Pseudomonas aeruginosa Lung Infection and Respiratory Muscle Weakness: Role of Cytokines in Diaphragm Muscle Dysfunction

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"Ignorance more frequently begets confidence than does knowledge: it is those who know little, not those who know much, who so positively assert that this or that problem will never be solved by science "

(Charles Darwin)

I dedicate this thesis to my father, **Farshid Divangahi**, grandfather, **Dr. Golamreza Shahroodi**, my sister in law, **Valerie Asselin**, and **Dr. David Price**, with all my love.

Contribution of Authors

I have chosen to present the current thesis as a dissertation which takes the format of a collection of research papers. In order to inform the external examiner of the faculty regulations concerning this format of thesis preparation, I have reproduced in full form the guideline for thesis preparation of McGill Faculty of Graduate Studies.

Candidates have the option of including as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicate text of one or more published papers. These text must be bound as an integral part of the thesis and reprints of published papers can include in appendices.

The thesis must be written as a cohesive unit with a logical progression from one chapter to the next rather than as a collection of manuscripts. Connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must still confirm with all other requirements of the "Guidelines for thesis preparation". The thesis must include: a table of contents, an abstract in both French and English, an introduction which clearly states the rational and objectives of the research, a review of literature, a final conclusion or summary, and a comprehensive reference list.

Additional materials must be provided, where appropriate (eg. in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of research reported in the thesis. In the case of co-author papers, the candidate is required to make an explicit statement in the thesis as who contributed to such work and to what extent. Supervisors must attest to the accuracy of this statement at the doctoral oral defense. Since the task of experiments is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the authors of the co-authored papers.

The data presented in this thesis have been published or are to be submitted to peer-reviewed journals for publication. The following individuals appears as co-authors, in addition to Maziar Divangahi on some of thses papers and their contributions are as follow:

<u>From Dr Petrof laboratory at the Meakins-Christie Laboratories</u>

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Former post-doctoral trainee in Dr Petrof's laboratory, he performed conjointly with me the majority of the muscle contractility experiments described in chapter 4.

Dr. Demoule, Alex:
Former post-doctoral trainee in Dr Petrof's laboratory, he performed conjointly with me the animal experiments involving adenovirus IL-10 gene delivery.

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The supervisor, his contribution to this thesis is intellectual, moral, and financial.

Maziar Divangahi:
My contribution as a second author in chapter 5, involved Perform/analysis of all the Rnase protection assay and revision of the manuscript.

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Principal investigator of the Critical Care and Respiratory Research. His contribution to this thesis is intellectual and moral.

From the Center for Gene Therapeutics at McMaster University

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From Montreal General Hospital

Dr. Radzioch, Dunata:
Principal investigator at Montreal General Hospital. CF mice were provided by her laboratory. She also intellectually contributed to this thesis.

This thesis contains the texts adopted from the following papers:

CHAPTER 2. PREFERENTIAL DIAPHRAGMATIC WEAKNESS DURING SUSTAINED PSEUDOMONAS AERUGINOSA LUNG INFECTION

Maziar Divangahi, *Stefan Mateckie, Roy W. Dudley, Stephanie A. Tuck, Weisheng Bao, Alain S. Comtois, Basil J. Petrof (Published in Am J of Resp Crit Care Med, 2004, 169(6): 679-86. Followed by editorial comments Am J of Resp Crit Care Med, 2004, 169(6): 662-3)*

CHAPTER 3. IL-10 GENE TRANSFER ALTERS CYTOKINE BALANCE AND REVERSES WEAKNESS OF THE DIAPHRAGM DURING PSEUDOMONAS LUNG INFECTION

Maziar Divangahi, *Alex Demoule, Gawiyou Danialou, Weisheng Bao, Basil J. Petrof (Submitted to Journal of Immunology)*

CHAPTER 4. INCREASED SUSCEPTIBILITY OF CFTR KNOCKOUT MICE TO DIPAHRAGM DYSFUNCTION AFTER PSEUDOMONAS AERUGINOSA LUNG INFECTION

Maziar Divangahi, *Weisheng Bao, Danuta Radzioch, Alain S. Comtois, Basil J. Petrof (To be submitted to Journal of Clinical Investigation)*

CHAPTER 5. DIFFERENTIAL CYTOKINE GENE EXPRESSION IN THE DIAPHRAGM IN RESPONSE TO STRENUOUS RESISTIVE BREATHING

*Theodoros Vassilakopoulos, **Maziar Divangahi**, George Rallis, Osama Kishta, Basil J. Petrof, Alain S. Comtois, Sabah N.A. Hussain (Published in Am J of Resp Crit Care Med, 2004, 170(2): 154-161)*

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List of Abbreviations

ABC	ATP-binding-cassette
Ad	Adenovirous
AM	Alveolar macrophage
ARDS	Acute respiratory distress syndrom
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CARS	Compensatory anti-inflammatory response syndrom
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CLP	Cecal ligation and puncture
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
DTH	Delayed-type hypersensitivity
ELF	Epithelial lining fluid
ERK	Extracellular signal regulated kinase
GM-CSF	Granulocyte/macrophage colony stimulating factor
GSH	Glutathione
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthases
IRAP	Interleukin-1 receptor antagonist protein
IRF	Interferon regulatory factor
KC	Keratinocyte-derived chemokine
Kda	Kilodalton
LBP	LPS-binding protein
LFA	Leukocyte function antigen
LIX	LPS-induced CXC chemokine
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
Mdx	Murine dystrophy X-linked
MHC	Myosin heavy chain
MC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor kappa B
NK	Natural killer cell
NO	Nitric oxide
PCR	Polymerase Chain Reaction

PMN	Polymorphonuclear leukocyts
RANTES	Regulated upon activation normal T cell Express sequence
ROS	Reactive oxygen species
SIRS	Sytemic inflammatory response syndrom
SR	Sarcoplasmic reticulum
STAT	Signal transducer and activator of transcription
sTNF	Soluble tumor necrosis factor
Th	T helper cell
TLR	Toll like receptor
TNF	Tumor necrosis factor

Abstract

The primary muscle of respiration is the diaphragm. Diaphragm muscle dysfunction and ventilatory pump failure are well documented phenomena in animal models of sepsis. However, the primary cellular mechanisms underlying respiratory muscle dysfunction in sepsis are poorly understood. In addition, most investigations of respiratory muscle dysfunction in sepsis have been performed in models involving high doses of bacterial endotoxin and these investigations have been criticized on the basis of questionable relevance to human sepsis. Therefore, the objective in the first study of this thesis was to study respiratory muscle dysfunction in a more clinically relevant animal model, namely, the *Pseudomonas aeruginosa* pulmonary infection model. Remote inflammatory processes in different diseases, such as cancer, arthritis, sepsis, and cystic fibrosis are known to contribute to muscle wasting and weakness through more widespread systemic effects. In keeping with the above notion, we hypothesized that sustained *P. aeruginosa* lung infection would cause diaphragmatic and limb muscle weakness. In this thesis, we demonstrate for the first time that persistent pulmonary infection with *P. aeruginosa* induces significant dose- and time-dependent contractile dysfunction of the diaphragm. By comparison, prototypical slow- and fast-twitch hindlimb muscles were not influenced by pulmonary *P. aeruginosa* infection.

Because skeletal muscles can express a variety of immune modulating molecules such as cytokines, chemokines, adhesion molecules, and major histocompatibility molecules, the objective of the second study in this thesis was to study the possible role of pro-inflammatory cytokines in diaphragm muscle dysfunction in our animal model. Our results indicate for the first time that intra-diaphragmatic pro-inflammatory cytokine gene expression (TNF-α, IL-1α, IL-1β, IL-6, and IL-18) is highly up-regulated in infected animals and the magnitude of such upregulation is dependent upon the dose of P. *aeruginosa* lung infection. Parallel to the absence of muscle contractile dysfunction in hindlimb muscle under the same conditions, *P. aeruginosa* infection did not alter the levels of pro-inflammatory gene expression within the hindlimb muscle. To further address the involvement of muscle-derived pro-inflammatory cytokines in diaphragmatic contractile dysfunction, we have employed recombinant adenovirus (Ad) as a vehicle for systemic delivery of the anti-inflammatory cytokine IL-10, in order to shift the balance between pro- and anti-inflammatory cytokines within

the diaphragm toward a more anti-inflammatory profile. We report here that systemic delivery of Ad-IL-10 suppresses pro-inflammatory gene expression and improves force generating capacity of the diaphragm in *P. aeruginosa* infected animals. This finding emphasizes the role of anti-inflammatory cytokines as beneficial immune modulators in respiratory muscle failure caused by pro-inflammatory cytokines.

P. aeruginosa lung infection is a major cause of morbidity and mortality among cystic fibrosis (CF) patients and many patients with CF have weak peripheral and respiratory muscles. Although the role of pro-inflammatory cytokines has been extensively studied within the lungs of CF patients, the involvement of these cytokines in skeletal muscle dysfunction in animal models of CF or in human CF patients has not been studied. Therefore, in the third study of this thesis we have used mice sharing the same genetic defect as CF patients (*Cftr* knockout mice), in combination with our model of *P. aeruginosa* lung infection, to address several fundamental questions related to muscle function in CF. Our first objective in this portion of the thesis was to determine if diaphragmatic skeletal muscle cells express the CFTR mRNA. Our second objective was to ascertain whether intrinsic differences between CF and wild-type muscle cells could be detected *in vitro*, which might differentially affect the regulation of pro-inflammatory mediators in the setting of infection/inflammation. Our third objective was to evaluate possible differences in the ability of respiratory muscles to generate force prior to and after *P. aeruginosa* lung infection in *Cftr* knockout mice, as compared to their wild-type littermates. Finally, we aimed to determine if the absence of CFTR expression would predispose to muscle dysfunction triggered by up-regulation of intra-diaphragmatic pro-inflammatory gene expression. Our major results indicate that: First, *in vitro* stimulation with pro-inflammatory cytokines (TNF-α, IL-1α, and IFN-γ) and LPS (extracted from *Pseudomonas aeruginosa*) triggered increased expression of pro-inflammatory mediators (iNOS, RANTES, MIP-1α, MIP-1β, MIP-2 and KC) in both *Cftr*$^{-/-}$ and wild-type diaphragmatic myotubes, but the magnitude of cytokine/chemokine upregulation was significantly greater in CF than in wild-type diaphragm muscle cells. Second, CF mice are more vulnerable to diaphragm contractile dysfunction and increased intra-diaphragmatic pro-inflammatory gene expression (TNF-α, IL-1α,

IL-β, IL-18, RANTES, MIP-1α, and MIP-2) after pulmonary *P. aeruginosa* infection in comparison with wild-type mice.

In the final study of this thesis, we sought to test the hypothesis that increased diaphragm muscle activation would lead to increased production of intra-diaphragmatic cytokine expression, since this could possibly explain the greater susceptibility of the diaphragm to express pro-inflammatory cytokines in response to pulmonary *P. aeruginosa* infection as compared with the hindlimb muscle. To test this hypothesis, we subjected rats to inspiratory resistive loading (IRL), corresponding to 45-50% of the maximum inspiratory pressure, and described that mRNA levels of IL-1β, IL-6, and to a lesser extent, IL-4, IL-10, TNF-α, and IFN-γ were all significantly increased in a time-dependent fashion in the diaphragm but not hindlimb muscle (gastrocnemius) of loaded animals. I n addition, elevated protein levels of IL-1β and IL-6 in response to loading were confirmed with immunoblotting and immunostaining. We also detected significant IL-6 protein to be localized inside diaphragmatic muscle fibers of loaded animals. We conclude that increased diaphragm muscle activity during resistive loading induces upregulation of pro-inflammatory cytokine gene expression in the diaphragm, which could also provide an explanation for the greater cytokine expression observed in the diaphragms of animals with *P. aeruginosa* lung infection.

RÉSUMÉ

Le diaphragme est le principal muscle respiratoire. Lors du sepsis, la dysfonction diaphragmatique et sa principale conséquence, la défaillance ventilatoire, ont été largement documentées dans les modèles animaux. Toutefois, les mécanismes cellulaires sous-jacents à cette dysfonction des muscles respiratoires associée au sepsis sont peu connus. De plus, la plupart des études consacrées à ce sujet ont été réalisées sur des modèles reposant sur l'administration de fortes doses d'endotoxines. Ces modèles ont été largement critiqués car fort éloignés de la réalité du sepsis dans l'espèce humaine. Dès lors, la première partie de cette thèse a eu pour but d'étudier la dysfonction des muscles respiratoires dans un modèle de sepsis cliniquement plus pertinent : la pneumonie infectieuse à *Pseudomonas aeruginosa*. Il est en effet largement démontré que lors de pathologies chroniques telles que certaines néoplasies, arthrites, infections, ainsi que dans la mucoviscidose, un processus inflammatoire distant peut contribuer à la genèse d'une amyotrophie et d'un déficit moteur. Nous avons donc formulé l'hypothèse qu'une pneumopathie subaiguë à *P. Aeruginosa* pourrait entraîner un déficit moteur atteignant le diaphragme tout comme les muscles des membres. Dans la présente thèse, nous montrons pour la première fois qu'une pneumopathie chronique à *P. Aeruginosa* induit une dysfonction diaphragmatique dose et temps dépendante. En revanche, cette même infection ne s'accompagne d'aucune dysfonction des muscles des membres.

Parce que les muscles squelettiques peuvent exprimer nombre de molécules immunomodulatrices telles que des cytokines, chémokines, molécules d'adhésion et molécules du complexe majeur d'histocompatibilité, l'objectif des cette seconde partie de la présente thèse était d'étudier le possible rôle de certaines cytokines pro inflammatoires dans la genèse de la dysfonction musculaire diaphragmatique observée dans notre model animal de pneumonie infectieuse. Nos résultats sont les premiers à montrer que l'expression intra-diaphragmatique de cytokines pro inflammatoires (TNF-α, IL-6, IL-18) est largement accrue chez les animaux infectés et que l'amplitude de cette surexpression dépend de la dose infectante de *P. Aeruginosa*. Tout comme elle ne s'accompagnait d'aucune dysfonction contractile des muscles des membres, l'infection pulmonaire à *P. Aeruginosa* ne s'accompagnait d'aucune modification du niveau d'expression des cytokines pro inflammatoires au sein de ces mêmes muscles. De façon à évaluer l'implication de ces cytokines pro inflammatoires dans la genèse de la dysfonction contractile du diaphragme, nous avons utilisé un adénovirus recombinant (Ad) comme véhicule permettant d'acheminer

la cytokine anti-inflammatoire IL-10 par voie systémique. Ceci avait pour but de déplacer, dans le diaphragme, l'équilibre entre cytokines pro et anti-inflammatoires vers un profil anti-inflammatoire plus marqué. Nous montrons ici que l'apport systémique d'IL-10 supprime l'expression des gènes pro inflammatoires et accroît, chez les animaux infectés par *P. Aeruginosa*, la capacité du diaphragme à produire une force. Ces résultats soulignent le rôle immunomodulateur bénéfique des cytokines anti-inflammatoires dans la défaillance des muscles respiratoires que produisent les cytokines pro inflammatoires.

La pneumopathie infectieuse à *P. Aeruginosa* est une cause majeure de morbidité comme de mortalité chez les patients atteints de mucoviscidose. Par ailleurs, beaucoup d'entre eux présentent un déficit moteur respiratoire et périphérique de nature musculaire. Bien que le rôle des cytokines pro inflammatoires ait été largement étudié dans les poumons de ces patients, l'implications de ces cytokines dans la dysfonction des muscles squelettiques n'a été étudié ni dans les modèles animaux de mucoviscidose, ni chez les patients atteints de cette maladie. Dès lors, dans la troisième partie de cette thèse, nous avons appliqué notre modèle de pneumopathie infectieuse à *P. Aeruginosa* à des souris porteuses de la même anomalie génétique que celle des patients atteint de mucoviscidose (délétion par recombinaison homologue [$CFTR^{-/-}$]), et ce dans le but de répondre à plusieurs questions fondamentales concernant la fonction musculaire des patients atteints de mucoviscidose. Dans cette troisième partie de la présente thèse, notre premier objectif était de savoir si les cellules musculaires diaphragmatiques exprimaient les ARN messagers codant pour le CFTR. Notre second objectif était de s'assurer qu'il existait des différences intrinsèques entre les cellules musculaires provenant de souris $CFTR^{-/-}$ et celles provenant de souris de phénotype sauvage, lesquelles pourraient s'accompagner de différences entre les profils de régulation des médiateurs pro inflammatoires dans le contexte inflammatoire/infectieux. Notre troisième objectif était de comparer l'impact d'une infection pulmonaire à *P. Aeruginosa* chez les souris $CFTR^{-/-}$ et chez les souris de phénotype sauvage. Enfin, nous avons tenté de savoir si l'absence d'expression du CFTR pouvait prédisposer à la dysfonction musculaire qu'induit la surexpression diaphragmatique des gènes codant pour certains médiateurs pro inflammatoires. Nos principaux résultats montrent premièrement que la stimulation in vitro de myotubes diaphragmatiques par une association de cytokines pro inflammatoires (TNF-α, IL-1α et IFN-γ) et de LPS (extrait de *Pseudomonas aeruginosa*) entraîne la surexpression de différents médiateurs pro inflammatoires (iNOS, RANTES, MIP-1α, MIP-1β et KC), laquelle s'avère

toutefois plus marquée dans les mytotubes provenant de souris *CFTR*$^{-/-}$ que dans ceux provenant de souris de phénotype sauvage. Nos résultats montrent deuxièmement que, lors d'une pneumonie infectieuse à *P. aeruginosa*, les souris atteintes de mucoviscidose sont plus vulnérables à la dysfonction contractile du diaphragme et à la surexpression diaphragmatique des gènes pro inflammatoires que les souris de phénotype sauvage.

Dans la dernière partie de cette thèse, nous avons formulé l'hypothèse qu'une augmentation de l'activité du diaphragme entraînerait une augmentation de la production de cytokines par ce même muscle. Un tel phénomène pourrait expliquer pourquoi, en réponse à une infection pulmonaire par *P. aeruginosa*, le diaphragme exprime de façon plus marquée nombre de cytokines pro inflammatoires, et ce comparé aux muscles des membres. De façon à tester cette hypothèse, nous avons soumis des rats à des charges résistives estimées à 45-50 % de la pression inspiratoire maximale. Nos résultats montraient que les niveaux d'expression des ARN codant pour IL-1β, IL-6 et dans une moindre mesure pour IL-4, IL-10, TNF-α et IFN-γ étaient augmentés de façon temps dépendante dans le diaphragme mais n'étaient pas modifiés dans les muscles des membres (gastrocnemius). De plus, au niveau protéique, la concentration en IL-1β et IL-6 était confirmée par immunoblott et immuno marquage. Nous avons par ailleurs constaté que les molécules d'IL-6 étaient localisées à l'intérieur des fibres diaphragmatiques. Nous concluons donc qu'une respiration au travers de charges resistives induit la surexpression de nombreuses cytokines pro inflammatoires au sein du diaphragme, laquelle pourrait contribuer à expliquer pourquoi le niveau des d'expression des cytokines dans le diaphragme est élevé chez les animaux infectés par *P. aeruginosa*.

CHAPTER 1

INTRODUCTION & REVIEW OF LITERATURE

I. INTRODUCTION

Sepsis affects 750,000 people in US annually, and more than 210,000 of them die (1). Sepsis is an infection-induced syndrome followed by systemic inflammation, fever/hypothermia, leukocytosis/leukopenia, tachycardia and tachypnea. Septic shock is defined as sepsis induced hypotension, hypoperfusion, and organ dysfunction (2).

It has been well established that sepsis is associated with significantly impaired diaphragm contractility, playing an important role in the high incidence of respiratory insufficiency in sepsis (3). There are several sites in the diaphragm force generating machinery that can reflect impaired muscle contractility, including sarcolemmal function, excitation-contraction coupling, and depression of contractile myofibrillar proteins (4). Several mechanisms have been postulated to account for ventilatory muscle contractile dysfunction, such as imbalance between energy utilization and supply (5) as well as direct cytotoxic effects of a number of pro-inflammatory mediators (cytokines, reactive oxygen species, reactive nitrogen species) associated with the systemic inflammatory response in sepsis (3). In addition, both cytokines and free radicals have been implicated in either myofiber injury or myofibrillar protein loss (6) (7).

To date the vast majority of studies aimed at investigating the effects of sepsis on respiratory muscle function have employed lipopolysaccharide (LPS) to produce a state of acute endotoxemia. However, there are several disadvantages with endotoxin animal models. First, a higher than physiologic dose of LPS is typically used to induce diaphragmatic dysfunction. Second, there are other virulence factors within the Gram-negative bacteria other than LPS, and absence of such an interaction between bacteria and host may not manifest a full range of immune responses. Third,

administration of LPS only reproduces an acute phase of sepsis syndrome.

The global objective of my thesis has been to study the acute and chronic effects of Gram-negative bacteria (*Pseudomonas aeruginosa*) lung infection on both respiratory and peripheral limb muscles. We also wished to determine the role of pro-inflammatory mediators in this more clinically oriented animal model. Finally, we also sought to address the effects of *Pseudomonas aeruginosa* lung infection on diaphragmatic contractile function and pro-inflammatory gene expression, in an animal model of cystic fibrosis (CF).

II. ANIMAL MODELS OF SEPSIS

Forty years of clinical trials using anti-inflammatory reagents in the treatment of sepsis have failed to show a therapeutic benefit (8). There are many factors which could account for this lack of benefit, including the complexity of the physiological factors, the timing of therapeutic intervention, heterogeneity of the patient population, genetic polymorphisms among the patients, and lack of proper animal models in sepsis.

A) Lipopolysaccharide (LPS): LPS is present in the bacterial outer membrane and is a hallmark of Gram-negative bacteria. LPS is composed of three covalently linked segments; (1) Lipid A, firmly embedded in the membrane; (2) Core polysaccharide, located at the membrane surface; and (3) O antigens, which are polysaccharides that extend like whiskers from the membrane surface to the surrounding environment. The lipid A portion of LPS is highly conserved between strains, and mediates the binding of LPS to its receptor on bacteria to trigger biological effects of LPS (9). LPS-binding protein (LBP) is produced constitutively by liver and binds to LPS (10), which facilitates

its binding to either membrane associated CD14 or to soluble CD14, and Toll-like receptor 4 (TLR-4) and MD-2 (11). Soluble CD14 which exists in plasma helps to convey LPS signaling in cells (endothelial and epithelial cells) lacking membrane-bound CD14. MD-2 is a secreted glycoprotein, which acts as an extracellular adaptor protein in the activation of TLR-4 by LPS and is essential for LPS signaling (12). Activation of TLR-4 results in LPS signaling events mainly involve the activation of nuclear factor-κB (NF-κB) (13). TLR, a protein with a single transmembrane domain was identified by Anderson and Nusslein-Volhard (14) in *Drosophila* and was required for the response to fungal infection in *Drosophila* (15). TLRs (ten human representatives are now known to exist) function in pattern recognition and restriction of innate immune responses to different microbes. For instance, TLR-2 is a major receptor for recognition of Gram-positive bacteria (16), TLR-5 is required for the recognition of flagellin (a protein component of Gram-positive and Gram-negative organisms) (17) and TLR-9 binds to bacterial unmethylated DNA fragments (specifically, oligomers containing CpG dinucleotides) (18). Therefore, TLRs function as primary sensors of the innate immune responses to bacterial, fungi, and protozoa. The vast majority of studies to date aimed at investigating sepsis have employed massive doses of LPS, which do not precisely mimic the physiological changes in sepsis. Importantly, the interaction between whole bacteria and host immune response is absent in LPS models.

B) Cecal ligation and puncture (CLP): CLP is an animal model of sepsis induced by ligation of the cecum distal to the ileocecal valve to conserve bowel continuity, and puncture of the antimesentric surface of cecum to create a state of ongoing bacterial peritonitis. In contrast to the LPS model, CLP may better reflect the predominant physiological changes observed in sepsis (19). The classical example of this discrepancy between LPS models and CLP or septic humans is the differing responses to anti-TNF-α therapy. Following LPS injection in humans (20) or mice (21),

the levels of TNF-α are increased in serum. Anti-TNF-α antibody protected mice from lethal doses of LPS (22). In contrast, anti-TNF-α antibody did not protect CLP mice from mortality, and actually increased morbidity (23). Comparison of murine LPS and CLP models revealed that the serum levels of TNF- α in LPS-treated mice were 200 times greater than in CLP mice (21), and the levels of circulating LPS were very low in CLP mice (23). Similar to the findings in CLP mice, anti-TNF-α antibody failed to protect septic humans from mortality (24). Therefore, the fundamental incorrect assumption that LPS replicates the inflammatory response to whole bacterial infection may have resulted in the failure of many therapeutic interventions in septic patients.

C) *Pseudomonas aeruginosa* model of bacterial pneumonia: Although Gram-negative bacteria account for a relatively small proportion of community-acquired pneumonias, they are the major cause of hospital-acquired pneumonias. *Pseudomonas aeruginosa* is a leading cause of such Gram-negative pneumonia (25). *Pseudomonas* is an opportunistic pathogen and usually becomes pathogenic in immune-compromised hosts caused by chemotherapy, mechanical ventilation, acquired immune deficiency syndrom (AIDS), severe burns, or chronic lung diseases such as cystic fibrosis (CF) (26). The majority of pseudomonal pneumonias occurring in immune-compromised hosts lead to an acute course, whereas a more chronic type of infection is seen in patients with CF.

Pseudomonas aeruginosa is motile, aerobic, and rod-shaped (approximately 0.6 x 2 μm) organism, which is widely present in moist environments. *P. aeruginosa* is frequently found in small numbers in normal intestinal flora, skin, and upper airway (27). Extracellular bacteria cause disease by two principal mechanisms. First, they induce inflammation, which results in tissue destruction at the site of infection. Second, many of these bacteria produce toxins, which have diverse pathological effects. Clinical isolates of *Pseudomonas aeruginosa* secrete exotoxin A which is a

potent protein synthesis inhibitor and is 10,000 times more toxic to experimental animals than *P. aeruginosa* LPS (27). The LPS of *P. aeruginosa* is structurally similar to other Gram-negative bacteria but differs in some chemical groupings, which determines its multiple immunotypes. Two major and distinct cell surface polysaccharides are expressed by different strains of *P. aeruginosa*. One is located on the O polysaccharide side chain of the outer membrane LPS, and the other is mucoid exopolysaccharide (MEP)/alginate, which is involved in the evasion of host defense (28). *P. aeruginosa* also is an adaptable microorganism that has the ability to respond to a variety of environmental changes. The nonmucoid strains of *P. aeruginosa* are frequently responsible for opportunistic infections, whereas mucoid strains are often recovered from CF patients (29). The mucoid strains demonstrate properties such as: 1) Decreased motility by loss of pili and flagella which provides a better opportunity to adhere to the respiratory tract (30) and resist phagocytosis by macrophages (31); 2) High intrinsic resistance to antimicrobial drugs, which could be associated with the low outer membrane permeability to antimicrobial agents and production of antibiotic modifying enzymes (32); 3) Properties of mucoid MEP/alginate which are anti-phagocytic (33), immuno-suppressive (34), and protective against reactive oxygen species (35). Moreover, MEP/alginate permits the bacteria to grow as a biofilm, which further protects the embedded bacteria from phagocytosis and antibiotic agents (36).

In general, intratracheal installation of *P. aeruginosa* in immunocompetent rodents results in transient infection, which generally resolves within 24 hr at non lethal doses (37). In order to establish a chronic rodent model of lung infection, *P. aeruginosa* must be trapped in the lungs by using different immobilizing agents, such as seaweed alginate (38), agarose (39), and agar (40). Cash et al., (40) established the first animal model of chronic lung infection with *P. aeruginosa* embedded in agar beads, which resulted in chronic lung infection up to 35 days. Similarly, a mouse model of

chronic *P. aeruginosa* lung infection was developed in outbred mice (Swiss CD-1) using agarose beads (39) and inbred mice by agar (41). Studies in inbred mice following lung infection with mucoid *P. aeruginosa* enmeshed with agar beads have shown that BALB/c mice were the most resistant (<2% mortality) strain, DBA/2 mice were the most susceptible strains (40% mortality), and both C57BL/6 and A/J mice were intermediate with a small percentage of mortality (42). Moreover, comparison of BALB/c to C57BL/6 mice revealed that the susceptibility of C57BL/6 was associated with predominant lung neutrophilic infiltration with lung damage (43), reduced levels of TNF-α expression and secretion (44), and decreased proliferation of T-cells upon stimulation *in vitro* (41). In addition, T-cell clones from lymph nodes of C57BL/6 immunized mice with heat-killed *P. aeruginosa* were Th-1 type that released IFN-γ without IL-4 and IL-10 (45).

In summary, this model offers several advantages; (1) it is probably more clinically relevant than high dose administration of LPS; (2) it involves inoculation of additional virulence factors other than LPS (eg., *P. aeruginosa* exoenzyme S which is a potent inducer of cytokine expression (46) such that the full range of microbes versus host interaction can be expressed; (3) it produces a predominantly neutrophilic inflammatory infiltrate within the lungs and associated tissue damage, which are both similar to changes observed in the infected human lung (47); and (4) by virtue of its more sustained nature, the chronic lung infection model offers the ability to study responses at different stages of the infection process.

III. PULMONARY HOST DEFENSE AGAINST GRAM-NEGATIVE BACTERIAL INFECTION

The lung represents the largest epithelial surface area of the body in contact with the external

environment. Epithelia provide a physical barrier between the internal milieu and the external environment. As normal respiration occurs, the upper and lower airways are repeatedly exposed to a multitude of airborne particles and microorganisms. Therefore, an elaborate system of defense mechanisms is in place to maintain the sterility of the lung. The surface epithelial cells are more than a simple physical barrier to infections, since they also produce chemical substances that are microbicidal or inhibit microbial growth. The innate immune response involves alveolar macrophages (AM), neutrophils, and natural killer (NK) cells, while the acquired immune response associate with T and B cells (48). Infections occur when pathogens cross the epithelial barrier, upon which they are immediately recognized by AM. First, AM attempt to trap, engulf, and destroy the pathogen and signal for polymorphonuclear leukocyte (PMN) recruitment. Second, the interaction between AM and the pathogen results in release of cytokines. Finally, AM can act as a professional antigen presenting cell by presenting microorganism antigens on the major histocompatibility complex II molecules, and by delivering a costimulatory signal through the expression of B7 molecules. Therefore macrophages are also important in induction of the adaptive immune response (49).

A) Innate immune responses against Gram-negative bacterial infection in the lung: Innate immunity focused on internal and exogenous signals has the ability to continuously discriminate between harmful and innocuous signals and to generate an immune response only when required. Hence when bacteria break the line of defense consisting of lung structural and mucocilliary barriers, the innate immunity plays a major role in controlling bacterial infection. The resident AM and polymorphonuclear leukocyte/neutrophil (PMN) are the essential cellular components of innate immunity and participate in the acute tissue inflammatory response against acute bacterial infection. The AM, which represents 85% of the cells retrieved by bronchoalveolar lavage (BAL), is the

primary phagocytic cell that responds to the daily challenge of bacteria that enter the terminal airways, and orchestrates the inflammatory response of the lung in the event that the microbial challenge is either too large or too virulent to be contained by AM alone. AM have multiple functions, including: i) Initiation of inflammation by the release of pro-inflammatory cytokines (IL-1, TNF-α)/chemokines (IL-8, MIP-1, RANTES), growth factors (GM-CSF, G-CSF), or expression of adhesion molecules (ICAM-1) on endothelial cells or epithelial cells; ii) The control of inflammation by the release of IL-1 receptor antagonist, TNF-soluble receptor (50) or IL-10 production which suppresses macrophage pro-inflammatory gene expression (51); iii) Macrophages are also involved in lung remodeling and repair by release of metalloelastases, collagenase, metalloproteases (MMP-1, MMP-9) and their inhibitors TIMPs (52). AMs are also the primary phagocytic cells in the uninflammed lower airway. Interestingly, animal experimental results during lung infection with *P. aeruginosa* suggest that AMs do not play an important bactericidal role, but instead play a major role in regulating inflammatory responses through cytokine/chemokine expression and recruitment of PMNs to the infected lower respiratory tract (53) (54). AMs were also found to be incompetent to phagocytose *P. aeruginosa, in vitro* (55).

PMNs normally represents less than 2% of the cells in BAL and their half life is about 8 h. PMNs have been shown to be essential in early phase of many bacterial infection such as *P. aeruginosa*. Indeed, granulocytopenic mice did not survive *P. aeruginosa* lung infection due to inadequate PMN recruitment (56). Studies in inbred strains of mice (BALB/c) resistant to *P. aeruginosa* also revealed that their resistant with phenotype was mainly dependent on both the recruitment of PMNs and their rapid activation to kill bacteria (57). PMN infiltration is a result of recruitment of blood-borne PMNs. The maintenance of AM is partially dependent on the lung differentiation of blood-derived monocytes. The number of peripheral PMNs and monocytes is

maintained through hematopoiesis in the bone marrow (57).

B) Acquired immune responses against Gram-negative bacterial infection in the lung: Acquired immune defense is the antigen-specific mechanism of host defense and includes cell-mediated and antibody-mediated immunity. Gram-negative bacilli, especially *P. aeruginosa*, is the major cause of chronic lung infection in CF patients, which is involved in the decline in respiratory function and accounts for much of the morbidity and mortality in these patients (58). Due to the fact that CF patients are more prone to chronic lung infection, the role of the cell-mediated and the humoral immune responses has been a subject of considerable interest. Hoiby et al (59), found that cell-mediated immunity in chronically infected CF patients did not significantly differ from normal subjects, whereas others (60) found that the cell-mediated immunity was depressed during an exacerbation of infection. Moreover, evaluation of T-cell function in CF patients demonstrated decreased T cell proliferation to *P. aeruginosa* compared to normal individuals, *in vitro* (61). Recently, it has been shown that the percentage of peripheral T helper (Th) cells is decreased in CF patients (62). Indeed, the function of these cells in terms of proliferation and help for antibody production by B-cells was declined (63).

In the athymic rat, Johansen and coworkers found that T-cells do not play a major role in the natural course of the acute and chronic lung infection with *P. aeruginosa* (59), while studies by Markham and colleagues (64) in mice revealed that T cell-mediated immunity was protective against to *P. aeruginosa* even in the absence of antibody response. The exact reason for the discrepancy between these studies is unclear, but methodological differences such as animal species, strain of *P. aeruginosa*, and the dose of infection might contribute. Moreover, lung T cell responses in two inbred mouse strains, namely BALB/c and C57BL/6, which are respectively resistant and susceptible

to chronic lung infection with *P. aeruginosa*, indicated that T cells from resistant mice (BALB/c) were more proliferative to heat killed *P. aeruginosa* than cells from susceptible mice (C57BL/6), *in vitro* (41). They also showed that resistant mice had high *Pseudomonas*-specific delayed-type hypersensitivity (DTH) and low antibody responses, while susceptible mice responded with low DTH and high antibody (41). Similar to this in CF patients, chronic infection is characterized by high antibody responses against a wide range of *Pseudomonas* antigens (65) (66). It has been speculated that the antibody response against *Pseudomonas* in CF might contribute to pulmonary deterioration by formation of immune complexes that subsequently stimulate chronic inflammation (65). These data all suggest that there might be a dysregulation of T helper cell ($CD4^+$) responses in *Pseudomonas* susceptible hosts. Kondratieva et al (45) tested this hypothesis in *Pseudomonas*-susceptible and resistant mice and demonstrated that a lung T cell clone from susceptible mice produced IFN-γ but not IL-4 and IL-10, whereas T cell clones from resistant mice were produced IL-4 and IL-10 but not IFN-γ. This result is also in accordance with CF patients with *Pseudomonas* infection, who were shown to have significantly lower IL-10 in BAL fluid than healthy subjects, while there were no differences between non-infected CF patients and healthy individuals (67). Moreover, T cell clones from CF patients also showed reduction of IL-10 production, *in vitro* (68).

In summary, the majority of the evidence from both human and animal studies suggests an important role of T cells in development of immunity to *Pseudomonas aeruginosa* and ultimately the profile of cytokine production could contribute significantly to the balance between control of infection versus tissue damage in the lung.

C) Importance of cytokines: Cytokines are the most important soluble polypeptide mediators that regulate and coordinate the host response against infectious agents, particularly at local tissue sites.

Cytokines send intracellular signals by binding to specific cell-surface receptors. Most cytokines can be released by multiple cell types except a few that are restricted primarily to lymphocytes. In host responses elicited by immunogenic antigens, antigen-specific T lymphocytes are the primary source of the selective cytokines, which depend on the nature of antigen and the genetic background of host. These cytokines may be grouped into Th1 (eg., IL-2 and IFN-γ) or Th2 (eg., IL-4 and IL-10) cytokines, which dictate the different pattern of tissue immune-inflammatory responses. In contrast, in inflammatory responses elicited by non-immune stressors, many cytokines may be released from multiple cellular sources other than lymphocytes (69). Cytokines that are involved in host defense against pulmonary bacterial infections may be divided into pro-inflammatory, anti-inflammatory and chemotactic cytokines (chemokines). Cytokines are the major regulatory elements in inflammatory responses and there is a dynamic relationship between pro-and anti-inflammatory cytokines that collectively determines the ability of the host to cope with infections.

i) Th1/Th2 dichotomy: $CD4^+$ T cells are the central regulatory cells of the immune system and their role became more evident when studies showed they are not a homogenous class of cells. The paradigm of $CD4^+$ T cells has arisen from the study of long term clones of murine $CD4^+$ T cells. Mosmann and colleagues (70) showed that $CD4^+$ T cell clones could be designated as either being Th1 type (which produced IL-2, and IFN-γ) or Th2 type (produced IL-4 and IL-10). Th1 clones are well known to enhance cellular immunity by augmentation of macrophage microbicidal activity, while Th2 clones help B cells develop into antibody producing cells. Classical studies obtained from *Leishmania major* infection in mice indicated the Th1/Th2 phenotypic distinction and its role in pathophysiology of the disease. Liew et al (71) showed that immune responses dominated by the production of IFN-γ by $CD4^+$ T cells would be protective against *L. major*, while responses

dominated by IL-4 production offer no protection. Similar to this, study in two different strains of mice (BALB/c and C57BL/6) also confirmed the important role of Th1 responses to *L. major* infection. C57BL/6 mice have been identified as *L. major* resistant mice and $CD4^+$ T cell clones prepared from the cells of infected C57Bl/6 mice produced IFN-γ and failed to produce IL-4 (72). In contrast, BALB/c mice were susceptible to *L. major* infection and $CD4^+$ T cells prepared from infected mice were found to produce IL-4. Human leprosy also has revealed a similar pattern: T cells from lepromatous leprosy, which has enormous accumulation of intracellular organisms, produced IL-4, whereas tuberculoid leprosy in which there are fewer organisms, was dominated by T cells that produced IFN-γ (73). In humans with HIV infection, the progression to AIDS is characterized by decreases of IFN-γ production and concomitant increases in IL-4 and IL-10 (74). As mentioned earlier, susceptibility to *Pseudomonas* infection appears to be associated with a Th1 cytokine profile, whereas resistant mouse strains show a Th2 phenotype (45).

ii) Pro-inflammatory cytokines: The response elements of the innate immune system are triggered by the release of pro-inflammatory cytokines, which have three major effects: (1) They induce the production of acute phase proteins by the liver, which can further bind to bacterial surface molecules and activate complement or phagocytosis; (2) They can elevate body temperature, reduce the host's spontaneous activity, and induce behavioral changes, which are thought to be deleterious to the growth of the microorganisms, while enhancing host anti-microbial functions; (3) They induce local inflammation, in which surface properties and permeability of blood vessels are changed, recruiting phagocytes and immune cells to the site of infection. All these mechanisms have an important role in preventing the systemic dissemination of microbial infection during its early phase (75). Interleukin-1 (IL-1) is a potent pro-inflammatory cytokine which is able at low levels to induce a dramatic inflammatory responses. IL-1 has two different forms: 1) IL-1α which is primarily

membrane-bound (76); 2) IL-1β which is secreted and binds to its receptor that have been cleaved from a cell membrane and floating in the plasma or interstitial fluid (77). IL-1 induces fever, acute phase protein synthesis, and increases lymphocyte responses to infection. IL-1 also locally induces the production of adhesion molecules for recruitment of leukocytes to the site of infection (77). Tumor necrosis factor-alpha (TNF-α) is another multifunctional cytokine involved in inflammatory and immune processes which plays a central role in initiating innate immune responses (48). It is primarily released by macrophages and PMNs and augments bactericidal activities of these cells, chemokine release by many cell types, and adhesion molecules on the endothelium (48). TNF-α, similar to IL-1, can elicit clinical symptoms of fever, fatigue, anorexia, and headache (78). It also initiates the synthesis and release of other inflammatory cytokines, such as IL-6 (77). IL-6 is another pleiotropic cytokine, which shows elevation with many inflammatory pathologies including arthritis, congestive heart failure, neoplasms, and infection (77). IL-6 plays a fundamental role in inflammatory process by inducing acute phase proteins (79). During the early phase of inflammation, IL-6 acts as a pro-inflammatory cytokine by activating PMNs, promoting differentiation and maintenance of natural killer cells, and increasing the expression of IL-1 and TNF-α (77). IL-6 also possesses anti-inflammatory properties (see below). IL-12 is a critical cytokine for Th1 immune response due to its effects in mediating IFN-γ production. IL-12 is released primarily by activated macrophages or dendritic cells upon interactions with infectious agents (80). IL-18 is also a potent inducer of IFN-γ and particularly acts in synergy with IL-12 to promote development of T helper 1 (Th1) responses (81). IL-18 also appears to enhance Th2 cytokine production in a mouse model of allergic asthma (82) or during *Trichinella spiralis* infection (83). IFN-γ is a cytokine which is typically involved in Th1 immune responses and it is mainly produced by activated T and NK cells via IL-12. In addition, IFN-γ is a potent macrophage activator and activated macrophages can also release this cytokine (84).

iii) Anti-inflammatory cytokines: Anti-inflammatory cytokines including IL-6 and IL-10 serve to control the level of pro-inflammatory cytokines and counteract their functions to reduce side effects to the host. In the late phase of inflammation, IL-6 displays anti-inflammatory properties by attenuating the production of IL-1 and TNF-α, and induces the synthesis of anti-inflammatory factors including IL-1 receptor antagonist, which antagonizes the effect of both IL-1α and IL-1β, and soluble TNF-α receptor (85).

