both strains of mice. C57BL/6 mice were also tested for hypoglycaemia on day 3 of infection but no effect was found. Unfortunately a technical fault prevented one group being tested at this time point however, as fluids had not been commenced at this stage, this group

was merely a repeat of the influenza group. In BALB/c (**Figure 4.15a**) and C57BL/6 (**Figure 4.15b**) mice, influenza induced significant weight loss ($p < 0.05$ versus naive group) that was unaffected by treatment with 20 ml kg^{-1} CSL or NS OD (**Table 4.1**). Follow up to day 14 in BALB/c mice given 20 ml kg^{-1} CSL OD showed the intervention did not affect recovery of weight loss (**Figure 4.15c;** $p < 0.05$ versus control group). Further experiments in BALB/c mice showed that twice daily (BD) 20 ml kg^{-1} CSL (**Figure 4.15d**) up to day 7 did not alter weight loss ($p > 0.05$ versus control group). Increasing the influenza dose by 33% (to 67 HA) did not affect whether treated with 20 ml kg^{-1} CSL BD affected weight loss (**Figure 4.15d**).

Influenza-induced pulmonary (BAL or lung) cell recruitment (total and subset numbers) was also unaffected on day 7 and 14 post-infection by treatment with 20 ml kg^{-1} CSL ($p < 0.05$; data not shown). Administration of 2 x 20 ml kg^{-1} CSL did not affect cell recruitment ($p > 0.05$ versus sham; **Figure 4.16**) or activation status and airway IL-6 levels ($p > 0.05$ versus sham; **Figure 4.16b**) from infection with either 50 HA or 67 HA units of influenza.
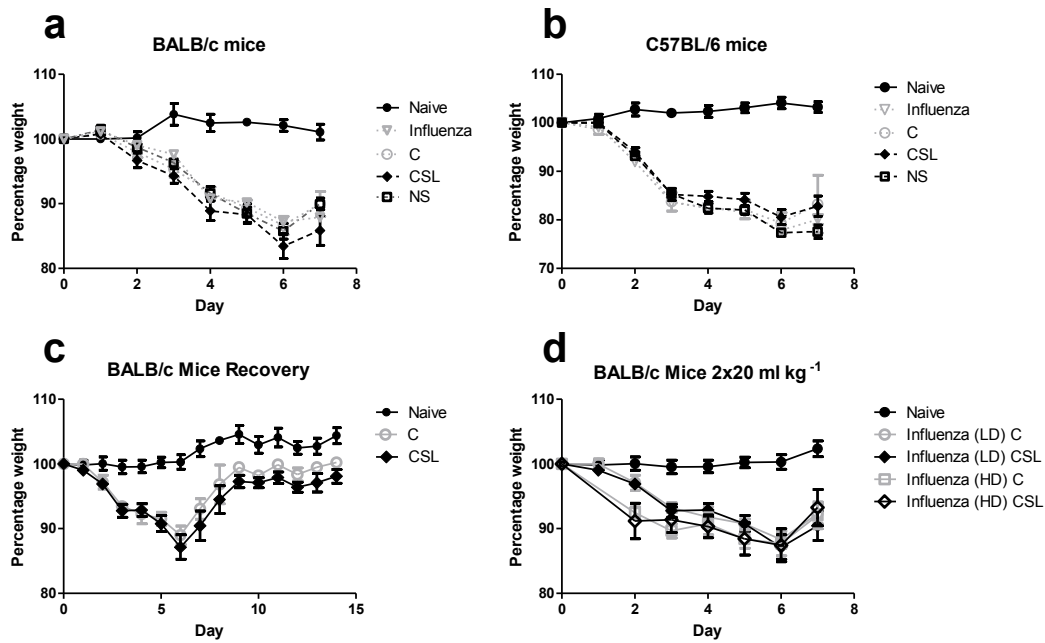
Figure 4.15 Fluid treatment does not affect weight loss in a murine influenza model.

Adult BALB/c (**a**) or C57BL6 mice (**b**) were infected with 3.7 x 10^3 plaque forming units (50 HA) of X31 (H3N2) influenza via the intranasal route and randomized to intraperitoneal treatment with 20 ml/kg compound sodium lactate (CSL), normal saline (NS) or no treatment with either sham control injection (C) starting on day three following infection and continued for four days (n = 8/group). An influenza infected group was also included that did not receive injections (Influenza) (n = 8/group) as were naïve (uninfected) mice. In a further experiment adult BALB/c were followed up to day 14 following infection with 3.7 x 10^3 plaque forming units of X31 (H3N2) influenza (**c**; n = 5/group). In a further cohort (**d**), BALB/c mice were given either low dose (LD; 3.7 x 10^3 plaque forming units) or high dose (HD; 4.9 x 10^3 plaque forming units/67 HA) of X31 (H3N2) influenza and rehydrated with 2 x 20 ml/kg CSL per day and followed for 7 days.

Parenteral fluid treatment during influenza did not affect the subsequent susceptibility to secondary bacterial pneumonia in BALB/c ($p = 0.39$) or C57BL/6 mice ($p = 0.8$) (**Figure 4.17a,b**). However weight loss during influenza correlated with time to death following secondary bacterial infection in C57BL/6 mice ($r^2 = 0.34$; $p = 0.03$) indicating that the severity of influenza infection may influence vulnerability to bacterial superinfection. Fluid treatment during influenza also did not affect bacterial load in the nasal wash, lung or blood ($p > 0.05$ versus control; **Figure 4.17c-e**) nor were there differences in cell recruitment to the lung following secondary bacterial pneumonia ($p > 0.05$ versus sham; **Figure 4.18**).
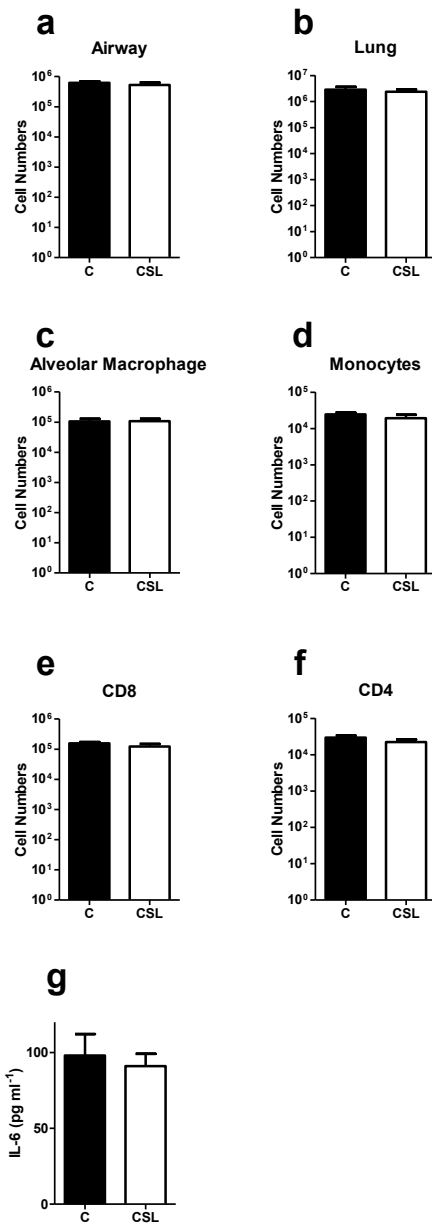
Figure 4.16 Fluid treatment during influenza does not affect immune cell recruitment or airway IL-6 levels on day 7 of infection.

Adult BALB/c mice were infected with 4.9 x 10^3 plaque forming units (67 HA) of X31 (H3N2) influenza and treated with 2 x 20 ml/kg CSL per day for 4 days (CSL) or sham injection (C). On day 7, cellular recruitment to the airway and lung was assessed. (**a**) Total airway counts, (**b**) total lung counts, airway numbers of (**c**) alveolar macrophage, (**d**) monocytes, (**e**) CD8 lymphocytes, and (**f**) CD4 lymphocytes. Airway IL-6 levels were also measured by ELISA (**g**).

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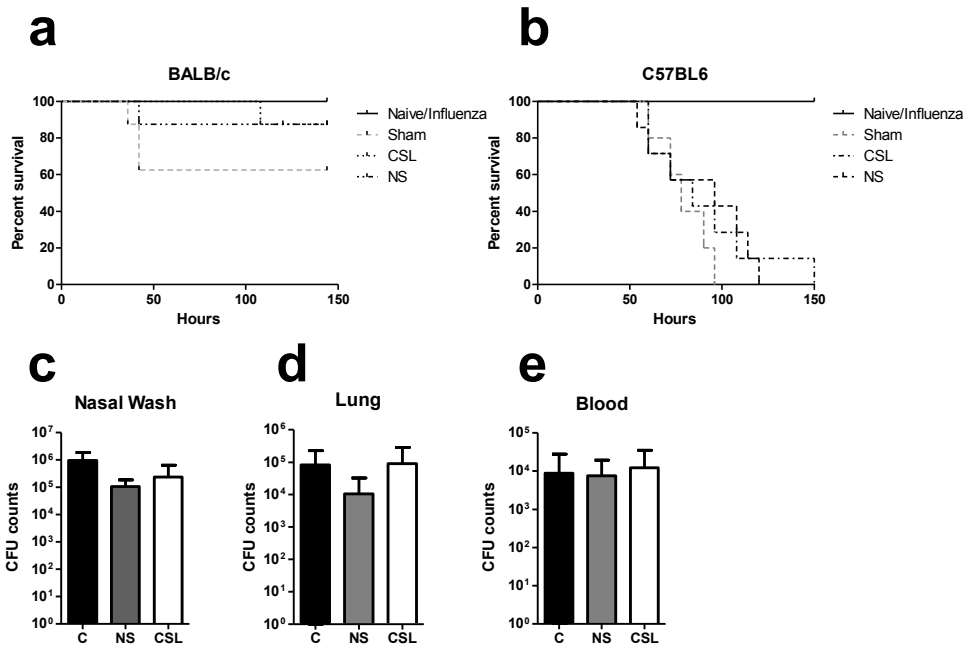
Figure 4.17 Fluid treatment during influenza did not affect susceptibility to subsequent bacterial superinfection.

Mice were infected with with 3.7 x 10^3 plaque forming units (50 HA) of X31 (H3N2) influenza and rehydrated between day 3 and 7 or not with 20ml/kg compound sodium lactate (CSL) or normal saline (NS). Controls received sham injection (C). On day seven, (**a**) BALB/c mice were challenged with 1 x 10^6 *Streptococcus pneumoniae* and (**b**) C57BL/6 mice with 1 x 10^4 *Streptococcus pneumoniae*. For the survival experiments, naïve and influenza alone groups were also included (but incurred no mortality). Survival following bacterial challenge was unaffected by fluid treatment in either species. In BALB/c mice, bacterial colony forming unit (CFU) counts in the (**c**) nasal wash, (**d**) lung and (**e**) blood were measured. Fluid treatment with 20 ml/kg CSL or NS during the influenza did not affect susceptibility to bacterial superinfection compared to sham.
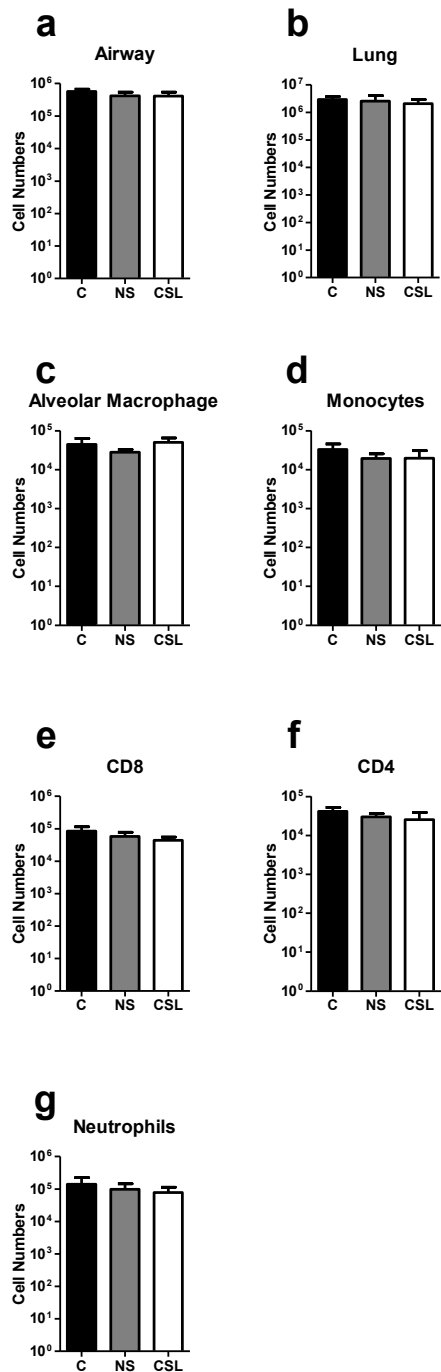
Figure 4.18 Immune cell recruitment at 48 hours following bacterial superinfection is unaffected by fluid treatment during influenza.

Cellular recruitment to the airway and lung was assessed. (**a**) total airway counts, (**b**) total lung counts, airway numbers of (**c**) alveolar macrophage, (**d**) monocytes, (**e**) CD8 lymphocytes, (**f**) CD4 lymphocytes and (**g**) neutrophils. Groups are control sham injection (C), normal saline (NS) and compound sodium lactate (CSL).

4.3 Discussion

Herein we have observed that diazepam increases susceptibility to both *Streptococcus pneumoniae* and influenza pneumonia. The enhanced susceptibility to *Streptococcus pneumoniae* is reversible with the $GABA_A$ antagonist bicuculline indicating dependence on $GABA_A$ receptor signaling. Augmenting $GABA_A$ receptor activity led to increased pathogen load and an exaggerated cytokine response to infection. As cell recruitment was unaffected it is likely that local responses were perturbed. Diazepam treatment throughout influenza led to a delay in weight loss on day 3 and increased weight loss between days 7 to 9. This increased weight loss coincided with a spike in IL-6 levels. While viral load appeared to be unaffected there was increased burden of secondary bacterial infections. The increase in bacterial load correlated with increased cell recruitment in the airway on day 10. In sum our data suggest that diazepam, acting via $GABA_A$ receptors, perturbs immunity *in vivo*. A consistent feature is an increase in bacterial load and mortality from infection. However intraperitoneal fluid treatment therapy did not affect the immune response to infection in our animal model.

4.3.1 Interpretation of the in vivo experiments with diazepam

There are several limitations to our experiments. Firstly the doses of diazepam used *in vivo*, if directly translated into man, would be lethal. We chose the dose of diazepam as we titrated the drug to pharmacodynamic effect, anxiolysis in

mice[75,76,78]. While diazepam's terminal half life is up to 48 hours in man it is much shorter in rodents with a half life of up to one hour[138,139]. Therefore we chose twice daily administration of diazepam. However pharmacokinetic data are not available for infected mice and therefore we cannot exclude that the dosing regimen led to accumulation of the drug. This may be important as diazepam has biologically active metabolites, such desmethyldiazepam, that also target the $GABA_A$ receptor[140]. The dose of bicuculline methiodide was again chosen based on previous *in vivo* work[79] but the pharmacokinetics are unknown in mice. Secondly we did not monitor the physiology of the animal throughout the infection. While physiological monitoring would not be widely used by people in the community at early stages of an infection, later when hospital admission may be common, some form of physiological monitoring would be routine. We cannot exclude that diazepam perturbed haemodynamics, blood gases and temperature in our studies. However it is plausible that derangement of these physiological variables would lead to a more global effect on immunity that we suspect would have affected cell recruitment as well as responsiveness. Nonetheless further studies are required to exclude derangement of non-immune physiology as contributing to this susceptibility to infection. We did attempt to build in factors such as fluid treatment and antibiotic treatment however these either proved to have no effect (fluids) or proved too effective in the treatment of bacterial pneumonia. It would be useful to understand whether diazepam can still increase susceptibility to infection when the pneumonia is treated with ampicillin (that leads to 100% survival). Thirdly it is unclear how our infection model, relates to clinical practice as these healthy young mice require large doses of pathogen to induce significant disease. However in the

previous chapter we have observed that the public health concern from the effect of sub-sedative dosing of benzodiazepines on infectious outcomes may lie in increasing mortality in patients with minimal comorbidity. Fourthly some of the experiments herein were likely underpowered. Notably the experiments with diazepam treatment during influenza should be repeated with a larger sample size to test whether diazepam effects viral clearance and increases lung damage. The next stage of enquiry, to further pursue the mechanism of the diazepam effect will be addressed in **Chapter 5**.

4.3.2 Interpretation of the effects of parental fluids on the immune response to influenza

Parenteral fluid administration did not affect immunity or pathophysiology during A/X31 influenza infection in two murine strains, thus significant dehydration may not be a confounder of disease severity in this model. Likewise hypoglycemia was not observed during the influenza infection, another important confounder in animal infection models[136]. Some important caveats that need mentioning. Firstly the fluids were administered via intraperitoneal rather than intravenous injection due to ease of administration and to avoid acute hemodynamic changes. Despite administration of up to 3.2g of fluid over four days (in the 2 x 20 ml/kg fluid treatment group) the mice did not gain weight and therefore the method of administration was not changed, especially as absorption occurs rapidly from the intraperitoneal route. These data suggest that dehydration and hypoglycaemia do not confound this disease model. Hence

immunomodulatory advances made in this murine models[2,137,141] may be more easily translated into the clinical domain paving the way for testing other therapies[23,54,69]. However our findings do not negate the importance of testing these variables in other species, especially larger animal models, where immune reactions are often different[23].

4.4 Conclusions

The studies in this chapter have provided additional biological plausibility for the clinical findings in **Chapter 3**, supporting previous preclinical studies[23]. In addition an *in vivo* mechanism of benzodiazepine induced susceptibility to infection has been identified – dependence on $GABA_A$ signaling. Next it will be important to ascertain which local response may be perturbed to impair pathogen clearance from the lung. Data shown herein suggest that epithelial mucus production is unlikely to be the target. This is consistent with very low levels of the γ2 subunit on epithelial cells[42,134]. Therefore our next approach is to survey the immune cell expression of the γ2 subunit to identify a plausible target for the diazepam effect and to investigate how activation of $GABA_A$ receptors leads to impairment in immune responses on the implicated cells.

Chapter 5 Immune regulation and function of Benzodiazepine sensitive GABA$_A$ receptor expression

5.1 Introduction

Given our clinical and preclinical findings in the previous chapters, we focused on the immune effects of benzodiazepine sensitive GABA$_A$ signaling. Benzodiazepines allosterically modulate GABA$_A$ receptors sensitizing them to GABA[35,76,77,142,143]. GABA$_A$ receptors are pentameric, ligand-gated channels that conduct chloride and bicarbonate anions[144]. The most prevalent GABA$_A$ receptor in the brain is the benzodiazepine sensitive α1β2γ2 receptor in a 2:2:1 stoichometry. This composition of the GABA$_A$ receptor is important as the γ2 confers some particular properties, such as low sensitivity to GABA and it contains the benzodiazepine recognition site at the interface between the α-γ2 subunit[35,143]. While monocytes and macrophage are thought to express α[41,43] and β[29,41,48] subunits (and this has been confirmed electrophysiologically[29,48]), studies of lymphocytes are contradictory[29,40,41]. Currently it is unclear whether immune cells express γ2 subunits, however based on the data in **Chapter 3** and **Chapter 4**, it was hypothesized that they would and therefore that their GABA$_A$ receptors are benzodiazepine sensitive. Of course other subunits, such as the δ subunit, may substitute for γ2; such receptors are highly sensitive to GABA but are insensitive to benzodiazepines.

Central nervous system expression of GABA$_A$ receptors is regulated by inflammation, with IL-1β upregulating the expression of GABA$_A$ receptor β2/3 subunits in hippocampal cultures and LPS upregulating their expression *in vivo*[44]. Presently it is unknown if immune cell expression is similarly regulated. Herein immune cell expression of GABA$_A$ receptor subunits was investigated in the naïve and stimulated state using inflammatory stimuli including cytokines and TLR agonists. Preliminary evidence suggests that macrophage must contain the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD), as GABA has been detected by magnetic resonance spectroscopy[145], therefore we also surveyed cells to confirm expression of GAD.

The function of GABA$_A$ immunomodulation remains relatively obscure though GABA$_A$ receptor stimulation has been shown to reduce cytokine release from peritoneal macrophage[29,43] but not T cells[29] in one experiment. However other studies have shown that T cell proliferation is reduced by the application of the GABA$_A$ agonists, GABA, pentobarbital and muscimol[40,146]. A similar link has been made to impairment of phagocytosis and chemoattraction in THP-1 cells[48], though these immortalized cells do not express the γ2 subunit[48] and so are benzodiazepine insensitive. Many other GABA$_A$ agonists have been linked to anti-inflammatory and impaired pathogen killing actions, though dependence on GABA$_A$ activity has not been proven[23]. Therefore the functional effects of GABA$_A$ stimulation were tested ex vivo.

5.2 Results

5.2.1 Immune cell expression of GABA$_A$ receptors

In order to address the expression of GABA$_A$ receptor subunits on immune cells in C57BL/6 mice flow cytometry was used; typical gating plans are available in **Figure 5.1** and **Figure 5.2**. Alveolar macrophage abundantly expressed γ2 (64±17% of cells) with evidence of α1β2γ2 receptors (**Figure 5.3; Table 5.1**). Lower levels of γ2 were present on splenic macrophage (36±5%; p<0.05) and monocytes (20±7%; p<0.05) with very low levels on CD4 cells (6±1%; p<0.05). γ2 subunits were not detected on other cell types. Expression of α1 and γ2 subunits was then confirmed on alveolar macrophage from BALB/c mice and in the immortalized MHS cell line (**Figure 5.4**). As benzodiazepines that are selective for specific α subunits are under development[75,77], we investigated α subunit expression on various cells. While α1 subunits were highly expressed on macrophages and monocytes, α2-4 subunits were rarely detected or absent (**Table 5.1**). Again α1 subunits were most abundant on alveolar macrophages. Neutrophils, natural killer B, CD8 and CD4 cells all expressed low levels of the α1 subunit and CD4 cells also expressed detectable γ2 expression (**Table 5.1**). Greater than 80% of all immune cells tested expressed the synthetic enzyme for GABA, glutamic acid decarboxylase (GAD) (**Table 5.1**).

5.2.2 Expression of the GABA$_A$ α1 and γ2 subunit on human immune cells

Based on the expression pattern in mice we focused on the α1 and γ2 subunits on human cells. Human alveolar macrophage and monocytes also expressed α1 subunits but neutrophils did not (**Figure 5.5**). While human monocytes expressed the γ2 subunit, it was absent from neutrophils (**Figure 5.6**). The γ2 subunit was not assessed on human alveolar macrophage due to the difficult in obtaining samples.
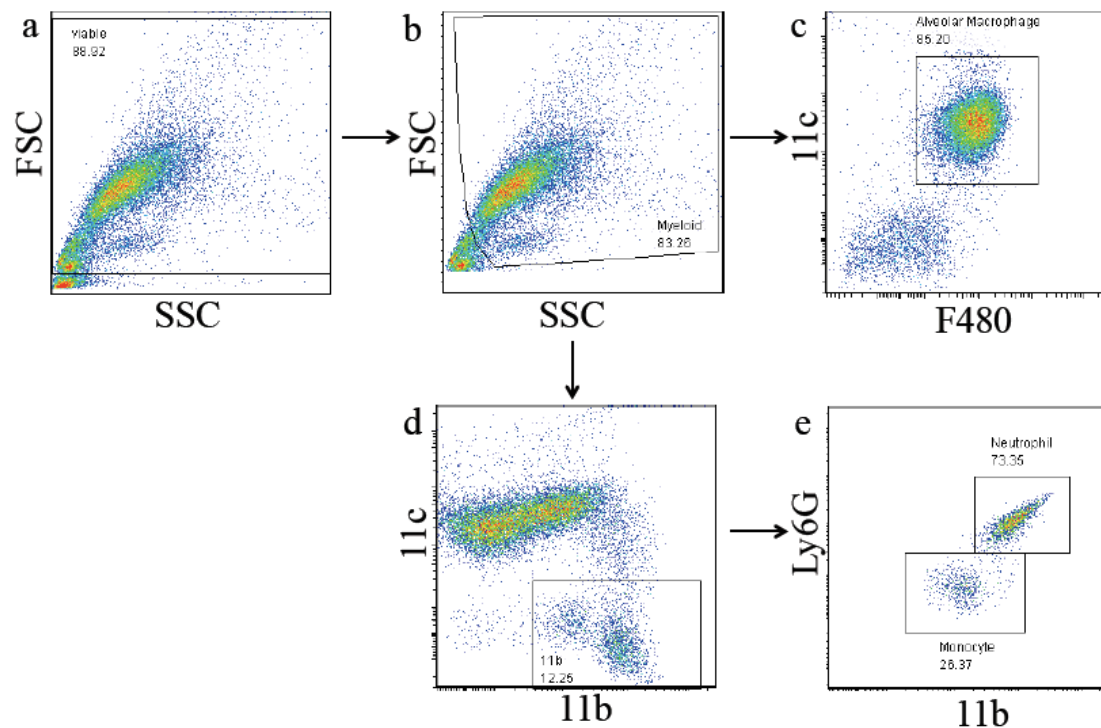
Figure 5.1 Sample gating plan from FlowJo showing the selection of innate immune cells (alveolar macrophages, neutrophils and monocytes) from the bronchoalveolar lavage.

Viable cells were first identified by (**a**) and then lymphocytes and other debris excluded based on forward scatter (FSC) and side scatter (SSC) (**b**). CD11c$^+$ F480$^+$ are alveolar macrophage (**c**). CD11c$^-$ CD11b$^+$ are recruited immune cells. Monocytes (CD11b$^+$ Ly6G$^-$) and neutrophils (CD11b$^+$ Ly6G$^+$) were discriminated by Ly6G positivity (**d**, **e**).
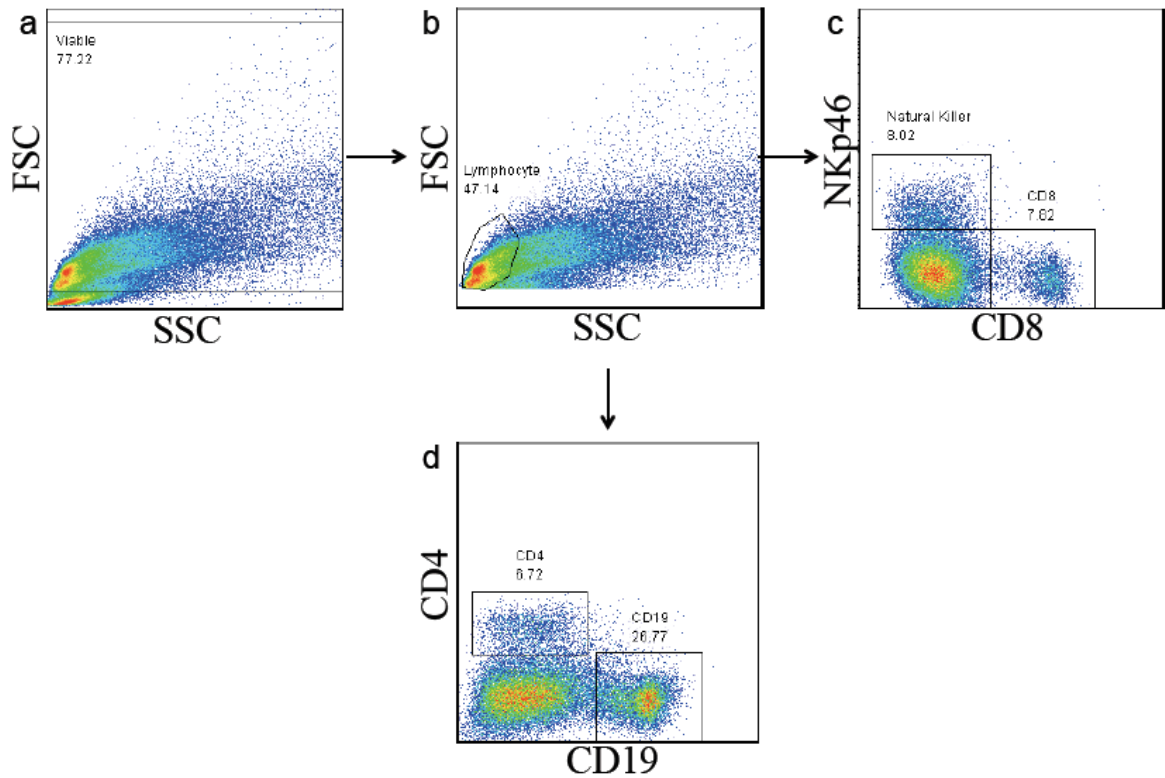
Figure 5.2 Gating strategy from Flowjo to identify natural killer cells (NKp46 high), CD8 lymphocytes (CD8 high), CD4 lymphocytes (CD4 high) or B cells (CD19 high).

Cells were identified by size on forward scatter (FSC) and side scatter (SSC) using a viable (**a**) and "lymphocyte" size (**b**) gates. Individual cell types were then identified using specific markers. Natural killer and CD8 cells are shown in (**c**) and CD4 and B cells are shown in (**d**).

a   b   c

α1   β2   γ2
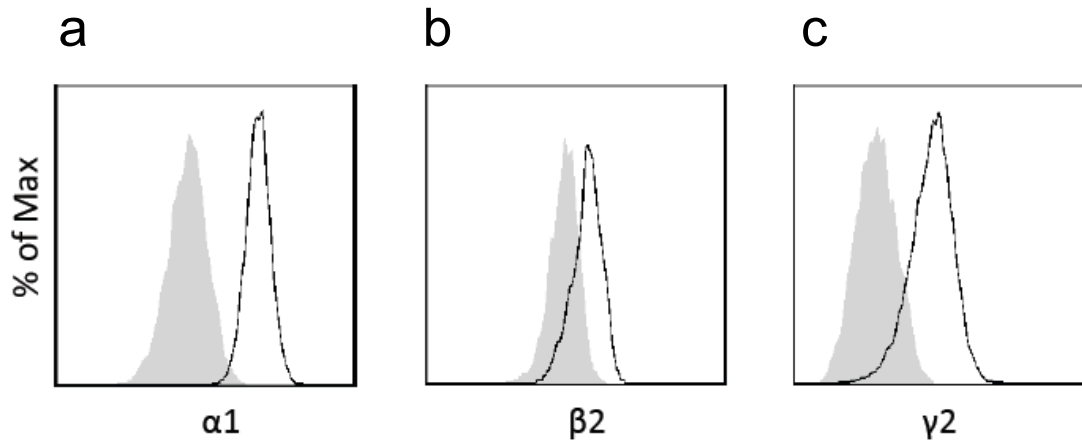
Figure 5.3 Representative histograms of alveolar macrophage GABA$_A$ subunit expression.

Cells were isolated by airway lavage from a C57BL/6 mouse and stained by antibodies for the α1 (**a**), β2 (**b**), and γ2 (**c**) subunits. The isotype antibody is shaded and positive GABA$_A$ subunit denoted by the black line.
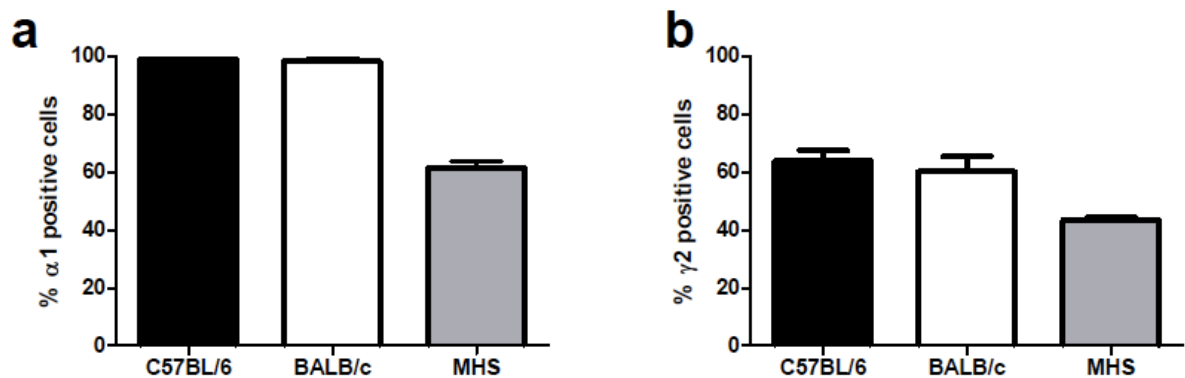
Figure 5.4 Quantative expression of α1 and γ2 subunit expression on alveolar macrophage from C57BL/6 and BALB/c mice and the immortalized murine alveolar macrophage cell line, MHS cells.

Data represents the mean ± s.e.m. of n=5 mice per group.
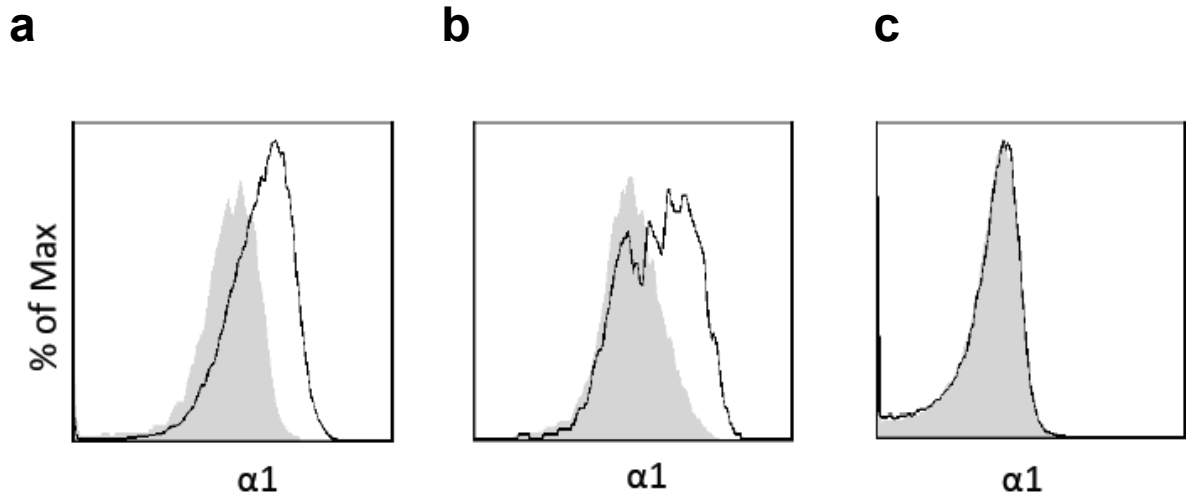
Figure 5.5 α1 subunit expression on human alveolar macrophage (a), monocytes (b) and neutrophils (c) assessed by flow cytometry.

Alveolar macrophage sample is representative of six subjects. Monocyte and neutrophil staining is representative of 8 subjects. Grey shading = isotype. Black outline = GABA$_A$ subunit.
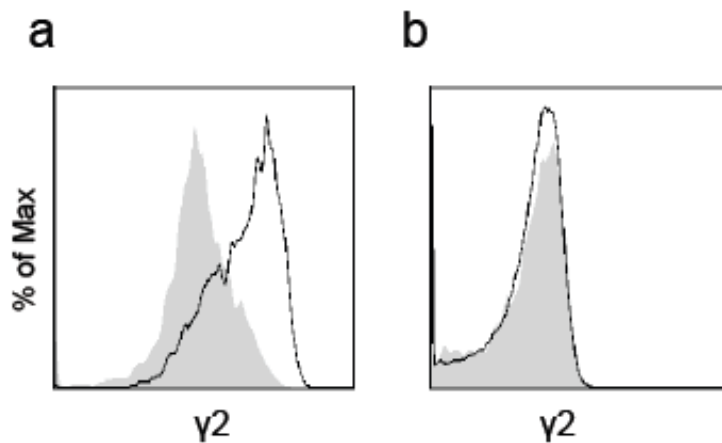
Figure 5.6 γ2 subunit expression on human monocytes (a) and neutrophils (b) assessed by flow cytometry.

Data are from one subject only. Grey shading = isotype. Black outline = GABA$_A$ subunit.

Table 5.1 Percentage expression of GABA$_A$ subunits and the synthetic enzyme for GABA, Glutamic Acid Decarboxylase (GAD) on cells from C57BL/6 mice.

Data are presented as mean (s.d.). ND = Not Detected. NT = Not Tested. *p<0.05 versus alveolar macrophage.