In previous studies examining the effects of IL-10 in animal models, exogenously supplied IL-10 has been found to be both beneficial and detrimental, depending on the nature of disease involved (infectious vs noninfectious origin), the extend of inflammation generated, and dose and route of IL-10 administration. For example, in animal models of endotoxemia, IL-10 can inhibit the expression of pro-inflammatory cytokines such as TNF-α and IL-6 in the lung and serum (86), as well as reduce mortality (87), whereas conflicting results were obtained in the cecal ligation and perforation model of polymicrobial sepsis (88). IL-10 inhibits multiple pathways involved in inflammation. The anti-inflammatory effects of IL-10 include (but are not limited to) inhibition of Th1 cytokine production by T cells, deactivation of monocyte/macrophage pro-inflammatory cytokine synthesis, and interference with cell surface presentation of major histocompatibility complex class II molecules, B7 accessory molecules, and the LPS signaling molecule CD14 (89). IL-10 suppresses the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IL-18, and TNF-α) by indirectly inhibiting the transcription factor nuclear factor (NF)-κB, which controls the expression of many pro-inflammatory genes (90) or promoting degradation of mRNA for the pro-inflammatory cytokines (91).

IV) RESPIRATORY MUSCLE FUNCTION IN SEPSIS

A) General aspects of respiratory muscle function: The respiratory system can be divided into two parts: the lung which, is a gas exchanging organ, keeps arterial blood gases within acceptable limits, and the ventilatory pump, which rhythmically moves air in and out of the lung through expansion/deflation of the chest wall. Displacement of the chest wall is achieved by several skeletal muscle groups (92).

The most important muscle of inspiration is the diaphragm. This consists of a thin, dome-shaped sheet of muscle that is inserted into the lower ribs (costal portion) as well as lumber vertebrae (crural portion). It is supplied by the phrenic nerves from cervical segments (C3-C5) of the spinal cord. When it contracts the abdominal contents are forced downward and forward, and the vertical dimension of the chest cavity increases. Moreover, the rib margins are lifted and moved out, causing an increase in transverse diameter of the thorax. In normal tidal breathing, the displacement of the diaphragm is about 1 cm, but can reach up to 10 cm during forced inspiration/expiration (93). There are two major pathways in central nervous system (CNS) that control breathing: corticospinal (voluntary) and bulbospinal (automatic) pathways. Motor fibers of the corticospinal tract originate in the cerebral cortex, whereas bulbospinal tract originate in the respiratory neurons of medulla (94).

There are four main causes for ventilatory pump failure: (1) insufficient activation form the CNS (e.g. anaesthesia, drug overdose, and disease of medulla), which results in inadequate activation of the respiratory muscles; (2) mechanical defects of chest wall which cause impaired mechanical function of the inspiratory muscles; (3) excessive inspiratory load which can fatigue the inspiratory muscles; and (4) diseases which directly reduce the intrinsic contractile strength of the respiratory muscles.

Respiratory failure is a major cause of death in severe septic patients which is traditionally assigned to lung injury (95). However, there is ample evidence suggesting that with sepsis there is also concomitant respiratory muscle insufficiency. Normal contractile function of ventilatory muscle is essential for survival, and respiratory muscle contractile dysfunction during sepsis is a well documented phenomenon. In experimental animals suffering from septic shock after endotoxin (LPS) administration, death occurs due to ventilatory muscle fatigue and failure (96). Several investigators also have demonstrated a severe decrease in the force generating capacity of the diaphragm within hours of LPS administration (97) (98) (4) (6). The mechanisms of LPS-induced diaphragmatic contractile dysfunction include increased energy utilization due to augmented ventilatory demands in concert with decreased muscle perfusion (99), as well as direct cytotoxic effects of various mediators released as part of the systemic inflammatory response (100) (101) (102) (103) (104) (105) (106). There is now evidence that cytokines belonging to the interleukin family and TNF are major elements in the pathophysiology of sepsis. The source of these pro-inflammatory mediators may be: a) organs distant from the diaphragm, such as lung (107); b) infiltrating leukocytes within the diaphragm muscle interstitium (98) (4) (100); c) the diaphragm muscle fibers themselves (6) (97).

B) Immunological properties of skeletal muscle: Although not conventionally considered as an "immune cell", there is evidence for active participation of skeletal muscle cells in the response to inflammatory stimuli. Skeletal muscle cells have the ability to express a large repertoire of immunological relevant molecules. In cultured human muscle cells, pro-inflammatory cytokines induced expression of class I and II major histocompatibility molecules, as well as several adhesion molecules (ICAM-1, LFA-3) involved in antigen presentation (108) (109). Biopsies have revealed the expression of the same molecules on muscle fibers of patients with inflammatory myopathies

(109) (110). Human skeletal muscle cells also demonstrate low level of constitutive expression of IL-1α and IL-6, which is increased upon exposure to pro-inflammatory cytokines (111) (112) (113). Similarly expression of TNF-α by myoblasts is induced by exposure to IL-1α, IL-1β, IL-6 and IFN-γ, as well as TNF-α itself (112) (113). *In vivo*, TNF-α (97) and IL-6 (114) were expressed at both the mRNA level and by immunostaining in diaphragm myofibers in endotoxin and resistive loading of rat models, respectively. Others found up-regulation of iNOS in septic diaphragm myofibers (6). Skeletal muscle cells are also capable of expressing chemokines. Under unstimulated conditions, cultured human myoblasts constitutively express RANTES, MIP-1α, and IL-8 at the transcript level (112) (113). After exposure to pro-inflammatory cytokines (TNF-α, IFN-γ), the mRNA for RANTES, IL-8, and MCP-1 was upregulated, and the corresponding proteins can also be detected at greatly elevated levels in the supernatants of cultured human myoblasts (113). Along these same lines, it was recently reported that RANTES and MCP-1 transcripts were up regulated after stimulation with TNF-α and IFN-γ, in both myoblasts and myotubes derived from a rat skeletal muscle cell line (Reyes-Reyna, S., 2000). Furthermore, MCP-1 protein was found in the supernatant of cultured cells as well as *in vivo* by immunohistochemistry within rat muscle fibers (115).Therefore, if skeletal muscle *per se*, which constitutes 40-45% of the total body mass, are able to express cytokines and chemokines, what would be their functional significance?

C) Functional implications of cytokine effects on skeletal muscle: Up-regulation of cytokine gene expression within the diaphragm muscle might contribute to several local and systemic functions, which could be adaptive or maladaptive.

i) Cytokines and muscle contractile dysfunction: There is considerable evidence that local expression of classical pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IFN-γ, and TNF-α) could

cause muscle weakness, either through contractile dysfunction or loss of muscle mass (116). LPS on the other hand, had no direct effects on the contractility of the dissected rat diaphragm, *in vitro* (117). The idea that pro-inflammatory mediators can directly affect the intrinsic contractile properties of skeletal muscle, independent of any changes in muscle mass, is strongly supported by a number of studies. Wilcox and coworkers (102)initially reported that activated monocyte secretory products, including TNF-α and IL-1, reduced diaphragm force generating capacity, and subsequently provided evidence that high dose TNF-α administration could reproduce the same phenomenon *in vitro* (103). Moreover, systemic administration of TNF-α depressed diaphragmatic contractility *in vivo* (103). The role of TNF-α also was examined in LPS-induced diaphragmatic hypocontractility. Three hours post-LPS administration, the mRNA transcript levels of TNF-α are significantly elevated in the diaphragm, and neutralizing TNF-α antibodies partially ameliorated LPS-induced diaphragmatic hypocontractility, in the rat (105). Tracey, et al. provided a potential mechanism for the force inhibition by showing that incubation of isolated skeletal muscle with TNF-α produced membrane depolarization, which impairs excitation-contraction coupling in muscle fibers (106). TNF-α alters intracellular calcium stores in many different nonmuscle cells (118) and TNF-α induces a negative inotropic effect on cardiac myocytes by reducing sarcoplasmic calcium levels during the systolic contraction sequence (119). However, a recent study by Reid et al. reported that TNF-α did not alter calcium transients in skeletal muscle, despite the skeletal muscle hypocontractility (120). The fact that TNF-α reduced the force production capacity of the muscle without affecting the calcium intracellular transients suggests that the dysfunction must be downstream of calcium signaling, perhaps at the myofilament level. Although the role of TNF-α in skeletal muscle contractile function has been extensively studied, the role of other pro-inflammatory cytokines has not been studied to the same extent.

Thus, it has been suggested that TNF-α effects on muscle may be mediated in large part by secondary generation of reactive oxygen species (ROS) or nitric oxide (NO) derivatives (121). In this regard, TNF-α stimulates generation of both superoxide (122) and NO (123) in skeletal muscle, and several studies have implicated both ROS (100) (97) and NO derivatives (6) (4) (3) in skeletal muscle dysfunction associated with endotoxemia. In addition, TNF-α-induced muscle dysfunction can be partially restored by antioxidants (122) or NO synthase inhibitors (123). Endogenous NO influences several processes involved in muscle contraction. For instance, it has been shown to augment maximal velocity of shortening during isotonic contractions of normal muscles (124). In contrast, several investigators have reported that NOS inhibition in normal isolated muscles increases sub-maximal isometric force generation, suggesting that the muscle NO pathway inhibits excitation-contraction coupling (125) (126) (3). While the influence of low levels of NO generated by nNOS on muscle contractility is still being evaluated, most investigators agree that muscle contractility and myosine ATPase and sarcoplasmic reticulum (SR) calcium-ATPase activities are significantly attenuated by high NO levels (such as those generated by iNOS) (127) and by peroxynitrite exposure (128). ROS are induced by activation of the cyclooxygenase pathway and stimulation of mitochondria (122). ROS can induce muscle contractile dysfunction by damaging SR (122), regulatory proteins of the sarcolemma, and myofilaments (120). Damage by ROS might blunt the response of myofilaments to calcium activation (120).

ii) Cytokines and skeletal muscle wasting: Muscle mass is determined by both the rate of protein synthesis and the rate of protein degradation, and alteration in the balance between these two events can lead to hypertrophy or atrophy of skeletal muscle. Skeletal muscle atrophy results in decreased protein content, fiber diameter, force, and fatigue resistance. There are different types of conditions that cause muscle atrophy, including aging, starvation, disuse (such as, muscle unloading,

denervation, and immobilization), and disease oriented atrophy (cachexia).

Cachexia is a condition that occurs in several diseases, including AIDS, congestive heart failure, cancer, sepsis, and CF (129) (130). Cachexia is mainly manifested by loss of skeletal muscle mass and adipose tissue which cannot be fully explained by lower food intake (131). However, skeletal muscle wasting is primarily associated with significant decrease in lean body mass (132). Muscle catabolism has two phases, acute and chronic. The acute phase of muscle catabolism might be beneficial to the host, since the release of amino acids from muscle tissues provides an important energy source for other tissues such as liver (133) intestinal mucosa (134), and immune cells (135). The chronic phase of muscle catabolism has significant deleterious effects and causes muscle wasting and weakness. In addition, chronic respiratory muscle cachexia has significant consequences for ventilatory function and pulmonary complications, including pneumonia (136).

Skeletal muscle contains several proteolytic systems responsible for the breakdown of proteins including lysosomal, calcium/calpain-dependent and ATP-ubiquitin-dependent pathways (137). However, skeletal muscle has few lysosomes, which contain several acid proteases (e.g. cathepsins B, D, H, and L) and other hydrolases, and cathepsins were not involved in degradation of myofibrillar proteins (actin and myosin), in a rat model of starvation (138). A recent study suggests that calcium/calpain-dependent pathway may serve as an initial or rate-limiting step in muscle cachexia (139). However, the ATP-ubiquitin-dependent proteolytic pathway is mainly involved in the development of muscle cachexia in most inflammatory diseases, including sepsis (140) (141).

Most proteins are degraded by the ubiquitin-proteasome pathway, after first being tagged by

the small protein cofactor, ubiquitin (142). Protein ubiquitination involves three main enzymes: activating enzyme, E1; ubiquitin conjugating enzyme, E2; and ubiquitin ligase, E3. First, ubiquitin is activated by E1, which requires ATP. Second, activated ubiquitin is transferred to E2. Third, the substrate protein interacts with E3 and this complex binds to E2 resulting in ubiquitination of the substrate protein. This cycle is repeated by forming a chain of five or more ubiquitin molecules linked to the targeted protein (142). The large proteolytic 26S proteasome are recognized and degrades marked ubiquitinated proteins. The 26S proteasome is composed of 19S complex and 20S proteasome. Ubiquitin proteins recognize by 19S complex, which is believed to be involved in release of the ubiquitin, unfolding of the proteins and their transportation to the 20S proteasome, which is the catalytic core of the 26S proteasome. Within the 20S proteasome, proteins are cut into small peptides of 6 to 12 amino acids (143). The mRNA expression of 14 kDa $E2_{14k}$ (144) and E3α (145) were both up-regulated in skeletal muscle of a rat CLP model of sepsis. Recent studies have provided data that other muscle-specific ubiquitin ligases, such as muscle ring finger 1 (MuRF1), and atrogin-1 (MAFbx) were increased in hindlimb muscle during CLP induced sepsis in rats (146). Sepsis in rats also resulted in a 50% increase in 20S proteasome activity in skeletal muscle (147) (148).

Although muscle cachexia is mediated by multiple factors, pro-inflammatory cytokines are believed to be among the most important factors regulating muscle catabolism in chronic inflammatory diseases. Since concentrations of TNF-α are often elevated in the circulation of patients with cancer, sepsis, or CF, its role in muscle cachexia has been extensively studied. TNF-α increases protein catabolism in skeletal muscle both *in vivo* (149) and *in vitro* (150) (151), which is associated with increased activation of the ubiquitin-dependent proteolytic pathway (150) (152). In addition, neutralizing TNF-α by antibody (153) and gene-deficient mice for TNF-α receptor type I

(p50) (154) have demonstrated less muscle wasting and significantly decreased activation of the ubiquitin-proteasome dependent pathway in rodent cancer cachexia models. Other pro-inflammatory cytokines like IL-1α (155), IL-1β (154), IL-6 (156), and IFN-γ (157) can also enhance muscle proteolysis through the activation of ATP-ubiquitin-dependent proteolytic system. Although none of these compounds seem to induce dramatic cachexia-like effects by themselves, in combination they can promote muscle wasting. Recently, Acharyya and coworkers have shown that combination of TNF-α and IFN-γ selectively triggers a reduction in expression of myosin heavy chain (MHC), via a MyoD-mediated block in gene transcription (158).

MyoD is a muscle transcription factor, which is a member of the myogenic regulatory factors (MRFs) involved in the determination and development of muscle, including activation of muscle specific genes (159). Guttridge and coworkers (160) also have shown that TNF-α and IFN-γ significantly downregulate the MyoD expression at transcriptional level in myotubes, which leads to reduced synthesis of MHC (fast-twitch), *in vitro*. MyoD is needed for expression of the MHC IIB in the diaphragm (161) and expression of MHC is dependent on binding of MyoD to the MHC IIB promoter, in fast twitch muscle (162). In addition, the diaphragmatic force generating capacity was significantly decreased in MyoD knockout mice compared to wild type mice (163). In addition to MyoD, other MRFs include myogenin, Myf-5 and MRF 4. For instance, TNF-α has been shown to inhibit cell cycle exit in differentiating mouse skeletal muscle cell line (C2C12) by suppressing accumulation of not only MyoD, but also Myf-5 and myogenin (164).

The NF-κB family of transcription factors play a central role in cytokine signaling. The family includes p65 (RelA), p105/p50, p100/p52, RelB, c-Rel and v-Rel. These proteins associate as homo or heterodimers to form transcriptional regulatory complexes known as NF-κB (165).

Transcriptional activity of NF-κB is determined by the composition of the dimers and their association with inhibitory proteins of the IκB family (166). This is dependent on phosphorylation of IκB, which triggers its ubiquitination and degradation by the proteasome and unmasks the nuclear localization signal to allow NF-κB translocation to the nucleus (166). The most prevalent cytoplasmic NF-κB complex contains p65 and p50 along with IκBα, but other combinations are possible and may result in different DNA binding specificities (167). NF-κB is activated in skeletal muscle during sepsis, *in vivo* (Penner, CG., 2001). *In vitro*, inhibition of NF-κB by a dominant negative form of IκB prevents TNF-α-induced skeletal muscle protein degradation (150).

Since cytokines are involved in skeletal muscle cachexia and they can activate the NF-κB pathway, the next question is what would be the target genes for NF-κB. Li et al (168) have demonstrated that UbcH2/$E2_{20k}$ promoter region contains a functional NF-κB binding site, and that TNF-α increases the expression levels of UbcH2/$E2_{20k}$ via an NF-κB-dependent pathway. This leads to increased ubiquitin-conjugating activity in skeletal muscle. Muscle specific activation of NF-κB in transgenic mice expressing activated IκB kinase (IKKβ) results in excessive muscle wasting via up-regulation of the ubiquitin E3 ligase MuRF1 (169). In addition, inhibition of NF-κB in muscle using transgenic mice that express IκBα suppressor, or using salicylates to inhibit IKKβ, reverses muscle cachexia (169). These studies show that activation of NF-κB in skeletal muscle, can up-regulate the expression of proteins that are involved ubiquitin-proteasome pathway. In addition, NF-κB (p65 subunit) was able to reduce the protein expression of MyoD in culture, and dominant negative expression of IκB maintained both MyoD and MHC expression in muscle cells exposed to TNF-α and IFN-γ.

iv) Chemokines and skeletal muscle: Chemokines are a large family of small molecular weight

cytokines, which have been broadly divided into CXC or α, CC or β, C or γ, and CX3C or δ subgroups based upon the positioning of amino acids relative to the first two conserved cysteine residues (170). The chemokines are diffusible molecules, which exert their biological effects by binding to G protein-coupled receptors, which then trigger the relevant signaling cascades within target cells (171). Chemokines play a major role in cell migration. In general, CXC chemokines act predominantly on neutrophils. CXC chemokines can be further divided into two groups on the basis of the presence or absence of a structural/functional domain which contains a glutamyl-leucyl-arginine (ELR) motif, that is crucial to their neutrophil binding and chemotactic functions (172). Several ELR^+ CXC chemokines exist in humans, including IL-8. Murine ELR^+ CXC have also been identified; these include MIP-2, KC, and LPS-induced CXC chemokine (LIX) (171). CC chemokines (eg., MCP-1, MIP-1α, MIP-1β, and RANTES) are chemotactic for monocytes, eosinophils, basophils, and lymphocytes (173).

Recently, it has been shown that skeletal muscle cells are able to express chemokines. *In vitro*, human myoblasts constitutively express IL-8, MIP-1α and RANTES (CC), (171). Several chemokines, mostly of CC subtypes (RANTES, MIP-1α, MIP-1β, and MCP-1), are expressed in muscle biopsies from myositis patients who suffer from muscle weakness manifested by inflammation, fibrosis and loss of muscle fibers (174) (175). Transgenic mice designed to express MCP-1 by cardiomyocytes induced leukocyte infiltration of the heart, which was associated with significant contractile dysfunction (176). In other animal models, such as a rat model of myasthenia gravis, up-regulation of MCP-1 was associated with increased macrophage as well as T-cell trafficking in affected muscles (177). Degeneration and necrosis of injured muscle initiates a rapid influx of inflammatory cells to the site of injury, in order to phagocytose the necrotic myofibers (178). This is followed by regeneration, that includes activation, proliferation, and differentiation

of satellite cells (precursor of muscle cells) to form myoblasts, which in turn fuse to form multinucleated myotubes (178). The influx of inflammatory cells appears to play an important role in muscle injury/recovery process. For example, interference with macrophage function impairs muscle regeneration after acute injury. Warren and colleagues (179) have also shown that MCP-1 and its receptor (CCR-2) are needed for the optimal regeneration and recovery after muscle injury. Recent study using gene microarray has revealed that injured muscles were associated by an increased CC chemokines expression, particularly MCP-1 (180). RANTES can act as a chemotactic factor for myoblasts (Corti, et al. 2001). LPS-inducible CXC chemokine (LIX) induced in satellite cells after injury activates satellite cells to enter the cell cycle from the normally quiescent state. LIX may also play a role in angiogenesis during tissue repair (181). It has recently reported that a novel chemokine-like cytokine accelerates transcription of several muscle specific genes (myosine light chain, muscle creatine kinase) when overexpressed in a murine satellite cell line, *in vitro* (182).

Chemokines also could play other important roles (eg. homeostatic) than their chemotactic effects on leukocytes. The fact that several chemokines are constitutively produced by muscle cells in the absence of any ongoing inflammatory process (183) (113), suggests homeostatic functions for chemokines in skeletal muscle, which are well described for other tissues. Recently, Demoule et al. (184) have reported that CC chemokines, as well as their receptors, are up-regulated in the diaphragm of the dystrophic (*mdx*) mouse. In addition, Yahiaoui et al. (185) reported that several CC chemokines (MIP-1α, MIP-1β, and MCP-1) induced myoblast proliferation and are able to activate the extracellular signal regulated kinase (ERK), mitogen-activated protein kinase (MAPK) signaling pathway. Taken together, these studies suggest that chemokines might have migratory, proliferative, or differentiative effects on skeletal muscle, which may play an important role in skeletal muscle homeostasis and the response to various insults.

V) ANTI-INFLAMMATORY TREATMENT IN SEPSIS

There are accumulating data suggesting that an equilibrium between the pro- and anti-inflammatory response is important for the final outcome of excessive inflammatory diseases, such as sepsis or CF. Sepsis has a multimodal nature, which is characterized by a systemic inflammatory response syndrome (SIRS) initially, followed by a compensatory anti-inflammatory response syndrome (CARS). SIRS is associated with nonspecific, systemic activation of the innate immune system and a pro-inflammatory cytokine (TNF-α, IL-1, and IL-6) cascade. In contrast, CARS is associated with a pattern of macrophage deactivation, reduced antigen presentation, T-cell anergy, and a shift towards Th2 anti-inflammatory cytokines (IL-10, soluble TNF receptor I and II, and IL-1 receptor antagonist) (186) (187). Patients with SIRS and presumed or known infection are considered to have sepsis. Some patients with infection have only mild sepsis or SIRS and minor organ dysfunction that resolves rapidly. Others exhibit a massive systemic inflammatory reaction and die early from profound shock. A third group have less severe initial course but a later deterioration, multiple organ dysfunction syndrome, and often death. This heterogeneity of patient groups is probably responsible in large part for the failure of anti-inflammatory therapies to show the beneficial effects in human clinical trials of sepsis (188). In addition, the redundancy of pro-inflammatory cytokine effects may prevent inhibition of only one cytokine from having a significant impact.

In animal model of *Pseudomonas aeruginosa* lung infection, mice receiving exogenous recombinant IL-10 (rIL-10) had improved survival (189), and IL-10 knockout mice also revealed higher bacterial load and dysregulated cellular inflammatory response (190) (191). This suggests that tight regulation for the recruitment of an appropriate number as well as subtype of inflammatory cells plays an important role in outcome of infection. It was previously shown, in mouse models of

Pseudomonas areuginosa lung infection, that an exaggerated inflammatory response dominated by PMN correlated with susceptibility to infection, whereas a modest inflammatory response dominated by macrophages correlated with resistance (192). Consistent with this finding, McClellan and coworkers (193) also reported that macrophages played an important role in the control of bacterial growth, PMN influx, and regulation of anti (IL-10) and pro-inflammatory cytokines balance in *Pseudomonas areuginosa* corneal infection. In patients with sepsis, the level of IL-10 concentration correlates both with the magnitude of inflammatory response and the plasma concentration of pro-inflammatory cytokines (194) (195). Several clinical investigations have examined the potential benefits of recombinant human IL-10 (rhIL-10) in human model of endotoxemia. Both Pjkrt and Kumar (196) (197) have demonstrated the attenuation of LPS-induced pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) in serum. Moreover, the concentration of IL-10 was significantly lower in patients who died of acute respiratory distress syndrom (ARDS) (88).

VI) CYSTIC FIBROSIS-A DISEASE WITH CHRONIC LUNG INFECTION AND MUSCLE DYSFUNCTION

A) General Features: Cystic fibrosis (CF) is the most common fatal autosomal recessive disorder with an incidence of 1 in 2,500 in Caucasian population (198). CF is more prevalent in certain geographical areas, as in the Saguenay-Lac St. Jean area in the province of Quebec, where the incidence of CF is 1 in 891 births (199). In 1938, the median survival age was less than one year old, but today median survival rate is 32 years (200).

CF is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR), which is located on chromosome 7, contains 27 exons and is 250 kb long (201). The CFTR protein is a

transmembrane glycoprotein of 1480 amino acid residues that functions as a cyclic adenosine monophosphate (cAMP) dependent chloride conductance channel (202). The CFTR protein is a member of the ATP-binding-cassette (ABC) membrane transport superfamily. It is made up of two homologous halves, with each half containing a nucleotide binding domain that binds ATP as well as a membrane-spanning domain with 6 segments that help form a channel pore spanning on the cell membrane. The two halves are connected by a regulatory domain (R), which is phosphorylated by a cAMP-dependent protein kinase (203). The CFTR channel also functions as a cAMP-dependent negative regulator of Na^+ channel (ENaC) (204). Moreover, a role for chloride transport across the membrane of mitochondrial organelles has also been suggested for CFTR (205).

Over 1,000 mutations in CFTR have been identified (206). These mutations have been divided into six classes: (1) defective CFTR protein production; (2) defective processing of CFTR, often due to misfolding of the CFTR protein; (3) defective regulation; (4) defective Cl^- channel conductance; (5) partially defective production or processing; (6) defective regulation of other channels (206). The most common mutation observed in CF patients is ΔF508, which is a class II mutation that is on 70% of CF chromosomes and thus affects more than 90% of Cf patients. The ΔF508 mutation is characterized by a deletion of three base pairs in exon 10, resulting in the deletion of phenylalanine (202).

CFTR is found primarily in the epithelial cells affected in CF, which includes lungs, nasal polyps, pancreas, sweat glands, liver, large intestine, colon and testes (47). CFTR as an ion channel is crucial in both absorption and secretion. Therefore, CF is characterized by abnormalities in salt and water transport, resulting in thick mucus secretion by the respiratory tract, pancreas, gastrointestinal tract, sweat glands and other exocrine tissues. For instance, a greatly elevated

concentration of chloride in sweat (〉 60 mmol/l) is a main diagnostic test with CF (207). In CF, the lungs lose their ability to maintain a sterile surface and are gradually destroyed by bacterial infection. The intestine secretes less fluid and becomes susceptible to blockage by poorly hydrated stool. The pancreas also secretes less fluid, which causes ductal blockage and consequently pancreatic degeneration. Other physiological manifestations in humans are male infertility due to blockage and eventual degeneration of the vas deferens and female reduced fertility by failure of the cervical mucus to show appropriate hydration during ovulation (207). However, the hallmark of CF disease is a progressive and ultimately fatal, inflammatory lung disease characterized by a vicious cycle of repeated acute and chronic bacterial infections.

B) CF Lung Disease: The mechanism by which a defect in the CFTR channel leads to CF lung disease is not well understood. However, the CF lung disease, unlike any other organs, is dominated by infection and inflammation. Infection associated with active and damaging host inflammatory responses starts early in the life of CF patients. Early in life, the lower respiratory tract of CF infants becomes colonized with opportunistic bacterial pathogens, particularly *Staphylococcus aureus*, and non-encapsulated *Haemophilia influenza*, and later on by Gram-negative bacteria, such as *Burkholderia cepacia* and *Pseudomonas aeruginosa* (208).

Chronic infection of the lungs with mucoid strains of *Pseudomonas aeruginosa* is the most persistent bacteria in the majority of CF patients. Over 80% of CF patients have *P. aeruginosa* infection, which contributes to the progressive decline in pulmonary function and early death (209). *P. aeruginosa* within the CF lungs develops three main irreversible phenotypic conversions, including LPS deficient in O-side chain (complement fixation occurs at this chain), decreased secretion of pyocins, and immotility due to loss of flagella (210). *P. aeruginosa* has the ability to

develop resistance to multiple antibiotics due to the presence of efflux pumps, which export antibiotics, detergents, and dyes (211). CF patients initially become infected with a non-mucoid phenotype of *P. aeruginosa*, but the bacteria ultimately produce mucoid exopolysaccharaide (MEP) or alginate, and form a biofilm. The formation of biofilm contributes to the persistence of *P. aeruginosa* by interfering with host defenses, as well as delivery of antibiotics to the bacterial cell (212). Interesting, the conversion to the mucoid phenotype typically is associated with reduced virulency. Most isolated *P. aeruginosa* from the early phase of chronic infection secrete fewer virulance factors, such as exotoxin A or proteases, than their clonal relative isolated during the initial phase of the infection, *in vitro* (213). However, during later stages of disease, with the advanced state of inflammation, remodeling and destruction of lung tissue, the bacteria grow under iron depletion, which triggers the production of virulence factors from *P. aeruginosa* (214). This suggests that the progressive deterioration in lung function in CF is largely due to the long term deleterious effects of host inflammatory responses rather than direct damage of the bacteria *per se*.

C) Role of CFTR in initiation of infection: There are at least three main hypotheses to explain CF lung disease caused by the absence of CFTR protein as a ion channel.

(1) The high salt hypothesis, which is based on CFTR as an anion channel. Missing or defective CFTR results in reduced transepithelial chloride conductance, as in the sweat duct. Therefore, the surface liquid of CF airways has higher levels of salt content, that interferes with bacterial killing by natural antibiotics such as defensins and lysozyme (215).

(2) The low volume hypothesis, which is based on CFTR's function as an inhibitor of ENaC on the apical surface. According to this hypothesis, absence of CFTR's inhibition of the epithelial ENaC

results in sodium transport. However, due to substantial chloride shunt pathways in the airways, the increased sodium transport results in an increased absorption of chloride and water. Therefore, CF airway surface liquid volume is reduced, which interferes with proper ciliary function and reduces mucociliary clearance (216).

(3) The low secretion hypothesis, which is based on CFTR's function in fluid secretion. This hypothesis emphasizes the role of CFTR in serous cells for the secretion of an antibiotic-rich fluid. Serous cells are located in the glands of larger airways and on the surface of the smaller airways and have the lung's highest levels of CFTR. Airway submucosal glands provide the majority of surface liquid when the lung is irritated. In CF, fluid secretion by serous glands is significantly reduced and results in a thicker airway mucus which is deficient in antibiotic substances. This hypothesis would exacerbate the defects proposed in two other hypothesis: natural antibiotics would be less abundant and effective, and the reduction in airway surface liquid would result from both decreased secretion and increased absorption (217).

In addition, CF patients also overproduce a particular ganglioside (asialo-GM1) receptor on epithelial cells, which can bind to *P. aeruginosa*, as well as other pathogens (218). Although some investigators speculate that adherence of *P. aeruginosa* to these residues is a critical factor in infection (218), others have questioned the importance of this adherence (219). Since many respiratory pathogens bind avidly to asialo-GM1, this enhanced accessibility might only partially explain the persistence of *P. aeruginosa* lung infection in CF. In addition, it has been described that CFTR is involved in recognition and elimination of *P. aeruginosa* from the respiratory tract and the expression of CFTR correlates with internalization of *P. aeruginosa* by epithelial cells (220). This CFTR-dependent internalization was specific to *P. aeruginosa* and not other common respiratory

pathogens. The outer core of *P. aeruginosa* LPS binds to the first extracellular domain of CFTR and the accumulation and turnover of CFTR was enhanced during *P. aeruginosa* infection (221).

In summary, CFTR mutations directly or indirectly impair the first line of innate host response, including mucociliary clearance, and natural antimicrobial peptides (defensins). In addition, adherence of *P. aeruginosa* to the cell surface of epithelial cells is markedly increased, but CFTR-dependent internalization into the epithelial cells is absent. The persistent bacterial proliferation results in a profound inflammatory response, which is dominated by neutrophilic infiltration into the airways. Since survival rate of neutrophils is short after leaving the blood circulation, there must be a constant stimulus to attract neutrophils. Indeed, CF neutrophils differ from healthy neutrophils in terms of an increased propensity to release granulocytes after stimulation (222). The excessive release of oxidants and proteases, like elastase, by infiltrating neutrophils plays a significant role in tissue damage (223). The anti-protease defenses of the CF lung are also overwhelmed by combination of endogenous and bacterial proteases, which leads to uninhibited proteolytic enzyme activity (224). Elastase directly damages the airway wall by digesting elastin and other structural proteins. It also augments mucus secretion, cleaves vital opsonins and receptors essential for phagocytosis, and promotes the generation of chemoattractants (58).

D) Role of CFTR in oxidant and cytokine regulation: Lung inflammation in CF is associated with increased production of pro-inflammatory cytokines in the airway. Several studies documented increased concentration of pro-inflammatory cytokines, (including IL-1, IL-6, IL-8, and TNF-α) in the sputum, BAL fluid, (225) or epithelial lining fluid (ELF) of CF patients (67).On the other hand, anti-inflammatory cytokines, such as IL-10 (in BAL), IL-1 receptor antagonist protein (IRAP), and

soluble TNF-α receptor (sTNF-α-R) in ELF was down regulated in CF patients (67). It also has been shown that CF epithelial cell lines produce more pro-inflammatory cytokines (TNF-α, IL-6, IL-8) than normal cell lines in response to *P. aeruginosa* infection (226) (227) (228). Similarly, CF primary bronchial gland epithelial cells also revealed exaggerated up-regulation of IL-6 and IL-8 expression both *in vivo* and *vitro* (229) (230). Augmentation of pro-inflammatory cytokines in CF, has been linked to alterations in NF-κB signaling pathway by several groups. Increased DNA binding of NF-κB (231),decreased IκBα (232), and decreased IκBβ (233) have been shown in stimulated CF cell lines. In sharp contrast, some other investigators found no differences in the expression of pro-inflammatory mediators (IL-6, IL-8, MCP-1) between CF and non-CF lines (234) (235) (236) (237). Although the reasons for these contradictory results are not clear, differences in cell types, number of passages, and the nature or duration of stimulation might account for these inconsistently (237). Together, these studies suggest that there might be intrinsic abnormalities in regulation of cytokine release by CF epithelial cells that contribute to an excessive inflammatory response in CF lung.

Parallel with increased inflammatory responses in CF, the levels of oxidative stress are also augmented, which results in pulmonary injury (58). Therefore, antioxidants, such as glutathione (GSH), play an important role in the inflammatory environment (238) (239). The levels of GSH are significantly lower in epithelial lining fluid (ELF) of CF patients (240) and mice (241). *In vitro* studies showed that GSH was permeable through a CFTR-dependent pathway (229) (242) which emphasizes a direct association between CFTR and GSH. Jungas and colleagues (Jungas, T., 2002) have reported that absence of CFTR resulted in increased levels of intracellular GSH and reduced pro-apoptotic proteins in human CF epithelial cells. More interesting, *P. aeruginosa* lung infection in *cftr*$^{-/-}$ have shown lower apoptotic cells compared to infected wild-type mice, and infected human CF epithelial cells also were less sensitive to apoptosis than non-CF cells (243). Extrusion of GSH

from epithelial cells caused hypersensitivity to apoptosis (244). These data provide evidence for the hypothesis that a shift from apoptosis to necrosis would promote chronic inflammation in CF.

Recently, it has been shown that the levels of exhaled-NO was decreased in CF patients, despite severe infection/inflammation (245). In addition, the expression of iNOS was also reduced in CF human (246) as well as murine airway epithelial cells (247) and iNOS reduction was influenced by CFTR expression (248). Several mechanisms that have been proposed to explain decreased exhaled NO levels in CF. There is some evidence of an intrinsic defect in iNOS expression in CF airway epithelial cells in both CF patients and CF murine model. This appears to involve alterations in the signaling pathways responsible for iNOS expression. iNOS is induced by two main pathways, IFN-γ and NF-κB. Beside NF-κB, there are several other transcriptional factors, including STAT-1, and IFN regulatory factor-1 (IRF-1) that interacts with iNOS promoter for full expression of iNOS (249). STAT-1 is essential for IRF-1 and iNOS expression in IFN-γ signaling pathway (250). Recently, Kelly et al., have reported that increased levels of the protein inhibitor of activated STAT-1 (PIAS1) in CF mouse nasal epithelium results in reduced activated STAT-1 and ultimately IRF-1 and iNOS expression in IFN-γ-dependent signaling (251). Thus, reduced NO and iNOS production in CF partially might be affected by alteration in IFN-γ signaling pathway. Moreover, other mechanisms such as increased consumption of NO by formation of NO byproducts or increased consumption by inflammatory cells such as neutrophils (252). Thus, decreased expression of iNOS and low levels of NO production potentially might contribute to the disordered host defense of CF patients by preventing the inhibition of bacterial (*P. aeruginosa*) growth (247) and also bronchial obstruction by impairment of airway smooth muscle function (253).

E) Animal model of cystic fibrosis: Our understanding of the molecular and biological basis of CF has become more comprehensive due to the study of CF animal models. It is to note that animal models of CF do not mimic all the pathophysiology observed in human CF. For instance, CF mice models do not show pancreatic insufficiency, which is a prominent manifestation of CFTR dysfunction in CF patients. Therefore, there might be differences between murine and human pancreas due to: I) the levels of CFTR expression which has been shown to be high in human pancreas (254) and low in mice pancreas (255); II) the presence of an alternative murine chloride channel that can partially compensate for the absence of CFTR (256). Nevertheless, animal models have proven extremely useful to understand the complexities of human CF. To date, eleven CF mouse models have been reported with varying degree of characterization. All of the CF mouse models have been created by two types of gene targeting: (1) those designed to disrupt CF gene with no normal CFTR production, absolute nulls; (2) models that mimic the human clinical mutation, like G551D (substitution of glycine with an aspartic acid) and ΔF508 (loss of a phenylalanine) by insertion into the target gene, without loss of any genomic sequence, resulting in mutants expressing low levels of normal CFTR mRNA (47). However, a disadvantage of the insertional mutants is the potential for reversion to wild type and the production of normal CFTR by various mechanisms (257).

Lung disease represents the primary concern in CF, since about 95% of the morbidity and mortality in CF humans is due to repeated acute exacerbations of pulmonary infection/inflammation (258). The classical pathophysiology of CF lung disease involves airway mucus plugging with bacterial infection, bronchiolitis, bronchiectasis, and goblet cell hyperplesia (259). Surprisingly, lack of pulmonary pathology was noted in most of the CF mice with the exception of one model. The $CFTR^{tm1UNC}$ knock out mice had pathological changes in the upper airways but there were no signs

of either inflammation or bacterial infection in the lung (255). Kent et al., hypothesized that $CFTR^{tm1UNC}$ had a mixed C57BL/6 and 129/J genetic background (C57BL/6/129), which might affect the development of lung pathophysiology (260). Therefore, $CFTR^{tm1UNC}$ mice were backcrossed onto the C57BL/6 background for 18 generation to generate congenic C57BL/6-$CFTR^{tm1UNC}$ /$CFTR^{tm1UNC}$ knockout mouse (260). Although no spontaneous infection of the lungs occurred, the C57BL/6-$CFTR^{tm1UNC}$ /$CFTR^{tm1UNC}$ mouse developed spontaneous and progressive lung disease, including failure of effective mucociliary clearance, postbronchiolar over inflation of alveoli, and parenchymal interstitial thickening with evidence of fibrosis and inflammatory cell recruitment (260). Therefore, apart from C57BL/6-$CFTR^{tm1UNC}$ /$CFTR^{tm1UNC}$ knockout mice, no CF mouse models develop spontaneous lung disease, and none develop spontaneous chronic bacterial infection, as observed in most human CF patients.

The model of chronic *P. aeruginosa* lung infection, by using immobilizing agents such as agar beads, has been employed in CF mice in order to mimic human CF lung infection. The entrapment of *P. aeruginosa* in agar beads seems to slow the growth of the bacteria in some ways resembling the biofilm state (261). Chronic *P. aeruginosa* lung infection in $CFTR^{tm1UNC}$ knockout mice causes increased bacterial proliferation associated with a decreased survival rate after infection compared to wild-type mice. There is also increased production of pro-inflammatory cytokines (TNF-α, MIP-2, and KC), as well as decreased production of anti-inflammatory cytokines (IL-10) and pulmonary iNOS (262). Chronic *P. aeruginosa* lung infection in congenic C57BL/6-$CFTR^{tm1UNC}$ /$CFTR^{tm1UNC}$ knockout mice also have demonstrated higher mortality and higher bacterial burden compared to wild-type mice (263).

F) Skeletal muscle function in CF: Exercise capacity is significantly reduced in patients with CF,

and impaired exercise performance is associated with a poorer prognosis in terms of quality of life (264) as well as survival (265). The reduction in exercise capacity in CF has traditionally been ascribed to a combination of diminished pulmonary function and decreased peripheral skeletal muscle mass, with the later being attributed primarily to malnutrition (266). In addition there is the problem of general deconditioning due to an overall decrease in daily activity levels of patients with impaired pulmonary function (266) (267) (268). Several investigators have demonstrated that peripheral limb muscle strength is significantly reduced in CF patients. For instance, in CF patients aged 16-28 yrs, Mier et al. found the quadriceps muscle strength averaged only 68% of normal (269). Lands et al. also reported significant diminished leg muscle strength (65% predicted) in subjects with stable CF and attributed these findings primarily to reduced muscle mass although it was noted that the patients were only mildly undernourished (266). Whether the reduction in peripheral muscle mass are related solely to nutritional factors is unclear. In addition, there is controversy as to whether the remaining muscle mass has normal physiological function. Thus, peripheral muscle weakness and a diminished capacity of performing work have been observed in CF patients with essentially normal spirometry and nutritional status (270). In addition, de Meer et al. (271) employed NMR spectroscopy during exercise in CF patients and reported a reduced efficiency of mitochondrial oxidative phosphorylation in comparison to healthy controls, suggesting the presence of intrinsic abnormalities of muscle function. Therefore, there is evidence indicating that in addition to the atrophic effects on skeletal muscle related to malnutrition, other factors are also important in the development of skeletal muscle wasting and dysfunction in CF patients.

Despite major improvements in the medical care and prognosis of CF, respiratory failure remains the cause of death in most patients. This fact, along with the aforementioned evidence for impaired peripheral muscle function in CF patients, has led a number of investigators to examine

respiratory muscle function in this patient population. The increased respiratory mechanical load associated with CF lung disease (272), particularly when combined with augmented ventilatory demands during exercise or with acute exacerbations of airflow obstruction, could predispose patients to the development of ventilatory insufficiency on the basis of respiratory muscle fatigue (273). Respiratory muscle fatigue is more likely to occur when underlying respiratory muscle weakness is present (274). In this regard, reductions in the capacity for maximal inspiratory pressure (MIP) generation by inspiratory muscles have been reported in many studies of CF patients (275) (276) (277) (278). Expiratory muscle strength, which is important for effective cough generation as well as for facilitating ventilation during the periods of increased ventilatory demand such as exercise, has also been found to be significantly impaired (275) (279). However, other investigators found little evidence for intrinsic weakness of the respiratory muscles, particularly when the effects of hyperinflation are taken into account (279) (280) (281). The seemingly contradictory findings of these studies is likely is related to differences in patient selection as well as different methodologies employed to assess respiratory muscle function. For instance, whereas the MIP measurement used in most early studies is a clinically convenient but crude index of diaphragm muscle strength, recent work using more invasive and sophisticated methods have indicated reduced diaphragm force-generating capacity in CF (276). The ability to sustain maximal inspiratory pressure has also been found to be reduced in CF patients despite relatively normal MIP values (278), suggesting that the standard MIP lacks adequate sensitivity for detecting abnormal respiratory function. It is important to emphasize that investigations of muscle performance in CF to date have involved patients who were felt to be clinically "stable" . Unfortunately, the specific criteria used to establish clinical stability were frequently vague or undefined in these studies. In particular, the time elapsed between patient evaluation and the last episode of acute infectious exacerbation or antibiotic administration were usually not stated. Therefore, different levels of pulmonary infection/inflammation might

explain the apparent contradictions among some studies with respect to respiratory muscle strength measurements in CF patients.

CFTR expression has been reported in rat skeletal muscle (282). CFTR plays an important role in autonomic of smooth muscle cells (283) as well as cardiac myocytes (284) (285) (286), but its function in skeletal muscle has not been elucidated. Exercise capacity is significantly reduced in patient with CF, and patients with milder CFTR mutation classes have better exercise tolerance (287). Many patients with CF have weak peripheral (270) (266) and respiratory muscles (276) (275) (278) (288) (289). The etiology of respiratory muscle weakness in CF is potentially multifactorial, including pulmonary hyperinflation (290) (291), airway obstruction (272) (292), malnutrition (293) (294), and muscle catabolism (Debigare, R., 2001). In fact, abnormalities of muscle function are not readily attributed to muscle atrophy in CF (271). Intrinsic abnormalities in the mitochondria of skeletal muscle of CF patients have been reported (270), but the clinical impact of these abnormalities is still unclear. Therefore, additional factors beyond diminished lung function or malnutrition and muscle atrophy are likely to be involved in producing skeletal muscle weakness in CF patients.

G) The link between lung inflammation and muscle dysfunction: There has been increasing recognition that pulmonary injury and inflammation may trigger more widespread systemic inflammatory effects throughout the body. Slutsky et al (295) (296)have shown that ventilator induced lung injury is associated with augmented levels of circulating inflammatory cytokines such as TNF-α, TNF-α receptor, and IL-6, leading to speculation that lung injury may contribute to cytokine-mediated multiorgan dysfunction. In chronic obstructive pulmonary disease (COPD) related to smoking, increased serum levels of C-reactive protein, LPS-binding protein, IL-8, and TNF-α

receptor have been reported (297). These abnormalities were correlated with an increase in resting energy expenditure, suggesting that inflammatory mediator release from the lungs could contribute to the weight loss often observed in COPD patients (297). Moreover, the TNF-α levels were approximately 10 times higher in COPD patients with cachexia as compared to another group of patients with similar lung function but no weight loss (298). In CF patients, several studies have also documented increased peripheral blood levels of various markers of inflammation (278) (299) (300). With one exception (301), different investigators have reported the serum levels of TNF-α are significantly elevated in clinically stable CF patients (299) (300) (302). Similar to COPD, there appears to be a significant relationship between raised TNF-α levels and increased resting energy expenditure found in certain CF patients (278). These findings have led to the suggestion that weight loss in CF may be at least partly related to sustained elevation of TNF-α caused by chronic pulmonary inflammation, even in the absence of clinically apparent infection (278). In addition, circulating TNF-α and C-reactive protein levels in CF are further increased in the setting of symptomatic respiratory exacerbation (300) (302), and can be reduced by two weeks of anti-*Pseudomonas* antibiotic therapy (Norman, D., 1991). This is consistent with the observation that circulating levels of endotoxin also increased in CF during acute pulmonary exacerbation, with a subsequent fall following the initiation of anti-*Pseudomonas* therapy (303).

In addition, increased circulating cytokines or bacterial products released from the lung can trigger pro-inflammatory cytokine production by the muscle fibers themselves. As mentioned earlier, this has been associated with contractile dysfunction in models of acute endotoxemia (105).

VII Summary

Local Pulmonary infection/inflammation may manifest more systemic inflammatory effects. Increased levels of various pro-inflammatory markers in blood circulation might cause skeletal muscle to express more pro-inflammatory mediators through autocrine or paracrine pathways. In return, augmented production of pro-inflammatory mediators within the skeletal muscle can contribute to muscle weakness by interfering with muscle contractile machinery or increasing muscle wasting.

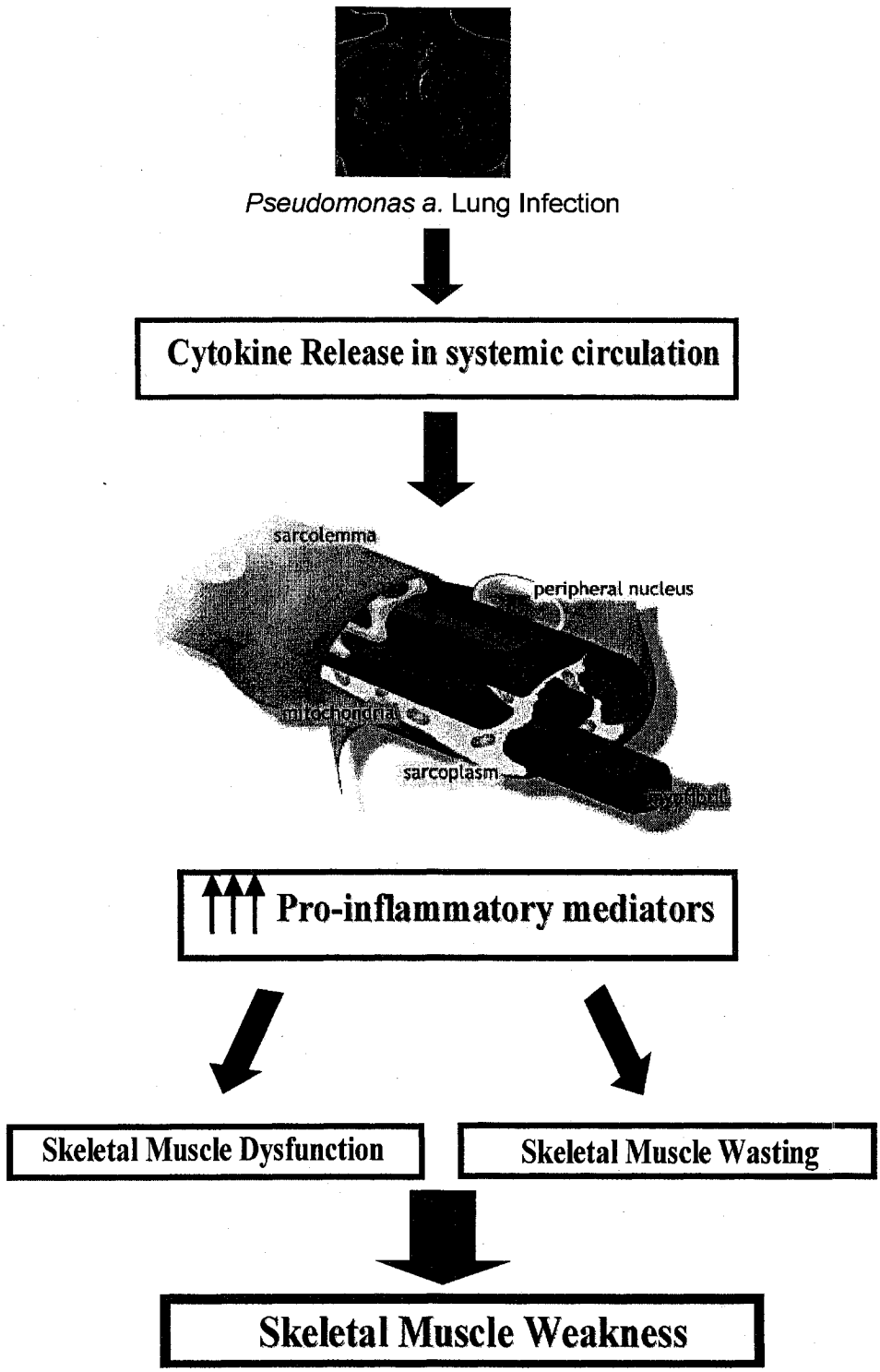

Figure 1. Schematic representation of the steps leading to skeletal muscle weakness following pulmonary lung infection.

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CHAPTER 2

PREFERENTIAL DIAPHRAGMATIC WEAKNESS DURING SUSTAINED PSEUDOMONAS AERUGINOSA LUNG INFECTION

2.I Prologue

There is a large literature implicating local pulmonary inflammation in the deterioration of lung function. Recently, there has also been increased interest in the idea that pulmonary inflammation and lung injury may trigger more widespread systemic inflammatory effects. Increased peripheral blood levels of various markers of inflammation have been documented in patients with CF as well as other forms of chronic obstructive pulmonary disease. Such findings have led to the suggestion that systemic manifestations of disease, including muscle wasting and weakness, may be caused by ongoing pulmonary inflammation. Therefore, we wished to determine the effcts of pulmonary *Pseudomnas aeruginosa* infection on both respiratory and peripheral muscles function.

2.II Abstract

Infection with *Pseudomonas aeruginosa* plays a major role in the pulmonary inflammation and injury associated with cystic fibrosis. Lung inflammation may also lead to more widespread systemic effects on other organs. We tested the following hypotheses: 1) ongoing *P. aeruginosa* lung infection produces diaphragmatic and limb muscle weakness; and 2) such muscle dysfunction is directly correlated with the level of pulmonary inflammation. Chronic bronchopulmonary infection with mucoid *P. aeruginosa* was induced in C57BL/6 mice. At day-2 post-infection, diaphragmatic force was decreased (37%) only in mice infected with a high dose of $1x10^6$ colony forming units, whereas by day-7 post-infection diaphragmatic force was similarly reduced (36%) even at a 5-fold lower inoculating dose. No significant correlations were found between diaphragmatic weakness and pulmonary inflammation as assessed by the number of neutrophils, macrophages, and lymphocytes in bronchoalveolar lavage fluid. Moreover, in marked contrast to the diaphragm, no effects of *P. aeruginosa* infection on contractile function were observed in prototypical slow- and fast-twitch hindlimb muscles. We conclude that sustained lung infection with *P. aeruginosa* induces preferential weakness of the diaphragm, which is not directly correlated with the degree of pulmonary inflammation induced under these conditions.

2.III Introduction

Cystic fibrosis (CF) is the most frequent autosomal recessive disorder in the Caucasian population, affecting approximately 1 in 2500 live births. Exercise capacity is significantly reduced in patients with CF, and this is associated with a worsened prognosis (1). Interestingly, muscle weakness and a diminished capacity for performing work have been reported in CF patients with essentially normal spirometry and nutritional status (2). This is also in keeping with the fact that abnormalities of muscle function not readily attributable to muscle atrophy have been observed (3). Therefore, additional factors beyond diminished lung function or malnutrition and muscle atrophy are likely to be involved in producing skeletal muscle weakness in CF patients.

Patients with CF are particularly prone to chronic or recurrent pulmonary infections with the mucoid strain of *Pseudomonas aeruginosa*. This pathogen plays a central role in the vicious cycle of lung infection and inflammation which ultimately culminates in irreparable lung damage, respiratory failure and death (see (4) for review). While the role of local pulmonary inflammation in the pathogenesis of CF lung disease is well established, it is unknown whether this also contributes to skeletal muscle dysfunction. However, there is increasing recognition that lung injury and pulmonary inflammation may trigger a systemic inflammatory response (5-7). In addition, several investigators have reported that serum levels of tumor necrosis factor (TNF)-α, a known inducer of muscle wasting and weakness (8,9), are significantly elevated in CF patients (10-12).

In the present study, we hypothesized that pulmonary inflammation triggered by *P. aeruginosa* infection could be an important cause of diaphragmatic as well as peripheral limb muscle dysfunction, thereby contributing to the global muscle weakness found in CF patients. In order to mimic the scenario found in CF, we employed a previously characterized murine model of chronic *P. aeruginosa* infection (13,14). In this model, bacteria are encapsulated within agar beads to impede pulmonary clearance of the organisms, which allows for the establishment of an ongoing but clinically tolerable infection (13,14). Here our specific objectives were three-fold: 1) to determine the effects of a sustained pulmonary infection with *P. aeruginosa* on the function of the diaphragm, as well as prototypical slow-twitch (soleus) and fast-twitch (extensor

digitorum longus, EDL) hindlimb muscles; 2) to examine the relationship between alterations in respiratory or limb muscle contractile function and pulmonary mechanics, pulmonary bacterial burden, and the level of lung inflammation induced by *P. aeruginosa* infection; and 3) to ascertain the extent to which these responses might differ at different stages of the infection process. Some of the results of this study have been previously reported in the form of an abstract (15).

2.IV Materials and Methods

Animal Model of Sustained *P. aeruginosa* Infection

Studies were performed in 8-10 week old C57BL/6 male mice weighing 20-25 g (Charles River Laboratories, Quebec, Canada), which were used in accordance with the guidelines established by the Canadian Council on Animal Care. The model of chronic pulmonary infection with *P. aeruginosa* was performed essentially as described by Starke and colleagues (13), with minor modifications. A mucoid strain of *P. aeruginosa* (strain 508) was used in these studies, which was originally isolated from a cystic fibrosis patient as previously described (16). Briefly, log-phase *P. aeruginosa* bacteria were concentrated and mixed with 1.5% trypticase soy agar pre-warmed to 52°C. This mixture was added to heavy mineral oil at 52°C and rapidly stirred for 6 min, followed by cooling for 10 min at 4°C. After removing excess oil, the bacteria-bead preparation was washed extensively and resuspended in sterile phosphate-buffered saline (PBS) at pH 7.4. The size (100-150 um) and uniformity of the beads were verified by light microscopy. In addition, the number of viable bacteria trapped within the agar beads was measured by homogenizing the beads and then plating 10-fold serial dilutions on trypticase soy agar plates. Sterile agar beads were produced in the same manner but with omission of bacteria, and were confirmed to be free of colony-forming units (CFU).

To deliver either bacteria-containing or sterile agar beads to mouse lungs, the mice were first anaesthetized with a combination of ketamine (130 mg/kg) and xylazine (20 mg/kg) injected intramuscularly. A small incision was made at the midline of the neck in order to expose the trachea. The trachea was then intubated with a sterile flexible 22-ga cannula attached to a 1 ml syringe, which was used to inject 50 ul of the agar bead suspension into the lungs. All animal procedures were approved by the Animal Care Committee of McGill University.

Bronchoalveolar Lavage (BAL)

The trachea was cannulated with a 22-ga catheter connected to two separate syringes via a three-way stopcock. One syringe was used to instill 5 ml of cation-free Hank's balanced salt solution (GIBCO, Burlington, Ontario) into the lungs, while the second syringe allowed the fluid to be collected by gentle aspiration. The total lavage fluid recovered was approximately 4 ml. Total

cell numbers were determined using a hemocytometer. Differential cell counts were performed on cytospin preparations stained with Diff-Quick (American Scientific Products, McGaw Park, IL). From 300-400 cells were counted on each cytospin preparation, and the cells were classified as polymorphonuclear leukocytes, macrophages, and lymphocytes using standard morphological criteria (16).

Myeloperoxidase (MPO) Assay

Myeloperoxidase (MPO) activity was measured essentially as described by Koike et al. (17), with minor modifications. Tissues were homogenized (PT10135; Brinkmann Instruments, Inc., Mississauga, ON., Canada) for 45 seconds in PBS and then centrifuged at 3000 rpm for 10 minutes at 4°C. The pellet was resuspended in 4 ml of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5 g/dl hexadecyltrimethyl ammonium bromide (Sigma Chemical Co., St. Louis, MO). Samples were sonicated for 3 minutes at full power, incubated in a 60°C water bath for 2 hours, and then centrifuged at 13,000 rpm for 10 min. Supernatant (0.1 ml) was added to 2.9 ml of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine hydrochloride (Sigma Chemical Co.) and 0.0005% hydrogen peroxide. Absorbance of 460 nm visible light was measured for 3 min, and MPO activity per gram wet tissue weight was calculated as previously described.

Lung Bacterial Colony Assay

Both lungs were removed and homogenized for 2 min at high speed (PT10135; Brinkmann Instruments Co., Mississauga, ON, Canada) in 4.0 ml of PBS. Serial dilutions (1:10) of homogenized lungs were then plated on petri dish containing trypticase soy agar. The number of *P. aeruginosa* colonies was counted after overnight incubation at 37°C (16).

Measurements of Respiratory Mechanics

Animals were anesthetized with xylazine hydrochloride (10 mg/kg) and sodium pentobarbital (30 mg/kg) delivered by intraperitoneal (i.p.) injection. The trachea was cannulated with a snug-fitting metal needle and connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Quebec) for measurement of respiratory system mechanics as previously described (18). The mice were paralyzed with pancuronium chloride (0.07 mg/kg i.p.) and ventilated in a quasi-sinusoidal fashion with a tidal volume of 0.18 ml at a rate of 150 breaths/min. A positive end-expiratory pressure (PEEP) level of 1.5 cm H_2O was established by connecting the expiratory line of the ventilator to a water trap. Two minutes prior to measurement of respiratory system mechanics, inflation to an airway pressure of 30 cm H_2O was performed in order to provide a standard volume history. After cessation of regular ventilation and exhalation to the above level of PEEP, a 2.5 Hz sinusoidal perturbation with an amplitude of 0.18 ml and a length of 1.2 sec was applied. Respiratory system resistance was then derived from the relationship between airway opening pressure and airflow. Quasi-static deflation pressure-volume curves were also collected to evaluate potential alterations in the compliance characteristics of infected lungs. Deflation proceeded in 0.1 ml increments, with a 2-3 s pause at each step. The measurements were repeated two times, and the mean values are reported.

Analysis of diaphragm contractile function

After euthanasia, the diaphragm, soleus and extensor digitorum longus (EDL) muscles were surgically excised for *in vitro* contractility measurements under isometric conditions as previously described in detail (19). After removal from the animal, the diaphragm was immediately placed into a chilled (4°C) and equilibrated (95% O2-5%CO2; pH 7.38) Krebs solution with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1 KH2PO4, 25 NaHCO3, and 11 glucose. A muscle strip approximately 2 mm wide was dissected free, taking care to leave the central tendon and adjacent rib cage margins intact. The excised diaphragm strip was mounted into a jacketed tissue bath chamber filled with equilibrated Krebs solution, using a custom-built muscle holder containing two stimulation electrodes located on either side. The central tendon was tied to an isometric force transducer using 4-0 silk thread. A thermoequilibration period of 15 min was observed prior to initiating contractile measurements at 23°C. After placing the diaphragm strip at optimal length (Lo), the force-frequency

relationship was determined by sequential supramaximal stimulation for 1 sec at 5, 10, 20, 30, 50, 100, 120, and 150 Hz, with 2 min between each stimulation train. The force data were acquired to computer at a sampling rate of 1000 Hz for later analysis. After completion of the above contractility studies, the muscles were removed from their baths, and muscle length was measured with a microcaliper accurate to 0.1 mm. Muscle cross-sectional area was determined by assuming a muscle density of 1.056 g/cm^3, which allowed specific force (force/cross-sectional area) to be calculated and expressed in $Newtons/cm^2$.

Statistical Analysis

All data are presented as mean values ± SE. Group mean differences were determined by ANOVA, with post-hoc application of the Tukey test where appropriate. Linear regression was performed using the least-squares method. A statistics software package was used for all analyses (SigmaStat V2.0, Jandel Scientific, San Rafael, CA). Statistical difference was defined as $P < 0.05$.

2.V Results

Clinical Status

Mice were sacrificed at either 2 or 7 days post-infection with *P. aeruginosa*-laden agar beads. Two different doses of inoculating bacteria ($2x10^5$ and $1x10^6$ CFU) were studied at 2 days post-infection, whereas only the lower inoculating dose was used for the 7-day time point due to unacceptable signs of ill health at the higher dose in the 7-day group. As has been previously reported (20), body weight was slightly reduced in infected mice at day 2 (-7% and -11 % for $2x10^5$ and $1x10^6$ CFU, respectively) and to a lesser extent at day 7 (-6%) compared to animals injected with sterile beads at the same time points.

Lung Bacteriology

Pulmonary bacterial counts at days 2 and 7 post-infection are shown in Fig. 1, along with values obtained from the lungs of control mice. The pulmonary bacterial burden did not differ significantly between the two inoculating doses evaluated at day 2 post-infection, although there was a trend toward increased CFU values with the higher dose (Fig. 1A). In addition, there was no significant change in pulmonary bacterial load between days 2 and 7 post-infection at the lower inoculating dose of $2x10^5$ CFU, indicating an inability to clear the bacteria-laden beads and persistent ongoing infection (Fig. 1B). Control mice in which no previous intervention had been made (CTL), as well as mice that had been instilled with sterile agar beads (CTL-Beads), were culture negative at both time points.

Bronchoalveolar Lavage (BAL)

The total number of inflammatory cells contained within BAL fluid was markedly increased at day 2 post-infection, particularly in mice inoculated with the higher dose of $1x10^6$ CFU (see Fig. 2A). This was due to increases in both PMNs and macrophages. In contrast, by day 7 post-infection the total number of cells found in infected mice was not significantly greater than the CTL-Beads group, although higher numbers of PMNs and lymphocytes were found in infected mice (Fig. 2B). There was no significant difference in BAL cell numbers between the CTL and CTL-Beads groups at either time point.

Respiratory Mechanics

In order to ascertain whether the instillation of agar beads (either sterile or bacteria-laden) into the lungs produced changes in resistance or compliance, respiratory mechanics were measured in a subset of mice from the different experimental groups. As can be seen from Fig. 3, there were no significant differences among the various experimental groups in either resistance or the pressure-volume relationship.

Diaphragmatic Contractile Function

Figure 4 illustrates the effects of *P. aeruginosa* infection on the diaphragmatic force-frequency relationship at both time points. With the inoculating dose of $2x10^5$ CFU, the force-frequency relationship of the diaphragm was unaltered at day 2 post-infection. However, the higher dose of $1x10^6$ CFU greatly depressed force-generating capacity of the diaphragm in comparison to the other 3 groups (CTL, CTL-Beads, and $2x10^5$ CFU). Moreover, in contrast to its lack of effect at day 2, the lower inoculating dose of $2x10^5$ CFU was associated with a marked decrease in diaphragmatic force production at day 7 post-infection (Fig. 4B). On the other hand, the ability of the diaphragm to resist fatigue *in vitro* was not significantly affected by *P. aeruginosa* infection under any of the conditions studied (Fig. 5).

Relationship Between Contractile Dysfunction and Inflammation

In order to determine whether there was any direct relationship between the level or nature of pulmonary inflammation and the observed impairment in diaphragmatic force production after *P. aeruginosa* infection, correlation analysis was performed (see Table 1). At day 2 post-infection, total BAL cell number showed the strongest correlation with diaphragmatic force impairment, although this did not achieve statistical significance (P=0.11). At day 7 post-infection, the best correlation with diaphragmatic force impairment was obtained for total lymphocytes in BAL, but once again this failed to reach statistical significance (P=0.21). There was also no significant correlation between diaphragmatic weakness and bacterial burden in the lungs at either day 2 (P=0.51) or day 7 (P=0.26) post-infection.

We next evaluated whether *P. aeruginosa* infection of the lungs was associated with inflammatory cell infiltration of the diaphragm. Diaphragm muscle sections stained with hematoxylin and eosin (not shown) did not reveal inflammatory cell infiltration at either day 2 or day 7 post-infection. In addition, in order to address this issue in a more quantitative manner, we also performed MPO assays (marker of neutrophil content) on the tissues of infected mice. In keeping with the BAL data, Fig. 6A shows a large increase in MPO activity within the lung tissue of infected mice at day 2 post-infection, with a subsequent decline toward control values by day 7. In contrast, Fig. 6B demonstrates that MPO activity within the diaphragm was negligible under control conditions, and remained so at both day 2 and day 7 post-infection.

Hindlimb Muscle Contractile Function

Figure 7 shows the effects of instilling *P. aeruginosa*- laden beads into the lungs on the force-frequency relationship of the soleus muscle at days 2 and 7 post-infection. In marked contrast to results obtained in the diaphragm, there was no significant effect of either $1x10^6$ CFU (day 2) or $2x10^5$ CFU (day 7) on specific force production by the soleus. Similarly, the endurance properties of the soleus muscle were not significantly altered by *P. aeruginosa* infection (Fig.8). Because the diaphragm contains a higher proportion of fast-twitch fibers than the soleus, we also determined the response of a fast-twitch limb muscle (the EDL) under the same conditions. Essentially identical results were obtained for the fast-twitch EDL, i.e., no significant effects of *P. aeruginosa* infection on either the force-frequency relationship (Fig.9) or endurance properties (Fig.10) of the muscle were found.

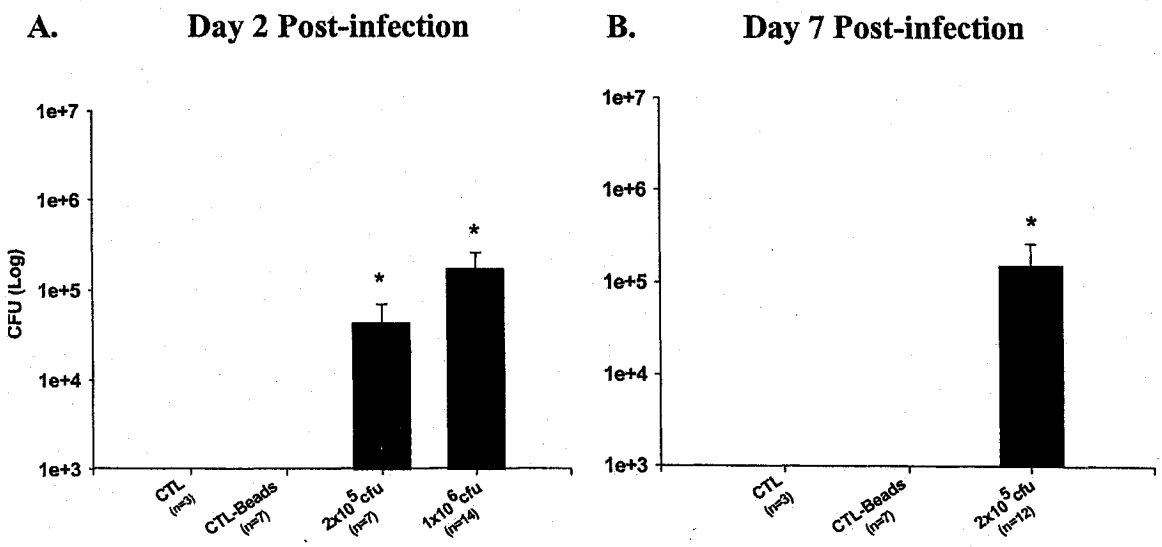

Figure 1. Pulmonary bacterial burden. Mice were sacrificed to quantify numbers of bacteria in the lung at: **(A)** Day 2, and **(B)** Day 7 post-infection. Values are group means ± SE. *P<0.05 compared to CTL and CTL-Beads groups.

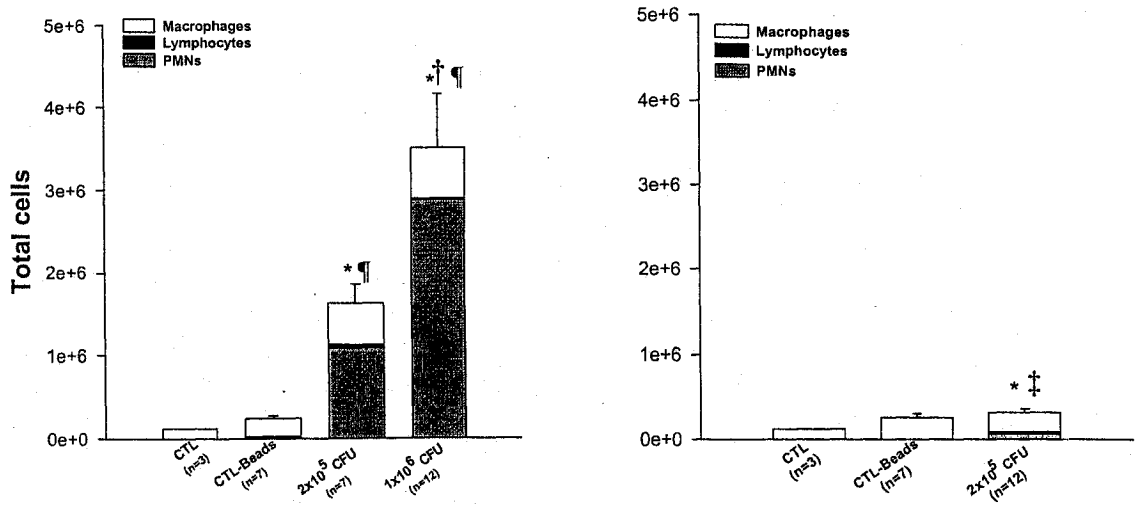

Figure 2. Pulmonary inflammatory response to *Pseudomonas* infection. Inflammatory cells in BAL fluid at: **(A)** Day 2, and **(B)** Day 7 post-infection. Values are group means ± SE. $P<0.05$ compared to CTL and CTL-Beads groups: *=PMNs, †=Macrophages, ‡=Lymphocytes, ¶=Total Inflammatory Cells.

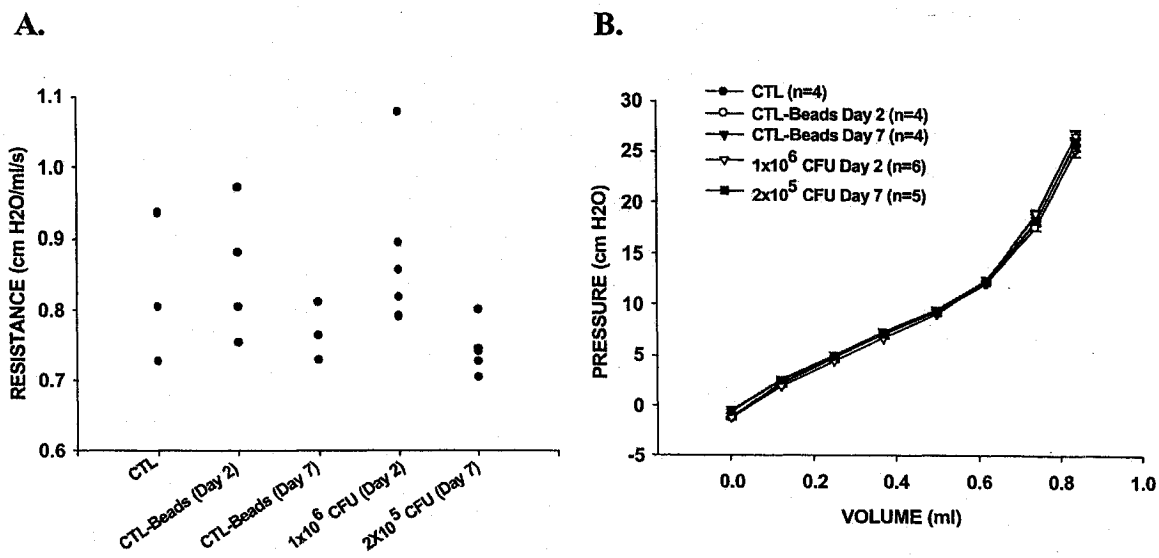

Figure 3. Respiratory system mechanics. **(A)** Individual values for resistance of the respiratory system at the indicated time points. **(B)** Group mean values (± SE) for quasi-static pressure-volume curves are shown. There were no significant differences among groups.

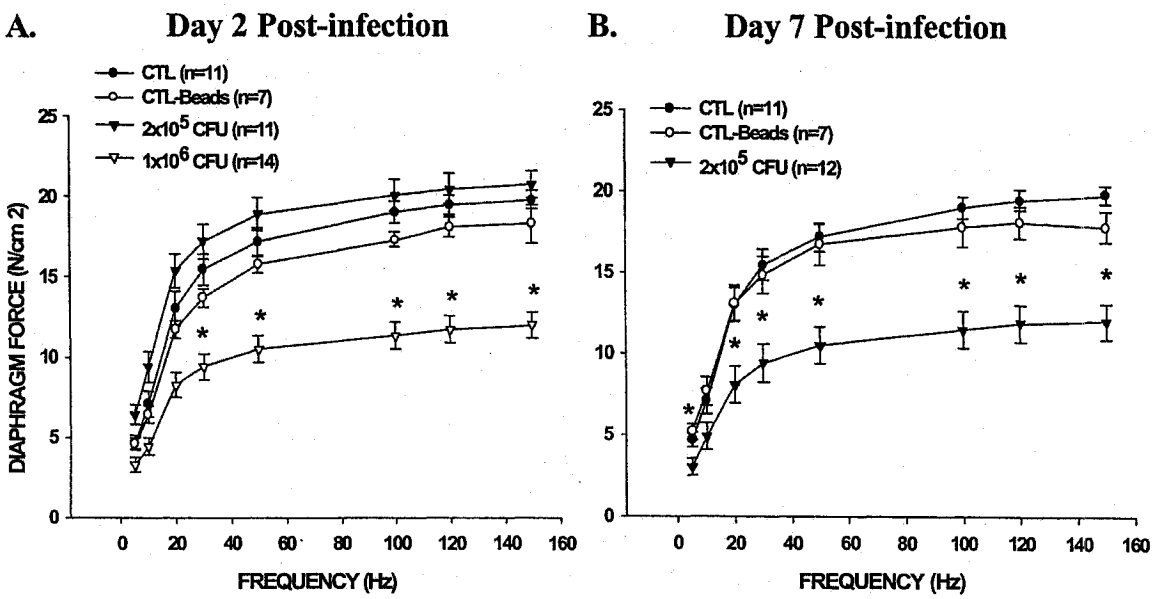

Figure 4. Effects of *Pseudomonas* lung infection on the diaphragmatic force-frequency relationship. **(A)** At Day 2 post-infection, diaphragmatic force was significantly decreased at a dose of $1x10^6$ but not with the lower dose of $2x10^5$ CFU. **(B)** At Day 7 post-infection, diaphragmatic force was also decreased at the lower infecting dose of $2x10^5$ CFU. Values are group means ± SE. *$P<0.05$ compared to CTL and CTL-Beads groups.