	GABA$_A$ signaling proteins							
	α1	α2	α3	α4	β2	Y2	σ	GAD
Alveolar Macrophage	99(1)	4(1)	4(2)	ND	42(19)	71(11)	ND	93(5)
Splenic Macrophage	37(7)*	5(2)	7(3)	NT	NT	36(5)*	NT	NT
Peritoneal Macrophage	21(8)*	NT	NT	NT	NT	13(4)*	NT	NT
Monocyte	34(12)*	6(2)	8(3)	ND	NT	20(7)*	ND	90(5)
Neutrophil	5(1)*	ND	ND	ND	NT	ND	ND	94(3)
CD4	10(2)*	NT	NT	ND	NT	6(1)*	ND	93(3)
CD8	4(1)*	NT	NT	ND	NT	ND	ND	91(1)
B Cell	8(3)*	NT	NT	ND	NT	ND	ND	84(4)
NK cell	9(2)*	NT	NT	ND	NT	ND	ND	89(3)

5.2.3 Immune regulation of GABA$_A$ subunit expression on alveolar macrophage ex vivo

Alveolar macrophages most abundantly expressed the γ2 subunit while splenic and peritoneal macrophage expressed lower levels (**Table 5.1**). Given this evidence for GABA$_A$ receptor regulation, we investigated what factors modulated alveolar macrophage γ2 subunit expression. *Ex vivo* stimulation with bacterial TLRs (TLR-4, -5, -6/2, and -9), but not viral TLRs (TLR-3 and -7), reduced γ2 subunit expression (**Figure 5.7a**). We also investigated the effects of cytokine signaling on alveolar macrophage GABA$_A$ γ2 subunit expression *ex-vivo*. IL-1β reduced, while IL-4 increased, expression (**Figure 5.7b**). It is interesting to note

that pro-inflammatory stimuli in general reduce γ2 subunit expression whereas those associated with dampening macrophage activity (IL-10) or for converting them to "alternatively activated" or wound healing phenotype (IL-4) do not.

5.2.4 Changes in α1 and γ2 subunit expression on alveolar macrophage and monocytes following infection with Streptococcus pneumoniae

We next determined $GABA_A$ subunit expression on alveolar macrophage following *Streptococcus pneumoniae* infection. Alveolar macrophage, monocyte and neutrophil expression of the α1 and γ2 subunit were followed at 24 and 48 hours post-intranasal infection with *Streptococcus pneumoniae* ($1x10^6$ CFU). Later time points were not assessed as animals begin to die at 48 hours. Consistent with the *ex-vivo* stimulations of alveolar macrophage, 48 hours following intranasal *Streptococcus pneumoniae* ($1x10^6$ CFU) infection *in vivo*, γ2 subunit expression decreased on alveolar macrophage by 46% (**Figure 5.8a, b**). In contrast α1 expression remained unchanged on alveolar macrophage. *Streptococcus pneumonia* infection did not affect monocyte expression of α1 or γ2 subunits (**Figure 5.8c, d**). Infection did not alter neutrophil expression of either subunit (data not shown).
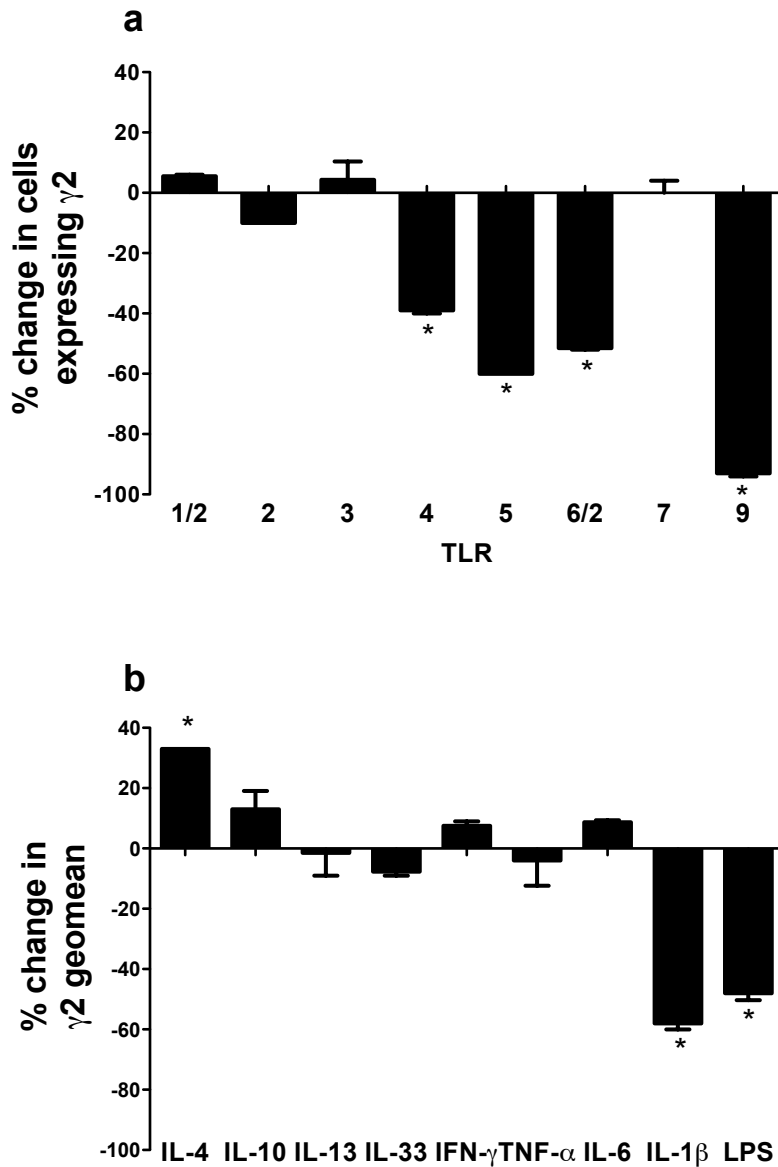
Figure 5.7 Ex vivo changes in γ2 subunit expression on alveolar macrophage.

Alveolar macrophage from C57BL/6 mice were incubated with TLR agonists (**a**) or cytokines (and lipopolysaccharide, LPS) (**b**) and γ2 subunit expression determined by flow cytometry (n = 3 wells/treatment; each well contained 100,000 cells) at 16 hours. *p<0.05 versus non-treated control.
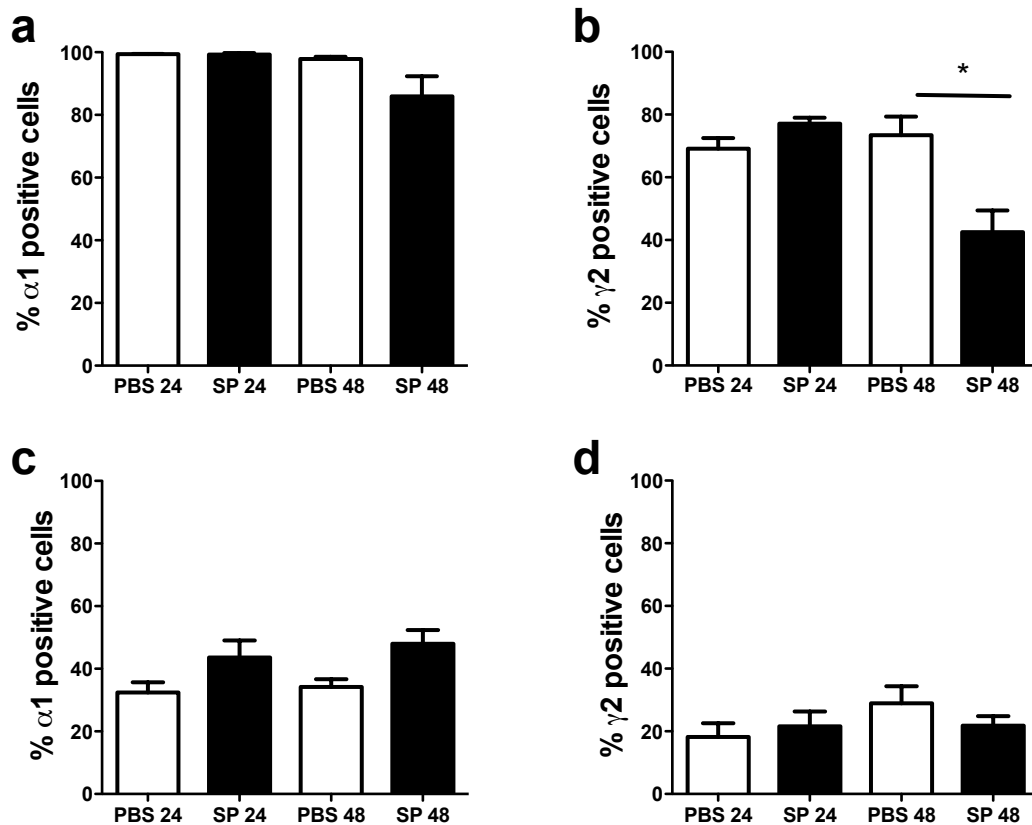
Figure 5.8 Alveolar macrophage and monocyte α1 and γ2 subunit expression following *Streptococcus pneumoniae* infection.

Mice were given $1x10^6$ CFU *Streptococcus pneumoniae* (SP) or PBS intranasally, alveolar macrophage α1 (**a**) and γ2 (**b**) and monocyte α1 (**c**) and γ2 (**d**) subunit expression was assessed at 24 and 48 hours by flow cytometry (n = 5/group). *$p<0.05$ versus PBS treated at 48 hours.

5.2.5 Changes in α1 and γ2 subunit expression on alveolar macrophage and monocytes following infection with Influenza H3N2 X31/A

While follow up in the bacterial infection model is limited by animal mortality, influenza (H3N2 X31/A) infection leads to a prolonged infection and recovery without animal death. We therefore studied the effects of influenza infection on expression of α1, β2 and γ2 subunits on alveolar macrophages and α1, and γ2

subunits on monocytes. Influenza infection led to a decrease in expression of all subunits on alveolar macrophage that resolved over time (**Figure 5.9**). These findings are of interest as **Figure 5.7** shows that viral TLR agonists alone do not affect GABA$_A$ subunit expression on alveolar macrophage whereas *in vivo* influenza does. It is likely that whole virus activates multiple TLRs, but also other pattern recognition receptors that we did not test including NOD proteins and MDA-S[1]. Finally *in vivo* TLR signaling occurs within a complex inflammatory milieu that may not be replicated by the *ex vivo* testing.

In contrast monocyte expression of the α1 subunit increased, and γ2 subunit expression was unchanged, by influenza infection (**Figure 5.10**). Again this difference to macrophage was not anticipated and is surprising. The mechanism behind this finding is unclear but it may be that monocyte regulation is different to that of alveolar macrophage or that monocytes are at a different level of activation/regulation compared to macrophage.

5.2.6 Changes in GABA$_A$ subunit expression on lymphocytes

Lymphocytic GABA$_A$ subunit expression has been studied by PCR[40] and western blot[29], though γ2 subunit expression (and therefore benzodiazepine sensitive GABA$_A$ receptor expression) has not been studied previously. CD4 expression of both α1 and γ2 subunits increased during influenza infection (**Figure 5.11**). While CD8 cells also upregulated the α1 subunit, they increased expression of

the δ subunit (**Figure 5.12**) but did not express the γ2 subunit. B and NK cell expression of GABA$_A$ receptors remained unchanged throughout the infection (data not shown).
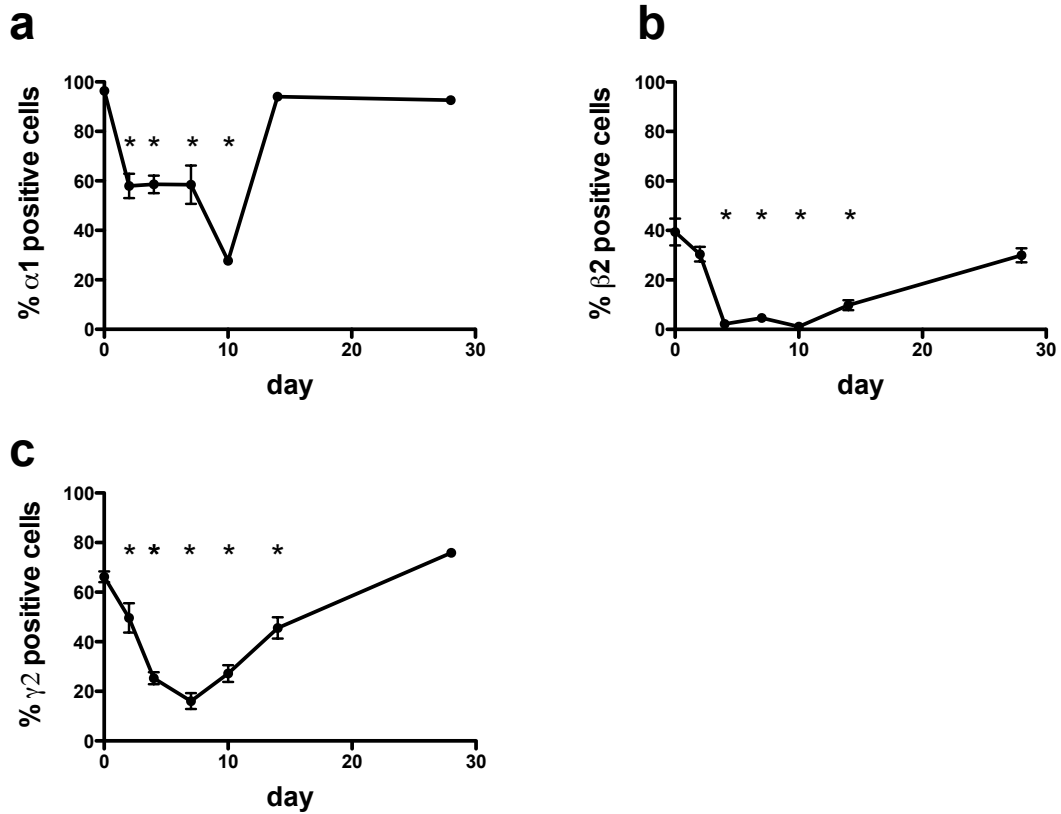
Figure 5.9 Alveolar macrophage α1, β2 and γ2 subunit expression following influenza infection.

Mice were given 50 HA of H3N2 X31 influenza or PBS intranasally, alveolar macrophage α1 (**a**), β2 (**b**) and γ2 (**c**) subunit expression was assessed in day 0, 2, 4, 7, 10, 14 and day 28 post-influenza (n = 5/group). *p<0.05 versus day 0.
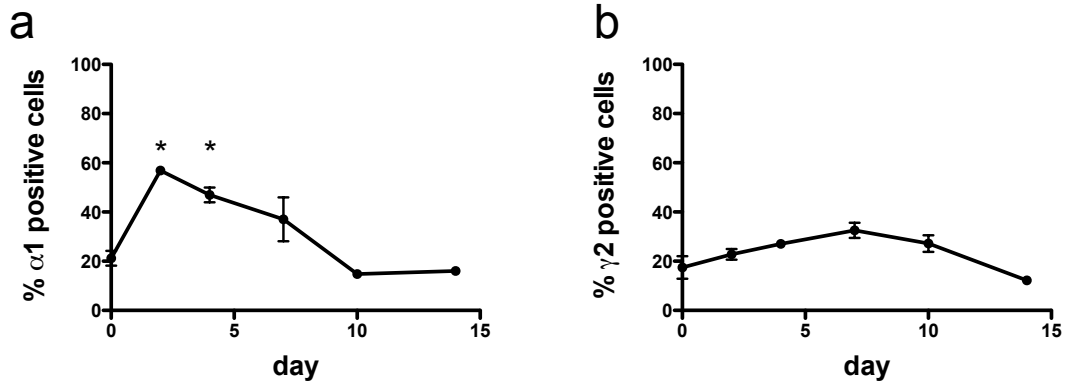
Figure 5.10 Monocyte α1 and γ2 subunit expression following influenza infection.

Mice were given 50 HA of H3N2 X31 influenza or PBS intranasally, monocyte α1 and γ2 subunit expression was assessed on day 0, 2, 4, 7, 10 and 14 post-infuenza (n = 5/group).*p<0.05 versus day 0.
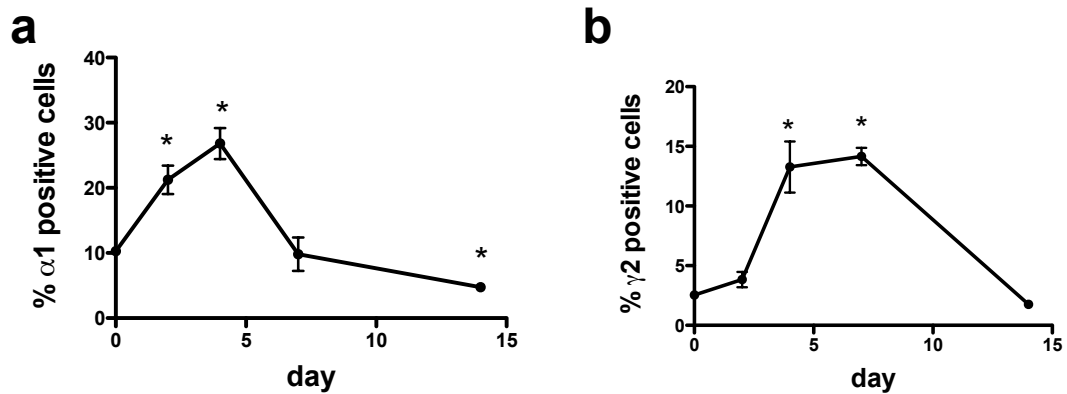
Figure 5.11 CD4 lymphocyte α1 and γ2 subunit expression following influenza infection.

Mice were given 50 HA of H3N2 X31 influenza or PBS intranasally, CD4 cell α1 (**a**) and γ2 (**b**) subunit expression was assessed on day 0, 2, 4, 7, and 14 (n = 5/group). *p<0.05 versus day 0.
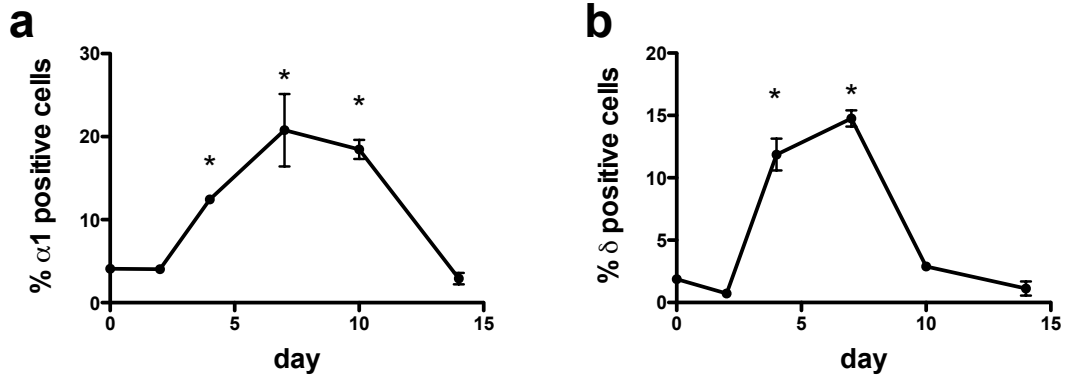
Figure 5.12 CD8 lymphocyte α1 and δ subunit expression following influenza infection.