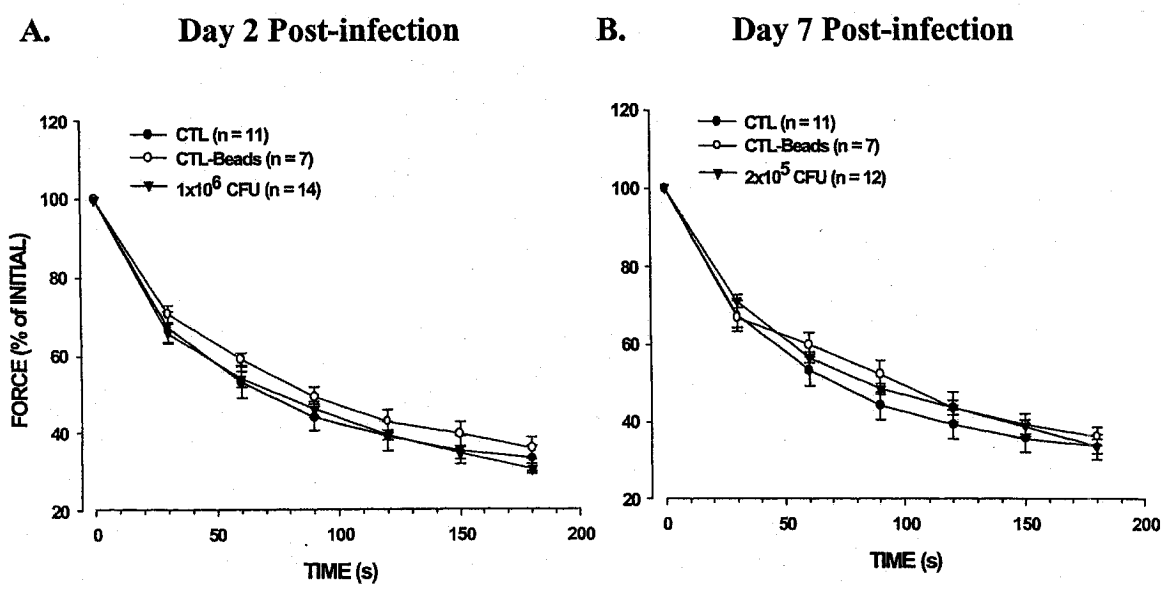

Figure 5. Effects of *Pseudomonas* lung infection on diaphragmatic endurance properties. Values are group means ± SE, and are expressed as a percentage of the initial force values obtained at the onset of the fatigue protocol. There were no significant differences among groups at either **(A)** Day 2, or **(B)** Day 7 post-infection.

	Total Cells (BAL)	Macrophages	Lymphocytes	PMNs
Max. Diaphragm Force Day 2 Post-infection	P=0.11 R=-0.36	P=0.87 R=-0.03	P=0.77 R=-0.11	P=0.17 R=-0.34
Max. Diaphragm Force Day 7 Post-infection	P=0.38 R=-0.12	P=0.85 R=-0.05	P=0.21 R=-0.37	P=0.93 R=-0.06

Table 1. Relationship between maximal force production by the diaphragm and BAL inflammatory cells. Data sets used for the calculations consisted of individual values obtained from the CTL-Beads and infected animal groups at the indicated time points. P=significance level; R=correlation coefficient.

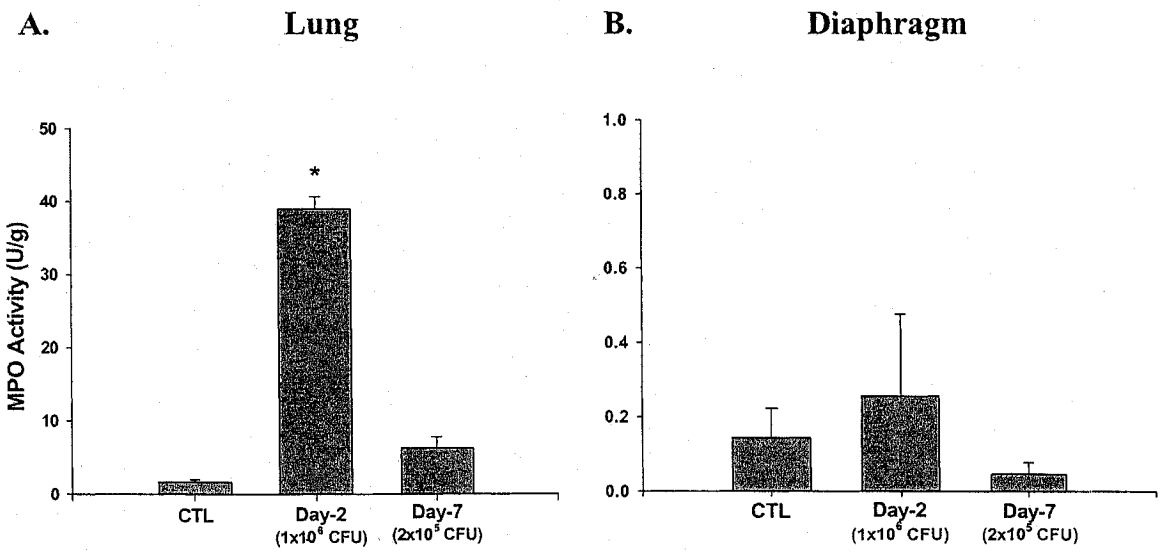

Figure 6. Myeloperoxidase (MPO) activity in lung and diaphragm after *Pseudomonas* infection. **(A)** In the lung, there was a large increase in MPO activity at day 2 post-infection, which then declined toward CTL values by day 7. **(B)** In the diaphragm, there was no significant effect of *Pseudomonas* lung infection on MPO activity, which remained extremely low (note the difference in y-axis scale as compared to the lung). Values are group means ± SE (n=6 per group). *P<0.05 compared to CTL.

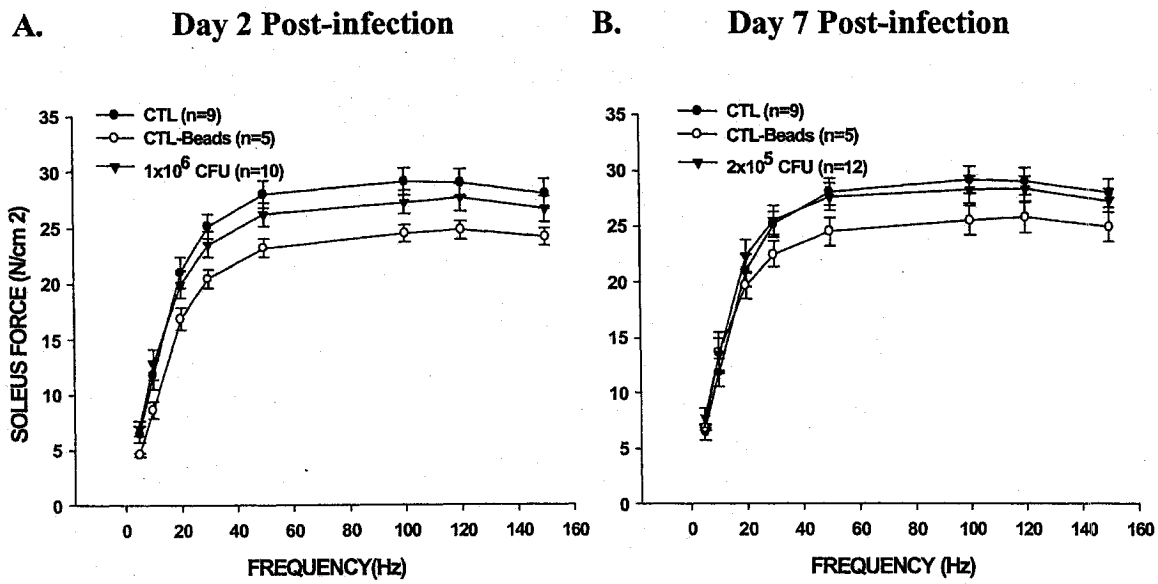

Figure 7. Effect of *Pseudomonas* lung infection on the limb muscle (soleus) force-frequency relationship. Values are group means ± SE. *Pseudomonas* lung infection had no significant effects on soleus muscle force production at either **(A)** Day 2, or **(B)** Day 7 post-infection.

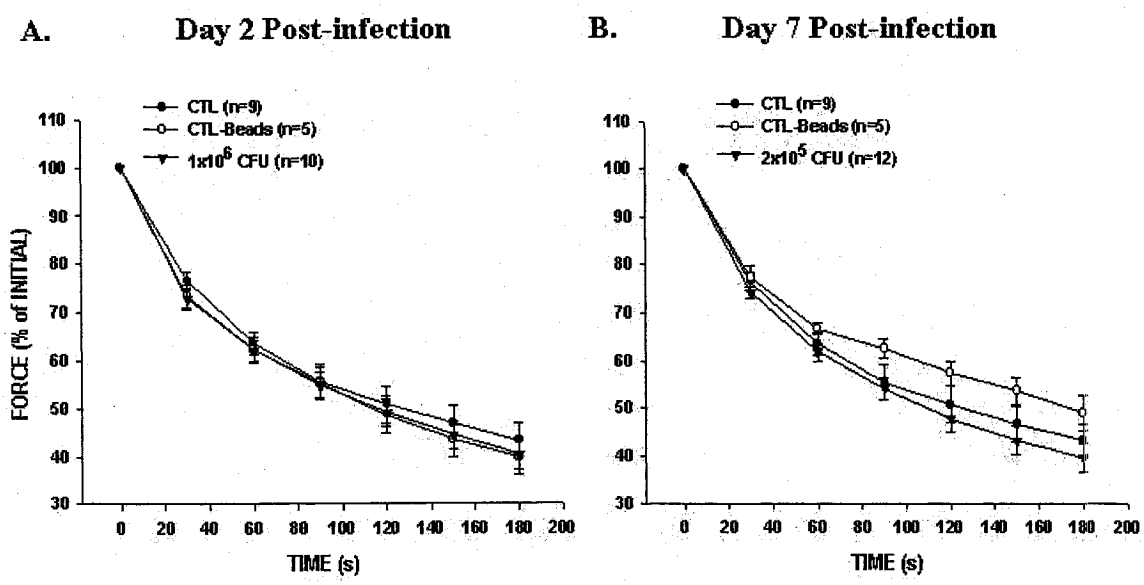

Figure 8. Effects of *Pseudomonas* lung infection on endurance properties of the soleus muscle. Values are group means ± SE, and are expressed as a percentage of the initial force values obtained at the onset of the fatigue protocol. There were no significant differences among groups at either **(A)** Day 2, or **(B)** Day 7 post-infection.

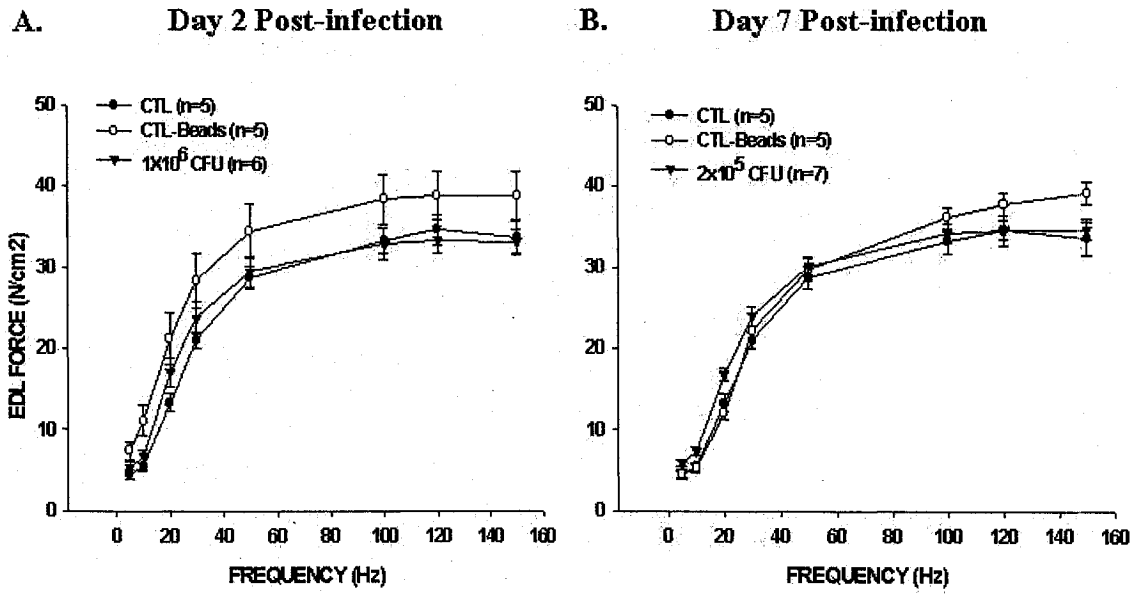

Figure 9. Effect of *Pseudomonas* lung infection on the force-frequency relationship of the extensor digitorum longus (EDL) muscle. Values are group means ± SE. *Pseudomonas* lung infection had no significant effects on EDL muscle force production at either **(A)** Day 2, or **(B)** Day 7 post-infection.

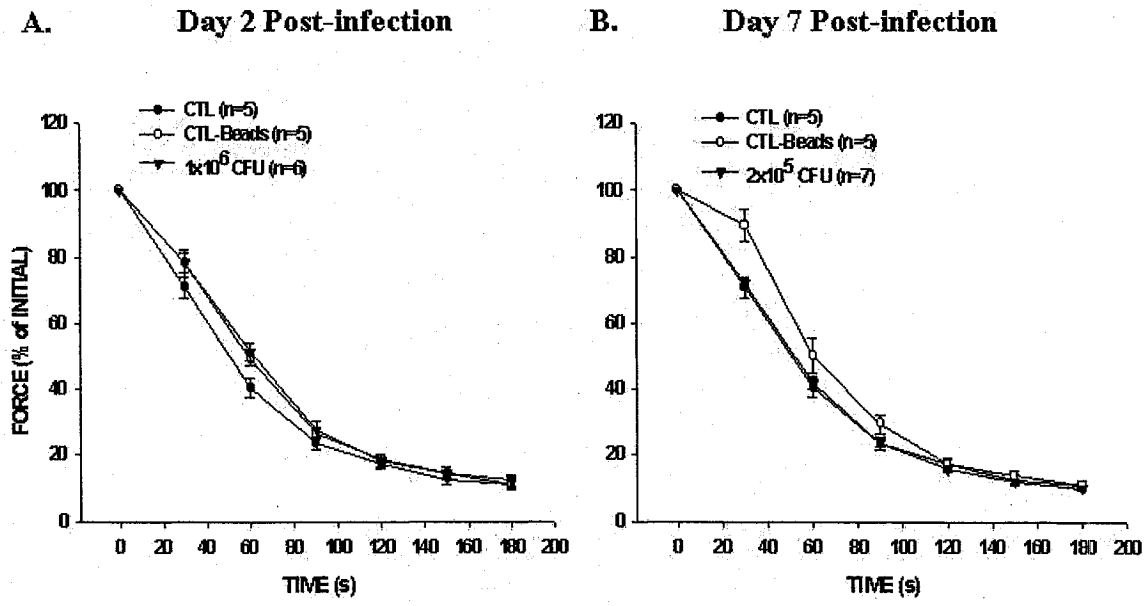

Figure 10. Effects of *Pseudomonas* lung infection on endurance properties of the extensor digitorum longus (EDL) muscle. Values are group means ± SE, and are expressed as a percentage of the initial force values obtained at the onset of the fatigue protocol. There were no significant differences among groups at either **(A)** Day 2, or **(B)** Day 7 post-infection.

2.VI Discussion

The present study demonstrates that persistent pulmonary infection with *P. aeruginosa* produces significant contractile dysfunction of the diaphragm. The major findings of our study can be summarized as follows: 1) the diaphragm was preferentially susceptible to the adverse effects of *P. aeruginosa* infection on skeletal muscle function, since hindlimb muscles displayed normal function under the same conditions; 2) the process was exacerbated by a more prolonged duration of infection; and 3) no statistically significant relationships were found between the level of diaphragmatic weakness after infection and either pulmonary bacterial burden or lung inflammatory cell counts within BAL fluid.

Pseudomonas Lung Infection Model

The *Pseudomonas* lung infection model utilized in the present study offers several advantages. First, it may be more clinically relevant than the high doses of lipopolysaccharide (LPS) typically used to induce diaphragmatic dysfunction in most studies. Second, it involves inclusion of additional virulence factors within the bacteria other than LPS (e.g., *Pseudomonas* exoenzyme S, a potent inducer of cytokine expression (21)), such that the full range of microbe versus host interactions can be expressed. Third, it produces a predominately neutrophilic inflammatory infiltrate within the lungs and associated tissue damage, which are both similar to changes observed in the infected CF lung (14). Finally, by virtue of its more sustained nature, the chronic lung infection model provides the ability to study responses at different stages of the infection process. On the other hand, the model used in our study also suffers from certain limitations. In particular, it does not precisely mimic the natural history of *P. aeruginosa* infection in CF from airway colonization to lung injury. In addition, the mice employed in our study lack impaired pulmonary defense mechanisms and other aspects of the multiorgan dysfunction found in CF patients.

To date, the vast majority of studies aimed at investigating the effects of sepsis on respiratory muscle function have employed LPS in order to produce a state of acute endotoxemia. Under these conditions, diaphragmatic dysfunction appears not to be caused by LPS itself, but rather by its ability to induce release of endogenous free radical species (22,23)

and other proinflammatory mediators (9,24). To our knowledge, the only study examining the effects of a chronic infection on diaphragmatic function was performed by Drew et al. (25), who found reduced specific force generation by the fast-twitch plantaris muscle, but not the diaphragm or soleus, at 7-12 weeks after infecting hamsters with the protozoan parasite *Leishmania donovani*. In addition, despite the high frequency of pneumonia as a clinical problem, few studies have examined the effects of pulmonary infection on diaphragmatic function. Desmecht et al. (26) performed intratracheal instillation of *Pasteurella haemolytica* in calves, and reported that a subset of animals displayed evidence of diaphragmatic dysfunction over a 10-hour period. Boczkowski et al. (27) also reported a significant reduction in diaphragmatic force production 3 days after subcutaneous inoculation of rats with *Streptococcus pneumoniae*, although there was no histological evidence of pneumonia in their model.

In immunocompetent mice, direct intratracheal inoculation or aerosolization of *P. aeruginosa* produces only transient infection, with essentially complete bacterial clearance from the lungs within 24-48 hrs (13,14). In order to induce a more sustained infection, we employed a model in which *P. aeruginosa* bacteria are first embedded in agar prior to intrapulmonary instillation. The ability of this method to achieve a chronic *Pseudomonas* lung infection has been validated in several animal species (14). However, because instillation of sterile agar beads alone can cause mild and transient mononuclear cell infiltration in the lungs (14), we also ascertained the effects of this intervention on BAL cell counts and skeletal muscle function. Importantly, no significant effects of sterile agar beads on these parameters were observed. In addition, we ascertained that intrapulmonary instillation of agar beads (either alone or combined with bacteria) had no significant effects on respiratory mechanics, thus confirming a previous report (20). Therefore, we believe that the changes found in our study can be attributed to *P. aeruginosa* infection rather than any non-specific effects related to the experimental procedure.

Role of Pulmonary Inflammation

There is a large literature implicating local pulmonary inflammation in the deterioration of lung function observed in CF patients (see (4) for review). Recently, there has also been increased interest in the idea that pulmonary inflammation and lung injury may trigger more widespread systemic inflammatory effects (5,7). Increased peripheral blood levels of various markers of

inflammation have been documented in patients with CF as well as other forms of chronic obstructive pulmonary disease (6,10-12,28). In addition, circulating TNF-α and C-reactive protein levels in CF are further increased in the setting of symptomatic respiratory exacerbations (11,12). Such findings have led to the suggestion that systemic manifestations of disease, including muscle wasting and weakness, may be caused by ongoing pulmonary inflammation.

In the present study, we sought to determine whether there is a direct relationship between either the number or type of inflammatory cells present within the lung and *P. aeruginosa*-induced diaphragmatic dysfunction. Previous studies have reported a significant correlation between BAL fluid neutrophils and infection-related weight loss in wild-type mice, as well as in genetically altered CF mice, following intrapulmonary instillation of *Pseudomonas*-laden agar beads (20,29). In our study, while there were trends relating total BAL cell count at day 2 post-infection and BAL lymphocyte count at day 7 post-infection with diaphragmatic weakness, none of the relationships examined were statistically significant. In addition, at the lower inoculating dose of $2x10^5$ CFU, severe diaphragmatic dysfunction developed between days 2 and 7 post-infection despite a fall in BAL inflammatory cell counts over the same time period.

There are several possible explanations for these findings. For example, a better correlation may have existed between diaphragmatic weakness and the levels of certain cytokines produced by pulmonary inflammation, rather than the numbers of inflammatory cells present within the lungs of infected animals. Although we cannot exclude this possibility, in the same model van Heeckeren et al. (20) reported that the correlations between infection-induced weight loss and either proinflammatory cytokine levels or absolute neutrophil counts within BAL fluid were of similar statistical strength. Another possibility is that inflammatory cells within the lung interstitium were not accurately reflected by the cells retrieved in BAL fluid, and that it is the former which are most involved in the systemic inflammatory response induced by *P. aeruginosa* lung infection. However, BAL fluid cell counts were previously found to be significantly correlated with infection-related weight loss as mentioned earlier (20,29). In addition, previous studies have generally reported a good relationship between BAL and whole lung inflammatory cell characteristics in this model (30-32).

We believe that differential regulation of the inflammatory response in the pulmonary and extra-pulmonary compartments is the most likely explanation for our findings. In support of

this proposition, it has recently been shown that in patients with chronic obstructive pulmonary disease (COPD), there is no direct correlation between sputum and serum levels of individual markers of inflammation, despite the fact that both sputum and serum show elevated levels of these markers compared to control subjects (6). This suggests that while there is no doubt cross-talk between the two compartments, the extra-pulmonary systemic inflammatory response does not simply reflect spillover from the lung, but is instead an independently regulated process. Moreover, it is important to note that proinflammatory mediators can be expressed by diaphragm muscle fibers themselves (9,22,33), and that the timing of such expression may differ from that found in neighboring inflammatory cells (22). Therefore, differences between the pulmonary and extra-pulmonary compartments in the specific mediators involved and/or the timing of their expression, likely accounts for the fact that certain aspects of the systemic response, such as contractile dysfunction of the diaphragm, do not correlate well with local pulmonary inflammation.

Preferential Weakness of the Diaphragm

A particularly interesting finding in our study was the presence of muscle-specific contractile impairment, i.e., in the diaphragm but not in limb muscles (EDL and soleus) of infected animals. The EDL is adapted for relatively infrequent bursts of phasic activity, whereas the soleus is tonically activated to maintain posture. The diaphragm is essentially always active except for very short pauses, even during sleep. However, differences in fiber type composition among these muscles are unlikely to have played a role in our findings, since the diaphragm is intermediate in this respect between the fast-twitch, glycolytic EDL and the slower-twitch, more oxidative soleus. In addition, our data do not support inflammatory cell infiltration into the muscle as a cause for the preferential diaphragmatic impairment, since neither histological nor biochemical (MPO activity) examination revealed any evidence of increased diaphragmatic inflammation in the infected mice.

Several prior studies (22,34,35) have reported a greater susceptibility of the diaphragm to the effects of endotoxemia in comparison to limb muscles. On the other hand, Supinski et al. (36) found equivalent reductions in force production by the diaphragm and flexor halluces

longus muscle after LPS injection. The precise reasons for these apparent discrepancies are not clear, but could relate to variations in the route, timing and dosage of LPS administration as well as species differences. In a transgenic mouse model of heart failure in which cardiac and serum (but not diaphragmatic) TNF-α levels are elevated, Li et al. (37) reported a major loss of force-generating capacity in the diaphragm, whereas the EDL and soleus muscles were unaffected. However, this same group also found no differences in the intrinsic susceptibility of isolated diaphragm and limb muscle fibers to tetanic force depression by TNF-α administered *ex vivo* (38).

We speculate that the greater activity level of the diaphragm *in vivo* may have contributed to its increased vulnerability to *P. aeruginosa* infection in our study. Muscle activity can potentially exacerbate diaphragmatic injury and weakness during sepsis through several mechanisms. These include: 1) an exaggerated generation of free radical species by contracting muscle fibers (23); 2) imposition of contraction-induced mechanical stress on muscle fiber membranes made hyperfragile by exposure to free radicals (39); and 3) increased exposure of muscle fibers to force-inhibiting cytokines, either through increased endogenous production of such molecules by the muscle fibers themselves (9,22,33) or via augmented flow of blood-borne molecules to working muscles (40). With regard to the latter, fever and increased respiratory rates associated with sepsis, although not directly documented in our study, would be expected to further increase blood flow to the diaphragm. In addition, while our data do not indicate an increased susceptibility to *in vitro* diaphragmatic fatigue after infection, this may not be the case *in vivo*. This is because the propensity to develop fatigue is inversely related to the maximal force-generating capacity of the muscle, as reflected by an increase in the tension-time index of the diaphragm (41). Therefore, diaphragmatic weakness *per se* favors the onset of diaphragmatic fatigue under conditions of spontaneous breathing *in vivo*.

It is also possible that the close proximity between the infected lung and the diaphragm contributed to the preferential impairment of diaphragmatic contractility. The peritoneal and pleural surfaces of the diaphragm are both lined by mesothelial cells, and beneath this layer lies a network of lymphatics (42-44). On the peritoneal side, small openings (stomata) connect the peritoneal cavity with these diaphragmatic lymphatics, and tracer studies have revealed that

substances injected intraperitoneally are capable of attaining the lymphatics as well as connective tissue spaces of the diaphragm (42). Similar but less frequent stomata have also been reported on the pleural surface of the diaphragm (43). Accordingly, it is conceivable that proximity and indeed direct communication between the diaphragmatic interstitial compartment and proinflammatory mediators induced within the pleural space by lung infection (45), might be involved in the loss of diaphragmatic force-generating capacity observed in our study.

Conclusions and Implications

In summary, we have shown that sustained lung infection with *P. aeruginosa* results in significant weakness of the diaphragm. Interestingly, even relatively mild respiratory tract infections have been found to cause further decreases in respiratory muscle strength, along with attendant hypercapnia, in patients with underlying respiratory muscle impairment (46). By impairing diaphragmatic function, chronic lung infection may similarly contribute to ventilatory insufficiency in patients with underlying lung disease from various causes, such as CF and COPD. To the extent that CF patients have a greatly reduced ability to clear *P. aeruginosa* from the lungs, this phenomenon could be particularly exaggerated in CF patients. Application of the *P. aeruginosa* infection model in genetically altered CF mice (16,29) could provide valuable insights into these questions.

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CHAPTER 3

IL-10 GENE TRANSFER ALTERS CYTOKINE BALANCE AND REVERSES WEAKNESS OF THE DIAPHRAGM DURING PSEUDOMONAS LUNG INFECTION

3.I Prologue

Sepsis is the systemic immune response to severe infections and is mediated through systemic release primarily of pro-inflammatory cytokines, like tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6 and secondarily of anti-inflammatory cytokines, like IL-10, soluble TNF receptor I and II, and IL-1 receptor antagonist. In addition, an equilibrium between the pro- and anti-inflammatory response is an important factor for the final outcome of patients with severe sepsis. There is considerable evidence that pro-inflammatory cytokines can contribute to muscle weakness through two major pathways: contractile dysfunction and muscle catabolism. Therefore, we sought to evaluate the levels of pro-inflammatory gene expression within the diaphragm of mice infected with *Pseudomonas aeruginosa*, and to determine the beneficial effects of suppressing such pro-inflammatory genes by an anti-inflammatory cytokine (IL-10) on diaphragmatic muscle weakness.

3.II Abstract

Dysfunction of the diaphragm can be produced by several pro-inflammatory mediators associated with sepsis. In the present study, we postulated two hypotheses: 1) pro-inflammatory gene expression would be upregulated in the diaphragm during lung infection with *Pseudomonas aeruginosa*; and 2) adenovirus-mediated IL-10 gene transfer would suppress intra-diaphragmatic pro-inflammatory gene expression and ameliorate diaphragmatic force-generating capacity under these conditions. Lung infection with high dose ($1x10^6$ CFU) of *P. aeruginosa*, highly up-regulates expression levels of cytokines (TNF-α, IL-1α, IL-1β, IL-6 and IL-18) in the diaphragm at day 2 post-infection. In contrast, the lower dose of infection at day 2 did not upregulate intra-diaphragmatic cytokine gene expression. Ad-IL-10 treatment of mice infected with the high dose of *P. aeruginosa* significantly inhibited induction of cytokines (TNF-α, IL-1α, IL-1β, IL-6 and IL-18) in the diaphragm, and also greatly improved diaphragmatic force production compare to control vector (Ad-dl-70). Taken together, our results suggest that lung infection with *Pseudomonas aeruginosa* increases pro-inflammatory gene expression by the diaphragm, which could contribute to diaphragm contractile dysfunction during sepsis. Adenovirus-mediated IL-10 gene delivery was able to restore normal diaphragmatic force-generating capacity under these conditions, suggesting a possible avenue for therapeutic intervention in severe pulmonary sepsis.

3.III Introduction

The diaphragm is the primary muscle of respiration. Impaired contractility of the diaphragm, leading to respiratory failure and death, is well-documented in animal models of sepsis (1). Sepsis can be defined as a systemic inflammatory response of the host to severe infection (2), which is mediated in large part through the production of pro-inflammatory effector molecules. Respiratory failure is a major clinical manifestation of sepsis, which greatly contributes to the high mortality associated with this condition (3). To date, most investigations of respiratory muscle dysfunction in sepsis have been performed in models involving high-dose endotoxin administration. However, we have recently reported that severe diaphragmatic weakness also occurs during a more low-grade and sustained form of Gram-negative infection, caused by instillation of *Pseudomonas aeruginosa* organisms into mouse lungs (4). *P. aeruginosa* is one of the most common causes of nosocomial pneumonia (5), and is also a major source of morbidity and mortality in patients with chronic lung diseases such as cystic fibrosis (6).

There is considerable evidence that diaphragmatic weakness may be induced by a number of pro-inflammatory mediators, including oxygen free radicals (7), nitric oxide (NO) (8), TNF- (9;10)and IL-1 (11). These pro-inflammatory mediators have the potential to cause muscle weakness via several pathways, including stimulation of muscle cell protein loss (12-16), interference with insulin receptor signaling (17;18), and direct depression of excitation-contraction coupling (19) or myofilament function (20). In addition to such pro-inflammatory mediators, anti-inflammatory effector molecules are also activated during sepsis (21). Indeed, an important determinant of the final clinical outcome in septic patients may be the systemic and tissue equilibrium between pro-inflammatory and anti-inflammatory effector molecules (22). With respect to the latter, IL-10 is the probably the most well-established of the anti-inflammatory cytokines, and was originally termed cytokine synthesis inhibitory factor (23). IL-10 exerts its anti-inflammatory effects via several complex mechanisms including inhibition of cytokine expression (24-26), interference with antigen presentation (27;28), and suppression of costimulatory molecules (29). In animal models of endotoxemia, IL-10 can inhibit the expression of pro-inflammatory cytokines such as TNF- and IL-6 in the lung and serum (30), as well as reduce mortality (31).

In the present study, we hypothesized that pulmonary infection with *P. aeruginosa* would lead to significant upregulation of pro-inflammatory effector molecules in the diaphragm, and that this would occur in a bacterial dose-dependent manner. Intriguingly, whereas diaphragmatic contractility was greatly impaired during *P. aeruginosa* lung infection in a previous study performed by our group, limb muscle function was unaffected under the same conditions (4). Therefore, we also postulated that pro-inflammatory effector molecule production by the diaphragm and limb muscles could be different in the setting of *P. aeruginosa* infection. Finally, we wondered whether pro-inflammatory mediator gene expression by the diaphragm, as well as associated diaphragmatic weakness, could be beneficially modulated by shifting the systemic balance between pro- and anti-inflammatory cytokines in favor of the latter. Accordingly, our specific objectives were as follows: (1) To ascertain the effects of different levels of *P. aeruginosa* lung infection on pro-inflammatory mediator gene expression by the diaphragm. The mediators targeted by our study (iNOS, TNF-, IL-1, IL-6, IL-18) were specifically selected on the basis of previous studies indicating their potential role in muscle wasting and weakness (8-12;14;15;32-34); (2) To compare the magnitude and pattern of pro-inflammatory mediator expression between diaphragm and limb muscle under identical conditions of *P. aeruginosa* lung infection; and (3) To determine whether systemic delivery of IL-10, achieved through adenovirus-mediated IL-10 gene transfer, could suppress pro-inflammatory mediator expression and improve force production in the diaphragms of *Pseudomonas*- infected mice.

3.IV Materials and Methods

Animal model of sustained *P. aeruginosa* infection

Studies were performed in 8-10 week old C57BL/6 mice, which were used in accordance with the guidelines established by the Canadian Council on Animal Care. The model of chronic pulmonary infection with *P. aeruginosa* employed in this study was performed essentially as described by Starke and colleagues (35), with minor modifications. A mucoid strain of *P. aeruginosa* (strain 508) was used, which was originally isolated from a cystic fibrosis patient (36). Briefly, log-phase *P. aeruginosa* bacteria were concentrated and mixed with 1.5% trypticase soy agar pre-warmed to 52°C. This mixture was added to heavy mineral oil at 52°C and rapidly stirred for 6 min, followed by cooling for 10 min at 4°C. After removing excess oil, the bacteria-bead preparation was washed extensively and resuspended in sterile phosphate-buffered saline (PBS) at pH 7.4. The size (100-150 um) and uniformity of the beads were verified by light microscopy. In addition, the number of viable bacteria trapped within the agar beads was measured by homogenizing the beads and then plating 10-fold serial dilutions on trypticase soy agar plates. Sterile agar beads were produced in the same manner but with omission of bacteria, and were confirmed to be free of colony-forming units (cfu). To deliver bacteria-containing agar beads to mouse lungs, the mice were first anesthetized with a combination of ketamine (130 mg/kg) and xylazine (20 mg/kg) injected intramuscularly. A small incision was made at the midline of the neck in order to expose the trachea. The trachea was then intubated with a sterile flexible 22-ga cannula attached to a 1 ml syringe, which was used to inject 50 ul of the agar bead suspension into the lungs. Mice were infected with two different dose of bacteria: $2x10^5$ cfu and $1x10^6$ cfu. Mice were euthanized at day 2 post-infection. All animal procedures were approved by the institutional animal care committee.

Evaluation of pro-inflammatory gene expression levels

RNase protection assays were employed to quantify tissue mRNA levels. Samples of total RNA were isolated from mouse tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). ^{32}P-labelled riboprobes were synthesized using a commercial mouse multiprobe kit (BD Biosciences, Pharmingen, San Diego, CA) containing templates against the following gene transcripts: iNOS, TNF-, IL-1, IL-1, IL-6, and IL-18. The riboprobes were hybridized with each RNA sample

overnight at 56°C according to the manufacturer's instructions, using 20 ug of RNA for muscle tissues (diaphragm, soleus) and 10 ug of RNA for the lungs. The protected RNA fragments were separated using a 5% polyacrylamide gel and detected by autoradiography. Bands representing the individual mRNA species were then quantified using an image analysis system (FluorChem 8000, Alpha Innotech Corp, San Leandro, CA), and the signals were normalized to the L32 housekeeping gene to control for loading in each lane.

IL-10 receptor expression

IL-10 receptor (IL-10R□) mRNA expression by the diaphragm *in vivo* and *in vitro* was evaluated by RT-PCR, using total RNA (1 ug) obtained from diaphragm tissues or cultured primary diaphragmatic myotubes, respectively. The latter were prepared from isolated single muscle fibers as previously described in detail (37). RT was performed using M-MLV reverse transcriptase and random primers (Promega, Madison, WI). PCR amplification of the cDNA was performed with primers which span intron one, and consist of the following sequences (5' to 3'): CCCATTCCTCGTCACGATCTC (forward), and TCAGACTGGTTTGGGATAGGTTT (reverse). Amplification was performed for 45 cycles with a denaturation step at 95°C, annealing at 57°C, and extension at 72°C. The resulting PCR product (predicted amplicon size of 141 bp) was visualized on an agarose gel containing ethidium bromide.

IL-10 gene transfer and measurement of serum IL-10 levels

Construction of the recombinant adenoviral vector (Ad5E1mIL-10), containing the murine IL-10 cDNA driven by the human cytomegalovirus promoter, has been previously described in detail (30). Ad5E1mIL-10 ($0.1x10^9$ pfu) was diluted in 100 μl PBS and injected intramuscularly into the hindlimbs (two injection sites per leg, 25 μl per site) at the time of lung infection. The above injection protocol was based upon a prior investigation showing successful inhibition of circulating pro-inflammatory cytokines in a mouse endotoxemia model (30). Mice injected under identical conditions with an empty adenoviral vector (Ad5dl70-3), i.e., lacking the IL-10 transgene, served as a control group. To quantify serum levels of IL-10, a commercially available double sandwich ELISA method (R&D Systems, Minneapolis, MN, USA) was employed according to manufacturer's instructions. The sensitivity of detection was 5 pg/ml or

less. Sera were prepared from blood samples obtained by retro-orbital bleeding at days 0, 1, and 2 post-injection of the adenoviral vectors.

Analysis of diaphragm contractile function

After euthanasia, the diaphragm muscle was surgically excised for *in vitro* contractility measurements under isometric conditions as previously described in detail (38). After removal from the animal, the diaphragm was immediately placed into a chilled (4°C) and equilibrated (95% O2-5%CO2; pH 7.38) Krebs solution with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1 KH2PO4, 25 NaHCO3, and 11 glucose. A muscle strip approximately 2 mm wide was dissected free, taking care to leave the central tendon and adjacent rib cage margins intact. The excised diaphragm strip was mounted into a jacketed tissue bath chamber filled with equilibrated Krebs solution, using a custom-built muscle holder containing two stimulation electrodes located on either side. The central tendon was tied to an isometric force transducer using 4-0 silk thread. A thermoequilibration period of 15 min was observed prior to initiating contractile measurements at 23°C. After placing the diaphragm strip at optimal length (Lo), the force-frequency relationship was determined by sequential supramaximal stimulation for 1 sec at 5, 10, 20, 30, 50, 100, 120, and 150 Hz, with 2 min between each stimulation train. The force data were acquired to computer at a sampling rate of 1000 Hz for later analysis. After completion of the above contractility studies, the muscles were removed from their baths, and muscle length was measured with a microcaliper accurate to 0.1 mm. Muscle cross-sectional area was determined by assuming a muscle density of 1.056 g/cm^3, which allowed specific force (force/cross-sectional area) to be calculated and expressed in $Newtons/cm^2$.

Statistical analysis

All data are presented as mean values ±SE. Group mean differences were determined by analysis of variance, with *post hoc* application of the Tukey test where appropriate. A statistics software package was used for all analyses (SigmaStat V2.0, SPSS, Chicago, IL). Statistical difference was defined as a p value less than 0.05.