Mice were given 50 HA of H3N2 X31 influenza or PBS intranasally, CD8 cell α1 (**a**) and σ (**b**) subunit expression was assessed on day 0, 2, 4, 7, 10 and 14 (n = 5/group). *p<0.05 versus day 0.

5.2.7 Changes in γ2 subunit expression on alveolar macrophage following house dust mite treatment

As IL-4 increased expression of the γ2 subunit *ex vivo* we investigated the effects of a three week house dust mite treatment on alveolar macrophage expression of the γ2 subunit *in vivo* in BALB/c mice. This allergen treatment promotes a Th2 environment with increased IL-4 levels[147]. Both the percentage of positive cells and geomean of γ2 increased on alveolar macrophage indicating a substantial increase in γ2 expression (**Figure 5.13**).

5.2.8 Anti-inflammatory effects of GABA$_A$ receptor stimulation on alveolar macrophage ex vivo

We next tested the functional relevance of GABA$_A$ receptor stimulation on naive alveolar macrophage. These cells were chosen due to their abundance of expression of GABA$_A$ receptors and importance in defense of the lung against pathogens. Alveolar macrophage were sampled by bronchoalveolar lavage and plated at a density $1x10^5$ cells/well. The cells were treated with a standard dose of LPS (100 ng ml^{-1}) and GABA$_A$ agonists and left in culture for 18 hours. The supernatants were removed and cytokine analysis completed by ELISA. Activation of the GABA$_A$ receptor with GABA, diazepam or muscimol reduced IL-6 release from alveolar macrophage (**Figure 5.14a, b**). In order to confirm that the alveolar macrophage effect was dependent on α1-containing GABA$_A$ receptors, we used a benzodiazepine that is selective for α2/3-, but lacks activity at α1-, containing GABA$_A$ receptors[77], L-838-417. Consistent with α1 subunit expression on alveolar macrophage, L-838-417 did not affect cytokine release (**Figure 5.14c**). In further experiments focusing on TNF-α release, diazepam and muscimol, but not L-838-417, reduced cytokine levels (**Figure 5.14d**). Therefore α1-γ2 but not α2/3-γ2 GABA$_A$ receptor activation affects inflammatory responses of alveolar macrophage.
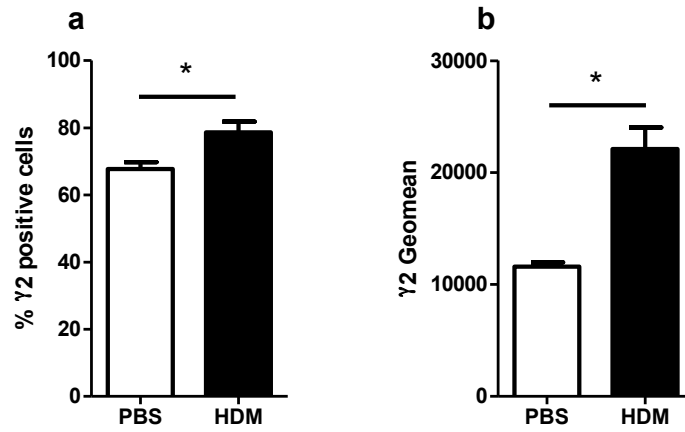
Figure 5.13 Alveolar macrophage γ2 subunit expression in response to three weeks of house dust mite treatment assessed by flow cytometry.

Data are expressed as the percentage of cells that are γ2 positive or the geometric mean of γ2 antibody fluorescence. * $p < 0.05$ versus control or vehicle. Graphs show mean ± s.e.m. (n = 5 BALB/c mice/group).
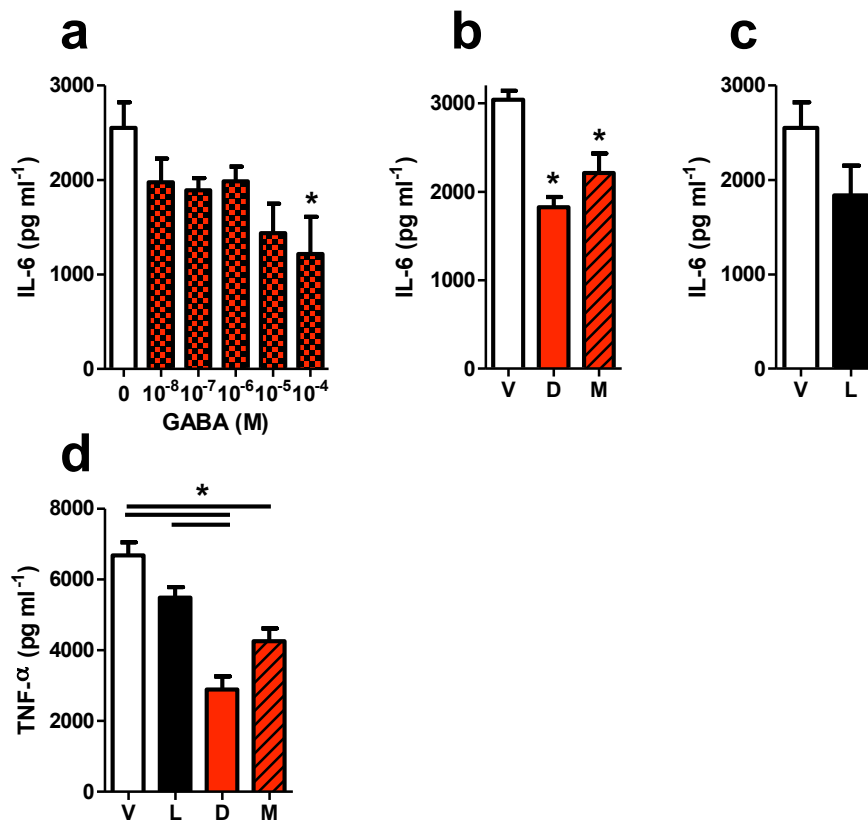
Figure 5.14 Effect of GABA$_A$ agonists on ex vivo cytokine production from stimulated alveolar macrophage.

Alveolar macrophages, purified from C57BL/6 mice, were incubated with GABA vehicle (V), diazepam (D, 10µM) muscimol (M, 100µM) or L-838-417 (L, 567nM; an equivalent Ki multiple to diazepam[6]) and LPS (100ng ml^{-1}) for 18 hours. IL-6 (**a-c**) and TNF-α release (**d**) were measured by ELISA (**a**) (n = 3-7 wells/group; 100,000 cells/well). *P<0.05 versus 0M GABA or vehicle unless otherwise stated.

5.2.9 Functional effects of GABA$_A$ receptor stimulation on alveolar macrophage on anti-pathogen responses ex vivo

Previous data from THP-1 cells[48] suggests that GABA$_A$ receptor activation reduces phagocytosis of FITC-labeled microspheres. This method is suboptimal for alveolar macrophages due to their auto-fluorescence in the FITC channel. Furthermore microspheres lack pathogen associated molecular patterns limiting their biological relevance. Instead we tested the effects of GABAergic modulators on phagocytosis of phrodo-labelled *Staphylococcus aureus ex vivo* (phrodo-labelled *Streptococcus pneumoniae* is not available) (**Figure 5.15a**). Diazepam and GABA reduced phagocytosis by 25% and 14% respectively following a two hour incubation.

Next we checked whether impaired phagocytosis correlated with impaired bacterial killing *ex vivo*. Bacterial killing by alveolar macrophage was stimulated by pretreatment with IFN-γ for 18 hours at 37°C. The cells were then mixed with *Streptococcus pneumoniae* that were opsonized in mouse serum and the remaining viable bacterial count was assessed 60 minutes later by serial dilutions onto blood agar. Diazepam inhibited bacterial killing by 28%, a defect blocked by the GABA$_A$ receptor antagonist bicuculline and not mimicked by L-838-417 (**Figure 5.15b**).
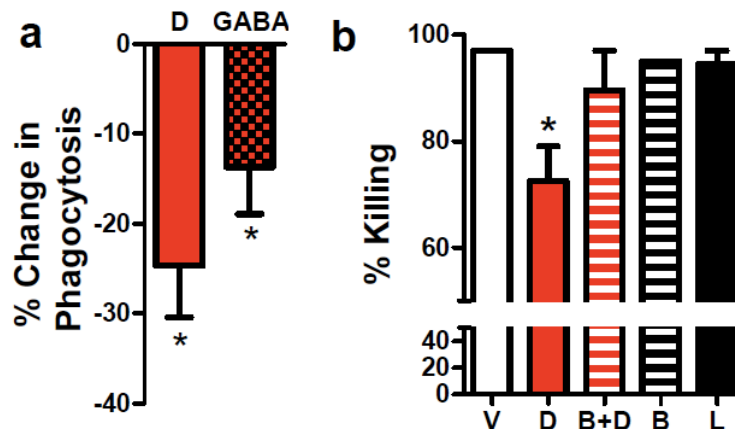
Figure 5.15 Effect of GABA$_A$ agonists on ex vivo alveolar macrophage phagocytosis and bacterial killing.

Change in phagocytosis of phrodo-labelled *Staphylococcus aureus* (n=3-6 wells/group) by alveolar macrophage assessed at 1 hour with diazepam (10μM) or GABA (100μM) (**b**). Alveolar macrophages were incubated with vehicle, diazepam, bicuculline and diazepam (B+D), bicuculline (B) or L-838-417 and killing of *Streptococcus pneumoniae* was assessed by counting bacterial colonies (n = 3/group). Results represent mean ± s.e.m. *p<0.05.

In order to provide supporting evidence that diazepam was working through GABA$_A$ receptor stimulation to suppress bacterial killing, the effects of diazepam on neutrophil killing of *Staphylococcus aureus* was tested *ex vivo* as neutrophils do not express the GABA$_A$ receptor (**Table 5.1**). Briefly human neutrophils were obtained by venipuncture and spun through a phycol gradient to obtain a pure neutrophil population. Neutrophils were then mixed in a 1:10 ratio with bacteria and incubated for 20 minutes at 37°C. The neutrophils were then lysed and bacterial counts obtained by plating on agar. In keeping with a lack of GABA$_A$ receptor expression on neutrophils (**Table 5.1**)[41], GABAergic drugs did not modify neutrophil bacterial killing *ex vivo*. Incubation of neutrophils with

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bacteria decreased the CFU count from 218±11 to 76.5±3.5 indicating active bacterial killing (**Figure 5.16**; $p < 0.05$). DMSO vehicle [CFU 83±9], diazepam 10µM [CFU 72±2] and muscimol 100µM [CFU 65±4] did not inhibit bacterial killing by neutrophils. However neutrophil killing was inhibited by the oxidase inhibitor diphenylene iodonium (DPI 5µM; [CFU 111±11]; **Figure 5.16**; $p < 0.05$). We also examined the effect of GABA modulators on neutrophil respiratory burst *ex vivo* (**Figure 5.16**) by incubating the neutrophils with an Amplex probe. Similarly respiratory burst was not affected by diazepam (10 µM) and muscimol (100 µM) but was attenuated by the NADPH oxidase inhibitor DPI ($p < 0.05$).
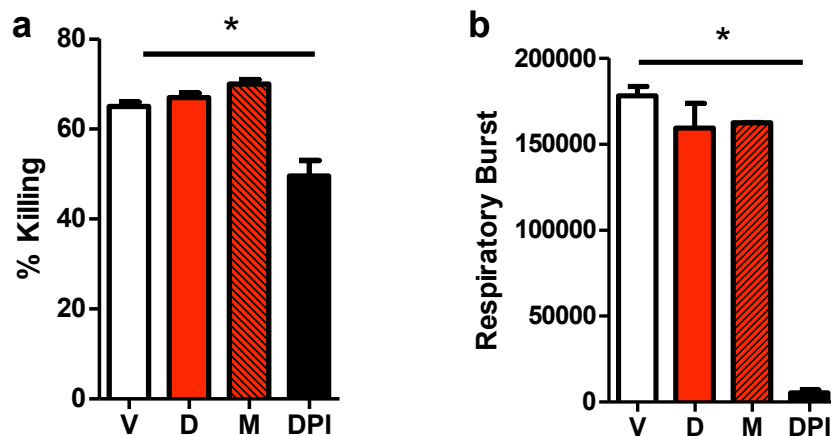
Figure 5.16 Neutrophil responses are not affected by GABA$_A$ modulators.

Human neutrophils were incubated with diazepam (D, 10µM) or muscimol (M, 100µM) and killing of *Staphylococcus aureus* (**a**) and Phorbol 12-myristate 13-acetate stimulated respiratory burst *ex-vivo* (**b**) was measured. As a positive control on both of these processes neutrophils were incubated with the NADPH oxidase inhibitor diphenyliodonium (DPI). Results represent the mean ± s.e.m. of n = 3/group.

5.2.10 Functional effects of GABA$_A$ receptor stimulation on alveolar macrophage on intracellular pH ex vivo

Activation of neuronal GABA$_A$ receptors leads to cytoplasmic acidification through bicarbonate efflux[144]. As acidification of the cytoplasm is known to reduce TNF-α production[148] and bacterial phagocytosis and killing[149] by alveolar macrophage, we hypothesized that GABA$_A$ receptor stimulation perturbed immune responses by altering intracellular pH. This was tested by incubation with a BCECF probe that changes fluorescence at varying pH. A standard curve was established based on permeabilising the cell in buffers at varying pH. Intracellular pH was then measured after ten minutes of equilibration with the drugs at 37°C in room air. Augmenting GABA$_A$ signaling with diazepam increased the intracellular H$^+$ concentration by 10% (baseline pH 7.13±0.03 vs. Diazepam pH 7.08±0.01; p=0.004; **Figure 5.17a-c**). α1 containing GABA$_A$ receptors were implicated as L-838-417 did not alter pH (pH 7.12±0.02; **Figure 5.17a-c**). In a separate experiment this effect was mimicked by treatment with the GABA$_A$ agonist muscimol (baseline pH 7.18 vs. muscimol pH 7.05; p<0.001; **Figure 5.17d**). Increasing GABA$_A$ receptor expression with IL-4 pretreatment increased H$^+$ concentration by 167% (pH 6.91±0.01; p<0.001 vs. baseline pH; **Figure 5.17b**). Cytoplasmic acidification is consistent with the "alternatively activated" macrophage phenotype induced by IL-4: reduced TNF-α production, phagocytosis and killing[150]. Diazepam (pH 6.89±0.01; p=0.01), but not L-838-417 (pH 6.90±0.00), still acidified the pH in IL-4 treated cells (**Figure 5.17b**). In contrast, reducing GABA$_A$ receptor expression with lipopolysaccharide led to

cytoplasmic alkalosis (pH 7.16±0.01; p<0.001 vs. baseline; **Figure 5.17c**). Despite reduced receptor expression, diazepam still increased the cytoplasmic H^+ concentration (pH 7.11±0.01; p<0.001), an effect antagonized by bicuculline (pH 7.13±0.01; p<0.001; **Figure 5.17c**). In contrast, L-838-417 (pH 7.17±0.01) and bicuculline (pH 7.16+0.02) exerted no effect alone. Furthermore H^+ concentration correlated with changes in $GABA_A$ subunit expression (r^2=0.80; p<0.001; **Figure 5.17e**). In sum, augmenting $GABA_A$ receptor activity acidifies the cytoplasm of alveolar macrophage through α1-γ2 containing $GABA_A$ receptors.

We next tested whether α1 subunit containing $GABA_A$ receptors are responsible for benzodiazepine increase in susceptibility to infection. Despite being used for the *ex vivo* studies, L-838-417 was not appropriate for *in vivo* work as it is rapidly metabolized in mice and humans and hence is unsuitable for comparison with diazepam that has a long half life. Rather than use L-838-417[77], we selected NS11394 as its pharmacokinetics are compatible with twice daily dosing in mice [75] and it produces anxiolysis at comparable doses to diazepam[75,76]. This is possible as anxiolysis is mediated by α2/3-, not α1-, containing $GABA_A$ receptors[76,142,151]. Unlike diazepam, NS11394 did not increase mortality from infection (HR 1.23 [0.56- 2.67]; p=0.6; **Figure 5.18**). In a further experiment bacterial load was also measured in these mice at 40 hours post-infection. A very severe infection meant the samples were harvested 8 hours earlier than anticipated. Compatible with a very severe infection, bacterial counts were higher in the lung but not the BAL of diazepam treated animals however

NS11394 had no effect (**Figure 5.19**). Cellular recruitment was not altered by diazepam or NS11394 (**Figure 5.20**).
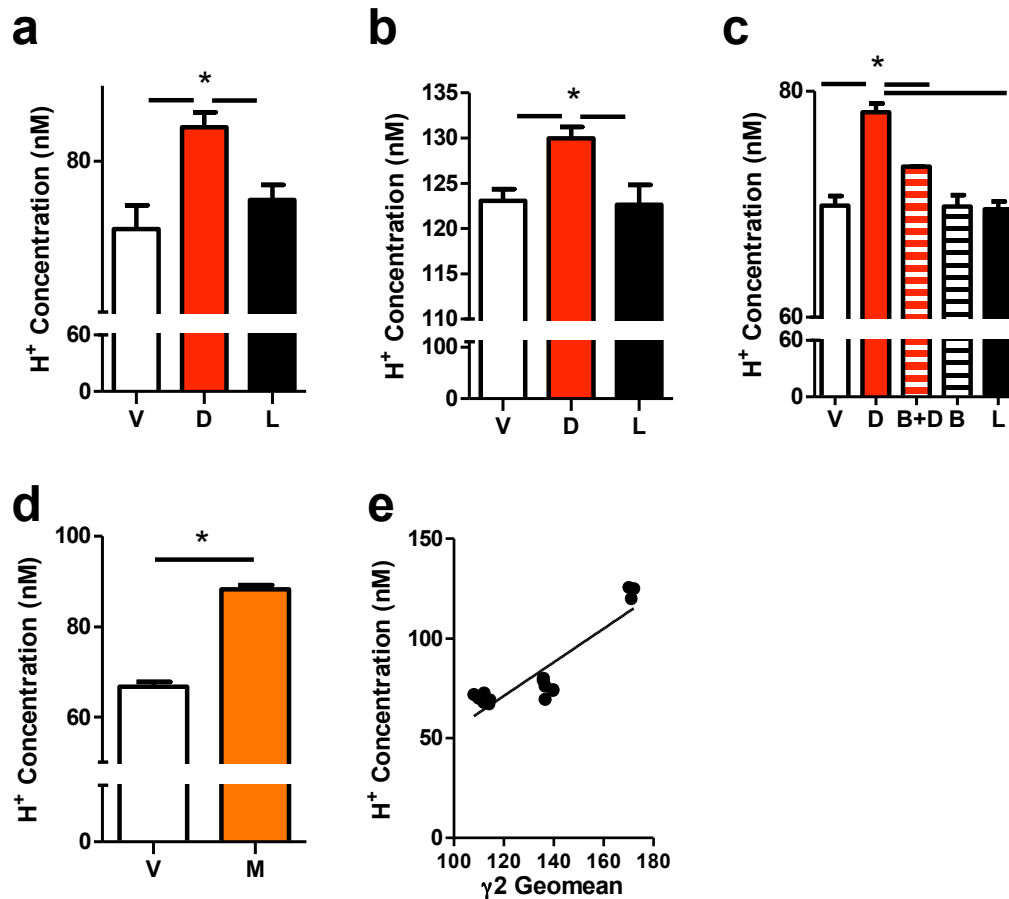
Figure 5.17 Effect of GABA$_A$ modulation on alveolar macrophage intracellular pH.

Cytoplasmic pH (H$^+$ concentration) was measured by BCECF probe in untreated (**a**), IL-4 treated (**b**) and LPS treated alveolar macrophage (**c**) (n=3 wells/treatment; 100,000 cells/well). Cells were treated with vehicle (V, DMSO 0.05%), diazepam (D, 10μM) or L-838-417 (L, 567nM) (**a, b**); in addition in (**c**) cells were treated with bicuculline methiodide (30 μM) with or without diazepam. In separate experiments, muscimol (M, 100 μM) also altered intracellular H$^+$ concentration (**d**). Correlation between γ2 subunit expression and H$^+$ concentration in alveolar macrophage (n = 15)(**e**).
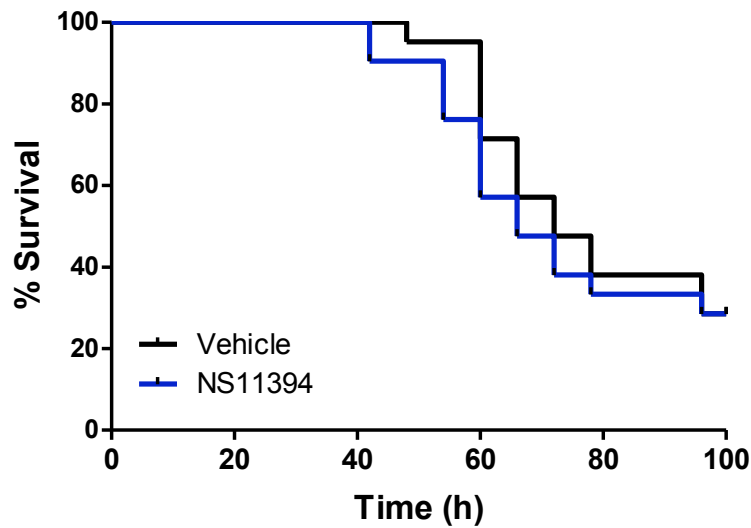
Figure 5.18 Kaplan-Meier survival curve of mice treated with NS11394 or vehicle prior to and during *Streptococcus pneumoniae* infection.

C57BL/6 mice were infected with *Streptococcus pneumoniae* and survival was monitored following infection (n = 20/group). NS11394 (2 mg kg^{-1} IP) or vehicle were given twice daily for seven days prior to the infection and continued throughout.
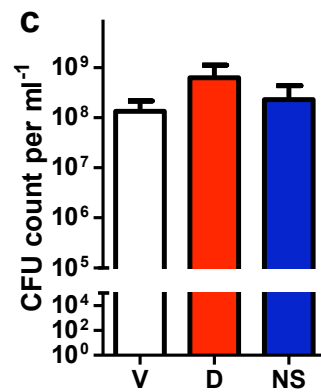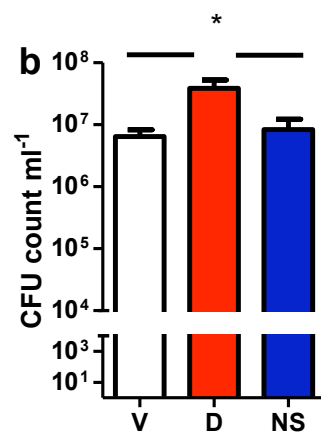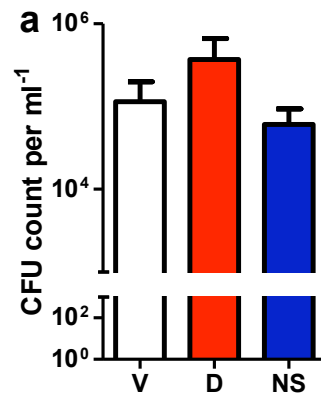
Figure 5.19 Bacterial load in the airway (a), lung (b) and blood (c) of animals infected with Streptococcus pneumoniae 48 hours earlier.

Mice were treated by IP injection with diazepam (D, 2 mg kg^{-1}), NS11394 (NS, 2 mg kg^{-1}) or vehicle (V, 4% ethanol in PBS) twice daily for 7 days before the infection and continued throughout the infection.
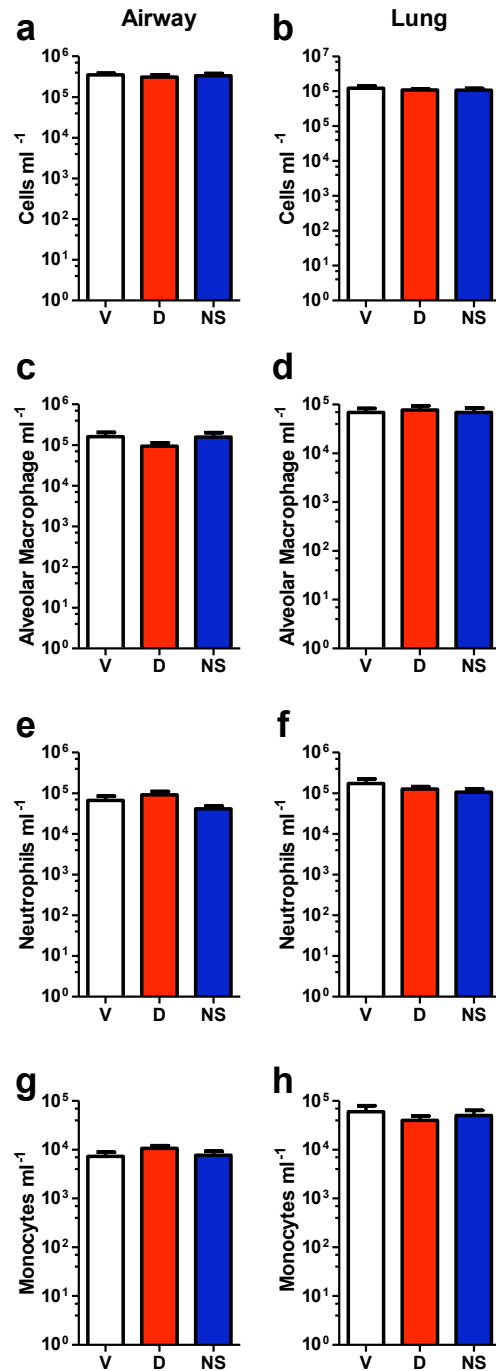
Figure 5.20 Cell numbers in the airway and lung following infection with *Streptococcus pneumoniae* 48 hours earlier.

Mice were treated by IP injection with diazepam (D, 2 mg kg^{-1}), NS11394 (NS, 2 mg kg^{-1}) or vehicle (V, 4% ethanol in PBS) twice daily for 7 days before the infection. Total cell concentration in the airway (**a**), and lung (**b**). Alveolar macrophage concentration in the airway (**c**) and lung (**d**), neutrophil concentration in the airway (**e**) and lung (**f**) and monocyte concentration in the airway (**g**) and lung (**h**).

5.3 Discussion

Macrophage and monocytes abundantly express $GABA_A$ receptors that act to negatively regulate their anti-pathogen responsiveness. In keeping with a fundamental role in macrophage physiology, $GABA_A$ receptor expression is tightly regulated and modulated by immune stimuli including TLR agonists and cytokines. Similar to neurons, activation of $GABA_A$ receptors on macrophage leads to cytoplasmic acidification which likely accounts for the reduced responsiveness to the pathogen. This is revealed as reduced cytokine release, phagocytosis and bacterial killing. Furthermore immune cells synthesize GABA, and the *ex vivo* experiments in which GABA was not co-applied with diazepam, suggest that alveolar macrophage likely can release GABA. This is because diazepam requires at least one GABA molecule to be bound to the receptor to activate the channel and hence there must be a source of GABA. Thus while it is unclear if or how they release GABA, the accumulating data point towards a system of $GABA_A$ immunomodulation.

5.3.1 Immune Cell Expression of $GABA_A$ receptors

It is currently unclear why macrophage and monocytes should express $GABA_A$ receptors much more abundantly than other immune cells. Functional studies reveal that $GABA_A$ receptor signaling acts to negatively regulate macrophage anti-bacterial effects. Consistent with this premise, bacterial TLRs downregulate

GABA$_A$ receptor expression on alveolar macrophage *ex vivo* and *in* vivo. We propose that GABA$_A$ receptors act to negatively regulate anti-bacterial function of the cell. Increased expression of GABA$_A$ receptors would be consistent with the highly regulated nature of alveolar macrophage that are kept in a subdued state to avoid "bystander damage" to the lung by any over exuberant response to a stimulus. While our *ex vivo* TLR studies imply a role for bacterial TLRs in the downregulation of GABA$_A$ receptor expression, influenza infection *in vivo* also decreased the expression on alveolar macrophages. This may be due to increased viral TLR burden (compared to *ex vivo*), the cytokine response to the infection, a combination of these two factors or another unappreciated factor such as signaling through other pattern recognition receptors.

In contrast upregulation of GABA$_A$ receptor expression was noted with IL-4 treatment *ex vivo* and with house dust mite treatment *in vivo* (to reproduce the allergic airways disease in the lung). Furthermore the *ex vivo* change in γ2 subunit expression, driven by IL-4, correlated with an intracellular acidosis. Amongst other Th2 cytokines, IL-4 is thought to polarize macrophages away from "classic activation" to "alternative activation". The latter state is important for wound healing and protection against helminthes however it is associated with a reduced ability to fight bacterial and viral pathogens[150]. As cytoplasmic acidification in alveolar macrophage is associated with reduced TNF-α production, bacterial phagocytosis and killing it is plausible that changes in GABA$_A$ receptor expression are important to the "alternatively activated"

macrophage phenotype induced by IL-4, via provocation of an intracellular acidosis.

Approximately a third of monocytes expressed the α1 subunit and a fifth the γ2 subunit. However it is clear that the regulation of monocytic expression of GABA$_A$ receptors needs further study. Whether GABA$_A$ receptors are expressed by a subset of monocytes dependent on different stages of maturation, regulation or stimulation needs enquiry. Furthermore monocyte regulation of GABA$_A$ receptor expression is potentially different from macrophage, as influenza increased expression of the α1 subunit initially as opposed to decreasing it. When challenged with IL-1β, neurons increase GABA$_A$ subunit expression[44] but the opposite response occurs in alveolar macrophage, so it may be that in response to certain stimuli monocytes behave more akin to neurons. Following *Streptococcus pneumoniae* infection there was also a trend to an increase in expression of the α1 subunit though this failed to reach significance.

Further *ex vivo* studies are also required to address whether monocytic function is altered by GABA$_A$ agonists as it is for macrophage. Based on our evidence in alveolar macrophage; evidence from THP-1 cells (an immortalized macrophage cell line)[48]; and data showing that GABA$_A$ agonists reduce calcium transients in peripheral blood mononuclear cells[41]; it is reasonable to suspect that monocyte function will be affected in a similar manner to macrophage.

The low level of expression of GABA$_A$ receptor subunits on neutrophils is also intriguing. Neutrophils do not seem to express GABA$_A$ receptor subunits, though only eight subunits from 19 were surveyed so the subunit screen was not exhaustive. Our data therefore suggest that neutrophils are not directly regulated by GABA$_A$ immunomodulation unlike macrophage and monocytes. Interestingly neutrophils express glycine receptors[152], and it has been suggested that these receptors may function equivalently to GABA$_A$ receptors on macrophage[41]. Further functional studies of glycinergic regulation of neutrophil function would be of interest.

The expression pattern of GABA$_A$ subunits on T cells is intriguing and may help explain the contradictory results in the literature. Herein CD4 cells were observed to upregulate both α1 and γ2 subunits; previously CD4 have been shown to upregulate γ3 subunit expression (γ2 subunit expression was not tested in that study)[40]. The same previous study has shown that CD4 cells contain the mRNA for the δ subunit in both naïve and activated conditions but under our conditions, based on in vivo sampling, this subunit was not observed on the cell surface by flow cytometry. However in contrast to CD4 cells, CD8 cells upregulated δ subunits during the influenza infection. Whether the previous results were caused by contamination of the CD4 population with CD8 cells, or whether δ subunit transcription does not lead to translation in these cells or whether our methods were too insensitive to detect δ subunit expression on the surface of CD4 cells requires further investigation.

Based on our data from influenza infection, sampling cells in the naïve state may not accurately inform us of the influence of GABA immunomodulation on T cells. Previous experiments have been unable to identify an effect on interferon-γ and TNF-α release from mixed T cell cultures[29] however those data showed significant variability. Further analysis of the effects of GABA immunomodulation isolated T cell subsets is required. In particular the observation that CD8 cells upregulate the δ subunit is intriguing. δ subunit containing $GABA_A$ receptors are highly sensitive to GABA but insensitive to benzodiazepines. Upregulation of the δ subunit implies that CD8 cells are highly regulated by GABA immunomodulation. The functional importance of this requires investigation as CD8 cells play a critical role in the defence against viral pathogens. At present the available agonists at δ subunits have relatively short half lives in humans (and are unknown in rodents) and so are potentially difficult to study *in vivo*. Though animal experiments using implanted drug pellets or mini-osmotic pumps are one way around this issue.

5.3.3 Immune Function of $GABA_A$ receptor signaling

Given the prevalence of $GABA_A$ receptors on alveolar macrophage, these cells were used for *ex vivo* assays to probe the function of $GABA_A$ signaling. The data obtained show reduced TNF-α and IL-6 release, bacterial phagocytosis and killing by alveolar macrophage in the presence of $GABA_A$ modulators. These effects mirror our in vivo findings with diazepam. We propose, consistent with the known role of $GABA_A$ receptors in neuronal function, activation of $GABA_A$

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receptors on alveolar macrophage leads to intracellular acidification potentially due to bicarbonate efflux from the cell. Indeed GABA$_A$ receptors are bicarbonate permeable, however our data do not exclude that changes in chloride ion concentration contribute to the changes in intracellular pH. These data are reproducible across various macrophage phenotypes including polarization with LPS (classic activation) or IL-4 (alternative activation). Furthermore GABA$_A$ receptor expression itself correlates with the change in intracellular pH and these changes can be driven by stimuli that drive the cell to either classic or alternative activation phenotype. It is also important that the GABA$_A$ antagonist bicuculline reversed the cytoplasmic acidification induced by diazepam. This indicates diazepam is acting in a GABA$_A$ receptor dependent manner. Functionally the finding that L-838-417 does not mimic diazepam indicates that α1 containing GABA$_A$ receptors mediated the impairment of macrophage function and intracellular pH change.

Nonetheless we are limited in understanding whether the changes to intracellular pH changes occur *in vivo*, as they do *ex vivo* and whether the magnitude of the change is similar *in vivo*. Indeed multiple differences between the *ex vivo* set up and in the *in vivo* environment make definite conclusions about the relevance of the magnitude in change in H$^+$ concentration uncertain. By necessity the pH experiments were conducted in atmospheric conditions, not a CO$_2$ enriched (5%) environment typical for *ex vivo* assays. Furthermore the multiple washing steps and even loading of the BCECF dye may alter macrophage function. Nonetheless we remain confident that the relative differences induced

by GABA$_A$ modulator treatment has value. Indeed we consider it plausible that multiple enzymatic processes that are pH sensitive may be affected by affected by a shift intracellular pH and that this may explain at least some of the *ex vivo* functional changes observed. Indeed cytoplasmic acidification has previously been implicated in reducing TNF-α production[148] and phagocytosis and killing of bacteria by alveolar macrophage[149]. However it is unclear as to whether the change in pH throughout the cytoplasm is the important determinant in impairing macrophage function or whether more localized changes in pH in particular subcellular compartments are required.

Given that GABA$_A$ receptor expression differs between neutrophils and macrophage, it is of interest that they use different mechanisms of bacterial killing (though both are pH sensitive). Macrophage recruit the autophagic machinery to kill pathogens[153,154] rather than mounting a potent respiratory burst as occurs in neutrophils[7]. Autophagy ("eating oneself") is a highly conserved homeostatic process that regulates turnover of cell macromolecules and organelles. It is stimulated by various conditions, notably starvation, but also by cellular alkalosis[155]. It is therefore conceivable that a TLR stimulated reduction in GABA$_A$ receptor expression may stimulate autophagy through changes in pH to enhance bacterial clearance by macrophage. Alternatively activated macrophage have reduced autophagic capacity, this may be due to the intracellular acidosis we have observed. Separately GABA$_A$ agonists have been linked to the inhibition of autophagy in neurons[156]; any role in the regulation of macrophage function should be followed up. The interplay between GABA$_A$

receptor expression, autophagy and intracellular pH requires further investigation.