3.V Results

Upregulation of pro-inflammatory genes in the diaphragm is bacterial dose-dependent

RNase protection assay (RPA) was used to compare expression levels of pro-inflammatory genes in three groups of mice: (1) Uninfected controls (CTL), (2) High-dose infection ($1x10^6$ cfu), and (3) Low-dose infection ($2x10^5$ cfu). Figure 1 shows a representative RPA performed on total RNA extracted from the diaphragm in all 3 experimental groups at 48 hours post-infection, whereas Fig. 2 depicts quantitative group mean data. In the uninfected CTL group, there was a degree of constitutive basal expression of all the cytokines examined, the magnitude of which varied depending upon the cytokine in question.

After high-dose infection, there was substantial upregulation of TNF-α, IL-1α, IL-β, IL-6, and IL-18 within the diaphragm. In contrast, low-dose infection did not significantly modify the expression levels of these same cytokines in the diaphragm. In addition, the response to *P. aeruginosa* infection was markedly different in the limb muscle as compared to the diaphragm. Hence even high-dose *P. aeruginosa* infection did not significantly affect the level of pro-inflammatory cytokine expression in the hindlimb muscle (see Fig. 3). Interestingly, iNOS showed the highest level of constitutive expression in the diaphragm but was not significantly modified by either high- or low-dose infection with *P. aeruginosa.*

Reduced pro-inflammatory gene expression and improved function of the diaphragm after IL-10 gene transfer

We confirmed by RT-PCR that the IL-10 receptor is expressed by the diaphragm in vivo as well as in cultured diaphragm muscle cells (see Figure 4). To determine whether adenovirus-mediated IL-10 gene transfer could modify cytokine responses in the diaphragm during P. *aeruginosa* lung infection, mice in the high-dose infection group received Ad-IL-10 ($0.1x10^9$ pfu by i.m. injection) at the time of pulmonary inoculation. Mice identically treated with an empty adenoviral vector (i.e., lacking the IL-10 transgene) served as a control group. There was no detectable IL-10 protein by ELISA in the serum of uninfected CTL mice (data not shown). Figure 5 shows the levels of IL-10 present in the serum at different time points after high-dose *P. aeruginosa* infection. In mice treated with Ad-IL-10, serum IL-10 levels were maximal at day 1

and then began to taper off by day 2. Serum levels of IL-10 were detectable but significantly lower in the infected mice treated with empty vector, and did not differ between days 1 and 2.

Figure 6 shows the effects of Ad-IL-10 on pro-inflammatory cytokine expression levels in the lung, as determined by RPA. As expected, high-dose *P. aeruginosa* infection led to marked upregulation of pro-inflammatory gene expression in the lung, with the exception of IL-6 (not detectable). However, there was no evident effect of IL-10 gene transfer on the expression levels of these different inflammatory cytokines in the lung, when compared to identically infected mice treated with either empty vector or PBS injection.

To determine the effects of IL-10 gene transfer on pro-inflammatory cytokine expression in the diaphragm under the same conditions, RPA was performed on diaphragms of the high-dose infected mice. As shown in Figs. 7 and 8, mice treated with Ad-IL-10 exhibited a significant blunting of the cytokine mRNA upregulation normally found after high-dose *P. aeruginosa* infection. With the exception of iNOS, this was true for all of the pro-inflammatory genes examined.

Finally, we wished to ascertain whether the above findings were associated with any alterations of diaphragmatic contractile function. Figure 9 illustrates the effects of Ad-IL-10 treatment on diaphragmatic force-generating capacity in the high-dose infection group. As we have previously reported (4), diaphragmatic force production was greatly depressed at 48 hours after high-dose pulmonary infection with *P. aeruginosa* in the control group. However, in mice identically infected mice and treated with Ad-IL-10, the force-generating capacity of the muscle was greatly ameliorated, as reflected by a major upward shift in the force-frequency relationship.

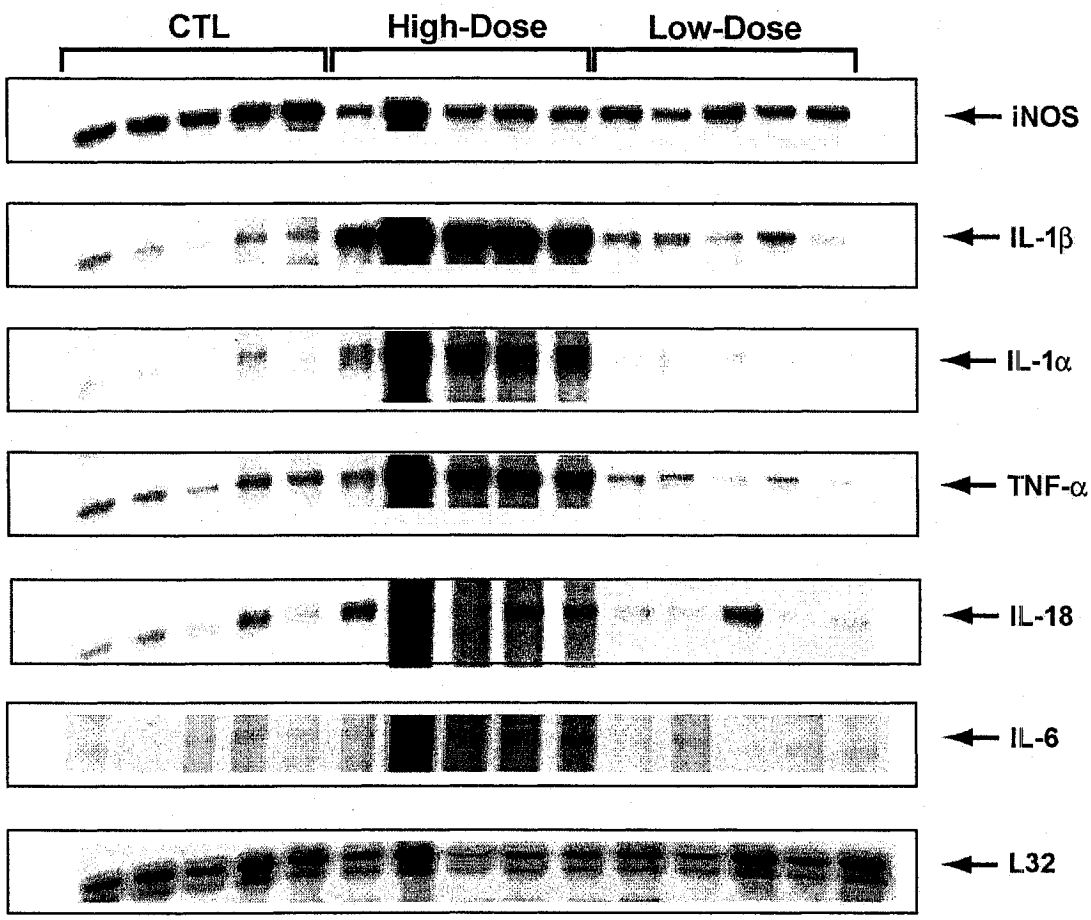

Figure 1. Representative autoradiograph of RNase protection assay showing the dose-dependent of pro-inflammatory gene expression in the diaphragm. Lanes 1-5: diaphragm samples from control non-infected mice. Lane 6-10: diaphragm samples obtained from infected animals with the high dose of *P. aeruginosa* ($1x10^6$ cfu). Lane 11-15: diaphragm samples collected from infected animals with low dose of *P. aeruginosa* ($2x10^5$ cfu).Mice were all sacrificed at day 2 post-infection. A total of 20 μg RNA was loaded in each lane.

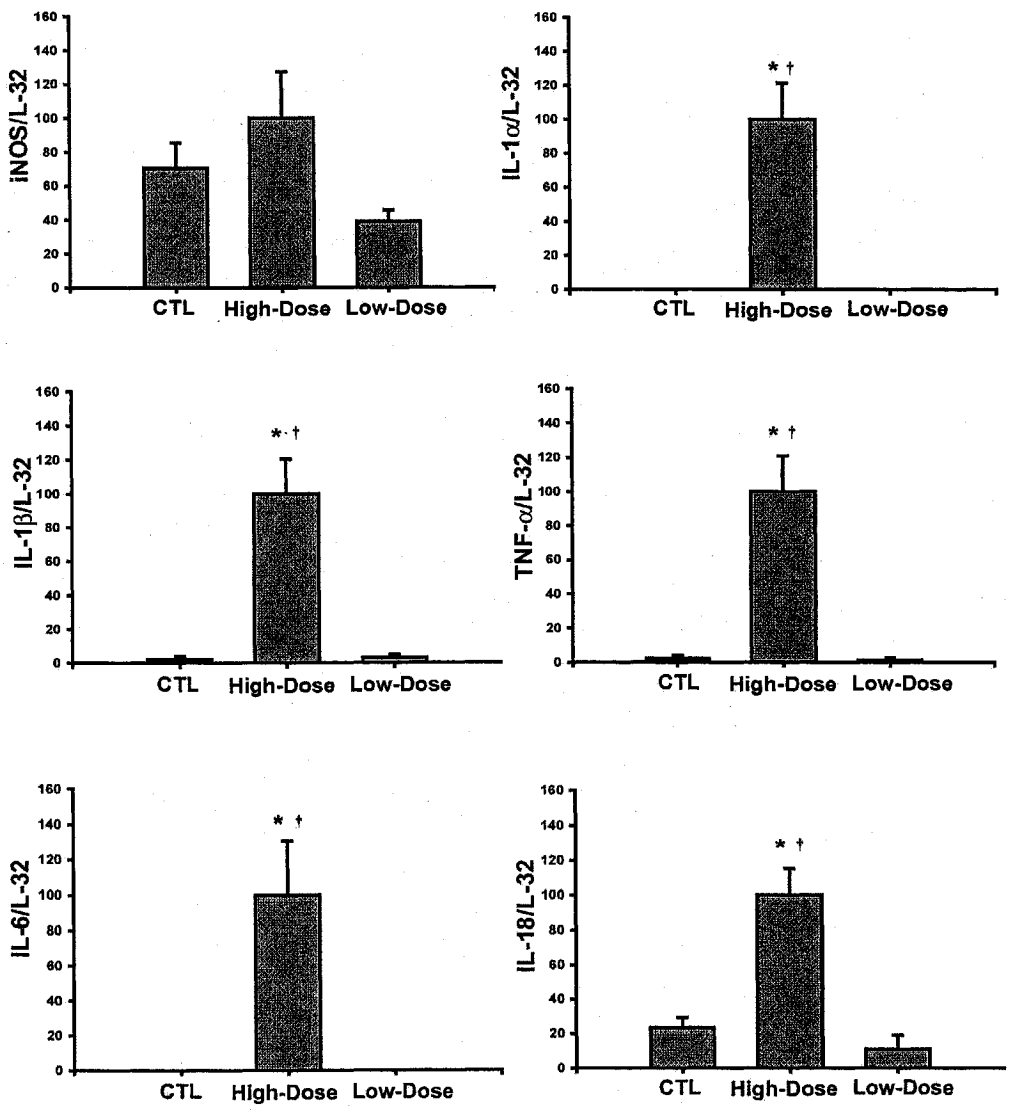

Figure 2. Group mean values for the RPA as shown in Fig.1. With the exception of iNOS, expression levels of cytokines (TNF-α, IL-1α, IL-β, IL-6, and IL-18) were significantly higher only in the diaphragm of infected group with the high dose of *P. aeruginosa* ($1x10^6$ cfu). Data are expressed as percentage increased above the infected group with high dose. All data were normalized to L-32 to account for potential variation in loading between lanes. *p⟨0.05 for diaphragm of infected groups versus control (non-infected group); † p⟨0.05 high dose versus low dose of infection.

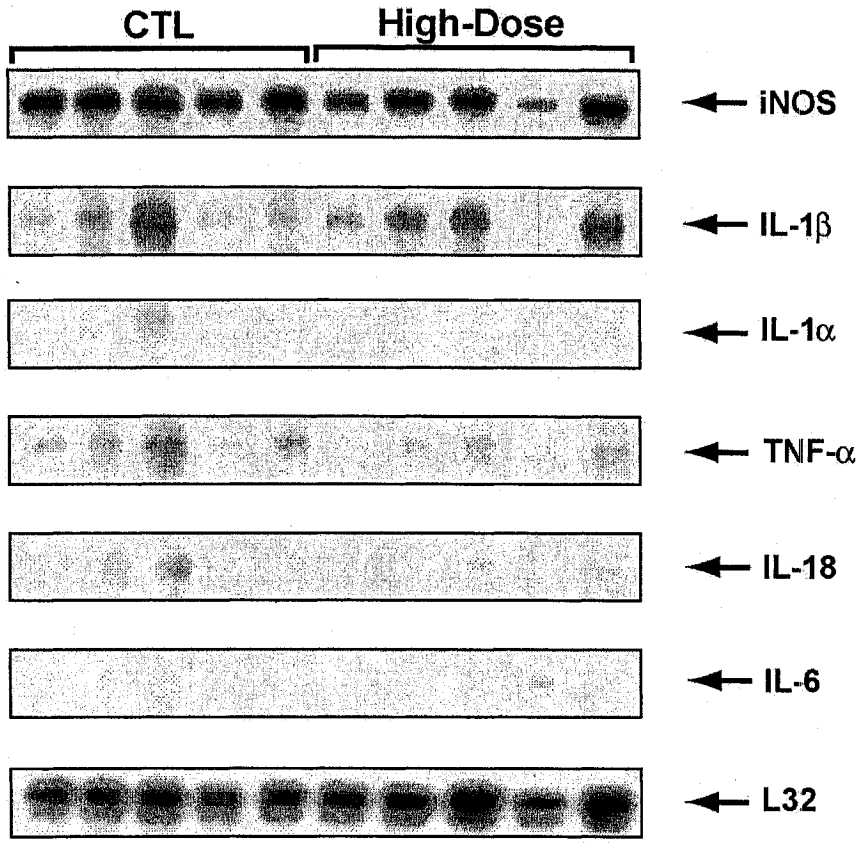

Figure 3. Representative autoradiograph of RNase protection assay from hindlimb muscle (soleus) muscle under basal (non-infected group) and infected group with the high dose of *P. aeruginosa* lung infection ($1x10^6$ cfu). RPA revealed no sign of up-regulation of pro-inflammatory genes within the soleus after infection. A total of 20 μg RNA was loaded in each lane.

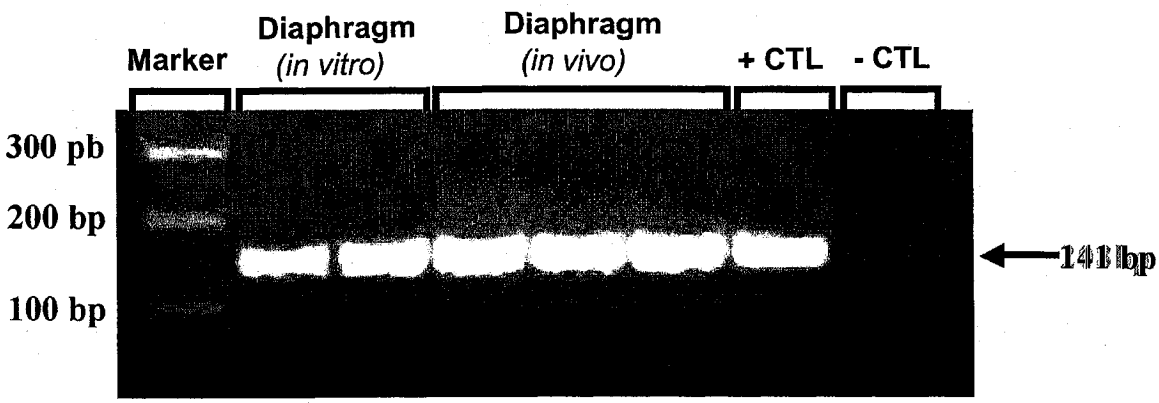

Figure 4. RT-PCR was performed on total RNA obtained from diaphragm tissue (*in vivo*) or primary cultures of diaphragm at the myotube stage (*in vitro*).

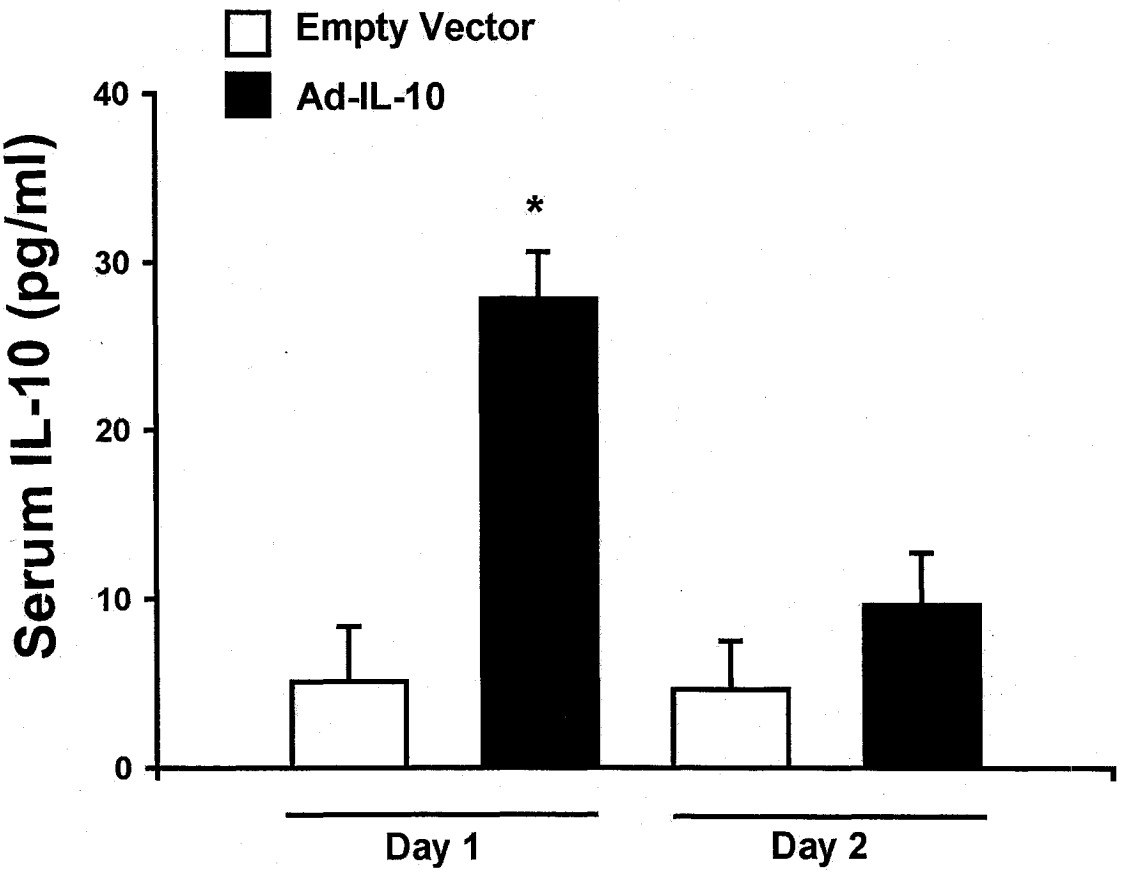

Figure 5. Transgene protein IL-10 expression after intramuscular gene transfer. Both Ad5E1mIL-10 and the control empty-vector Ad5E1dl70-3 with the dose of $0.1x10^9$ pfu were given i.m. to mice and sera collected at different time-points and assayed for mIL-10 by ELISA. The protein expression of IL-10 was significantly higher after day 1 post-injection with Ad5E1mIL-10. Data are expressed as means ± SEM; n=6 mice per group. *p⟨0.05 for Ad5E1mIL-10 treated versus control empty-vector (Ad5E1dl70-3) group.

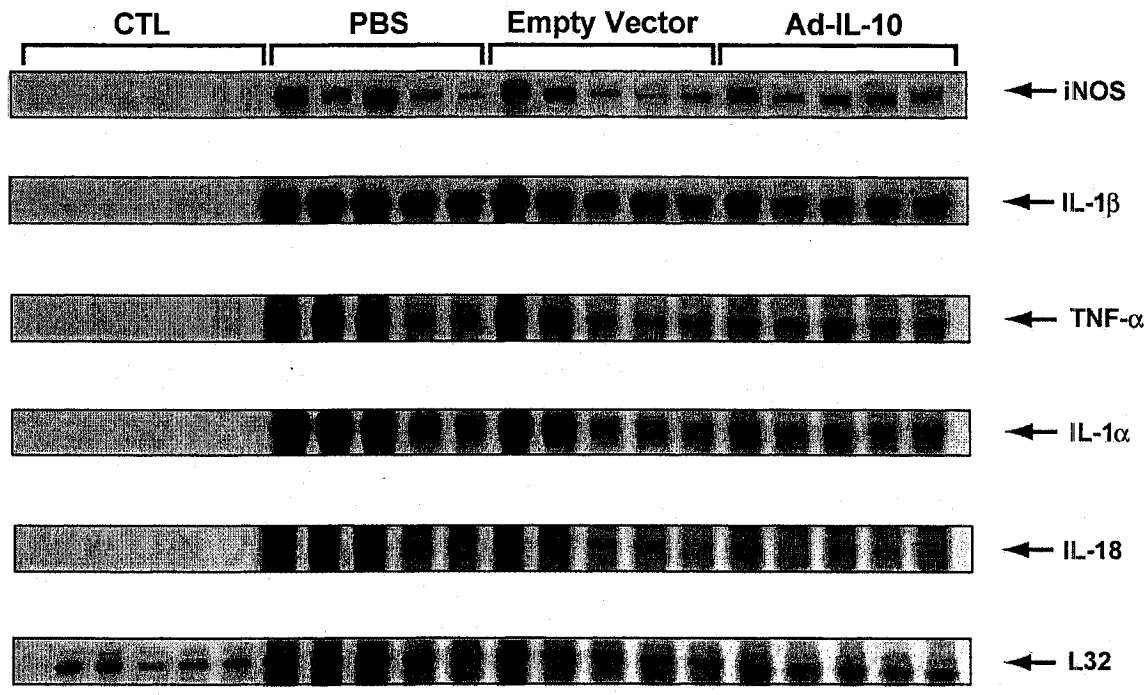

Figure 6. Representative autoradiograph of RNase protection assay from the lungs in four groups of mice: (1) uninfected control (CTL), (2) infected group treated with PBS, (3) infected group treated with empty-vetor, and (4) infected group treated with Ad-IL-10. *P. aeruginosa* lung infection ($1x10^6$ cfu) increased the levels of expression of pro-inflammatory mediators and there was no differences between infected animals treated with Ad-IL-10, empty-vector or PBS groups. A total of 10 μg RNA was loaded in each lane.

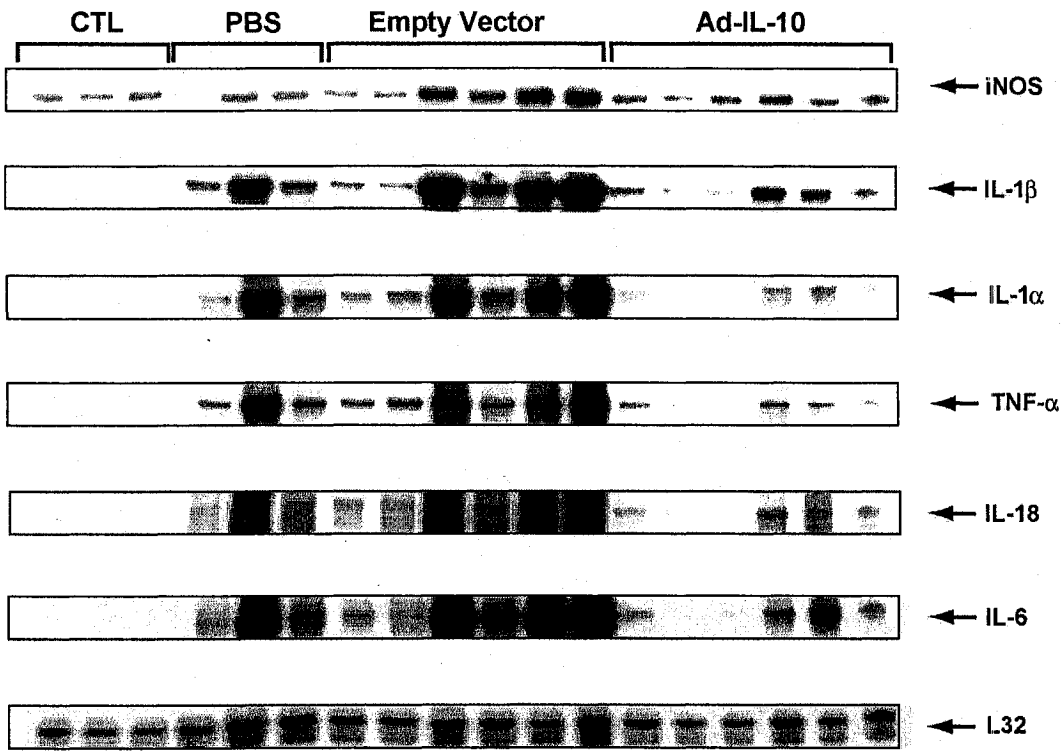

Figure 7. The effect of Ad5E1mIL-10 gene expression on diaphragmatic pro-inflammatory gene expression. Lanes 1-3: diaphragms of non-infected mice. Lanes 4-6: diaphragms obtained from infected mice that was injected (i.m.) with PBS. Lanes 7-12: diaphragms collected from infected mice that was treated (i.m.) with control empty-vector (Ad5E1dl70-3). Lanes 13-18: diaphragm obtained from infected mice injected (i.m.) with Ad5E1mIL-10. All mice were infected with the high dose of *P. aeruginosa* ($1x10^6$ cfu) and sacrificed day 2 post-infection. A total of 20 μg RNA was loaded in each lane.

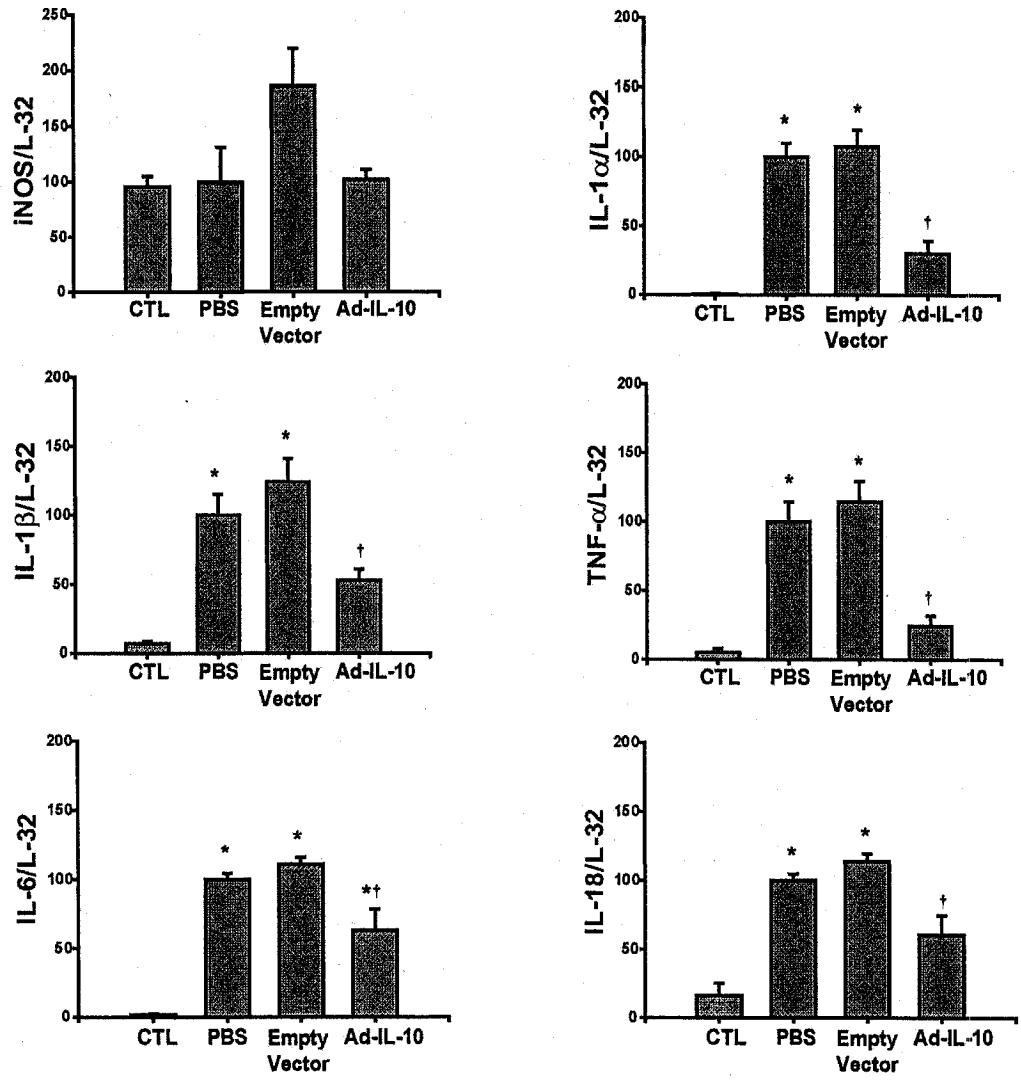

Figure 8. Expression levels of pro-inflammatory cytokines were suppressed after Ad5E1IL-10 gene transfer. Group mean values of pro-inflammatory gene expression as shown in Fig. 5. With the exception of iNOS, the expression levels of pro-inflammatory cytokines (TNF-α, IL-1α, IL-β, IL-6, and IL-18) were significantly attenuated after IL-10 gene transfer. All data were normalized to L32 to account for potential variations in loading between lanes. *p⟨0.05 infected groups (PBS, empty-vector, and Ad5E1IL-10) versus control (non-infected group); † p⟨0.05 for infected mice treated with Ad5E1IL-10 versus infected mice treated with empty vector (Ad5E1dl70-3).

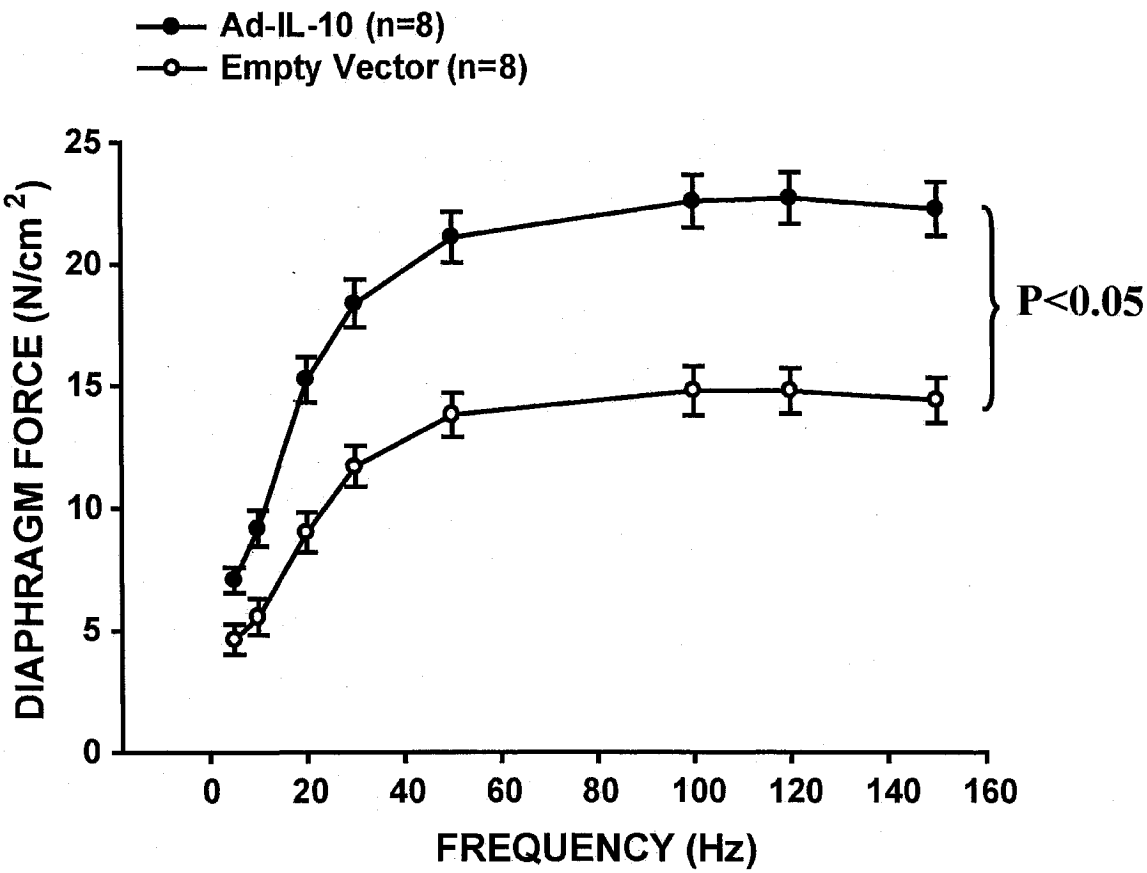

Figure 9. Effects of IL-10 gene transfer on the diaphragmatic force-frequency relationship. At day 2 after infection, diaphragmatic force was significantly ameliorated at each stimulation frequency in Ad5E1IL-10 compared to empty-vector treated (Ad5E1dl70-3) groups. All mice were infected with the high dose of *P. aeruginosa* ($1x10^6$ cfu). Values are group means ± SEM; n=8 mice per group. *p⟨0.05 compared with control empty-vector treated groups.

3.VI Discussion

Given the fact that reductions in skeletal muscle mass and function are associated with increased morbidity as well as mortality in patients with chronic lung disease (39), there is a need to better understand this process and its possible link to pulmonary infection. Despite its potential clinical importance, there is very little information available regarding the relationship between pulmonary infection and skeletal muscle properties. Skeletal muscle dysfunction, including that involving the diaphragm, is frequent in patients with cystic fibrosis and other forms of chronic pulmonary disease. The frequency of lung infection with *P. aeruginosa* is also greatly increased in such patients, as well as being one of the most common etiologies of nosocomial pneumonia in general.

In the present study, our primary objective was to determine the nature and magnitude of pro-inflammatory gene expression within the diaphragm and limb muscle after pulmonary infection with *P. aeruginosa*. We also wished to determine whether anti-inflammatory therapy with IL-10 gene transfer could suppress intra-diaphragmatic pro-inflammatory mediator expression and thus ameliorate diaphragmatic muscle function in this setting. The principal findings of our study were as follows: (1) Pro-inflammatory cytokine mRNA levels (TNF-α, IL-1α, IL-1β, IL-6, IL-18) were highly induced within the diaphragm during *P. aeruginosa* lung infection, and this occurred in a bacterial dose-dependent manner; (2) Induction of cytokine expression did not occur in the limb muscle under the same conditions, indicating a preferential sensitivity of the diaphragm to cytokine upregulation by pulmonary infection; and (3) IL-10 gene transfer led not only to greatly attenuated cytokine upregulation within the diaphragm during *P. aeruginosa* lung infection, but also restored diaphragmatic force-generating capacity to normal levels.

Pro-inflammatory gene expression in the diaphragm during pulmonary infection

This is the first study to specifically examine the effects of pulmonary infection on pro-inflammatory cytokine expression within the diaphragm. Most investigations of sepsis effects on skeletal muscle have been carried out in models of high-dose LPS exposure (40) (7) (41). However, the plasma cytokine profile in such models differs greatly from *P. aeruginosa* lung

infection (42), and one might therefore anticipate a similar divergence for skeletal muscle effects. We believe the lung infection model employed in our study offers a number of advantages. These include: (1) a more "realistic" model, to the extent that high doses of LPS typically employed in sepsis models greatly exceed what is encountered in most clinical infections; (2) inclusion of additional virulence factors within the bacteria other than LPS; and (3) by virtue of its more sustained nature, the ability to study a richer spectrum of responses at different stages of the infection. With regard to the latter, we found persistent upregulation of multiple pro-inflammatory cytokines within the diaphragm after 48 hours of ongoing pulmonary infection. This is likely to be functionally important, since the adverse effects of pro-inflammatory cytokines on muscle are more potent when multiple cytokine members are present at the same time (43).

An intriguing finding in our study was the observation that the increased cytokine mRNA levels found in the diaphragm were not mirrored by similar changes in the limb muscle. This differential response of diaphragm and limb muscle to *P. aeruginosa* lung infection is consistent with a previous study by our group (4), in which we found a greater vulnerability of the diaphragm to contractile dysfunction under the same conditions. Some (8;41) (44) but not all (45) studies by other groups have also reported a greater susceptibility of the diaphragm to LPS effects in comparison with limb muscles. The reason for such heterogeneous responses are not well understood, but could be related to intrinsic differences at the cellular level as well as different levels of exposure to environmental factors. With respect to the former, oligonucleotide microarrays have revealed major differences in constitutive gene expression between diaphragm and limb muscles, which go well beyond what can be accounted for simply on the basis of fiber type differences (46). Indeed, the gene expression pattern observed in the diaphragm suggests a higher presence of antigen-presenting cells and other immune-responsive elements (46). In addition, the diaphragm could be more vulnerable due to its greater inherent workload, and this difference would be further exaggerated during pulmonary infection. It has recently been reported that even in the absence of sepsis, inspiratory resistive loading causes significant upregulation of cytokines within the diaphragm (47). Because an increased workload will increase blood flow to the muscle, this could also result in greater exposure of diaphragm

muscle fibers to blood-borne pro-inflammatory effectors, such as bacterial breakdown products or circulating cytokines.

Implications for diaphragmatic function

In a previous study we observed that diaphragmatic weakness was present after *P. aeruginosa* pulmonary infection at the high dose of $1x10^6$ cfu, but not the low dose of $2x10^5$ cfu (4). In addition, even the higher dose of pulmonary infection did not cause weakness of the limb muscles (4). Therefore, the same conditions which failed to cause muscle weakness in the previous study also failed to induce pro-inflammatory cytokine upregulation within the corresponding muscles in the present investigation. Hence, under each of the scenarios of pulmonary infection studied thus far, experimental conditions which produced higher levels of pro-inflammatory gene expression in muscle were also associated with increased muscle weakness. Taken together, these findings suggest a relationship between pro-inflammatory cytokine upregulation in muscle and the presence of contractile impairment. This is further supported by the fact that IL-10 gene transfer was able to largely abrogate cytokine upregulation in the diaphragm and simultaneously prevent diaphragmatic dysfunction during high-dose pulmonary infection.

These findings are in agreement with existing evidence that the pro-inflammatory cytokines upregulated within the diaphragm in our study can cause muscle wasting and contractile dysfunction (48). For example, TNF-α has myriad adverse effects on muscle, including the ability to directly inhibit force production (20), reduce protein synthesis (49), enhance protein degradation (50), and destabilize myogenic transcription factors such as MyoD (guttridge for mRNA) (51). IL-1β also inhibits muscle protein synthesis (14), possibly by inducing resistance to IGF-1 (18). To our knowledge, IL-18 expression in skeletal muscle has not been previously reported. IL-18 is another member of the IL-1 cytokine family, and its upregulation in cardiac muscle has been associated with myocardial dysfunction (52), (53). IL-6 upregulates the cathepsin as well as ubiquitin pathways and favors muscle proteolysis (33). Interestingly, while iNOS has been implicated in the pathogenesis of diaphragmatic contractile dysfunction in LPS models (8) (54) iNOS mRNA levels in the diaphragm were not significantly modified by *P. aeruginosa* lung infection in the present study. Many of the adverse effects of

cytokines on skeletal muscle cells may be mediated through their common ability to activate the transcription factor NF-kB (12) (43), which induces muscle wasting and a loss of intrinsic force-generating capacity when constitutively overactivated in muscle (55). Therefore, all of the above strongly support the concept that simultaneous upregulation of multiple pro-inflammatory cytokines within the diaphragm during pulmonary infection is likely to favor synergistic interactions between these mediators at a cellular level, allowing them to act in a collective fashion to suppress global diaphragmatic function

IL-10-mediated suppression of cytokine production in the diaphragm

There is compelling evidence that the balance between pro- and anti-inflammatory cytokines determines the severity of the systemic response to infection. Particularly during the early stages of sepsis, a relative excess of pro-inflammatory mediators is believed to play an important role in the pathogenesis of multi-organ dysfunction. Nonetheless, attempts to suppress the systemic inflammatory response in sepsis with anti-inflammatory therapies (e.g., anti-TNF antibodies or soluble receptors, IL-1 receptor antagonist) have thus far produced disappointing results in clinical trials (56). One reason may be the presence of redundant biological effects of the various pro-inflammatory cytokines, such that interference with any single cytokine pathway is insufficient to significantly impact upon the process. Accordingly, in the present study we used a more broad-based approach by employing IL-10, which inhibits multiple pathways involved in inflammation. The anti-inflammatory effects of IL-10 include (but are not limited to) inhibition of Th1 cytokine production by T cells, deactivation of monocyte/macrophage pro-inflammatory cytokine synthesis, and interference with cell surface presentation of major histocompatibility complex class II molecules, B7 accessory molecules, and the LPS signaling molecule CD14 (57). In previous studies examining the effects of IL-10 in sepsis, exogenously supplied IL-10 has been found to be both beneficial and detrimental, depending on the infection model employed. For example, IL-10 treatment improved survival in acute endotoxemia (31), whereas conflicting results were obtained in the cecal ligation and perforation model of polymicrobial sepsis (58). In addition, IL-10 can paradoxically promote inflammation under certain conditions, and this appears to be critically dependent upon the timing and dosage of IL-10 administration (59) (30).

In the present investigation, we employed IL-10 gene transfer as a method for producing and delivering IL-10 to the systemic circulation. This method allows for more sustained elevations of IL-10 levels than can be achieved with single injections of recombinant protein, and the vector/gene dosage employed in our study was based upon previous work using the same approach in acute endotoxemia (30). The beneficial effects of IL-10 gene delivery on diaphragmatic cytokine expression and contractile function observed in our study may have resulted from direct local effects of IL-10 on muscle fibers. In this regard, here we show for the first time that the IL-10 receptor is expressed in the diaphragm at the tissue level as well as in primary muscle cell cultures obtained from the diaphragm. These findings are consistent with a prior report that IL-10 prevents IL-1β-induced intracellular adhesion molecule (ICAM)-1 expression by human myoblasts *in vitro* (60). IL-10 could also exert beneficial effects on diaphragmatic function by suppressing pro-inflammatory cytokine production by distant organs. However, under the experimental conditions employed in our study, we did not find evidence for significant downregulation of cytokine mRNA levels in the lung by IL-10 during *P. aeruginosa* infection. On the other hand, previous studies of animals with acute *P. aeruginosa* pneumonia found a reduction in lung injury as well as TNF-α release from the lungs into the systemic circulation when the animals were treated with recombinant IL-10 shortly before bacterial inoculation (61) (62). In addition, although not specifically examined, it is tempting to speculate that improved diaphragmatic function in these studies may have contributed to the enhancement of survival reported with IL-10 therapy (61).

Conclusions

In summary, we have shown that ongoing pulmonary infection with *P. aeruginosa* results in significant upregulation of diaphragmatic cytokine expression, as well as diaphragm muscle weakness, which could play a major role in contributing to respiratory muscle failure. In addition, adenovirus-mediated IL-10 gene delivery was able to largely suppress diaphragmatic cytokine expression under these conditions and simultaneously restore diaphragmatic force-generating capacity. Given that respiratory failure is commonly associated with pneumonia and other forms of sepsis, these findings may have important clinical implications. In particular, a strategy of altering the balance between pro-and anti-inflammatory cytokine expression in the

diaphragm may represent a promising avenue for the treatment and prevention of respiratory muscle weakness.

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CHAPTER 4

INCREASED SUSCEPTIBILITY OF CFTR KNOCKOUT MICE TO DIPAHRAGM DYSFUNCTION AFTER PSEUDOMONAS AERUGINOSA LUNG INFECTION

4.I Prologue

Pseudomonas aeruginosa lung infection is a major cause of mortality and morbidity in CF patients and respiratory muscle weakness is one of the distinguishing characteristics among such patients. However, the mechanism of this pathological defect has not been studied. We previously demonstrated that *P. aeruginosa* lung infection causes diaphragmatic dysfunction and that pro-inflammatory cytokines contribute to diaphragmatic muscle weakness. In order to mimic CF patients, we have employed a mouse model of CF to investigate the possible link between a defective *Cftr* gene and a predisposition to diaphragm muscle weakness after lung infection with *P. aeruginosa*.

4.II Abstract

Chronic infection with *Pseudomonas aeruginosa* is a major contributor to morbidity and mortality among cystic fibrosis (CF) patents. In the present investigation, we hypothesized that diaphragmatic muscle cells from CF ($cftr^{-/-}$) mice intrinsically have a greater propensity to up-regulate pro-inflammatory gene expression when exposed to pro-inflammatory cytokines or bacterial products (LPS), *in vitro*. In addition, we hypothesized that lack of expression of CFTR would predispose to diaphragm dysfunction and upregulation of intra-diaphragmatic pro-inflammatory cytokines/chemokines after pulmonary *P. aeruginosa* infection, *in vivo*. To more closely mimic the sustained infection found in CF patients, we employed a previously described model of *Pseudomonas*-impregnated beads instillation into the lungs. Both $cftr^{-/-}$ and wild-type littermates ($cftr^{+/+}$) mice were assigned to either: 1) uninfected group (control); or 2) infected group with *P. aeruginosa* at a dose of $1x10^5$ cfu. The lungs and diaphragm muscles were removed at day 2 post-infection. The diaphragm *in vivo*, as well as cultured primary diaphragmatic myotubes, *in vitro*, constitutively expressed CFTR mRNA. Stimulation of myotubes by pro-inflammatory cytokines (TNF-α, IL-1α, IFN-γ) and bacterial LPS (*Pseudomonas*-derive) upregulated pro-inflammatory cytokine/chemokine expression in both $cftr^{-/-}$ and $cftr^{+/+}$ cultures, but the magnitude of cytokine/chemokine upregulation was significantly greater in $cftr^{-/-}$ diaphragmatic muscle cells. *In vivo*, the maximal diaphragmatic force was reduced by approximately one-third, which was associated along with increased intra-diaphragmatic pro-inflammatory gene expression. Our results indicate an exaggerated pro-inflammatory cytokine expression by the diaphragm in $cftr^{-/-}$ mice, together with an increased vulnerability of mice lacking CFTR to diaphragm dysfunction.

4.II Introduction

Cystic fibrosis (CF) is the most common fatal autosomal recessive disorder in the Caucasian population (1). The genetic basis of CF is a defect in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), which functions as a chloride channel regulated by cyclic AMP (2). Despite major improvements in the medical care and prognosis of CF patients, respiratory failure remains the main cause of death (1). Exercise capacity is significantly reduced in CF patients, and is an important predictor of survival (3). Many patients with CF have weak peripheral (4) (5) and respiratory muscles (6-10). The etiology of respiratory muscle weakness in CF is likely multifactorial, with pulmonary hyperinflation (11;12) and malnutrition (13;14) playing a role. However, abnormalities of muscle function which are not readily attributed to these factors have also been documented (15). In this regard, impaired skeletal muscle metabolism during exercise has been reported in CF patients despite minimal changes in pulmonary function or nutritional status (16;17) (4) (18). Therefore, additional factors beyond diminished lung function or malnutrition and muscle atrophy are likely to be involved in producing skeletal muscle weakness in CF patients.

There is considerable evidence that a number of pro-inflammatory cytokines can cause peripheral and respiratory muscle weakness through several mechanisms, including direct interference with contractile or metabolic function and accelerated protein loss (19). Therefore, one plausible etiology of skeletal muscle dysfunction in CF patients is an increased exposure to pro-inflammatory effector molecules. Concentrations of pro-inflammatory mediators are markedly elevated in the sputum, bronchoalveolar lavage fluid, and serum of CF patients (20-22). The pronounced inflammatory response in the CF lung is the result of a complex interaction between bacterial infection and impaired or dysregulated host defense mechanisms. With respect to the former, *Pseudomonas aeruginosa* is a frequent pulmonary pathogen, which plays an important role in the pathogenesis of CF lung disease. In addition, some investigators have reported that epithelial cells lacking CFTR have an intrinsically greater propensity to produce cytokines, such as IL-8 and TNF-α, after being exposed to *P. aeruginosa* and other pro-

inflammatory stimuli (23-25). This suggests the possible existence of a positive feedback loop, in which *P. aeruginosa* infection could initiate dysregulated cytokine production in cells lacking CFTR, thereby triggering sustained and disproportionate inflammation. Intriguingly, Fiedler et al (26) reported that CFTR is normally expressed in rat skeletal muscle tissue, although the precise cellular origin (i.e., muscle cells versus other tissue components) was not determined, and to date there is no known function for CFTR in skeletal muscle cells. However, in view of the fact that a lack of CFTR expression may be associated with excessive cytokine elaboration in other cell types, it is conceivable that a similar situation could exist in skeletal muscle. Accordingly, an increased exposure of muscle fibers to pro-inflammatory mediators in CF could potentially originate not only from the lung, but also through an excessive production of pro-inflammatory cytokines by the muscle fibers themselves.

The diaphragm is the primary muscle of respiration. In the present study, we have employed a murine model of CF (*Cftr*$^{-/-}$ mice) to investigate whether a link exists between defective CFTR gene expression and the predisposition to diaphragm muscle weakness observed in CF patients. More specifically, we have utilized two separate approaches to examine the influence of CFTR on the response of skeletal muscle to infection, with a particular focus on the inflammatory gene expression profile and contractile function of the diaphragm. Firstly, an *in vitro* model of primary diaphragmatic skeletal muscle cell culture was established from both *Cftr*$^{-/-}$ and wild-type mice, in order to determine whether CFTR expression modulates the level of cytokine expression by diaphragm muscle cells per se. Since it has been shown that CFTR-deficient cell lines produce more pro-inflammatory cytokines than CFTR-expressing cells following exposure to *P. aeruginosa* (23), our first hypothesis was that diaphragm muscle cells from *Cftr*$^{-/-}$ mice would have a greater propensity to upregulate cytokines when exposed to pro-inflammatory mediators such as bacterial products (*Pseudomonas*-derived endotoxin), or other cytokines known to be released from the infected CF lung into the systemic circulation. Secondly, in view of the fact that lung infection with *P. aeruginosa* is a major cause of morbidity and mortality in CF patients, we have used *Cftr*$^{-/-}$ mice to examine the effects of *P. aeruginosa* lung infection on diaphragmatic muscle function *in*

vivo. Here we wished to determine whether the nature or magnitude of pro-inflammatory gene expression by the diaphragm during *in vivo* pulmonary infection would differ between *Cftr*$^{-/-}$ and normal wild-type mice. Our specific hypothesis was that lung infection with *P. aeruginosa* would lead to exaggerated pro-inflammatory cytokine expression by the diaphragm in *Cftr*$^{-/-}$ mice, and that this would be associated with significantly greater diaphragm muscle weakness than in identically infected wild-type mice with normal CFTR expression.

4.IV Materials and Methods

CFTR knockout (*Cftr*$^{-/-}$) mice

Studies were performed in 8-10 week old male knockout (*Cftr*$^{-/-}$) and wild-type (*Cftr*$^{+/+}$) littermates, used in accordance with the guidelines established by the Canadian Council on Animal Care. Mice used in these studies represented offspring from BL/6-Cftr$^{unc\ (+/-)}$ mice backcrossed to C57BL/6 mice for 10-12 generations (27) and kept under specific pathogen-free conditions. The mice were housed on corn cob bedding (Anderson, Maumee,OH) and fed sterile Peptamen (Clintec Nutrition, Deerfield, IL), a low-residue liquid diet which was previously shown to prevent intestinal obstruction in *Cftr*$^{-/-}$ mice [28]. The caloric content of the diet is 420 KJ/100 ml and the adult mouse consumes approximately 15 ml/day.

P. aeruginosa lung infection

The model of pulmonary inoculation with *P. aeruginosa* employed in this study allowed for a sustained infection over 2 days, and was performed essentially as described by Starke and colleagues (55). A mucoid strain of *P. aeruginosa* (strain 508) was used, which was originally isolated from a cystic fibrosis patient (27). Briefly, log-phase *P. aeruginosa* bacteria were concentrated and mixed with 1.5% trypticase soy agar pre-warmed to 52°C. This mixture was added to heavy mineral oil at 52°C and rapidly stirred for 6 min, followed by cooling for 10 min at 4°C. After removing excess oil, the bacteria-bead preparation was washed extensively and resuspended in sterile phosphate-buffered saline (PBS) at pH 7.4. The size (100-150 um) and uniformity of the beads were verified by light microscopy. In addition, the number of viable bacteria trapped within the agar beads was measured by homogenizing the beads and then plating 10-fold serial dilutions on trypticase soy agar plates. Sterile agar beads were produced in the same manner but with omission of bacteria, and were confirmed to be free of colony-forming units (cfu). To deliver bacteria-containing agar beads to mouse lungs, the mice were first anesthetized with a combination of ketamine (130 mg/kg) and xylazine (20 mg/kg) injected intramuscularly. A small incision was made at the midline of the neck in order to expose the trachea. The trachea was then intubated with a sterile flexible 22-ga

cannula attached to a 1 ml syringe, which was used to inject 50 ul of the agar bead suspension into the lungs. Mice were infected with an inoculation dose of $1x10^5$ cfu, and euthanized at day 2 post-infection. All animal procedures were approved by the institutional animal care committee.

Lung bacterial colony assay

Both lungs were removed and homogenized for 2 min at high speed (PT10135; Brinkmann Instruments Co., Mississauga, ON, Canada) in 4.0 ml of PBS. Serial dilutions (1:10) of homogenized lungs were then plated on petri dishes containing trypticase soy agar. The number of *P. aeruginosa* colonies was counted after overnight incubation at 37°C.

Bronchoalveolar lavage (BAL)

The trachea was cannulated with a 22-ga catheter connected to two separate syringes via a three-way stopcock. One syringe was used to instill 2.5 ml of cation-free Hank's balanced salt solution into the lungs, while the second syringe allowed the fluid to be collected by gentle aspiration. The total lavage fluid recovered was approximately 2 ml. Cell pellets were resuspended in PBS for total and differential cell counting and supernatants stored at -70□C for cytokine assay. Total cell numbers were determined using a hemocytometer. Differential cell counts were performed on cytospin preparations stained with Diff-Quick (American Scientific Products, McGaw Park, IL). From 300-400 cells were counted on each cytospin preparation, and the cells were classified as polymorphonuclear leukocytes, macrophages, and lymphocytes using standard morphological criteria.

TNF-a assay in BAL and serum

The concentrations of TNF- protein in BAL and serum were determined by double sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems, Minneapolis, MN), according to manufacturer's instructions. The sensitivity of detection was 5 pg/ml or less.

Pro-inflammatory gene expression

Samples of total RNA were isolated from tissues and cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). ^{32}P-labelled riboprobes were synthesized using a commercial mouse multiprobe kit (BD Biosciences, Pharmingen, San Diego, CA) containing templates against the following gene transcripts: MIP-2, KC, RANTES, MIP-1, MIP-1, iNOS, TNF-, IL-1, IL-1, and IL-18. The riboprobes were hybridized with each RNA sample (for muscle: 20 ug; for lung: 10 ug; for primary cell cultures: 5 ug) overnight at 56°C according to the manufacturer's instructions. The protected RNA fragments were separated using a 5% polyacrylamide gel and detected by autoradiography. Bands representing the individual mRNA species were then quantified using an image analysis system (FluorChem 8000, Alpha Innotech Corp, San Leandro, CA), and the signals were normalized to the L32 housekeeping gene to control for loading in each lane.

CFTR gene expression

CFTR expression was evaluated by RT-PCR using total RNA (1 ug) obtained from muscle tissues (diaphragm, tibialis anterior), lung, and primary diaphragm muscle cell cultures. RT was performed using M-MLV reverse transcriptase and random primers (Promega, Madison, WI). PCR amplification of the cDNA was performed with primers targeted to exons 9 and 10, and consisting of the following sequences, respectively (5' to 3'): TCTCTGCCTTGTGGGAAATC (forward), and AGTACCCGGCATAATCCAAG (reverse). Amplification was performed for 45 cycles with a denaturation step at 95°C, annealing at 60°C, and extension at 72°C. The resulting PCR product (predicted amplicon size of 208 bp) was visualized on an agarose gel containing ethidium bromide.

Primary diaphragm muscle cell cultures

Primary diaphragm muscle cell cultures were established essentially as per Rosenblatt et al (1995), using single living muscle fibers to isolate adult myoblast precurosors (also known as satellite cells). Briefly, excised diaphragm muscle strips from 8 week old wild-type and *Cftr*$^{-/-}$ mice were subjected to collagenase digestion (0.2% collagenase at 37°C for 60 minutes), followed by trituration with heat-polished Pasteur pipettes of decreasing bore size to liberate individual fibers. The individual fibers were washed in DMEM and

PBS, collected, and then transferred into Matrigel (Becton Dickinson, Franklin Lakes, NJ)-coated (1 mg/ml in DMEM) 6-well plates. All culture media contained 1% penicillin/streptomycin and 0.2% amphotericin B (Invitrogen, Carlsbad, CA). The cultures were maintained in DMEM with 10% horse serum and 0.5% chick embryo extract (MP Biomedical, Aurora, OH) for 4 days, during which myoblasts attached to the substratum. Diaphragmatic myoblasts were then expanded in growth medium (20% fetal bovine serum, 10% horse serum, 1% chick embryo extract in DMEM) until attaining approximately 75% confluence. At this point, the cultures were placed in differentiation medium (2% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract in DMEM) in order to induce myoblast fusion into differentiated myotubes. All experiments were performed on the 5th day of maintenance in differentiation medium. Diaphragmatic myotubes were washed with DMEM prior to stimulation, which consisted of combined exposure for 4 hours to the following: TNF- 1 ng/ml, IL-1 5U/ml, IFN- 20U/ml (all from R&D Systems, Minneapolis, MN), and *P. aeruginosa* LPS 1 ng/ml (Sigma, St. Louis, MO).

Diaphragmatic contractile function

The diaphragm muscle was surgically excised for *in vitro* contractility measurements under isometric conditions as previously described in detail (29). After removal from the anesthetized animal, the diaphragm was immediately placed into a chilled (4°C) and equilibrated (95% O2-5%CO2; pH 7.38) Krebs solution with the following composition (in mM): 118.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.0 KH2PO4, 25.0 NaHCO3, and 11.0 glucose. A muscle strip approximately 2 mm wide was dissected free, taking care to leave the central tendon and adjacent rib cage margins intact. The excised diaphragm strip was mounted into a jacketed tissue bath chamber filled with equilibrated Krebs solution, using a custom-built muscle holder containing two stimulation electrodes located on either side. The central tendon was tied to an isometric force transducer using 4-0 silk thread. A thermoequilibration period of 15 min was observed prior to initiating contractile measurements at 23°C. After placing the diaphragm strip at optimal length (Lo), the force-frequency relationship was determined by sequential supramaximal stimulation for 1s at 5, 10, 20, 30, 50, 100, 120, and 150 Hz, with 2 min between each

stimulation train. The force data were acquired to computer at a sampling rate of 1000 Hz for later analysis. After completion of the above contractility studies, the muscles were removed from their baths, and muscle length was measured with a microcaliper accurate to 0.1 mm. Muscle cross-sectional area was determined by assuming a muscle density of 1.056 g/cm^3, which allowed specific force (force/cross-sectional area) to be calculated and expressed in Newtons/cm^2.

Statistical analysis

All data are presented as mean values ± SE. Group mean differences were determined by analysis of variance, with *post hoc* application of the Tukey test where appropriate. A statistics software package was used for all analyses (SigmaStat V2.0, SPSS, Chicago, IL). Statistical difference was defined as a p value less than 0.05.

4.V Results

I. In Vitro Studies

Expression of CFTR by diaphragm muscle cells

RT-PCR was employed to determine whether CFTR is expressed by intact muscle tissues. As shown in Fig. 1, CFTR was found to be expressed in the diaphragm and limb (tibialis anterior) muscles of wild-type (*Cftr*$^{+/+}$), but not *Cftr*$^{-/-}$ mice. In order to ascertain whether CFTR can be expressed by muscle cells per se (as opposed to other cell types present within muscle tissue), RT-PCR was also performed on RNA extracted from *in vitro* cultures of differentiated diaphragmatic myotubes. As was the case for the whole muscles, CFTR expression was readily detectable in myotubes derived from wild-type, but not *Cftr*$^{-/-}$ mice.

Responsiveness of *Cftr*$^{-/-}$ diaphragm muscle cells to pro-inflammatory mediator stimulation

To determine whether a lack of CFTR is associated with altered sensitivity to pro-inflammatory mediator stimulation, we next compared the responses of wild-type and *Cftr*$^{-/-}$ diaphragmatic myotubes to a mixture of pro-inflammatory cytokines (TNF-α, IL-1α, and IFN-γ) and LPS derived from *P. aeruginosa*. The CXC chemokines, KC and MIP-2, are the murine analogues of IL-8, which has been found to be abnormally regulated in certain cell types lacking CFTR (REF). Therefore, we evaluated the mRNA expression levels of KC and MIP-2, as well as several CC chemokines (RANTES, MIP-1α, MIP-1β) and iNOS (see Fig. 2). Although a low level of constitutive expression of RANTES and KC could be detected under non-stimulated baseline conditions in both wild-type and *Cftr*$^{-/-}$ myotubes with prolonged film exposure times (data not shown), there were no apparent differences between the two groups. However, after 4 hours of stimulation with cytokines and LPS, all of the pro-inflammatory genes examined showed significantly greater upregulation in *Cftr*$^{-/-}$ than in wild-type diaphragmatic myotubes, as can be seen in Figs. 2 and 3.

II. In Vivo Studies

Pulmonary and systemic inflammatory response to *P. aeruginosa* lung infection

Pulmonary bacterial counts after infection are shown in Fig. 4A, together with values obtained from the lungs of control (CTL) non-infected wild-type and *Cftr*$^{-/-}$ mice. At 2 days after *P. aeruginosa* infection ($1x10^5$ cfu), the pulmonary bacterial burden was significantly higher in *Cftr*$^{-/-}$ than in wild-type mice. In addition, Fig. 4B shows that the total number of inflammatory cells contained within the bronchoalveolar lavage (BAL) fluid was also significantly higher in *Cftr*$^{-/-}$ than in wild-type mice at the same time point. This consisted largely of PMNs, which represented 87% of BAL cells in *Cftr*$^{-/-}$ mice, as compared to 58% in wild-type mice.

To determine the impact of these elevated bacterial and inflammatory cell counts on pulmonary cytokine production, RNase protection assays were performed on the whole lung. As shown in Fig. 5 (representative film) and Fig. 6 (group mean data), there was marked upregulation of multiple cytokines and chemokines within the infected lungs of *Cftr*$^{-/-}$ and wild-type mice. However, there were no significant differences in the mRNA expression levels for these pro-inflammatory mediators between the infected *Cftr*$^{-/-}$ and wild-type groups. Since the majority of studies in *Cftr*$^{-/-}$ mice have shown greater cytokine levels in BAL fluid following *P. aeruginosa* infection, we also measured the levels of TNF-α protein in BAL samples. In contrast to the lung mRNA findings, BAL from infected *Cftr*$^{-/-}$ mice revealed significantly greater TNF-α protein expression than in infected wild-type mice (see Fig. 7A). In addition, serum levels of TNF-α were also significantly elevated in infected *Cftr*$^{-/-}$ as compared to infected wild-type mice (see Fig. 7B) .

Diaphragmatic pro-inflammatory gene response to *P. aeruginosa* lung infection

RNase protection assays were performed to evaluate the effects of *P. aeruginosa* lung infection on pro-inflammatory gene expression by the diaphragm (see Fig. 8). Diaphragms of non-infected mice showed a low basal expression of iNOS, TNF-α, IL-1β, IL-18 and RANTES, which did not differ significantly between *Cftr*$^{-/-}$ and wild-type

mice. Pulmonary infection with *P. aeruginosa* resulted in significant pro-inflammatory gene upregulation within the diaphragms of both *Cftr*$^{-/-}$ and wild-type mice. However, as can be seen from Figs. 8 and 9, the mRNA expression levels of multiple cytokines (TNF-α, IL-1α, IL-1β, and IL-18) as well as chemokines (RANTES, MIP-1α, and MIP-2) were substantially higher in infected *Cftr*$^{-/-}$ mice than in their infected wild-type littermates. The one exception to this general pattern of greater pro-inflammatory gene expression in the *Cftr*$^{-/-}$ diaphragms was iNOS expression, which was increased by pulmonary infection in both *Cftr*$^{-/-}$ and wild-type mice, but more so in the latter.

Diaphragmatic dysfunction after *P. aeruginosa* lung infection

To determine whether the greater pro-inflammatory gene expression in the *Cftr*$^{-/-}$ diaphragms was associated with contractile dysfunction, measurements of isometric force production were performed on isolated strips of diaphragm muscle. Figure 10 illustrates the effects of *P. aeruginosa* lung infection on force production by the diaphragm at day 2 post-infection. No differences were found between the uninfected CTL wild-type and *Cftr*$^{-/-}$ groups. In addition, with the pulmonary inoculation dose of $1x10^5$ cfu employed in the current study, force-generating capacity of the wild-type diaphragm was also normal. In contrast, the response to pulmonary infection was markedly different in *Cftr*$^{-/-}$ diaphragms. Hence force production was significantly depressed in infected *Cftr*$^{-/-}$ mice across the entire force-frequency relationship. In *Cftr*$^{-/-}$ mice with *P. aeruginosa* lung infection, maximal isometric strength of the diaphragm was on average 33% lower than in identically infected wild-type mice.

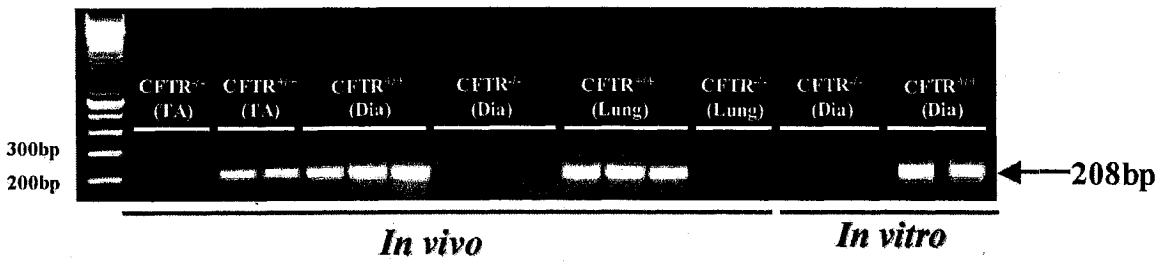

Figure 1. Expression of CFTR by muscle cells. RT-PCR revealed the presence of CFTR transcripts in diaphragm tissue (*in vivo*) and in culture myotubes derived from wild-type ($cftr^{+/+}$) diaphragm (*in vitro*). The positive control (+) consists of RT-PCR product obtained from wild-type lung *in vivo*.

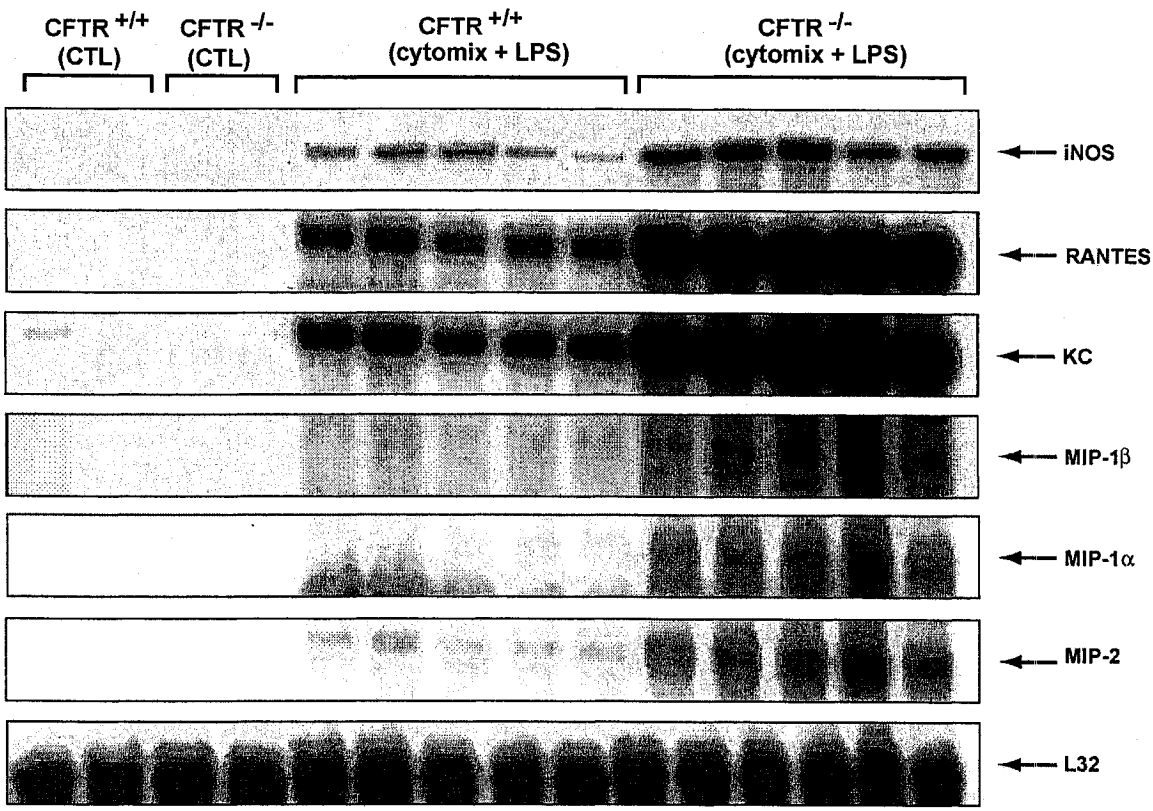

Figure 2. Upregulation of multiple cytokine mRNAs in diaphragm muscle cells by pro-inflammatory mediators. Representative autoradiograph of Rnase protection assay showing the pro-inflammatory gene expression in the absence (CTL) or presence (cytomix + LPS) of stimuli. Expression levels of pro-inflammatory mediators (iNOS, RANTES, KC, MIP-1α, MIP-1β, and MIP-2) were higher after simulation in both *cftr*$^{+/+}$ and *cftr*$^{-/-}$ groups. A total of 5 μg RNA was loaded in each lane.

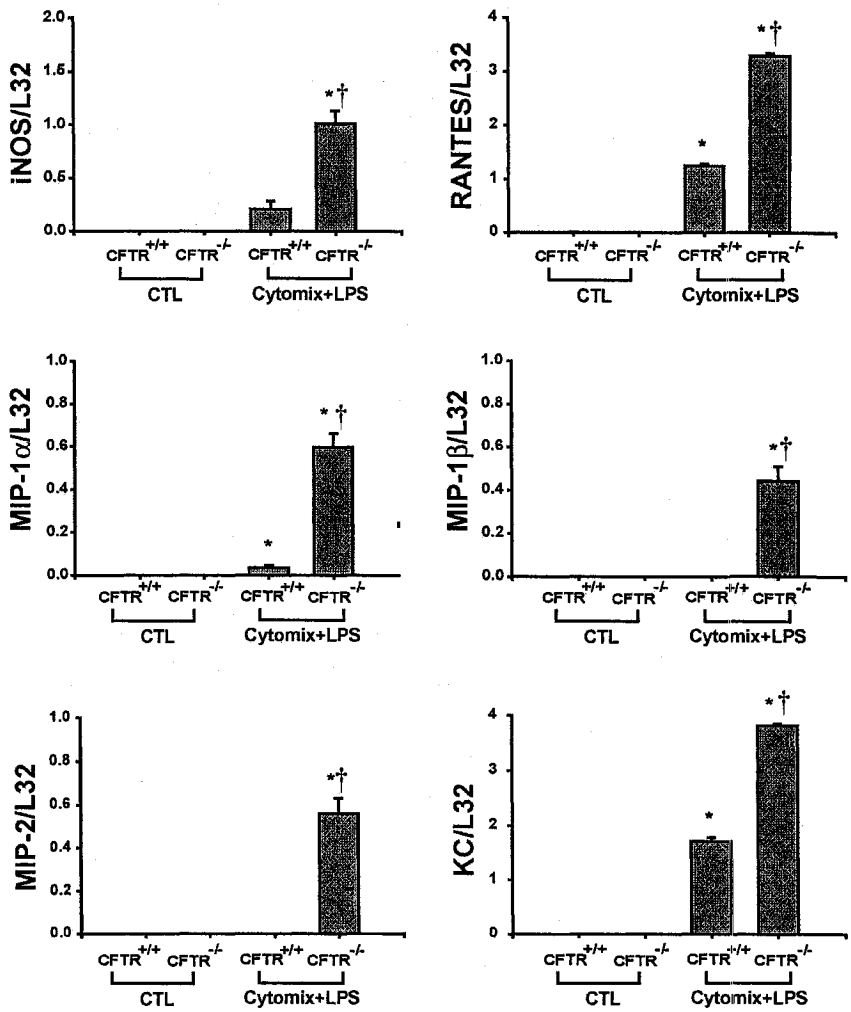

Figure 3. Group mean values for the RPA as shown in figure 2. Expression levels of pro-inflammatory mediators ((iNOS, RANTES, KC, MIP-1α, MIP-1β, and MIP-2) were significantly upregulated after stimulation in *cftr*$^{-/-}$ group. All data were normalized to L32 to control for loading across lanes. * $p<0.05$ for control versus stimulations; † $p<0.05$ for *cftr*$^{+/+}$ versus *cftr*$^{-/-}$ groups after stimulation.

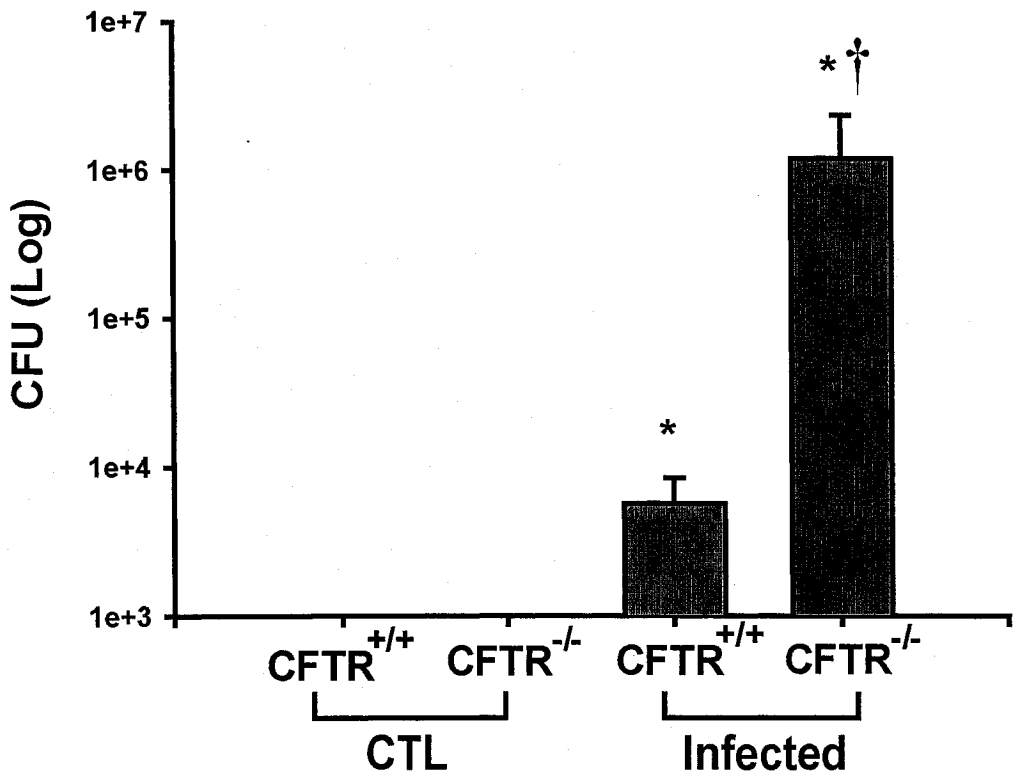

Figure 4A. Pulmonary bacterial burden. Mice were sacrificed to quantify numbers of bacteria in the lung at day 2 post-infection. Values are group means ± SE. * $p<0.05$ control (uninfected) versus infected groups; † $p<0.05$ infected *cftr*$^{+/+}$ versus infected *cftr*$^{-/-}$ groups.

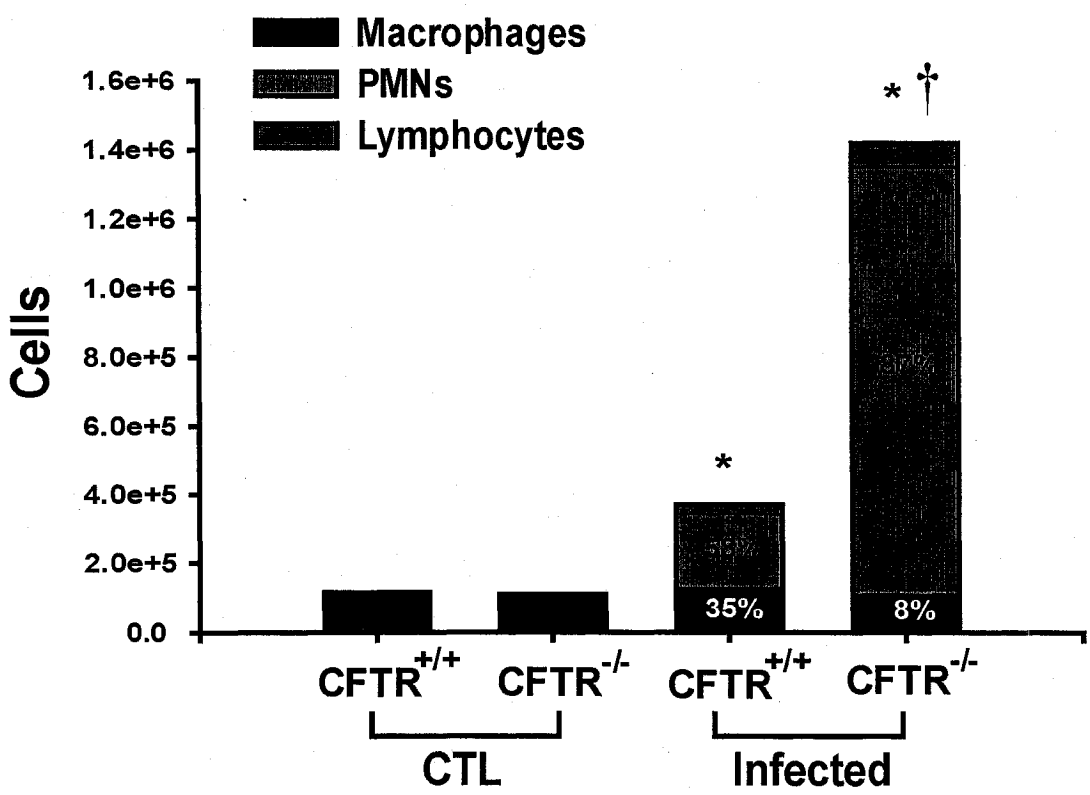

Figure 4B. Pulmonary inflammatory cell response to *Pseudomonas* infection. Bronchoalveolar lavage (BAL) performed at day 2 after infection. Values are group means ± SE. * $p<0.05$ compared control (uninfected) to infected groups; † $p<0.05$ infected *cftr*$^{+/+}$ versus infected *cftr*$^{-/-}$ groups.