5.3.4 The importance of α1-γ2 containing GABA$_A$ receptors

Given the abundant expression of α1-γ2 containing GABA$_A$ receptors on macrophage and monocytes, we tested whether benzodiazepines that do not augment GABA signaling at α1-γ2 containing receptors would cause impairments in macrophage function *ex vivo* and increase susceptibility to infection *in vivo*. For the *ex vivo* experiments we used, L-838-417 a commercially available selective benzodiazepine for α2/3-γ2 GABA$_A$ subunits. Unfortunately ultra rapid metabolism in the mouse makes it unsuitable for our *in vivo* work. NS11394 was therefore obtained from Neurosearch, Denmark for the *in vivo* work. NS11394 has prolonged receptor occupancy in rodents and exerts similar anxiolytic effects to diazepam at the same concentrations and therefore while the pharmacokinetics of both diazepam and NS11394 are unknown in mice (especially in the setting of infection), we considered them comparable for pilot preclinical investigations[75,157]. Our finding that L-838-417 did not affect cytokine release or bacterial killing *ex vivo* unlike diazepam supported our studies of GABA$_A$ receptor expression. However *in vivo* data were required to inform whether drug development of more selective benzodiazepines may reduce the immune side effects of this class of drug if they are used for anxiolysis. This is possible as anxiolysis is largely transduced through α2/3 GABA$_A$ subunit containing receptors (due to their localization in limbic areas) rather than via α1

GABA$_A$ subunit containing receptors. The drugs are being developed to separate the anxiolytic effects of the drugs from the sedative effects of the drugs as sedation is predominantly mediated by α1 GABA$_A$ subunit containing receptors[142,151]. This is likely due to the abundant expression of α1 GABA$_A$ subunit containing receptors at synapses in the central nervous system[142,151].

Consistent with our hypothesis NS11394 did not increase mortality from *Streptococcus pneumoniae* infection and did not increase bacterial counts relative to the vehicle. Due to a combination of a more aggressive baseline infection than predicted and the multiple comparisons required when comparing three groups, diazepam was not shown to increase bacterial counts in the airway as in previous experiments. However a difference was observed in the lung and in this setting NS11394 did not similarly increase the pathogen load. This suggests that selective benzodiazepines being developed to separate the anxiolytic and sedative actions of benzodiazepines may also have utility in separating the immune and anxiolytic actions of these drugs.

By extension, sedation and immune suppression may not be disentangled by the development of more selective benzodiazepines. As the available non-benzodiazepine GABAergic sedative, propofol, also activates α1β2γ2 GABA$_A$ receptors[151] a similar immune dysfunction can be expected from this drug. This should be confirmed in future studies. However these data support the idea that

non-GABAergic sedatives may be required to improve outcomes from infection in critically ill patients[69].

5.4 Conclusions

Based on the work presented in this chapter, the abundant expression of $GABA_A$ receptors on macrophage and monocytes and their functional role in inhibiting responsiveness to a pathogen suggests that augmenting $GABA_A$ receptor signaling with a benzodiazepine may lead to increased susceptibility to an infection through direct effects on these cells. In order to fine tune alveolar macrophage responses $GABA_A$ immunomodulation is tightly controlled by inflammatory signaling. The subtype of $GABA_A$ receptors expressed by macrophage and monocytes is important as benzodiazepines that lack activity at $\alpha 1$ subunits lack the immunosuppression of non-selective drugs; thus enhanced benzodiazepine selectivity should improve the safety profile of this widely used class of drug. However benzodiazepines are also the most commonly used intensive care sedative and it is unclear from our data how the sedative actions of GABAergic drugs can be disentangled from their immunosuppressive effects.

Chapter 6 Final Discussion

6.1 Summary of findings

The hypothesis was "GABA$_A$ receptor signaling plays a role in the response to infection and that augmentation of GABA$_A$ signaling, with drugs such as benzodiazepines, may compromise the immune responses to infection." Herein we provide data to support this hypothesis however the evidence is not definitive and further investigation is required. In particular the limitations of the clinical work necessitate further study.

Nonetheless data has been presented that indicates that exposure to GABAergic medication, such as benzodiazepines, may increase susceptibility to infection in both clinical and preclinical settings. By using a translational research approach, benzodiazepines have been associated with increased mortality from sepsis (in comparison to dexmedetomidine), community acquired pneumonia in humans and pneumonia in preclinical animal models. The mechanism is dependent on GABA$_A$ signaling as it is reversed by the GABA$_A$ antagonist, bicuculline; this is consistent with review of the data showing that the tested GABA$_A$ agonists exert similar effects as a class[23] and the clinical pneumonia data showing a similar effect of benzodiazepines and zopiclone (**Chapter 3**). The immune cell expression of GABA$_A$ subunits, in combination with the functional *ex-* and *in vivo* work we have done suggests that the development of subunit selective

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GABAergic drugs may lead to agents with an improved immune safety profile. This is achievable for endpoints such as anxiolysis that are mediated through α2/3 subunit containing GABA$_A$ receptors[151]. However benzodiazepines are also the most commonly used intensive care sedative and it is unclear from our data how the sedative actions of GABAergic drugs (mediated by α1β2γ2 GABA$_A$ receptors[142,151]) can be separated from their immunological effects. Therefore non-GABAergic sedatives may be required to improve outcomes from infection in critically ill patients[69].

Specific responses to the objectives for the thesis are listed below:

1. *Does GABAergic sedation worsen mortality in sepsis relative to an alternate non-GABAergic sedative dexmedetomidine?*

The GABAergic drug lorazepam was shown to increase mortality relative to non-GABAergic sedation with dexmedetomidine in septic patients. This secondary analysis of a randomized controlled trial provided preliminary evidence for an effect on survival in septic patients.

2. *Are sub-sedative doses of benzodiazepines and zopiclone associated with an increased incidence of, and mortality from, pneumonia?*

Using case-control and cohort study designs preliminary evidence was obtained for a deleterious effect of benzodiazepines and zopiclone on the incidence of, and mortality from, pneumonia.

3. *Does diazepam impair the immune response to Streptococcus pneumoniae pneumonia in a GABAA receptor dependent manner?*

Diazepam was shown to impair the immune response to *Streptococcus pneumoniae* pneumonia. This effect could be reversed with bicuculline indicating dependence on a $GABA_A$ receptor signalling. Diazepam delayed the cytokine response to the bacterial infection and inhibited bacterial clearance *in vivo*.

4. *Does diazepam impair the immune response to influenza?*

Diazepam increased weight loss from influenza and led to increased spontaneous bacterial infection in the mouse model. This was associated with an exacerbated inflammatory response.

5. *Do parenteral fluids during influenza affect the immune response?*

Parenteral fluids were shown not to affect weight loss or the immune response to influenza.

6. *Which immune cells express GABA$_A$ receptors?*

We showed that macrophage and monocytes highly expressed $GABA_A$ receptor subunits. $GABA_A$ receptor *s*ubunits could be detected at a low level on some other immune cells such as CD4 cells.

7. *What are the functional effects of GABA$_A$ receptor signaling in alveolar*

macrophage?

GABA$_A$ stimulation led to the suppression of cytokine production, and impaired phagocytosis and bacterial killing by alveolar macrophage *ex vivo*. The mechanism was linked to alterations of intracellular hydrogen ion concentration.

6.1 Preclinical Data

An important caveat to the preclinical work is the lack of evidence definitively demonstrating that the benzodiazepine effect is mediated by a specific cell type. Nonetheless the findings may be explained by perturbed function of alveolar macrophage based on (i) their abundant expression of GABA$_A$ receptors, (ii) the immune regulation of their expression and (iii) our *ex vivo* functional studies. Future studies should employ genetic manipulation of GABA$_A$ receptor expression in individual cell populations to definitively identify the cell type responsible. This may be achieved by generating Cre-Lox recombination transgenic mice[158], for example with Cre (F480) to target mouse macrophages and Lox (GABA$_A$ γ2 subunit) to delete the GABA$_A$ γ2 subunit from these cells. Deletion of the γ2 subunit will prevent benzodiazepine binding and activation of the GABA$_A$ receptors.

Nonetheless from a drug development perspective, the exact cellular target may not be critical given that (i) 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

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lung and (ii) therapeutically, selective benzodiazepines that do not target the α1 GABA$_A$ subunit did not increase susceptibility to infection *in vivo*. Further studies are needed to confirm our findings in other species and in other infection animal models before clinical translation of our findings.

It is intriguing that GABA$_A$ subunits were detected on macrophage and monocytes, but not neutrophils, indicating that neutrophil function does not require GABA$_A$ regulation. This may be due to the limited lifespan of the neutrophil, perhaps they are intended for a "burst" of activity that is rapidly turned off by apoptotic cell death[7] and hence does not require "fine tuning". As such, neutrophil regulation maybe considered a kin to a "binary switch". Tissue resident and longer living cells such as macrophage and monocytes may require more "fine tuning", analogous to a "dimmer switch" or "rheostat"[1,3]; this may allow site-specific regulation of macrophages. Consistent with this premise, alveolar macrophage, that need to be tightly regulated to avoid collateral damage to the lung, have higher GABA$_A$ receptor expression than macrophage at splenic and peritoneal sites. Perhaps peritoneal macrophages have very low expression of GABA$_A$ receptors to allow them to rapidly respond to invading gut pathogens, especially since collateral damage will be less costly in the peritoneal cavity than in the lung. Splenic macrophage may require an activation status that lies in between alveolar and peritoneal macrophage, as collateral damage may still be costly but the spleen also needs to play an active role in clearance of bacteria, particularly those with a capsule, from the blood. Therefore it maybe that GABAergic mechanisms help restrain macrophage function in differing tissues to

different levels. It would be of interest to understand what mechanisms control this GABAergic signaling, for example whether there are higher endogenous levels of IL-4 in the lung than in the spleen or peritoneal cavity.
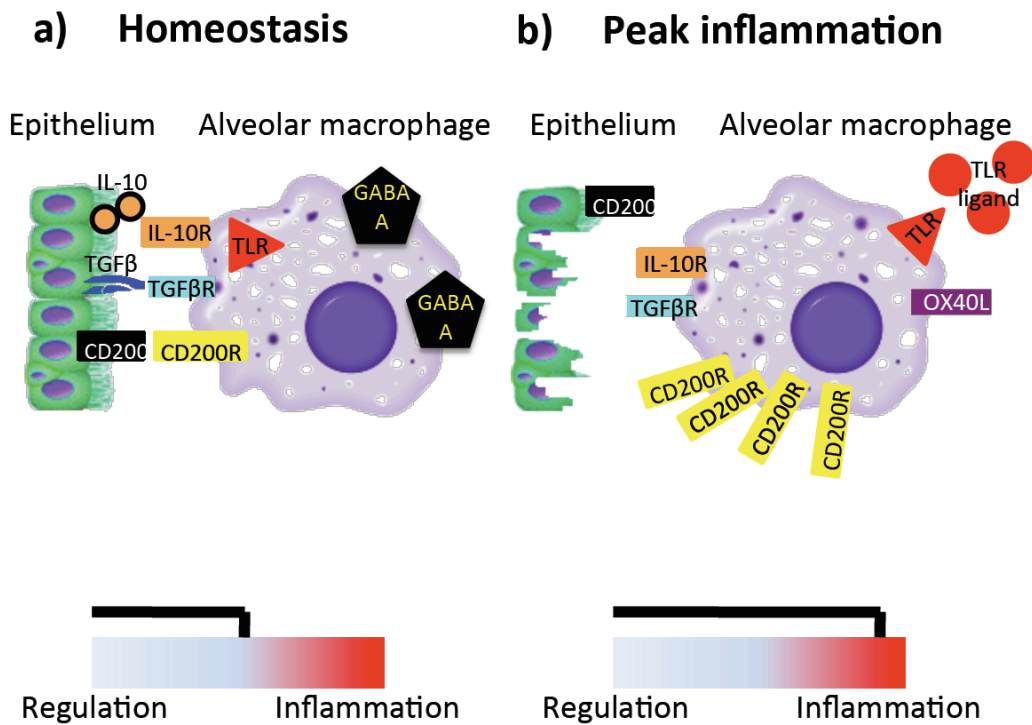
Figure 6.1 Schematic describing the effects of inflammation on negative regulators of alveolar macrophage function including GABA$_A$ receptors.

A) At homeostasis negative regulators, including IL-10, TGF-β, CD200 and GABA signaling restrain alveolar macrophage activity and TLR signaling is suppressed. B) Following inflammatory challenge, epithelial integrity and these negative regulatory signals are lost allowing TLR and OX40L signaling to potentiate inflammation. Notably loss of GABA$_A$ receptor expression, driven by TLR stimulation, leads to a shift in intracellular pH to more alkaline values that alter macrophage responsiveness.

The difference between macrophage and neutrophils may relate to their contrasting mechanisms of bacterial killing. Neutrophils kill by mounting a potent respiratory burst, leading to alkalosis of the vacuole, enzyme activation and then profound acidosis of the vacuole, essentially digesting the pathogen[7]. In contrast macrophage kill bacteria by recruiting the autophagy (literally "eating oneself") machinery to help "digest" the pathogen[153,154]. Autophagy is the process through which unwanted cellular organelles are digested if damaged or in times of starvation. Macrophages harness this system in order to allow them to kill bacteria. Interestingly autophagy is activated by alkaline pH in the cytosol[155] and inihibited by IL-4[159] that causes intraellular acidosis perhaps via increasing $GABA_A$ receptor expression (**Chapter 5**). Given that $GABA_A$ signaling impairs bacterial killing and cause cytosolic acidosis, it may regulate bacterial killing capability through effects on autophagy. Indeed the $GABA_A$ agonist, propofol has been shown to reduce autophagy in models of ischemia reperfusion injury of the heart[156] and LPS injury of pulmonary epithelial cells[160]. Further studies are required to understand how $GABA_A$ receptor signaling affects autophagy in macrophage.

The mechanism through which $GABA_A$ activity and pH changes affect cytokine production and phagocytosis remains unclear. It is plausible that these processes are affected by shifts in H^+ concentration, given the precise range of pH required to maintain enzymatic function, however the exact mechanisms will need to be elucidated in future work. Augmenting $GABA_A$ receptor signaling has been shown to reduce stimulated Ca^{2+} bioavailability in the cytoplasm of peripheral

blood mononuclear cells[41]. This may contribute to impaired phagocytosis[48] and cytokine production[161]. It is plausible that Ca^{2+} bioavailability is affected by anion flux (either Cl^- or HCO_3^-) as induced cytoplasmic alkalosis increases Ca^{2+} bioavailability[162]. $GABA_A$ receptor signaling reduces the alklaine pH shift that follows TLR-4 activation (**Chapter 5**) potentially blunting the immune response to infection by impairing Ca^{2+} responses.

There may be other effects that have not been addressed involving other anti-pathogen defences such as surfactant proteins, anti-bacterial peptides and complement. As yet the effects of GABA signaling on these mechanisms have not been investigated but given the lack of effect on immune cell recruitment of diazepam treatment, these targets remain plausibly involved in the detrimental effect on the immune system.

A final caveat to the interpretation of the preclinical data is the source and requirement of the endogenous ligand for the $GABA_A$ receptor, GABA. As immune and epithelial cells express GAD and synthesize GABA[39,145], and macrophage $GABA_A$ currents are activated by GABA[29], it seems plausible that GABA is the endogenous ligand for the receptor. Thus GABA may act as an "immunotransmitter" as a parallel for "neurotransmitter". However attempts to measure GABA release from immune cells have failed[29]. Nonetheless our ex vivo suggest that GABA must be released by macrophage otherwise diazepam could not activate the $GABA_A$ receptor. An alternative possibility is that immune cell

GABA$_A$ channels have tonic activity[64] as well as ligand-induced activity. Arguing against a role of tonic activity, the studies with the GABA$_A$ antagonist, bicuculline, did not reveal changes in baseline function or cytoplasmic pH. Nonetheless future studies should not discount the possibility of tonic activity of the GABA$_A$ channel on immune cells.

6.2 Clinical Data

An important caveat of the clinical data, that is worth reemphasizing, is that residual confounding may have affected the clinical results as drug therapy was not randomized. Randomized controlled trials are needed to address this significant limitation of these data. Nonetheless our data indicate benzodiazepines and zopiclone may exert clinically relevant affects on the response to infection and are supported by the preclinical mechanistic studies.

It is of interest, that at subsedative doses the impact of benzodiazepines waned with increasing patient comorbidity. This is a potentially important finding, as it may influence where the public health impact of the drugs can be noticed. However this finding does not mean that benzodiazepines are without importance in this group. Our finding may also reflect loss of statistical power in the subgroup with increased comorbidity as the impact of the benzodiazepines on mortality from pneumonia may be smaller. These data also highlight a pitfall of the translational approach, emphasizing the limitations of studying preclinical

disease in healthy adolescent mice. This is thought to be a significant limiting factor when extrapolating preclinical discoveries into the clinical arena[136]. Future studies should focus on whether benzodiazepines exert a meaningful impact on mortality and pathogen load from infection in mouse models with associated comorbidity such as diabetes and vascular disease. While we have done some preliminary work to check whether diazepam still has an effect on vulnerability to *Streptococcus pneumoniae* when it is preceded by an influenza infection, further data are required on the impact of more chronic co-morbidities.

In humans, further studies are underway to address whether benzodiazepines increase the risk of pneumonia in patients with stroke, as a randomized controlled trial of diazepam therapy for stroke observed an increase in the incidence of pneumonia in the diazepam treated group[163]. This study is being conducted by Prof Ken Lees, Dr Benedikt Frank, Miss Rachael Fulton and myself by analyzing the Virtual International Stroke Trials Archive (VISTA). VISTA is a database of randomized controlled trials of stroke therapy.

While increasing inflammation may reduce the impact of GABAergic drugs on alveolar macrophage responses (as $GABA_A$ receptor expression decreases), sedative doses of GABAergic drugs may still exert significant effects on immunity, as suggested by our sepsis data[69]. This is supported by Riker et al.,[24] who showed a doubling of secondary infections with midazolam sedation compared to dexmedetomidine sedation in a secondary analysis of their randomized

controlled trial of critically ill patients and the presented secondary analysis of the MENDS trial (**Chapter 3**). However while alveolar macrophage reduce $GABA_A$ receptor expression, the receptor is still present indicating they may still mediate at least some of the immunotoxic effects of benzodiazepines. However impairments of monocyte or adaptive immune function likely also contribute. Notably CD4 lymphocyte responses are known to be disabled in septic critical illness[9,10] and a significant proportion of CD4 cells upregulated $GABA_A$ receptor subunits during influenza infection. Likewise monocyte responses are defective in critical illness and sepsis[9,10] and $GABA_A$ receptor stimulation may contribute to this. Of course we also cannot exclude that central nervous system or indeed other non-immune and non-neuronal responses are responsible.