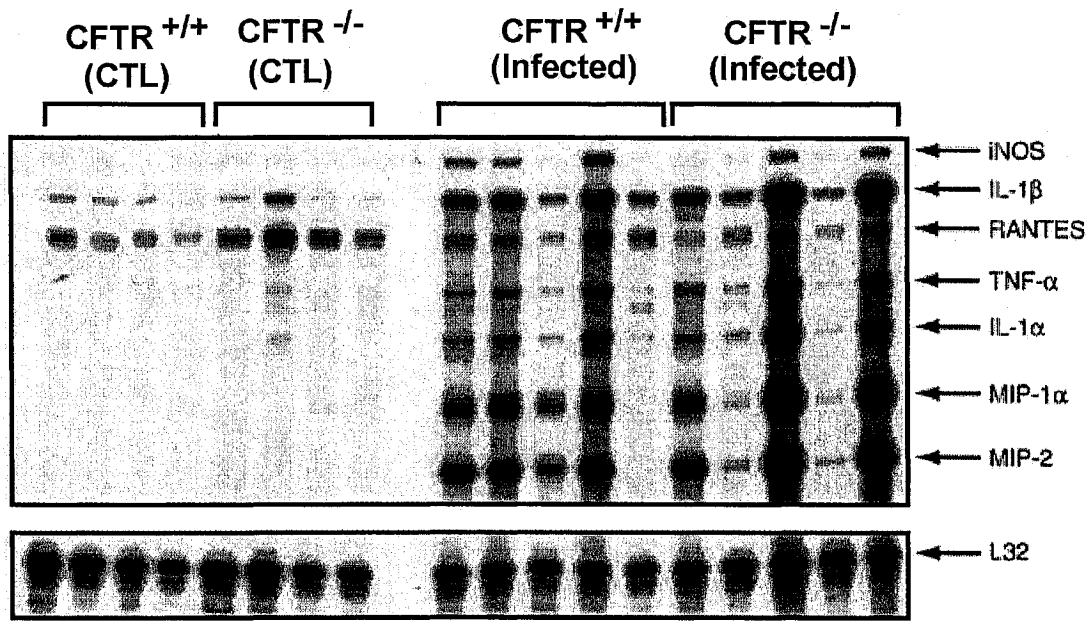

Figure 5. Representative of Rnase protection assay from the lungs under basal (uninfected) and pulmonary infection with *P. aeruginosa*. The pro-inflammatory gene expression were markedly upregulated in infected groups. A total of 10 μg RNA was loaded in each lane.

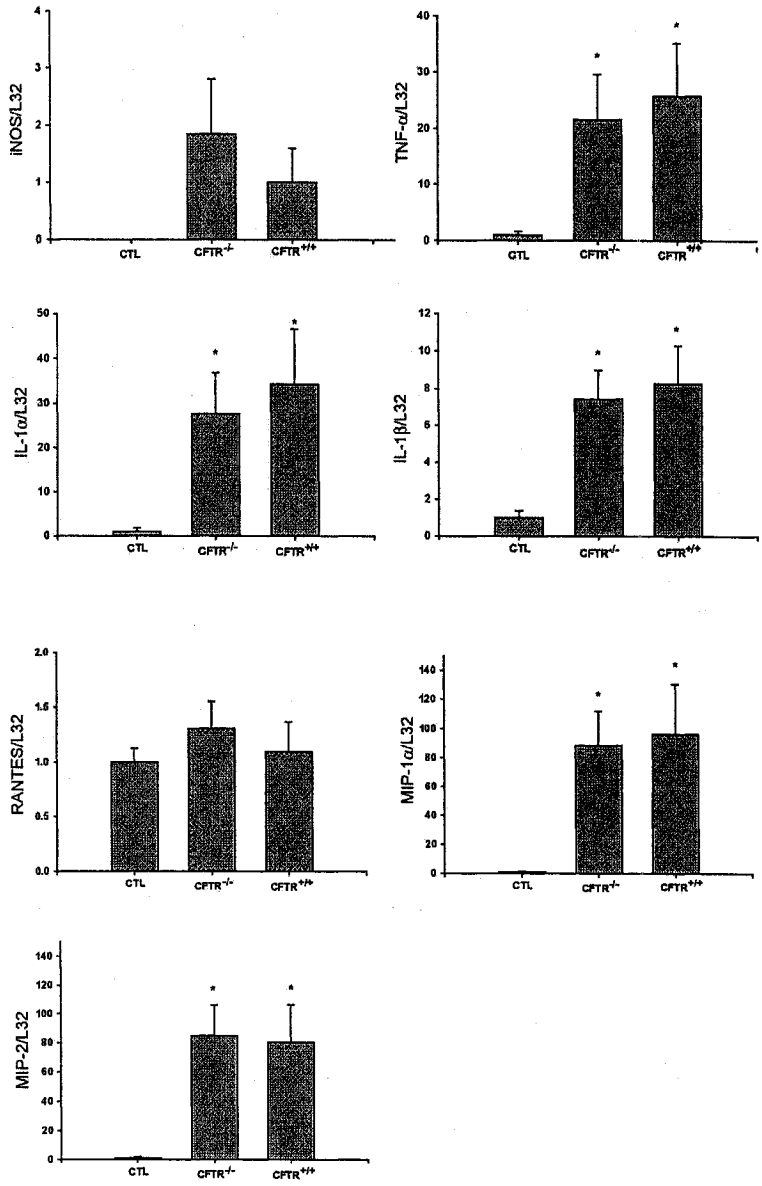

Figure 6. Group mean values of the RPA as shown in figure 5. With the exception of iNOS and RANTES, expression levels of cytokines (IL-1α, IL-1β, and TNF-α) and chemokines (MIP-1α, and MIP-2) were significantly increased in both *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice after *P. aeruginosa* lung infection. All data were normalized to L32 to control the loading across lanes. *$p<0.05$ compared control (uninfected) to infected groups.

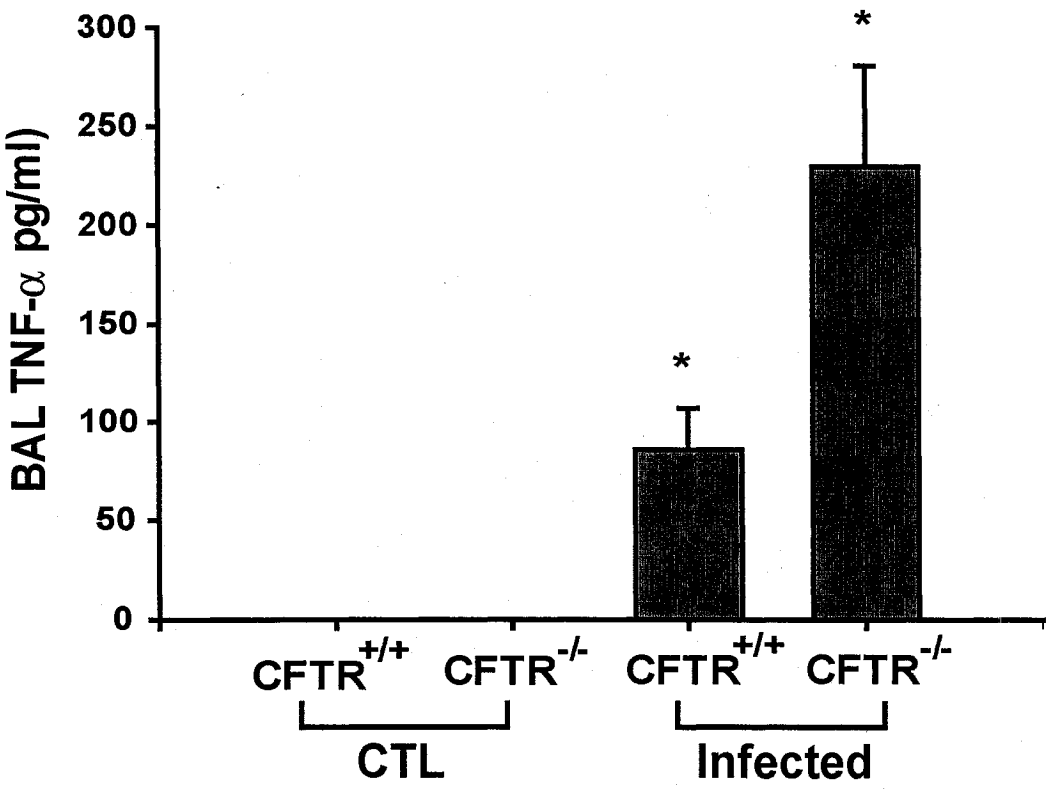

Figure 7A. TNF-α protein in BAL. ELISA showed greater levels of TNF-α protein in lungs of the *cftr*$^{-/-}$ mice. Values are group means ± SE. *$p<0.05$ compared control (uninfected) to infected groups.

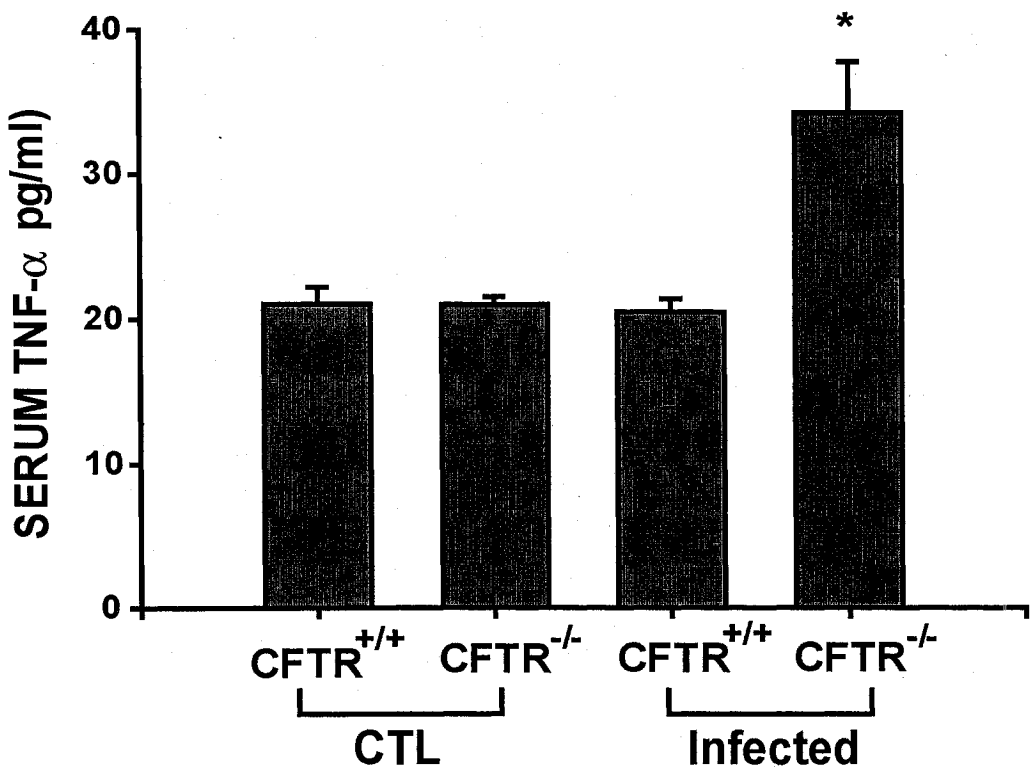

Figure 7B. TNF-α protein in serum. ELISA revealed higher levels of TNF-α protein in serum of the *cftr*$^{-/-}$ mice. Values are group means ± SE. *$p<0.05$ compared control (uninfected) to infected groups.

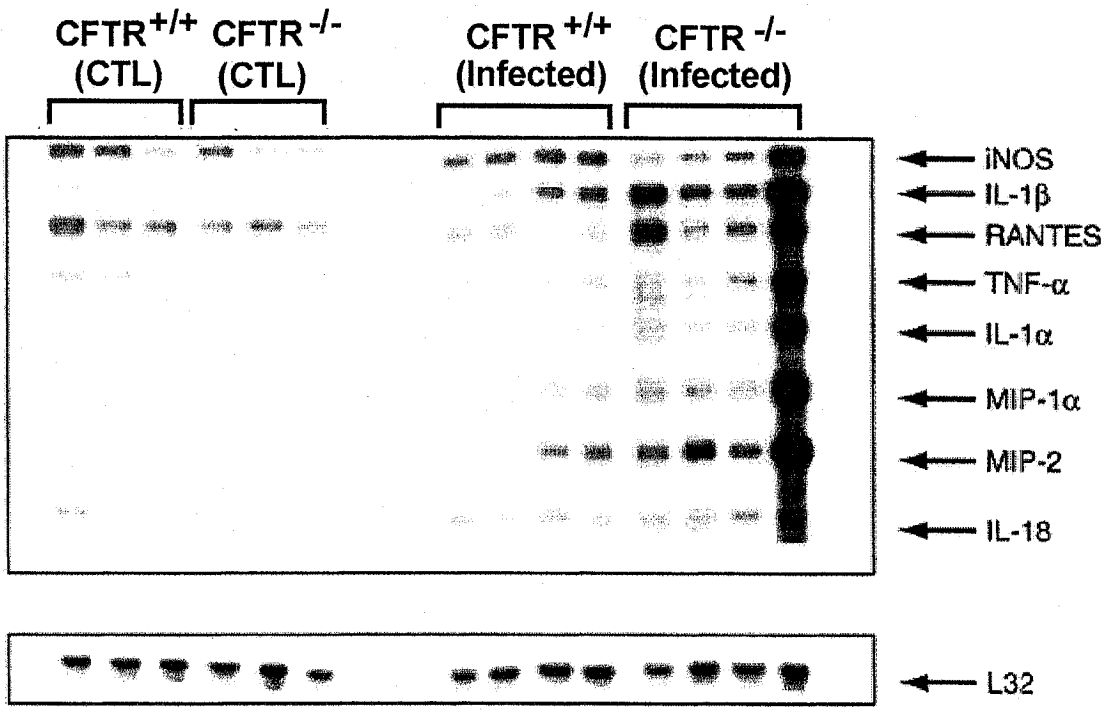

Figure 8. Representative Rnase protection assay from the diaphragm under basal (uninfected) and lung infection with *P. aeruginosa*. The expression levels of pro-inflammatory mediators (iNOS, IL-1α, IL-1β, IL-18, TNF-α, RANTES, MIP-1α, and MIP-2) were all up-regulated within the diaphragm of infected mice. A total of 20 μg RNA was loaded in each lane.

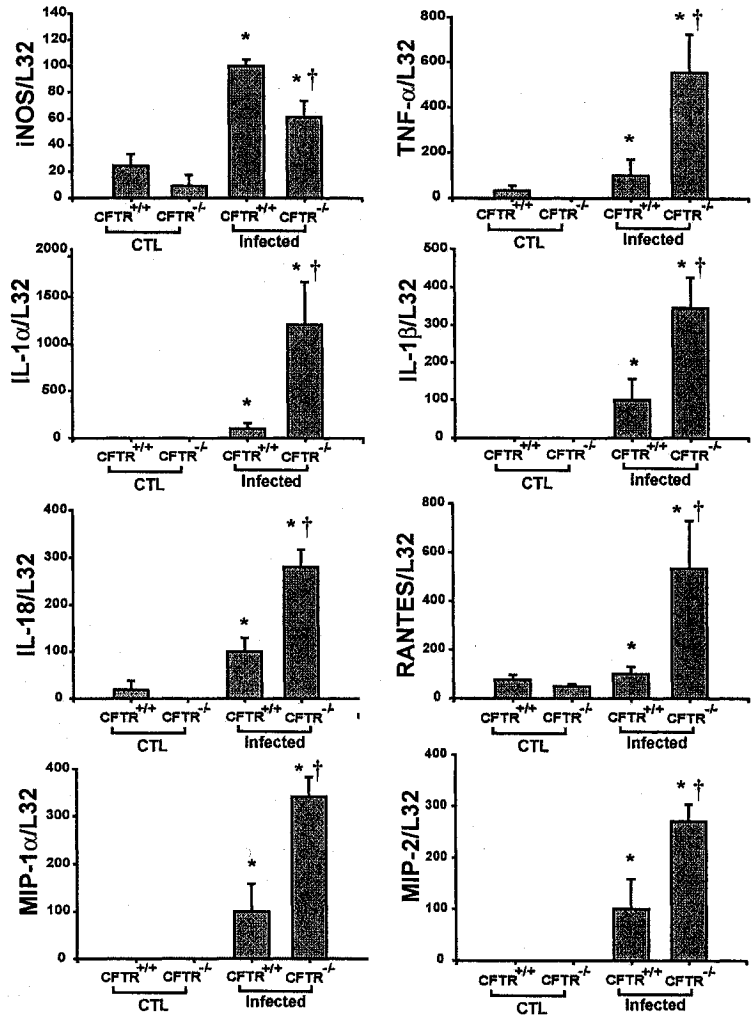

Figure 9. Group mean values of the RPA as shown in figure 8. Expression levels of pro-inflammatory mediators (iNOS, IL-1α, IL-1β, IL-18, TNF-α, RANTES, MIP-1α, and MIP-2) were significantly increased in the diaphragm of both *cftr*$^{+/+}$ and *cftr*$^{-/-}$ mice after pulmonary *P. aeruginosa* infection. However, with the exception of iNOS, the magnitude of multiple cytokines (IL-1α, IL-1β, IL-18, and TNF-α) and chemokines (RANTES, MIP-1α, and MIP-2) were significantly higher in infected *cftr*$^{-/-}$ mice than infected *cftr*$^{+/+}$ mice. * $p<0.05$ compared control (uninfected) to infected groups; † $p<0.05$ infected *cftr*$^{+/+}$ versus infected *cftr*$^{-/-}$ groups.

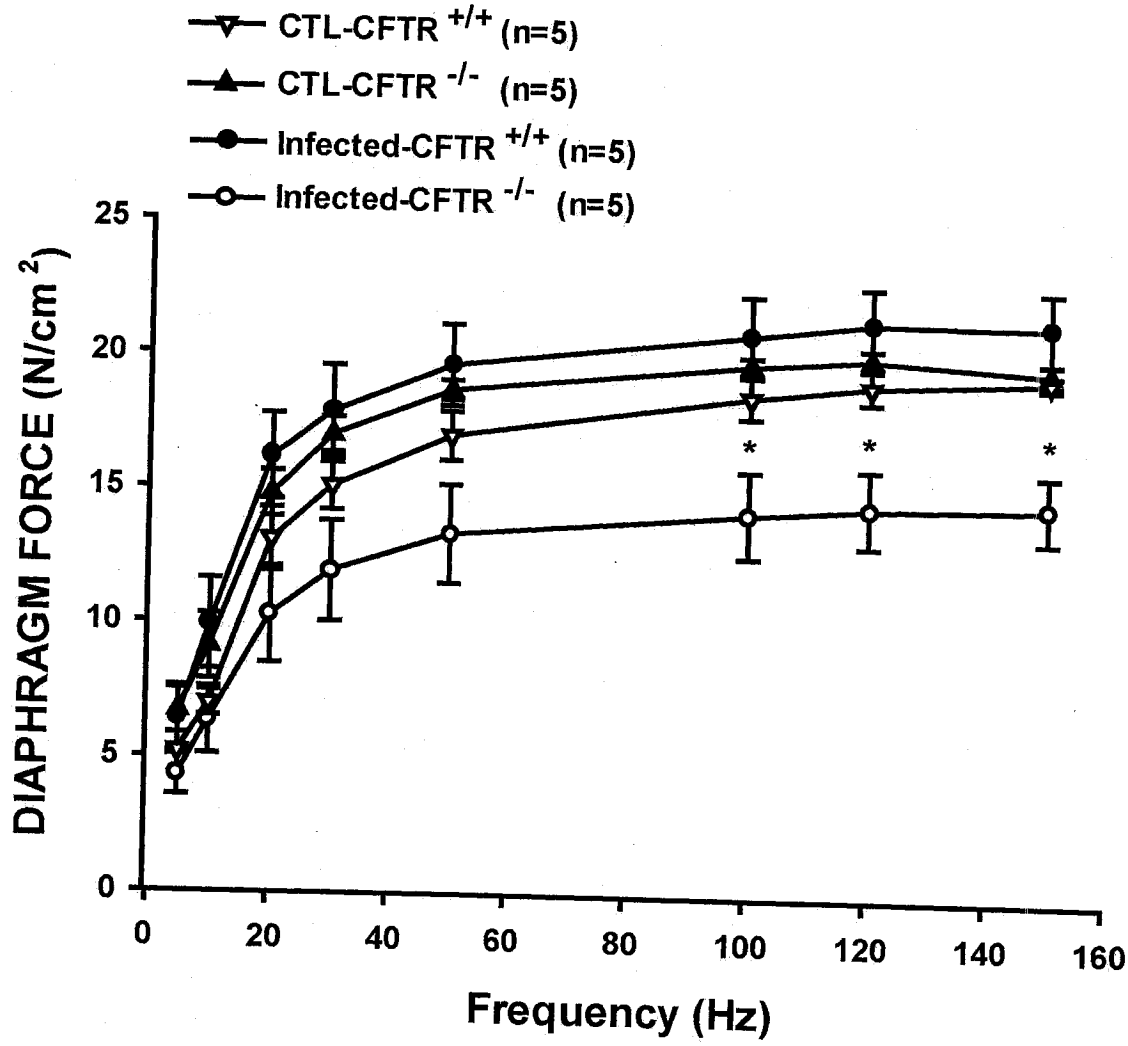

Figure 10. Effects of *P. aeruginos* lung infection on the diaphragmatic force-frequency relationship. At day 2 after infection, the diaphragmatic force was significantly decreased only in infected *cftr*$^{-/-}$ mice. Values are group means ± SE; n=5 per group. *$p<0.05$ compared control (uninfected) to infected groups.

4.VI Discussion

CF is a disease in which skeletal muscle weakness and wasting are prominent features. This process may involve not only the limb musculature, but also the diaphragm and other respiratory muscles. Because the respiratory muscles constitute a ventilatory pump which is necessary for survival, any pathological process which compromises diaphragmatic function is potentially of great clinical relevance. This is particularly the case in CF, since the underlying pulmonary disease is characterized by airway obstruction and repeated infections, which place an additional burden on the diaphragm.

In the present study, we have used a *Cftr*$^{-/-}$ mouse model to address several fundamental questions relating to the skeletal muscle weakness found in CF patients. Our main overall hypothesis was that diaphragm muscle cells lacking CFTR would exhibit an exaggerated pro-inflammatory response after being exposed to inciting agents such as cytokines and bacteria. The major novel findings of the present study are the following: (1) CFTR mRNA is expressed in whole muscle tissue from the diaphragm as well as in cultured diaphragmatic muscle cells; (2) *In vitro*, stimulation of diaphragmatic myotubes with pro-inflammatory effector molecules (TNF-α, IL-1α, IFN-γ, LPS) triggers greatly exaggerated cytokine and chemokine expression in *Cftr*$^{-/-}$ as compared to identically treated wild-type muscle cells; (3) *In vivo*, diaphragmatic expression levels of cytokines and chemokine in *Cftr*$^{-/-}$ mice are also significantly greater than in wild-type mice during *P. aeruginosa* lung infection; and (4) The increased diaphragmatic pro-inflammatory gene expression response to *P. aeruginosa* lung infection in *Cftr*$^{-/-}$ mice is associated with substantial contractile weakness of the diaphragm. Taken together, our findings provide the first evidence that skeletal muscle cells lacking CFTR have an increased propensity to express pro-inflammatory effector molecules, which may in turn contribute to the global skeletal muscle dysfunction found in CF patients.

In Vitro: Exaggerated pro-inflammatory gene expression by CF diaphragm muscle cells

CF leads to pathological changes in organs which normally express CFTR. In the present study, we report for the first time that skeletal muscle cells lacking CFTR demonstrate an

exaggerated upregulation of pro-inflammatory gene expression after being exposed to other pro-inflammatory effector molecules such as cytokines and LPS. This points to the potential for a positive feedback loop, in which circulating cytokines or bacterial products released from the infected lung could trigger cytokine production by the muscle fibers themselves, thereby initiating autocrine/paracrine effects that would serve to sustain pro-inflammatory gene expression in the muscle. Overall, our findings suggest that absence of CFTR in muscle causes a greater inherent sensitivity of CF muscle cells to pro-inflammatory stimuli, which might contribute to the imbalance between various effector molecules (e.g., pro- and anti-inflammatory cytokines, proteases and anti-proteases, oxidants and anti-oxidants) implicated in the muscle weakness and wasting found in CF patients.

In the present investigation, we show that CFTR is normally expressed in the diaphragm, both within intact muscle tissue *in vivo* and in differentiated myotubes *in vitro*. The CFTR gene is known to exhibit complex patterns of tissue-specific expression, with multiple *in vivo* transcription start sites and alternative splicing (30). To our knowledge, there is only one prior report of CFTR expression in skeletal muscle. Hence Fiedler et al (26) performed Northern blotting using a probe corresponding to nucleotides 2226-3714 of human CFTR, and found an mRNA species of approximately 7.5 kb in rat skeletal muscle, as compared to about 6.3 kb in the lung. Expression of CFTR has also been reported in cardiac (31-33) and smooth (34) muscle. It is interesting to note that in the only report directly comparing the two types of muscle (26), CFTR mRNA expression in rat skeletal muscle appeared to exceed that in the heart. The precise functions of CFTR in muscle tissues are not well understood. Chloride channel function is known to be important for maintaining the normal resting membrane potential of skeletal muscle fibers, but whether CFTR itself plays any role in this process is entirely unknown. In cardiac muscle, there is evidence that CFTR is responsible for normal cAMP-activated chloride currents in the heart (33). In addition, it was recently reported that the hearts of *Cftr*$^{-/-}$ mice are abnormally vulnerable to injury caused by repetitive ischemia, due to a relative inability to adopt an ischemic preconditioning response (35).

While the normal physiological role of CFTR in muscle remains unclear, dysregulation of pro-inflammatory gene expression has been reported in several studies performed in non-muscle cell types. For example, it has demonstrated that CF cell lines produce more pro-inflammatory cytokines (TNF-α, IL-6, IL-8) than normal cell lines in response to *P. aeruginosa* (23-25). Similarly, CF bronchial gland epithelial cells revealed relative upregulation of IL-6 and IL-8 expression, both *in vivo* and *vitro* (36;37). On the other hand, some other studies have failed to find such differences between CF and non-CF cell lines (38-41). The exaggerated pro-inflammatory gene expression found in cells or tissues lacking CFTR has been associated with hyperactivation of the NF-kB pathway (24; 42-45). Such a mechanism would also be consistent with the findings of the present study, since most of the cytokines and chemokines found to be upregulated in our study are known targets of NF-kB. Oxidant stress also plays an important role in inflammation, and CFTR has been implicated in regulating glutathione, which is the major anti-oxidant system of most cells. *In vitro* studies have suggested that the normal CFTR channel is permeable to glutathione (36) (46), and the absence of CFTR was associated with abnormally increased levels of intracellular glutathione, along with a concomitant reduction in pro-apoptotic proteins (47). It has been proposed that dysregulated glutathione metabolism in cells lacking CFTR may shift the balance between apoptosis and necrosis towards the latter, thereby further contributing to tissue injury and chronic inflammation in CF (47).

In Vivo: Greater pro-inflammatory gene expression and muscle weakness in the CF diaphragm during *P. aeruginosa* lung infection

Although *Cftr*$^{-/-}$ mice do not accurately model all aspects of human CF lung disease, they have proved to be a valuable tool for providing a better insight into CF pathogenesis. In this study, we have used homozygote congenic C57BL/6-*Cftr*^{UNC} knockout mice, which develop spontaneous lung disease including elements of hyperinflation, fibrosis, and inflammatory cell recruitment (48) (49). As aother group has documented (27), in our study the *Cftr*$^{-/-}$ mice were more susceptible to *P. aeruginosa* lung infection than littermate controls, as indicated by the higher bacterial counts in the lung at 2 days post-inoculation. Similar to what is observed in patients with CF lung disease, we also

observed an increase in inflammatory cells (predominantly PMNs) in the BAL of *Cftr*$^{-/-}$ versus wild-type mice during infection. Recently, van Heeckeren and coworkers (50) reported that any functional defect in CFTR causes comparable inflammatory responses to lung infection with *P. aeruginosa*. In addition, the level of pro-inflammatory mediators, including TNF-α and MIP-2 (analogue of IL-8 in human), in BAL of CF patients (21) and *Cftr*$^{-/-}$ mice (51) has been reported to be elevated in comparison to healthy subjects and wild-type mice, respectively. Similarly, we also found increased levels of TNF-α in the BAL of *Cftr*$^{-/-}$ versus wild-type mice after pulmonary infection with *P. aeruginosa*. Interestingly, the RNase protection assay from the whole lung showed a markedly elevated level of pro-inflammatory gene expression in both *Cftr*$^{-/-}$ and wild-type mice post-infection, which did not differ between the two groups. This could indicate that the regulation of pro-inflammatory gene expression in the infected *Cftr*$^{-/-}$ lung has an important post-transcriptional component, or that BAL does not accurately reflect cytokine expression within other compartments of the whole lung.

To our knowledge, this is the first study to examine the effects a CFTR gene defect on diaphragm muscle function. At the inoculating dose used in this study, *P. aeruginosa* lung infection caused severe diaphragmatic weakness only in the *Cftr*$^{-/-}$ mice, and this was associated with markedly higher levels of cytokine/chemokine expression in the *Cftr*$^{-/-}$ mouse diaphragms. Therefore, our *in vivo* data are entirely consistent with the *in vitro* diaphragmatic myotube studies, in the sense that both experimental systems point to a substantially more vigorous level of cytokine upregulation in *Cftr*$^{-/-}$ muscles in response to pro-inflammatory stimuli. In addition, it should be noted that within the *in vivo* context, a greater inherent sensitivity of *Cftr*$^{-/-}$ diaphragm muscle cells to stimulation by pro-inflammatory mediators could be compounded by the greater level of pulmonary and systemic inflammation observed in infected *Cftr*$^{-/-}$ mice. Hence a greater release of bacterial products or cytokines from the lung into the systemic circulation could serve to further amplify the already exaggerated pro-inflammatory gene expression by diaphragm muscle cells. Indeed, we observed higher serum levels of TNF-α in infected *Cftr*$^{-/-}$ mice. Serum levels of TNF-α were also reported to be significantly elevated in clinically stable

CF patients, which was further increased in setting of symptomatic respiratory exacerbation (52) (53) (22).

Upregulation of cytokine gene expression within the diaphragm muscle might contribute to muscle weakness and wasting through several mechanisms. There is considerable evidence that local expression of classical pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) could cause muscle weakness either through direct interference with contractile mechanisms or accelerated loss of muscle mass (19). Chemokines such as KC and MIP-2 (analogues of human IL-8) also have the potential to exacerbate diaphragmatic weakness by attracting neutrophils to the muscle (54). These effects were reviewed in detail in the previous chapter, in which we also demonstrated that the suppression of pro-inflammatory cytokine gene expression in the diaphragm during *P. aeruginosa* lung infection by IL-10 was associated with greatly improved force production by the diaphragm.

Conclusions and Implications

In summary, we have established that murine skeletal muscle normally expresses CFTR at the mRNA level (both *in vivo* and *in vitro*) and that CFTR expression is absent from the diaphragm in CF mice. In the setting of exposure to pro-inflammatory effector molecules associated with *P. aeruginosa* lung infection, diaphragmatic muscle cells from CF mice demonstrate an exaggerated upregulation of pro-inflammatory cytokines and chemokines, which is also associated with significant diaphragmatic weakness. Therefore, by similarly upregulating cytokine expression and impairing diaphragmatic function, chronic lung infection with *P. aeruginosa* could contribute to ventilatory insufficiency, as well as more generalized muscle weakness and wasting, in CF patients. Determining the mechanism of how a loss of CFTR function leads to increased pro-inflammatory gene expression in skeletal muscle will likely be a key to understanding and preventing muscle weakness and wasting in CF patients.

4.VII References

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CHAPTER 5

DIFFERENTIAL CYTOKINE GENE EXPRESSION IN THE DIAPHRAGM IN RESPONSE TO STRENUOUS RESISTIVE BREATHING

5.I Prologue

In the preceding chapters, we have found preferential weakness of the diaphragm and increased cytokine/chemokine expression in the diaphragm in comparison to limb muscles. This could be due to its increased activity level. In this study, , we have utilized a model of inspiratory restive loading (IRL) in rats to test the hypothesis that increased activity of the diaphragm might lead to augmented intra-diaphragmatic pro-inflammatory cytokine gene expression and this help explain its greater vulnerability compared to limb muscle.

5.II Abstract

Strenuous resistive breathing induces plasma cytokines that do not originate from circulating monocytes. We hypothesized that cytokine production is induced inside the diaphragm in response to resistive loading. Anesthetized, tracheostomized, spontaneously breathing Sprague-Dawely rats were subjected to 1, 3 or 6 hours of inspiratory resistive loading, corresponding to 45-50% of the maximum inspiratory pressure. Unloaded sham operated rats breathing spontaneously served as controls. The diaphragm and the gastrocnemius muscles were excised at the end of the loading period and messenger ribonucleic acid expression of tumor necrosis factor-alpha and beta, interleukin-1alpha, interleukin -1beta, interleukin-2, interleukin -3, interleukin-4, interleukin -5, interleukin -6, interleukin-10, interferon-gamma and 2 housekeeping genes was analysed using multi-probe ribonuclease protection assay. Interleuhin-6, interleukin-1beta and to lesser extent tumour necrosis factor-alpha, interleukin-10, interferon-gamma and interleukin-4 were significantly increased in a time-dependent fashion in the diaphragms but not the gastrocnemius of loaded animals or in the diaphragm of control animals. Elevation of protein levels of interleukin-6 and interleukin-1β in the diaphragm of loaded animals was confirmed with immunoblotting. Immunostaining revealed interleukin-6 protein localization inside diaphragmatic muscle fibers. We conclude that increased ventilatory muscle activity during resistive loading induces differential elevation of pro- and anti-inflammatory cytokine gene expression in the ventilatory muscles.

5.III Introduction

Strenuous resistive breathing has been recently shown to lead to elevation of the plasma levels of interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) (1, 2). Resistive breathing-induced plasma cytokines might serve several functions: They stimulate the hypothalamic pituitary adrenal axis (3) leading to β-endorphin release (1), and alterations in breathing pattern (4). They affect brain functions including sleep (5) and sensation of fatigue (6, 7). IL-6 has a hormone-like glucoregulatory role (6), whereas TNF-α depresses muscle and especially diaphragm contractility (8), and induces insulin resistance (9). IL-6, IL-1β and TNF-α also enhance protein degradation and have been implicated in muscle wasting (10) of chronic diseases such as chronic obstructive pulmonary disease (11, 12, 13). Whole body exercise has also been shown to induce an increase in plasma levels of cytokines such as IL-6, IL-1β, TNF-α, IL-1 receptor antagonist and IL-10 (14).

The cellular origin of these cytokines remains unknown. Monocytes, a major source of immuno-inflammatory mediators (15), have been excluded as sources of the resistive breathing-induced or whole body exercise-induced elevation of plasma cytokines (2, 16, 17, 18, 19). Myocytes have been suggested as a potential source of the exercise-induced cytokines. Indeed, muscle contraction during marathon running or knee extension increases IL-6 but not TNF-α gene expression within the exercising muscles, (20, 21), secondary to increased transcriptional activity (22) and leads to IL-6 protein release into the circulation (23). However, these results were not confirmed by other investigators who could not detect intramuscular cytokine up-regulation secondary to treadmill running (24, 25) or electrical stimulation (24). These conflicting results suggest that activation-induced intramuscular cytokine expression might be exercise- and muscle-type specific, given that different types of exercise activate different transcription factors in a manner specific to the type of muscle (26, 27). Furthermore, the cells of origin of the exercise-induced muscle derived cytokines are not known, and both resident and blood-derived invading cells are potential candidates.

Since resistive breathing is a form of exercise for the respiratory muscles associated with plasma cytokine elevation, and some forms of skeletal muscle activation lead to intramuscular IL-6 production (21) and release into the circulation (23) we hypothesized that the expressions of pro-and anti-inflammatory cytokines are up-regulated in the respiratory

muscles secondary to resistive loading and that this up-regulation is dependent on the duration of muscle activation. We evaluated in this study the nature and the time course of cytokines expression within the ventilatory muscles in response to increased activation secondary to inspiratory resistive loading. We have also identified the cellular sources of cytokines produced during strenuous ventilatory muscle contraction. We propose that myocytes are the main source of cytokine production in response to ventilatory muscle activation. Some of the results of these studies have been previously reported in the form of an abstract (28).

5.IV Materials and Methods

Animal Preparation

Male Sprague-Dawley rats (300-325 g) were anesthetized with pentobarbital sodium and tracheostomized with polyethylene tubing connected to a two-way non-rebreathing valve. The inspiratory line delivered 100% O_2 to prevent hypoxemia. After a short stabilization period, animals (n=8 in each group) were randomly assigned to a period of 1, 3 or 6 hrs of moderate inspiratory resistive loading (peak inspiratory tracheal pressure of about 50% of maximum). Other animals (n=6 per group) were exposed to either inspiratory loading for 1 hr followed by 2 hrs unloaded breathing, or intermittent loading (20 mins loading followed by 30 min recovery, repeated 3 times). Sham operated animals breathing against no load for 1, 3 and 6 hrs served as controls (n=8). Animals were euthanasized at the end of the experiment and the diaphragm and gastrocnemius muscles were quickly excised and frozen either in liquid nitrogen or in cold isopentane (20 s) before liquid nitrogen.

Ribonuclease Protection Assay

Total RNA was isolated with Proteinase K and DNaseI treatments (RNeasy kit, Qiagen, USA) and mRNA expression of IL-1α , IL-1β, TNF-α, TNF-β, IL-3, IL-4, IL-5, IL-6, IL-10, IL-2, interferon (IFN)-γ and 2 housekeeping genes (L32 and GADPH) was measured by Multi-Probe Ribonuclease Protection Assay System (RiboQuant, PharMingen, CA, USA). Briefly, the multiprobe set was hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNA were digested with RNases. The remaining RNAase-protected probes were purified, resolved on a denaturing polyacrylamide gel, and detected by autoradiography. Optical densities of various mRNAs in the scanned autoradiography films were quantified with ImagePro Plus software (Media Cyberetics Inc.).

Immunohistochemistry

Frozen tissues sections (5 μm thickness) were incubated overnight at 4°C with primary goat anti-rat IL-6 or rabbit anti-rat IL-6 antibodies. After three rinses with phosphate buffered saline (PBS), sections were incubated with biotin-conjugated anti-goat or anti-rabbit secondary antibodies followed by Cy3-labelled streptavidin. Sections were then examined under fluorescence microscopy and photographed with a digital camera.

Immunoblotting

Frozen muscle samples were homogenized in a homogenization buffer, centrifuged at 1000g for 10 min and supernatants (crude muscle homogenates, 80 μg total protein per sample) were separated onto tris-glycine SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes and probed overnight with rabbit anti-rat IL-6 and IL-1β antibodies. Specific proteins were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody and an enhanced chemiluminescence kit and quantified with ImageProPlus software (Media Cybernetics Inc.).

Myeloperoxidase activity assay

Crude muscle homogenates (in 0.5% hexadecyltrimethylammonium bromide), were mixed with 50 mM potassium phosphate buffer (pH 6.0) containing o-dianisidine dihydrochloride and H_2O_2 {Marcinkiewicz, 1998 2761 /id}. Absorbance was measured at 460 nm for 60 min. Myeloperoxidase activity was calculated in units (U): change in absorbance/minute/g protein.