The cumulative data contained herein also challenge the notion of "*ventilator* associated pneumonia" in critically ill patients and suggest that in fact this may relate, at least partly, to "*sedation* associated pneumonia". As sedatives exert profound effects on immunity, it is likely that they contribute to "ventilator associated pneumonia". This is difficult to study as all sedatives influence immune function and ventilation largely mandates sedative therapy. Opioids, given to most critically ill patients for analgesia or analgosedation, have profound immune effects, increasing susceptibility to infection[111,112], similar to GABAergic drugs. Dexmedetomidine has potent anti-inflammatory properties but effects on pathogen clearance are unknown, though other α_2 adrenergic drugs have been suggested to enhance bacterial clearance *in vitro*[94,95,97]. However dexmedetomidine is difficult to study in the preclinical setting as it is

has a relatively short half life. Further preclinical studies are required of clinical relevant sedative infusions with physiological support to address the relative importance of the immune effects of different sedative regimens.

Clinically dexmedetomidine is often difficult to use as the sole sedative for a patient. New approaches to potentiating dexmedetomidine sedation are required to reduce the use of GABAergic and opioid drugs in critically ill patients. One potential way of achieving this is to antagonize the arousal promoting orexinergic signaling pathway (**Figure 6.2**)[92]. Dexmedetomidine does not suppress orexin signaling[105], this may explain the unique rousability of patients sedated with dexmedetomidine[89,92]. By extension, it offers a potential route to enhance dexmedetomidine sedation, especially since orexinergic antagonists are under development[164]. However even if this approach did work, the immune effects of orexinergic drugs are poorly explored (though studies to date suggest limited effects[165]) and so this would not necessarily guarantee an improved immune profile of sedation.

It would also be of interest to investigate whether polymorphisms in the $GABA_A$ receptor contribute to different immune phenotypes, as they do for diverse neuropsychiatric disorders such as temporal lobe epilepsy, eating disorders, alcohol dependence and insomnia[166,167]. While this has not been explored as yet, it is important to realize there may not be an association due to the endogenous immune regulation of $GABA_A$ subunit expression.

a) Awake

b) Sedated with a GABAergic agent

c) Sedated with an α_2-adrenoceptor agonist
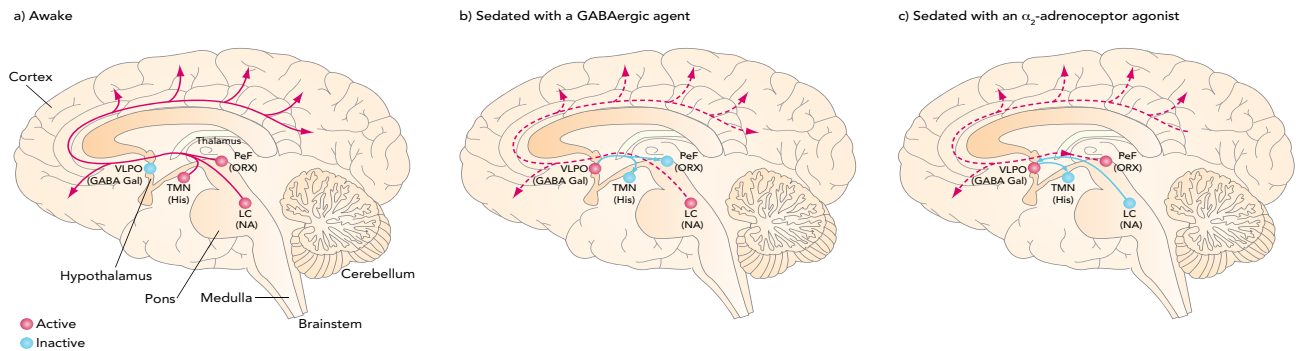
Figure 6.2 The sedative effects of GABA$_A$ and α_2 adrenoceptor agonists involve different neural networks: a schematic demonstrating some important neural nuclei involved in producing the sedative state.

Active nuclei are depicted in red and inactive nuclei are depicted in blue. (a) In the awake state, certain "awake-active" neural nuclei, including the noradrenergic locus ceruleus (LC), the orexinergic perifornical nucleus (PeF) and the histaminergic tuberomamillary nucleus (TMN), provide excitatory input to higher centres such as the cortex. When awake a "sleep-active" nucleus the venterolateral preoptic nucleus (VLPO) is silent. (b) During GABAergic sedation, potentiated inhibitory actions of the VLPO reduce neural activity in both the PeF and TMN but allow activity to proceed unimpeded in the LC (resulting in intact noradrenergic signaling; active signaling shown with a dotted red line). (c) During α_2 adrenoceptor agonist sedation activity is reduced in the LC and TMN while activity is enhanced in the inhibitory VLPO. Activity in the PeF is unaffected by α_2 adrenoceptor agonist sedation (resulting in intact orexinergic signaling; active signaling shown with a dotted red line). Reproduced with permission from Sanders et al.[168]

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6.3 Clinical Implications

Given the importance of GABAergic drugs to control of diverse central nervous system disorders as well as sedation and anaesthesia it would be premature to call for a moratorium on their use. Rather clinicians must use the information herein to guide their judgment about using these drugs in patients at risk from infection. Further data are required on the impact GABAergic drugs have on infection in various clinical situations and the possibility of alternative therapies. It is also unclear as to the benefit of stopping a GABAergic drug in a tolerant patient who has an infection. Studies are needed to specifically address this potential strategy.

6.4 Conclusions

GABAergic drugs exert significant effects on immunity, likely at least in part, on actions on endogenous $GABA_A$ immunomodulation. Indeed the endogenous regulation of $GABA_A$ receptor expression implies a potentially important role in immune function. Augmenting endogenous GABAergic signaling, with a benzodiazepine, increases mortality in various settings of infection. This has important ramifications for healthcare, as these drugs are widely used and therapeutic alternatives, such as subunit selective GABAergic drugs should be developed to potentially reduce the immune burden imposed by their non-selective counterparts.

References

1.   Wissinger E, Goulding J, Hussell T. Immune homeostasis in the respiratory tract and its impact on heterologous infection. Semin Immunol 2009;21:147-55.
2.   Snelgrove RJ, Goulding J, Didierlaurent AM, et al. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. Nat Immunol 2008;9:1074-83.
3.   Hussell T, Goulding J. Structured regulation of inflammation during respiratory viral infection. Lancet Infect Dis 2010;10:360-6.
4.   Snelgrove RJ, Godlee A, Hussell T. Airway immune homeostasis and implications for influenza-induced inflammation. Trends Immunol 2011;32:328-34.
5.   Maris NA, Dessing MC, de Vos AF, et al. Toll-like receptor mRNA levels in alveolar macrophages after inhalation of endotoxin. Eur Respir J 2006;28:622-6.
6.   Fernandez S, Jose P, Avdiushko MG, Kaplan AM, Cohen DA. Inhibition of IL-10 receptor function in alveolar macrophages by Toll-like receptor agonists. J Immunol 2004;172:2613-20.
7.   Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197-223.
8.   Didierlaurent A, Goulding J, Patel S, et al. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 2008;205:323-9.
9.   Boomer JS, To K, Chang KC, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. JAMA 2011;306:2594-605.
10.   Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med 2003;348:138-50.
11.   Myles PR, McKeever TM, Pogson Z, Smith CJ, Hubbard RB. The incidence of pneumonia using data from a computerized general practice database. Epidemiol Infect 2009;137:709-16.
12.   Lim WS, Baudouin SV, George RC, et al. BTS guidelines for the management of community acquired pneumonia in adults: update 2009. Thorax 2009;64 Suppl 3:iii1-55.
13.   Harrison DA, Welch CA, Eddleston JM. The epidemiology of severe sepsis in England, Wales and Northern Ireland, 1996 to 2004: secondary analysis of a high quality clinical database, the ICNARC Case Mix Programme Database. Crit Care 2006;10:R42.
14.   Bautista E, Chotpitayasunondh T, Gao Z, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N Engl J Med 2010;362:1708-19.
15.   Myles PR, Hubbard RB, Gibson JE, Pogson Z, Smith CJ, McKeever TM. The impact of statins, ACE inhibitors and gastric acid suppressants on pneumonia mortality in a UK general practice population cohort. Pharmacoepidemiol Drug Saf 2009;18:697-703.
16.   van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 2009;374:1543-56.
17.   Dockrell DH, Marriott HM, Prince LR, et al. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. J. Immunol. 2003;171:5380-8.

18.	Hotchkiss RS, Swanson PE, Freeman BD, et al. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. Crit. Care Med. 1999;27:1230-51.
19.	Angus DC. The search for effective therapy for sepsis: back to the drawing board? JAMA 2011;306:2614-5.
20.	Sprung CL, Annane D, Keh D, et al. Hydrocortisone therapy for patients with septic shock. N Engl J Med 2008;358:111-24.
21.	Moreno R, Sprung CL, Annane D, et al. Time course of organ failure in patients with septic shock treated with hydrocortisone: results of the Corticus study. Intensive Care Med 2011;37:1765-72.
22.	Pagani L, Afshari A, Harbarth S. Year in review 2010: Critical Care - infection. Critical care 2011;15:238.
23.	Sanders RD, Hussell T, Maze M. Sedation & immunomodulation. Crit Care Clin 2009;25:551-70, ix.
24.	Riker RR, Shehabi Y, Bokesch PM, et al. Dexmedetomidine vs midazolam for sedation of critically ill patients: a randomized trial. JAMA 2009;301:489-99.
25.	Jakob SM, Ruokonen E, Grounds RM, et al. Dexmedetomidine vs midazolam or propofol for sedation during prolonged mechanical ventilation: two randomized controlled trials. JAMA 2012;307:1151-60.
26.	Gleason PP, Schulz R, Smith NL, et al. Correlates and prevalence of benzodiazepine use in community-dwelling elderly. J Gen Intern Med 1998;13:243-50.
27.	Hak E, Bont J, Hoes AW, Verheij TJ. Prognostic factors for serious morbidity and mortality from community-acquired lower respiratory tract infections among the elderly in primary care. Fam Pract 2005;22:375-80.
28.	van de Nadort C, Smeets HM, Bont J, Zuithoff NP, Hak E, Verheij TJ. Prognosis of primary care patients aged 80 years and older with lower respiratory tract infection. Br J Gen Pract 2009;59:e110-5.
29.	Bhat R, Axtell R, Mitra A, et al. Inhibitory role for GABA in autoimmune inflammation. Proc Natl Acad Sci U S A 2010;107:2580-5.
30.	Yano M, Kuroda N, Han H, et al. Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. J Exp Med 2008;205:2827-38.
31.	Maemura K, Yanagawa Y, Obata K, et al. Antigen-presenting cells expressing glutamate decarboxylase 67 were identified as epithelial cells in glutamate decarboxylase 67-GFP knock-in mouse thymus. Tissue Antigens 2006;67:198-206.
32.	Buddhala C, Hsu CC, Wu JY. A novel mechanism for GABA synthesis and packaging into synaptic vesicles. Neurochem Int 2009;55:9-12.
33.	Uusi-Oukari M, Korpi ER. Regulation of GABA(A) receptor subunit expression by pharmacological agents. Pharmacol Rev 2010;62:97-135.
34.	Franks NP. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 2008;9:370-86.
35.	Pritchett DB, Sontheimer H, Shivers BD, et al. Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. Nature 1989;338:582-5.
36.	Baur R, Sigel E. Benzodiazepines affect channel opening of GABA A receptors induced by either agonist binding site. Molecular pharmacology 2005;67:1005-8.

37.   Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DW. Extrasynaptic GABAA receptors: form, pharmacology, and function. J Neurosci 2009;29:12757-63.

38.   Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 2008;60:243-60.

39.   Xiang YY, Wang S, Liu M, et al. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. Nat Med 2007;13:862-7.

40.   Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL. Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. J Immunol 2004;173:5298-304.

41.   Alam S, Laughton DL, Walding A, Wolstenholme AJ. Human peripheral blood mononuclear cells express GABAA receptor subunits. Mol Immunol 2006;43:1432-42.

42.   Jin N, Kolliputi N, Gou D, Weng T, Liu L. A novel function of ionotropic gamma-aminobutyric acid receptors involving alveolar fluid homeostasis. J Biol Chem 2006;281:36012-20.

43.   Reyes-Garcia MG, Hernandez-Hernandez F, Hernandez-Tellez B, Garcia-Tamayo F. GABA (A) receptor subunits RNA expression in mice peritoneal macrophages modulate their IL-6/IL-12 production. J Neuroimmunol 2007;188:64-8.

44.   Serantes R, Arnalich F, Figueroa M, et al. Interleukin-1beta enhances GABAA receptor cell-surface expression by a phosphatidylinositol 3-kinase/Akt pathway: relevance to sepsis-associated encephalopathy. J Biol Chem 2006;281:14632-43.

45.   Brask J, Owe-Larsson B, Hill RH, Kristensson K. Changes in calcium currents and GABAergic spontaneous activity in cultured rat hippocampal neurons after a neurotropic influenza A virus infection. Brain Res Bull 2001;55:421-9.

46.   Brask J, Kristensson K, Hill RH. Exposure to interferon-gamma during synaptogenesis increases inhibitory activity after a latent period in cultured rat hippocampal neurons. Eur J Neurosci 2004;19:3193-201.

47.   Winder TR, Minuk GY, Sargeant EJ, Seland TP. gamma-Aminobutyric acid (GABA) and sepsis-related encephalopathy. Can J Neurol Sci 1988;15:23-5.

48.   Wheeler DW, Thompson AJ, Corletto F, et al. Anaesthetic impairment of immune function is mediated via GABA(A) receptors. PloS one 2011;6:e17152.

49.   Kim SN, Son SC, Lee SM, et al. Midazolam inhibits proinflammatory mediators in the lipopolysaccharide-activated macrophage. Anesthesiology 2006;105:105-10.

50.   Wu XJ, Zheng YJ, Cui YY, Zhu L, Lu Y, Chen HZ. Propofol attenuates oxidative stress-induced PC12 cell injury via p38 MAP kinase dependent pathway. Acta Pharmacol Sin 2007;28:1123-8.

51.   Chiu WT, Lin YL, Chou CW, Chen RM. Propofol inhibits lipoteichoic acid-induced iNOS gene expression in macrophages possibly through downregulation of toll-like receptor 2-mediated activation of Raf-MEK1/2-ERK1/2-IKK-NFkappaB. Chem Biol Interact 2009;181:430-9.

52.   Wu GJ, Chen TL, Chang CC, Chen RM. Propofol suppresses tumor necrosis factor-alpha biosynthesis in lipopolysaccharide-stimulated macrophages possibly through downregulation of nuclear factor-kappa B-mediated toll-like receptor 4 gene expression. Chem Biol Interact 2009;180:465-71.
53.   Taniguchi T, Yamamoto K, Ohmoto N, Ohta K, Kobayashi T. Effects of propofol on hemodynamic and inflammatory responses to endotoxemia in rats. Crit Care Med 2000;28:1101-6.
54.   Qiao H, Sanders RD, Ma D, Wu X, Maze M. Sedation improves early outcome in severely septic Sprague Dawley rats. Crit Care 2009;13:R136.
55.   Laschi A, Descotes J, Tachon P, Evreux JC. Adverse influence of diazepam upon resistance to Klebsiella pneumoniae infection in mice. Toxicol Lett 1983;16:281-4.
56.   Domingues-Junior M, Pinheiro SR, Guerra JL, Palermo-Neto J. Effects of treatment with amphetamine and diazepam on Mycobacterium bovis-induced infection in hamsters. Immunopharmacol Immunotoxicol 2000;22:555-74.
57.   Galdiero F, Bentivoglio C, Nuzzo I, et al. Effects of benzodiazepines on immunodeficiency and resistance in mice. . Life Sciences 1995;57:2413-23.
58.   Huemer HP, Lassnig C, Nowotny N, Irschick EU, Kitchen M, Pavlic M. Diazepam leads to enhanced severity of orthopoxvirus infection and immune suppression. Vaccine 2010;28:6152-8.
59.   Kelbel I, Koch T, Weber A, Schiefer HG, van Ackern K, Neuhof H. Alterations of bacterial clearance induced by propofol. Acta Anaesthesiol Scand 1999;43:71-6.
60.   Marino F, Cattaneo S, Cosentino M, et al. Diazepam stimulates migration and phagocytosis of human neutrophils: possible contribution of peripheral-type benzodiazepine receptors and intracellular calcium. Pharmacology 2001;63:42-9.
61.   Papadopoulos V, Lecanu L. Translocator protein (18 kDa) TSPO: an emerging therapeutic target in neurotrauma. Exp Neurol 2009;219:53-7.
62.   Klegeris A, McGeer EG, McGeer PL. Inhibitory action of 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxam ide (PK 11195) on some mononuclear phagocyte functions. Biochem Pharmacol 2000;59:1305-14.
63.   Finnerty M, Marczynski TJ, Amirault HJ, Urbancic M, Andersen BR. Benzodiazepines inhibit neutrophil chemotaxis and superoxide production in a stimulus dependent manner; PK-11195 antagonizes these effects. Immunopharmacology 1991;22:185-93.
64.   McCartney MR, Deeb TZ, Henderson TN, Hales TG. Tonically active GABAA receptors in hippocampal pyramidal neurons exhibit constitutive GABA-independent gating. Mol Pharmacol 2007;71:539-48.
65.   Hsu DZ, Liu MY. Bicuculline methiodide attenuates hepatic injury and decreases mortality in septic rats: role of cytokines. Shock 2004;22:347-50.
66.   Lubick K, Radke M, Jutila M. Securinine, a GABAA receptor antagonist, enhances macrophage clearance of phase II C. burnetii: comparison with TLR agonists. J Leukoc Biol 2007;82:1062-9.
67.   Pandharipande PP, Pun BT, Herr DL, et al. Effect of sedation with dexmedetomidine vs lorazepam on acute brain dysfunction in mechanically ventilated patients: the MENDS randomized controlled trial. JAMA 2007;298:2644-53.

68.	Ely EW, Inouye SK, Bernard GR, et al. Delirium in mechanically ventilated patients: validity and reliability of the confusion assessment method for the intensive care unit (CAM-ICU). JAMA 2001;286:2703-10.

69.	Pandharipande PP, Sanders RD, Girard TD, et al. Effect of dexmedetomidine versus lorazepam on outcome in patients with sepsis: an a priori-designed analysis of the MENDS randomized controlled trial. Crit Care 2010;14:R38.

70.	The Health Improvement Network. The Health Improvement Network, 2011. (Accessed 23/06/2011, 2011, at http://www.thin-uk.com/.)

71.	Charlson M, Pompei P, Ales K, MacKenzie C. A new method of classifying prognostic comorbiditty in longitudinal studies: development and validation. J. Chron. Dis. 1987;40:373-83.

72.	Adams J, Ryan V, White M. How accurate are Townsend Deprivation Scores as predictors of self-reported health? A comparison with individual level data. J. Pub. Heal. Adv. Acc. 2004:1-6.

73.	Charlson F, Degenhardt L, McLaren J, Hall W, Lynskey M. A systematic review of research examining benzodiazepine-related mortality. Pharmacoepid. Drug Safety 2009;18:93-103.

74.	StataCorp. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP 2007.

75.	Mirza NR, Larsen JS, Mathiasen C, et al. NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile], a unique subtype-selective GABAA receptor positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo anxiolytic efficacy. The Journal of pharmacology and experimental therapeutics 2008;327:954-68.

76.	Low K, Crestani F, Keist R, et al. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 2000;290:131-4.

77.	McKernan RM, Rosahl TW, Reynolds DS, et al. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. Nat. Neurosci. 2000;3:587-92.

78.	Griebel G, Belzung C, Perrault G, Sanger DJ. Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology 2000;148:164-70.

79.	Elsersy H, Mixco J, Sheng H, Pearlstein RD, Warner DS. Selective gamma-aminobutyric acid type A receptor antagonism reverses isoflurane ischemic neuroprotection. Anesthesiology 2006;105:81-90.

80.	Jacobi J, Fraser GL, Coursin DB, et al. Clinical practice guidelines for the sustained use of sedatives and analgesics in the critically ill adult. Crit. Care Med. 2002;30:119-41.

81.	Kollef MH, Levy NT, Ahrens TS, Schaiff R, Prentice D, Sherman G. The use of continuous i.v. sedation is associated with prolongation of mechanical ventilation. Chest 1998;114:541-8.

82.	Girard TD, Jackson JC, Pandharipande PP, et al. Delirium as a predictor of long-term cognitive impairment in survivors of critical illness. Crit Care Med 2010;38:1513-20.

83.	Pandharipande P, Shintani A, Peterson J, et al. Lorazepam is an independent risk factor for transitioning to delirium in intensive care unit patients. Anesthesiology 2006;104:21-6.

84.   Sanders RD, Pandharipande PP, Davidson AJ, Ma D, Maze M. Anticipating and managing postoperative delirium and cognitive decline in adults. BMJ 2011;343:d4331.

85.   Weinhouse GL, Schwab RJ, Watson PL, et al. Bench-to-bedside review: delirium in ICU patients - importance of sleep deprivation. Crit Care 2009;13:234.

86.   Pisani MA, Murphy TE, Araujo KL, Slattum P, Van Ness PH, Inouye SK. Benzodiazepine and opioid use and the duration of intensive care unit delirium in an older population. Crit Care Med 2009;37:177-83.

87.   Carson SS, Kress JP, Rodgers JE, et al. A randomized trial of intermittent lorazepam versus propofol with daily interruption in mechanically ventilated patients. Crit Care Med 2006;34:1326-32.

88.   Breen D, Karabinis A, Malbrain M, et al. Decreased duration of mechanical ventilation when comparing analgesia-based sedation using remifentanil with standard hypnotic-based sedation for up to 10 days in intensive care unit patients: a randomised trial [ISRCTN47583497]. Critical care 2005;9:R200-10.

89.   Sanders RD, Maze M. Alpha2-adrenoceptor agonists. Curr Opin Investig Drugs 2007;8:25-33.

90.   Nelson LE, Guo TZ, Lu J, Saper CB, Franks NP, Maze M. The sedative component of anesthesia is mediated by GABA(A) receptors in an endogenous sleep pathway. Nat Neurosci 2002;5:979-84.

91.   Nelson LE, Lu J, Guo T, Saper CB, Franks NP, Maze M. The alpha2-adrenoceptor agonist dexmedetomidine converges on an endogenous sleep-promoting pathway to exert its sedative effects. Anesthesiology 2003;98:428-36.

92.   Sanders RD, Maze M. Contribution of sedative-hypnotic agents to delirium via modulation of the sleep pathway. Can J Anaesth 2011;58:149-56.

93.   Miles BA, Lafuse WP, Zwilling BS. Binding of alpha-adrenergic receptors stimulates the anti-mycobacterial activity of murine peritoneal macrophages. J Neuroimmunol 1996;71:19-24.

94.   Weatherby KE, Zwilling BS, Lafuse WP. Resistance of macrophages to Mycobacterium avium is induced by alpha2-adrenergic stimulation. Infect Immun 2003;71:22-9.

95.   Gets J, Monroy FP. Effects of alpha- and beta-adrenergic agonists on Toxoplasma gondii infection in murine macrophages. J Parasitol 2005;91:193-5.

96.   Nishina K, Akamatsu H, Mikawa K, et al. The effects of clonidine and dexmedetomidine on human neutrophil functions. Anesth Analg 1999;88:452-8.

97.   Hofer S, Steppan J, Wagner T, et al. Central sympatholytics prolong survival in experimental sepsis. Crit Care 2009;13:R11.

98.   Venn RM, Bryant A, Hall GM, Grounds RM. Effects of dexmedetomidine on adrenocortical function, and the cardiovascular, endocrine and inflammatory responses in post-operative patients needing sedation in the intensive care unit. Br J Anaesth 2001;86:650-6.

99.   Ma D, Hossain M, Rajakumaraswamy N, et al. Dexmedetomidine produces its neuroprotective effect via the alpha 2A-adrenoceptor subtype. Eur J Pharmacol 2004;502:87-97.

100.   Sanders RD, Xu J, Shu Y, et al. Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. Anesthesiology 2009;110:1077-85.

101.   Smith IM, Kennedy LR, Regne-Karlsson MH, Johnson VL, Burmeister LF. Adrenergic mechanisms in infection. III. alpha-and beta-receptor blocking agents in treatment. Am J Clin Nutr 1977;30:1285-8.

102.   Almirall J, Bolibar I, Balanzo X, Gonzalez CA. Risk factors for community-acquired pneumonia in adults: a population-based case-control study. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology 1999;13:349-55.

103.   Girard TD, Kress JP, Fuchs BD, et al. Efficacy and safety of a paired sedation and ventilator weaning protocol for mechanically ventilated patients in intensive care (Awakening and Breathing Controlled trial): a randomised controlled trial. Lancet 2008;371:126-34.

104.   Sanders RD. Hypothesis for the pathophysiology of delirium: Role of baseline brain network connectivity and changes in inhibitory tone. Med Hypotheses 2011.

105.   Zecharia AY, Nelson LE, Gent TC, et al. The involvement of hypothalamic sleep pathways in general anesthesia: testing the hypothesis using the GABAA receptor beta3N265M knock-in mouse. J Neurosci 2009;29:2177-87.

106.   Taniguchi T, Kidani Y, Kanakura H, Takemoto Y, Yamamoto K. Effects of dexmedetomidine on mortality rate and inflammatory responses to endotoxin-induced shock in rats. Crit Care Med 2004;32:1322-6.

107.   Taniguchi T, Kurita A, Kobayashi K, Yamamoto K, Inaba H. Dose- and time-related effects of dexmedetomidine on mortality and inflammatory responses to endotoxin-induced shock in rats. J Anesth 2008;22:221-8.

108.   Herr DL, Sum-Ping ST, England M. ICU sedation after coronary artery bypass graft surgery: dexmedetomidine-based versus propofol-based sedation regimens. J. Cardioth Vasc Anesth 2003;17:576-84.

109.   Memis D, Hekimoglu S, Vatan I, Yandim T, Yuksel M, Sut N. Effects of midazolam and dexmedetomidine on inflammatory responses and gastric intramucosal pH to sepsis, in critically ill patients. Br J Anaesth 2007;98:550-2.

110.   Tasdogan M, Memis D, Sut N, Yuksel M. Results of a pilot study on the effects of propofol and dexmedetomidine on inflammatory responses and intraabdominal pressure in severe sepsis. J. Clin Anesth. 2009;21:394-400.

111.   Wang J, Barke RA, Charboneau R, Roy S. Morphine impairs host innate immune response and increases susceptibility to Streptococcus pneumoniae lung infection. J. Immunol. 2005;174:426-34.

112.   Wang J, Barke RA, Charboneau R, Schwendener R, Roy S. Morphine induces defects in early response of alveolar macrophages to Streptococcus pneumoniae by modulating TLR9-NF-kappa B signaling. J. Immunol. 2008;180:3594-600.

113.   Weinert CR, Kethireddy S, Roy S. Opioids and infections in the intensive care unit should clinicians and patients be concerned? J Neuroimmune Pharmacol 2008;3:218-29.

114.   Pandharipande P, Cotton BA, Shintani A, et al. Prevalence and risk factors for development of delirium in surgical and trauma intensive care unit patients. J Trauma 2008;65:34-41.

115.   Sharshar T, Gray F, Lorin de la Grandmaison G, et al. Apoptosis of neurons in cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. Lancet 2003;362:1799-805.

116. Dellinger RP, Carlet JM, Masur H, et al. Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock. Crit Care Med 2004;32:858-73.

117. Pandharipande P, Girard T, Sanders R, Thompson J, Maze M, Ely E. Comparison of sedation with dexmedetomidine versus lorazepam in septic ICU patients. Crit Care 2008:P275.

118. Moher D, Schulz KF, Altman DG. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomized trials. Ann Int Med 2001;134:657-62.

119. Collins R, MacMahon S. Reliable assessment of the effects of treatment on mortality and major morbidity, I: clinical trials. Lancet 2001;357:373-80.

120. Assmann SF, Pocock SJ, Enos LE, Kasten LE. Subgroup analysis and other (mis)uses of baseline data in clinical trials. Lancet 2000;355:1064-9.

121. Wedel H, Demets D, Deedwania P, et al. Challenges of subgroup analyses in multinational clinical trials: experiences from the MERIT-HF trial. Am Heart J 2001;142:502-11.

122. Knol W, Van Marum RJ, Jansen PAF, Souverein PC, Schobben AFAM, Egberts ACG. Antipsychotic Drug Use and Risk of Pneumonia in Elderly People. Journal of the American Geriatrics Society 2008;56:661-6.

123. Almirall J, BolõÂbar I, BalanzoÂ X, lez CAG. Risk factors for community-acquired pneumonia in adults: a population-based case-control study. European Respiratory Journal 1999;13:349-55.

124. Vergis EN, Brennen C, Wagener M, Muder RR. Pneumonia in long-term care: a prospective case-control study of risk factors and impact on survival. Arch Intern Med 2001;161:2378-81.

125. Hak E, Bont J, Hoes A, Verheij T. Prognostic factors for serious morbidity and mortality from community-acquired lower respiratory tract infections among the elderly in primary care. Family Practice 2005;22:375-80.

126. James D, Schinnar R, Warren B, Wang X, Strom B. Validation studies of the health improvement network (THIN) database for pharmacoepidemiology research. Pharmacoepidemiology and Drug Safety 2007;16:393-401.

127. Macfarlane J, Colville A, Guion A, Macfarlane R, Rose D. Prospective study of aetiology and outcome of adult lower-respiratory tract infections in the community. Lancet 1993;341:511-4.

128. Macfarlane J, Holmes W, Gard P, et al. Prospective study of the incidence, aetiology and outcome of adult lower respiratory tract illness in the community. Thorax 2001;56:109-14.

129. Woodhead M, Macfarlane J, McCracken J, Rose D, R F. Prospective study of the aetiology and outcome of pneumonia in the community. Lancet 1987;1:671-4.

130. Schneeweiss S, Maclure M. Use of comorbidity scores for control of confounding in studies using administrative databases. International Journal of Epidemiology 2000;29:891-8.

131. Deyo R, Cherkin D, Ciol M. Adapting a clinical comorbidity index for use with ICD-9-CM administrative databases. J Clin Epidemiology 1992;45:613-9.

132. Righi DA, Pinheiro SR, Guerra JL, Palermo-Neto J. Effects of diazepam on Mycobacterium bovis-induced infection in hamsters. Braz J Med Biol Res 1999;32:1145-53.

133.   Krumholz W, Endrass J, Hempelmann G. Inhibition of phagocytosis and killing of bacteria by anaesthetic agents in vitro. British journal of anaesthesia 1995;75:66-70.

134.   Wang G, Wang R, Ferris B, et al. Smoking-mediated up-regulation of GAD67 expression in the human airway epithelium. Respir Res 2010;11:150.

135.   Evans CM, Koo JS. Airway mucus: the good, the bad, the sticky. Pharmacol Ther 2009;121:332-48.

136.   Dyson A, Singer M. Animal models of sepsis: why does preclinical efficacy fail to translate to the clinical setting? Crit Care Med 2009;37:S30-7.

137.   Hussell T, Pennycook A, Openshaw PJ. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. Eur J Immunol 2001;31:2566-73.

138.   Yoong YL, Lee HS, Gwee MC, Wong PT. Acute tolerance to diazepam in mice: pharmacokinetic considerations. Clin Exp Pharmacol Physiol 1986;13:153-8.

139.   Friedman H, Abernethy DR, Greenblatt DJ, Shader RI. The pharmacokinetics of diazepam and desmethyldiazepam in rat brain and plasma. Psychopharm 1986;88:267-70.

140.   Gobbi M, Barone D, Mennini T, Garattini S. Diazepam and desmethyldiazepam differ in their affinities and efficacies at 'central' and 'peripheral' benzodiazepine receptors. J Pharm Pharmacol 1987;39:388-91.

141.   Humphreys IR, Walzl G, Edwards L, Rae A, Hill S, Hussell T. A critical role for OX40 in T cell-mediated immunopathology during lung viral infection. J. Exp. Med. 2003;198:1237-42.

142.   Rudolph U, Crestani F, Benke D, et al. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 1999;401:796-800.

143.   Gunther U, Benson J, Benke D, et al. Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. PNAS 1995;92:7749-53.

144.   Kaila K, Voipio J. Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. Nature 1987;330:163-5.

145.   Stuckey DJ, Anthony DC, Lowe JP, et al. Detection of the inhibitory neurotransmitter GABA in macrophages by magnetic resonance spectroscopy. J. Leuk. Biol. 2005;78:393-400.

146.   Tian J, Chau C, Hales TG, Kaufman DL. GABA(A) receptors mediate inhibition of T cell responses. J Neuroimmunol. 1999;96:21-8.

147.   Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol 2011;32:402-11.

148.   Bidani A, Wang CZ, Saggi SJ, Heming TA. Evidence for pH sensitivity of tumor necrosis factor-alpha release by alveolar macrophages. Lung 1998;176:111-21.

149.   Bidani A, Reisner BS, Haque AK, et al. Bactericidal activity of alveolar macrophages is suppressed by V-ATPase inhibition. Lung 2000;178:91-104.

150.   Gordon S. Alternative activation of macrophages. Nat Rev Immunol. 2003;3:23-35.

151.   Rudolph U, Antkowiak B. Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci 2004;5:709-20.

152.   Wheeler M, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG. Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. FASEB J. 2000;14:476-84.

153.   Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004;119:753-66.

154.   Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 2006;313:1438-41.

155.   Suk J, Kwak SS, Lee JH, et al. Alkaline stress-induced autophagy is mediated by mTORC1 inactivation. J Cell Biochem 2011;112:2566-73.

156.   Noh HS, Shin IW, Ha JH, Hah YS, Baek SM, Kim DR. Propofol protects the autophagic cell death induced by the ischemia/reperfusion injury in rats. Mol Cells 2010;30:455-60.

157.   Munro G, Lopez-Garcia JA, Rivera-Arconada I, et al. Comparison of the novel subtype-selective GABAA receptor-positive allosteric modulator NS11394 [3'-[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitr ile] with diazepam, zolpidem, bretazenil, and gaboxadol in rat models of inflammatory and neuropathic pain. JPET 2008;327:969-81.

158.   Leppa E, Linden AM, Vekovischeva OY, et al. Removal of GABA(A) receptor gamma2 subunits from parvalbumin neurons causes wide-ranging behavioral alterations. PloS one 2011;6:e24159.

159.   Harris J, Master SS, De Haro SA, et al. Th1-Th2 polarisation and autophagy in the control of intracellular mycobacteria by macrophages. Vet Immunol Immunopathol 2009;128:37-43.

160.   Gu X, Lu Y, Chen J, et al. Mechanisms mediating propofol protection of pulmonary epithelial cells against lipopolysaccharide-induced cell death. Clin Exp Pharmacol Physiol 2012;39:447-53.

161.   Conboy IM, Manoli D, Mhaiskar V, Jones PP. Calcineurin and vacuolar-type H+-ATPase modulate macrophage effector functions. Proceedings of the National Academy of Sciences of the United States of America 1999;96:6324-9.

162.   Heming TA, Bulayeva NN, Bidani A. Cell alkalosis elevates cytosolic Ca2+ in rabbit resident alveolar macrophages. Clin Sci (Lond) 2003;105:21-8.

163.   Lodder J, Luijckx G, van Raak L, Kessels F. Diazepam treatment to increase the cerebral GABAergic activity in acute stroke: a feasibility study in 104 patients. Cerebrovasc Dis 2000;10:437-40.

164.   Gompf H, Chen J, Sun Y, Yanagisawa M, Aston-Jones G, Kelz MB. Halothane-induced hypnosis is not accompanied by inactivation of orexinergic output in rodents. Anesthesiology 2009;111:1001-9.

165.   Ichinose M, Asai M, Sawada M, Sasaki K, Oomura Y. Induction of outward current by orexin-B in mouse peritoneal macrophages. FEBS Lett 1998;440:51-4.

166.   Hamet P, Tremblay J. Genetics of the sleep-wake cycle and its disorders. Metabolism 2006;55:S7-12.

167.   Heilig M, Goldman D, Berrettini W, O'Brien CP. Pharmacogenetic approaches to the treatment of alcohol addiction. Nat Rev Neurosci 2011;12:670-84.

168.   Sanders RD, Hussell T, Maze M. Sedation & Immunity: Optimisation for Critically Ill Patients. Intense Times 2010;9:2-5.