Statistical analysis

Values reported are means ± SEM. Comparisons were made using Friedman analysis of variance (ANOVA) followed by Wilcoxon Matched Pairs Tests for post-hoc comparisons. A p-value of 0.05 was initially considered as statistically significant, and was accordingly adjusted using a Bonferroni-type procedure for multiple comparisons (30).

5.V Results

Ribonuclease protection assay detected weak expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ, IL-4, (highest to lowest mRNA concentration) in the diaphragm of quietly breathing (unloaded) rats. Different periods of unloaded breathing (1, 3 or 6 hours) did not change the expression of these cytokines. IL-6 mRNA was 3 times more abundant ($p< 0.05$) than the mRNAs of IL-1β, IL-10, TNF-α, IFN-γ which were equally abundant, whereas the expression level of IL-4 was one order of magnitude less than the other cytokines ($p<0.05$). A very weak expression for these cytokines was detected in the gastrocnemius, which did not change at any time point in the unloaded animals. Expression of TNF-β, IL-1α, IL-2, IL-3, IL-5 mRNAs could not be detected at any time point in the diaphragm and gastrocnemius of quietly breathing rats.

Maximum peak tracheal pressure measured prior to resistive loading averaged 75.2±11.7 cmH_2O. Peak inspiratory tracheal airway pressure developed by the animals during loading averaged 35.5±1.96 cmH_2O (46±8% of maximum peak tracheal pressure). Loaded breathing resulted in worsening hypercapnia and acidosis in a time dependent fashion, without concomitant hypoxemia, which was prevented due to the enriched inspired oxygen used (see online data supplement).

Loaded breathing resulted in a significant differential up-regulation of the expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ, and IL-4 in the diaphragm but not the gastrocnemius (Figure 1). The increase in the cytokine mRNA expression (expressed as fold-increase above the respective value of equal duration unloaded breathing) in the diaphragms of loaded animals is presented in Figure 2. With the exception of IL-1β which exhibited a nearly constant up-regulation at different time points, the other cytokines were up-regulated in a time-dependent manner, exhibiting the greatest increase after 6 hrs of loaded breathing (Figures 2, 3). IL-6 exhibited the greatest -fold increase both at 3 and at 6 hrs of loaded breathing. At each time point of loaded breathing, IL-6 mRNA was the most abundant (expressed as a percentage of the housekeeping gene L32 or GADPH), whereas the mRNA for IL-4 exhibited the weakest expression (Figure 4).

To evaluate the time course of cytokine gene expression after termination of muscle activation, a group of animals (n=6) completed 1 hr of loaded breathing followed by 2 hrs of recovery prior to tissue collection (Figure 5). With the exception of IL-10, all other cytokines were further upregulated after the termination of 1 hr of resistive loading ($p< 0.05$), suggesting that once initiated, contraction-induced diaphragmatic cytokine upregulation is a long lasting process (see online data supplement). To evaluate the influence of total duration of muscle activation on cytokine gene expression, another group of animals (n=6) underwent intermittent inspiratory resistive loading for 3 periods of 20 min separated by 30 min-periods of unloaded breathing for a total duration of muscle activation of 1 hr. This intermittent activation pattern resulted in marked up-regulation of cytokine expression (figure 5).

Figure 6 illustrates representative examples and mean values (n=5) of the changes in IL-6 and IL-1β protein expression in the diaphragm of rats exposed to 3 and 6 hrs of severe inspiratory resistive loading. No detectable IL-6 and IL-1β proteins were found in the diaphragms of animals breathing against no load. Inspiratory resistive loading for 3 hrs elicited a significant rise in diaphragm protein expression of these cytokines (figure 6). Six hours of inspiratory resistive loading elicited even greater rise in protein expression of IL-6 and IL-1β, which averaged about 10-fold higher than that observed after 3 hrs of inspiratory resistive loading (figure 6). No detectable protein expression of these cytokines was found in the gastrocnemius muscle samples in the three groups of animals (results not shown).

Figure 7 illustrates localization of IL-6 protein expression in rat diaphragms. Both goat anti-rat IL-6 (panel A) and rabbit anti-rat IL-6 antibody (panel B) detected positive IL-6 protein staining in the diaphragms of rats exposed to 6 hrs of inspiratory resistive loading. Both punctuate cytosolic and membrane-associated positive IL-6 staining (white arrows in panels A and B) was evident inside small muscle fibers, whereas large muscle fibers showed no IL-6 staining. Blood vessels were negative for IL-6 protein (white arrow in panel C). Very weak IL-6 staining was detectable in the diaphragm of quietly breathing rats (panel D). Replacement of primary antibodies with non-specific antibodies completely eliminated positive IL-6 staining (not shown).

Inspiratory resistive loading elicited no change in the myeloperoxidase activity in the diaphragms, which averaged 72.9 ± 6.2 U in animals breathing against no load, 91.3±18.0 U

after 3 hrs of inspiratory resistive loading and 80.1±9.7 U after 6 hrs of inspiratory resistive loading (p = NS).

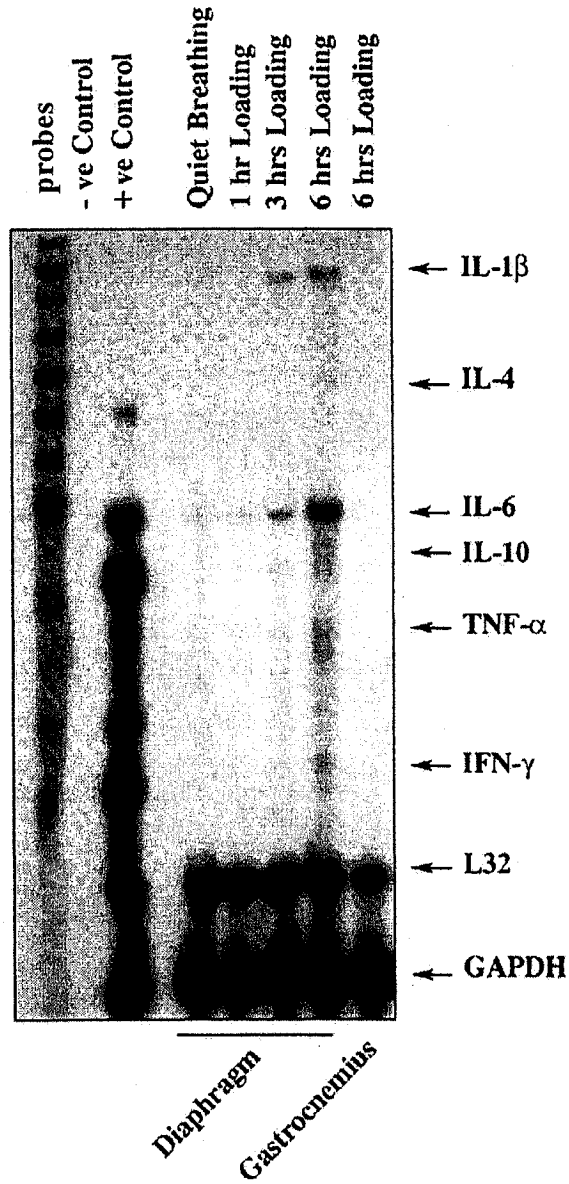

Figure 1: Representative autoradiograph of ribonuclease protection assay showing the time course of cytokine gene expression in the diaphragm and gastrocnemius muscles. Lanes 1-3: probe, the negative (-ve) and positive (+ve) control, respectively. Lane 4: diaphragm sample from control rat (quiet breathing). Lanes 5-7: diaphragm samples obtained from animals exposed to 1, 3 and 6 hrs of resistive loading, respectively. Lane 8: gastrocnemius sample obtained from rats exposed to 6 hrs of inspiratory resistive loading. A total of 10 μg RNA was used in each lane.

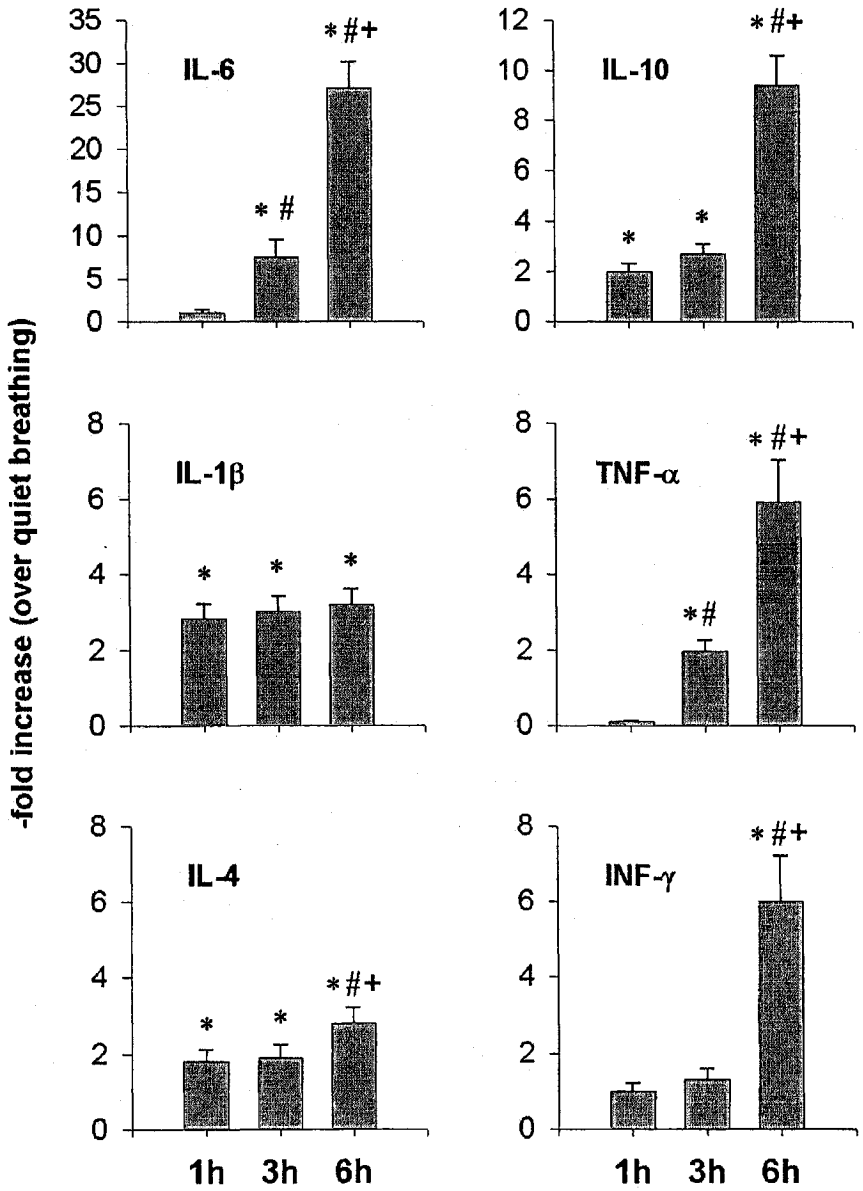

Figure 2. Time course of differential cytokine gene expression in the diaphragm secondary to inspiratory resistive loading. Data are expressed as fold increase over equal duration of unloaded (quiet) breathing, normalized to L32 mRNA. *P <0.05 compared with quiet breathing.

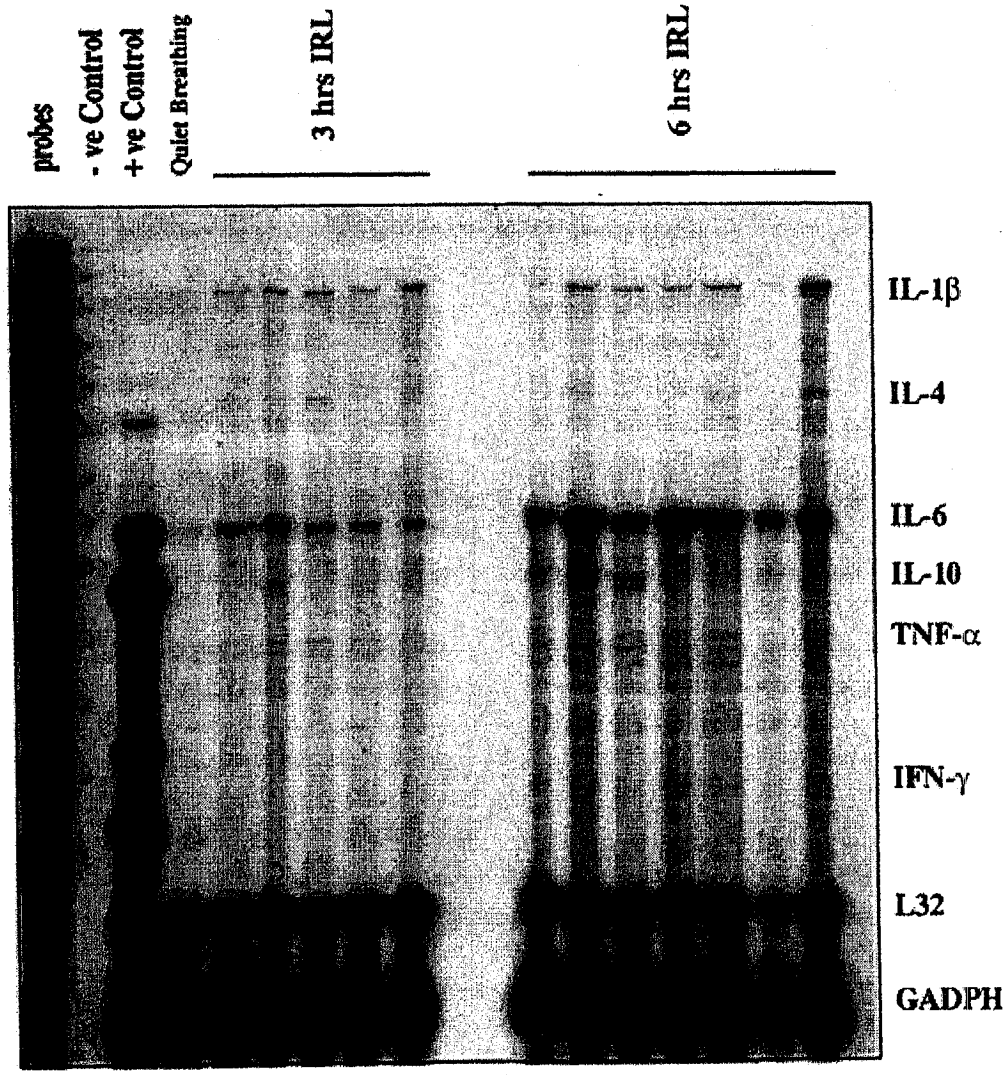

Figure 3. Representative autoradiograph of ribonuclease protection assay performed on diaphragm muscle samples obtained after 3 (lanes 5-9) and 6 hrs (10-16) of inspiratory resistive loading. Lanes 1-3: probe, the negative (-ve) and positive (+ve) control, respectively. Lane 4: diaphragm of a quietly breathing rat. A total of 10 μg RNA was used in each lane.

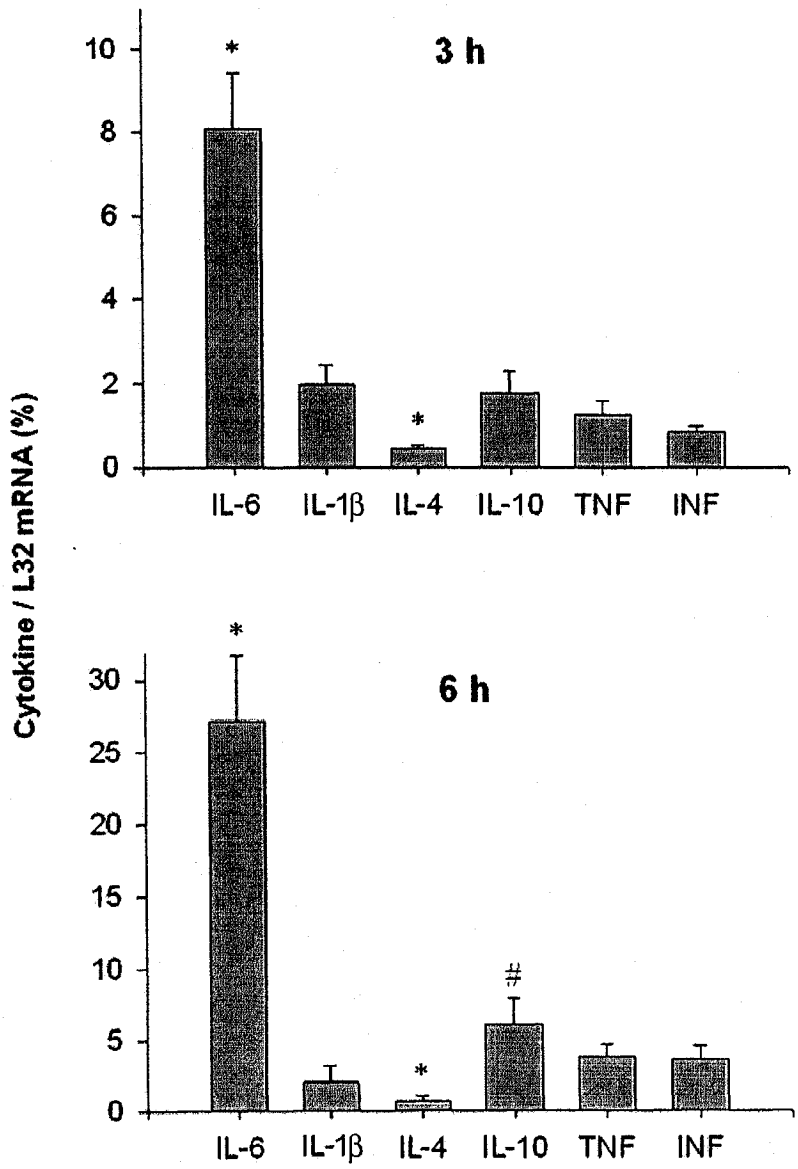

Figure 4. Relative abundance of cytokine mRNAs in the diaphragm after 3 (upper panel) and 6 (lower panel) hrs of inspiratory resistive loading (data normalized to L32 mRNA expression). *P <0.05. Please note that the scale of the upper panel is triple (0-10) compared to the scale of the lower panel (0-30).

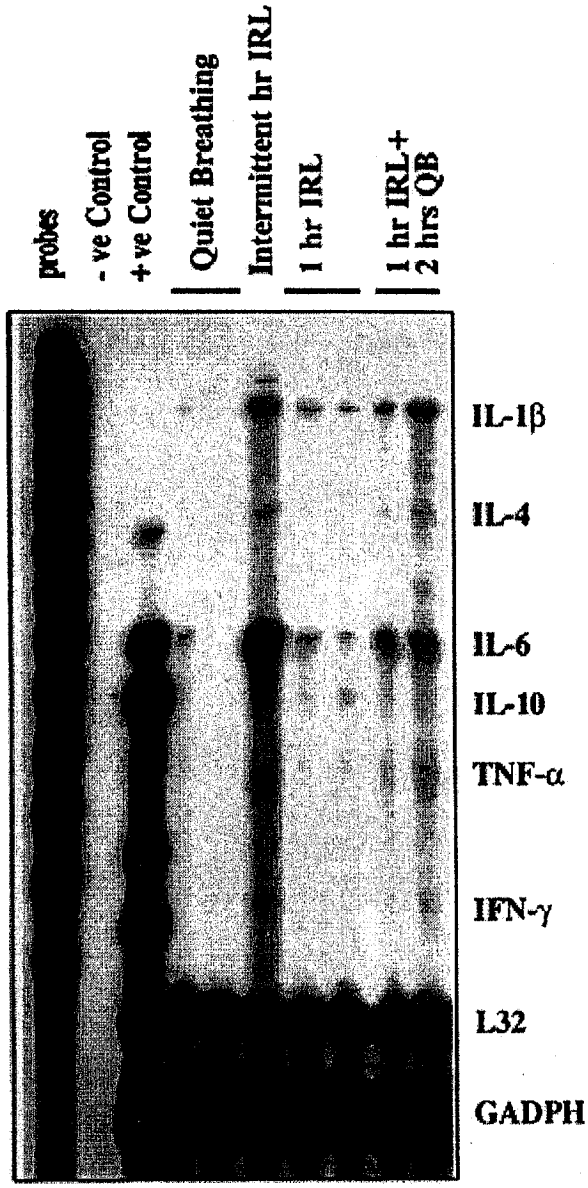

Figure 5. The influence of muscle activation pattern on diaphragmatic cytokine gene expression. Lanes 1-3: probe, the negative (-ve) and positive (+ve) control, respectively. Lanes 4 & 5: diaphragms of quietly breathing rats. Lane 6: diaphragm sample obtained after intermittent resistive loading (20 min loading-30 min quiet breathing, repeated 3 times with a total of 1 hr of inspiratory resistive loading). Lanes 7 & 8: diaphragm samples obtained immediately after 1 hr of inspiratory resistive loading. Lane 9 & 10: diaphragm samples obtained from rats exposed to 1 hr resistive loading followed by 2 hrs of quiet breathing.

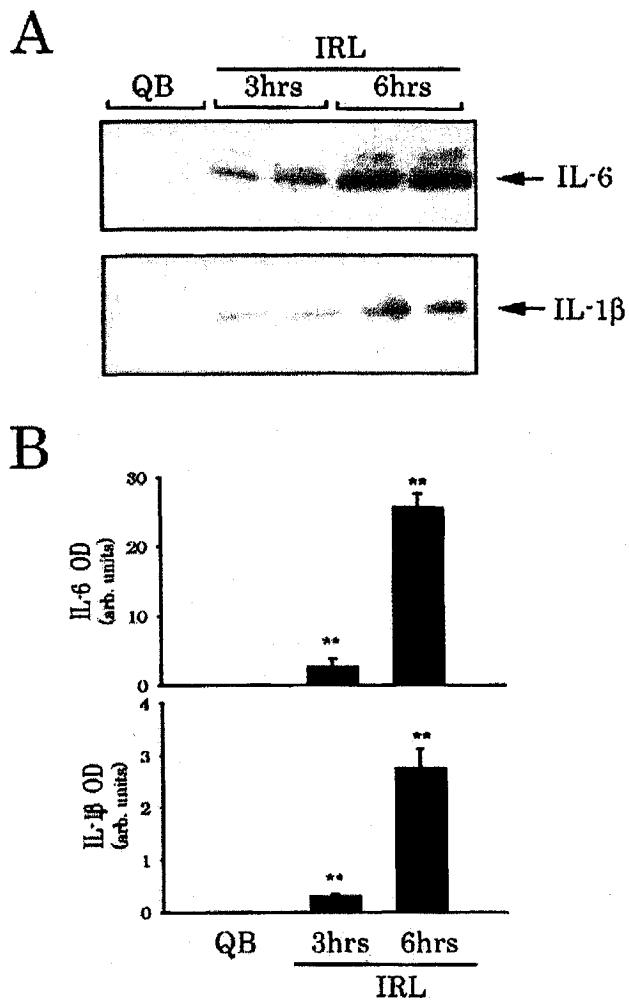

Figure 6: Representative examples of immunoblotting (upper panel, A) and mean optical density values (lower panel, B) of IL-6 and IL-1β protein expression in the diaphragm of rats exposed to 3 and 6 hrs of inspiratory resistive loading. No detectable IL-6 and IL-1β proteins were found in the diaphragms of animals breathing against no load (A, lanes 1-2). Inspiratory resistive loading for 3 hrs elicited a significant rise in diaphragm protein expression of these cytokines (A, lanes 3-4). Six hrs of inspiratory resistive loading elicited even greater rise in protein expression of IL-6 and IL-1β (A, lanes 5-6), which averaged about 10-fold higher than that observed after 3 hrs of IRL (B). QB: quiet (unloaded) breathing, IRL: inspiratory resistive loading, OD: optical density.

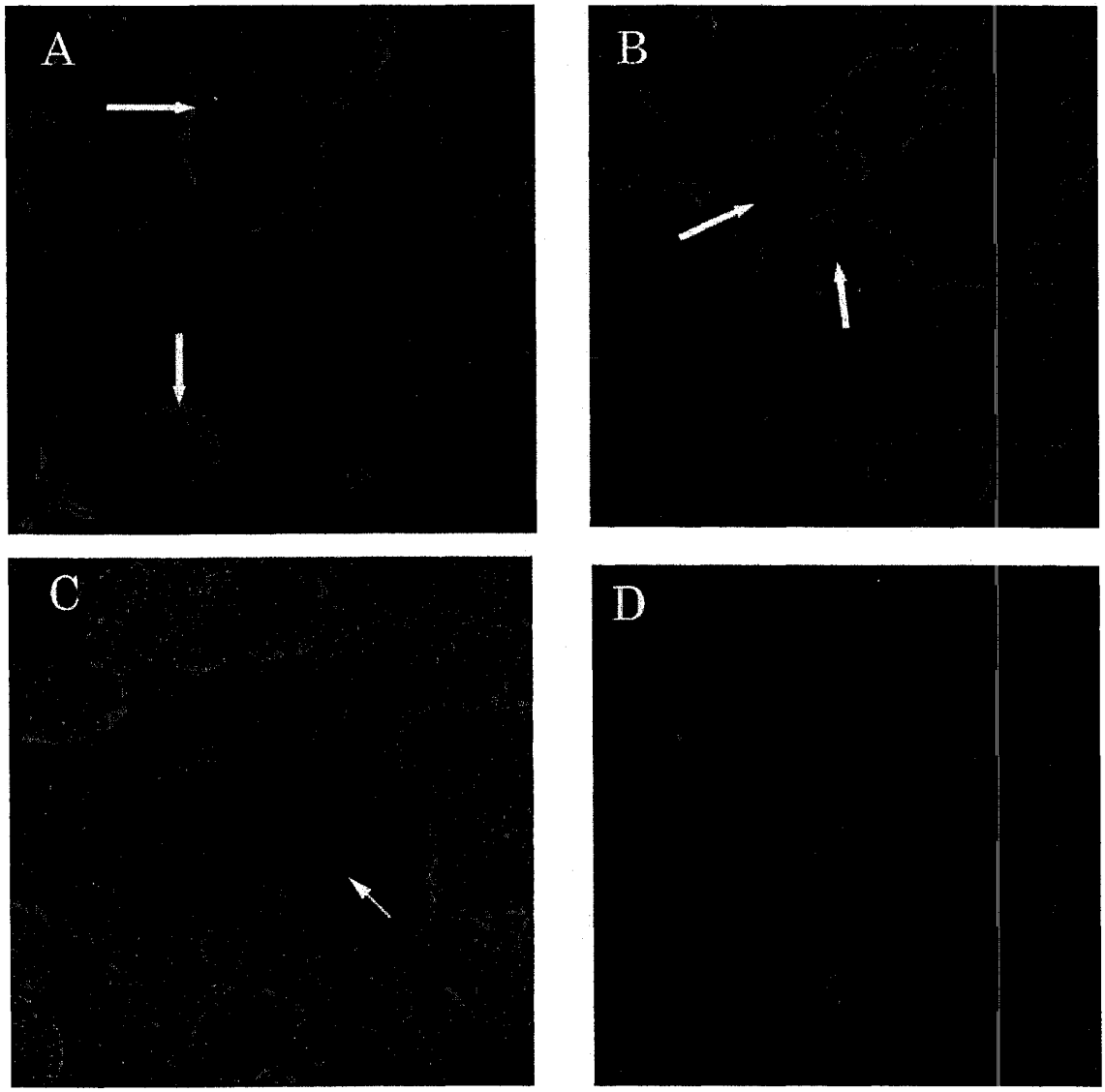

Figure 7: Localization of IL-6 protein expression in rat diaphragms. Both goat anti-rat IL-6 (panel A) and rabbit anti-rat IL-6 antibody (panel B) detected positive IL-6 staining in the diaphragms of rats exposed to 6 hrs of inspiratory resistive loading. Both membrane-associated (white arrows in panel A) and punctuate cytosolic positive IL-6 staining (white arrows in panel B) was evident inside small muscle fibers, whereas large muscle fibers showed no IL-6 staining. Blood vessels were negative for IL-6 protein (white arrow in panel C). Very weak IL-6 staining was detectable in the diaphragm of quietly breathing rats (panel D).

5.VI Discussion

The major finding of this study is that IL-6 and to a lesser extent IL-1β, TNF-α, IL-10, IL-4 and IFN-γ were significantly increased in a time- dependent manner in the diaphragms of animals subjected to inspiratory resistive loading. Immunohistochemical analysis and absence of any change in myeloperoxidase activity during resistive loading suggest that cytokines are produced inside muscle fibers and are not derived from infiltrating inflammatory cells up to six hours after inspiratory resistive loading.

To our knowledge, this is the first study showing that pro- and anti-inflammatory cytokines exhibit a low level of constitutive expression within the respiratory muscles under conditions of quiet-unloaded breathing, similar to what is observed in peripheral skeletal muscles (9, 21, 31, 32). More importantly, strenuous contraction of the respiratory muscles resulted in significant up-regulation of IL-6 expression and to a lesser extent expressions of IL-1β, TNF-α, IL-10, IL-4 and IFN-γ. The upregulation of intradiaphragmatic cytokine expression was not due a generalized increase in transcription, since no up-regulation was observed in the non-contracting gastrocnemius. Furthermore, it was not due to surgical manipulation (as previously demonstrated for the soleus (24)), since no increase was observed in the diaphragms of the animals that were subjected to the same surgical procedures without inspiratory loading. Thus, the intradiaphragmatic cytokine up-regulation was a specific response to increased activation of the diaphragm secondary to resistive loading.

It should be emphasized that we detected the messenger RNA expression of cytokines using a multi-probe ribonuclease protection assay, which does not amplify the RNA signal, is less prone to variability and errors and is significantly less sensitive from the usually used reverse transcription polymerase chain reaction (RT-PCR). The ribonuclease protection assay requires 10^4-10^5 larger quantities of RNA to be present in the tissues for positive signal detection (33) compared to the reverse transcription polymerase chain reaction that has been used for RNA detection in peripheral skeletal muscles (20, 21, 25). Since ribonuclease protection assay is less sensitive than reverse transcription polymerase chain reaction, some cytokine expression that was below the detection limit of the method might have been missed. On the other hand this secures that the upregulation of cytokine expression within the diaphragm secondary to resistive loading that we observed represents relatively abundant tissue messenger RNA levels.

The mRNA up-regulation was accompanied by commensurate increases in the cytokine protein levels, at least for the IL-6 and IL-1β. Although we have not detected the rest of the cytokines at the protein level, (which is a limitation of our study), there is no reason to expect a different response for these cytokines, since, whenever cytokine messenger RNA levels change within muscles, similar changes of protein levels occur (34, 35, 36, 37, 38, 39).

Cellular origins of muscle activation-induced cytokine expression are not yet established. Our results show that IL-6, the most abundantly expressed and upregulated cytokine secondary to increased muscle activation originates from the myocytes themselves. In fact, IL-6 exhibited both a cytoplasmic and a peri-sarcolemmal staining pattern, which is characteristic of a secreted protein. This finding is in keeping with *in vitro* results showing that myocytes are capable of producing IL-6 (38, 40, 41) secondary to stimuli relevant for exercise, such as exposure to reactive oxygen species (53) and increased intracellular Ca^{++} (41). Similar to what we found in the diaphragm, cytokines are upregulated within cardiac myocytes secondary to loading (35, 42), which suggest that IL-6 upregulation is a general response of myocytes to increased muscle activation. We have not evaluated the cellular origin of the rest of the cytokines, however, since myocytes are capable of producing a variety of cytokines *in vitro* (38), it is likely that myocytes are the sources of the augmented cytokine expression within the diaphragm, though other cells couldn't be excluded.

The stimulus for the up-regulation of cytokine expression during diaphragmatic activation is not known. We speculate that reactive oxygen species are important modulators of muscle cytokine production as indicated by the blunting by antioxidants of the elevation in plasma IL-6, IL-1β and TNF-α (2, 19) induced by either resistive loading (2) or whole body exercise (19) and by the induction of IL-6 production from cultured myocytes exposed to reactive oxygen species (40). Depletion of glycogen muscle stores during muscle activation could also regulate cytokine production as indicated by augmentation of muscle IL-6 expression after glycogen depletion (22, 23). Finally, preliminary data suggests that the rise in intracellular Ca^{++} can also lead to IL-6 secretion by myocytes (41).

Implications: Resistive breathing induced intra-diaphragmatic cytokine production may serve several local and systemic functions, which could be both adaptive and maladaptive. For instance, cytokines may play an important role at the local level by promoting muscle fiber injury. Resistive loading achieved in our study was of such magnitude that likely produces diaphragmatic injury (43, 44, 45, 46, 47). Our results raise the

interesting possibility that intra-diaphragmatic cytokine induction could be involved in mediating the injurious process by upregulating the expression of adhesion molecules on the surface of endothelial cells (48) and by enhancing transendothelial migration of blood-derived inflammatory cells (49), responses that would augment infiltration of neutrophils and promotion of muscle fiber injury. Although myeloperoxidase activity- an index of tissue infiltration by neutrophils was not increased in the diaphragms of animals up to six hours of resistive loading, this might be due to inadequate time (neutrophilic influx taking place later) or to inadequate power of our study to document a statistically significant response (a 25% increase in myeloperoxidase activity observed would require 70 animals per group). Pro-inflammatory cytokines such as TNF-α may also promote fiber injury by augmenting muscle reactive oxygen species production (10). These species are well known players in ventilatory muscle injury (50). The majority of evidence suggests that TNF-α also suppresses diaphragmatic contractility (51, 52, 8), (though earlier studies had suggested that TNF-α has either no effect (53) or affects diaphragmatic contractility only at high doses (54)) which might explain the observation that force decline after resistive loading is proportionally greater than the observed muscle injury (44).

We should emphasize that not only pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ were induced inside the diaphragm during resistive loading but anti-inflammatory cytokines such as IL-4, IL-10, and IL-6 (which has some pro- but mainly anti-inflammatory properties (55)) were also upregulated, suggesting that few of these cytokines may serve to oppose local muscle inflammation (55). Cytokines are also essential in orchestrating muscle recovery after injury. Cytokines such as TNF-α, IL-6 and LIF (leukemia inhibitory factor) and IL-1β (56, 57, 58, 31) and their cognate receptors (59) are up-regulated in skeletal muscle after injury. These cytokines enhance proteolytic removal of damaged proteins (60, 61) and damaged cells (through recruitment and activation of phagocytes). TNF-α and LIF are important signaling molecules for the regeneration of muscle fibers after injury (57, 62). TNF-α receptor double knock-out mice or mice receiving TNF-α neutralizing antibodies exhibit a reduced muscle strength recovery after injury compared to wild-type mice, associated with a reduced expression of the myogenic transcription factor MyoD (57). This is in concert with data suggesting that TNF-α promotes differentiation of myoblasts by increasing NF-κB activity (63) and both activates satellite cells to enter the cell cycle from the normally quiescent state and enhances their proliferation once it has been initiated (64). Nevertheless, it has to be acknowledged that the differentiation promoting effect of TNF-α

has been debated (65, 66, 67). More studies are needed to elucidate the exact role of cytokines in skeletal muscle injury and recovery.

The significantly greater induction of IL-6 within the diaphragm compared with other cytokines suggests that IL-6 might be involved in physiological muscle signaling (68). Diaphragmatic contraction leads to glycogen depletion, which greatly augments IL-6 production from skeletal muscles (23, 22). IL-6 has an hormone-like role, signaling that glycogen stores are reaching critically low levels in the contracting muscles and stimulating hepatic glucose output to maintain glucose homeostasis and muscle glucose supply (6, 69). IL-6 also mobilizes free fatty acids from triglycerides stored in fat tissue, thus increasing the energy that is available to the muscle.

We also speculate that diaphragm-derived cytokines might spill into the circulation leading to elevation of plasma cytokine levels. Ventilatory muscle production of cytokines could have been the source of elevated plasma cytokines observed after resistive loading in normal humans (1, 2) or in diseases of increased respiratory load, such as chronic obstructive pulmonary disease (70, 71) and sleep apnea (72). Elevation of circulating cytokines derived from the ventilatory muscles might have systemic effects including changes in breathing pattern (1) and sensation of fatigue (6, 7). Muscle-derived cytokines may also contribute to the cachexia observed in some chronic obstructive pulmonary disease patients (11, 12, 13). Further studies are needed to elucidate these interesting possibilities.

In conclusion, we have shown that inspiratory resistive loading results in differential cytokine expression in the diaphragm. Both pro-inflammatory and anti-inflammatory cytokines are expressed in a time dependent manner, which might have both local and systemic effects.

5.VII References

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CHAPTER 6

CONCLUSIONS

Conclusions

Respiratory failure is a hallmark of patients with sepsis or cystic fibrosis. Although respiratory insufficiency was traditionally recognized as a lung disease, there is growing evidence that the respiratory pump, especially the diaphragm, plays a major role in respiratory insufficiency. Diaphragm muscle dysfunction and ventilatory pump failure are well documented after acute endotoxin (LPS) administration. However, animal models of endotoxin have three major limitations: (i) in order to show muscle contractile dysfunction, a massive dose of LPS must be used which may be physiologically irrelevant; (ii) LPS is only one component of a whole Gram-negative bacteria, and many other interactions between host immune response and bacteria are absent; (iii) the endotoxin animal model is only suitable to study an acute response, while understanding the chronic aspects of respiratory muscle insufficiency is very important. Therefore, we have tried to overcome some of these limitations by studying respiratory muscle function in a more clinically relevant animal model of *Pseudomonas aeruginosa* lung infection.

In the present thesis, we are the first to have reported that either subacute (2 days) or more sustained (7 days) pulmonary infection with *P. aeruginosa* causes severe diaphragmatic weakness, and this would occurs in a time and dose-dependent manner. Intriguingly, whereas diaphragmatic contractility was greatly impaired during *P. aeruginosa* lung infection, limb muscle function was unaffected under the same conditions. We also found that there is no correlation between diaphragmatic weakness and the number of inflammatory cells or bacterial burden in the lung. This is consistent with observations in other diseases, such as COPD, showing there is no direct correlation between sputum and serum levels of individual markers of inflammation, despite the fact that both sputum and serum have elevated levels of these markers compared with control subjects (1). This suggests that although there might be cross talk between two compartments, differential regulation exists between the inflammatory response in the pulmonary and extrapulmonary compartments, which may explain the absence of correlation between contractile dysfunction of the

diaphragm and local pulmonary inflammation in our study (Chapter 2).

We also revealed that the preferential impairment of diaphragmatic contractility was not due to muscle fiber type (slow versus fast) composition. However, several other factors, such as location, and the level of muscle activity might explain differences between the diaphragm and limb muscle responses. Regarding to latter, greater activity of the diaphragm might contribute to its increased vulnerability by an exaggerated generation of cytokines within the contracting muscle fibers. In chapter 5, we attempted to explain such a possibility by subjecting animals to inspiratory resistive loading (IRL), which increases activity of the diaphragm. This resulted in significantly increased pro- as well as anti-inflammatory cytokine gene expression in a time-dependent manner within the diaphragm. This is the first study to show that intra-diaphragmatic cytokine up-regulation is a specific response to increased activation of the diaphragm secondary to resistive loading. Moreover, no up-regulation was observed in hindlimb muscle of the animals subjected to IRL. We also have shown that muscle fibers are the source of cytokine production at least up to six hours of IRL. These findings support the possibility that greater muscle activity by the diaphragm is one, but not necessarily the only factor, which contributes to its greater vulnerability.

Based on these findings, we also wished to study the possible role of cytokines in diaphragm and hindlimb muscle following *P. aeruginosa* lung infection. This is the first study to show that diaphragmatic pro-inflammatory cytokine gene expression is highly up-regulated within infected animals, and the magnitude of such up-regulation is dependent upon the dose of *P. aeruginosa*. In contrast to the diaphragm, the levels of pro-inflammatory gene expression were not altered within the hindlimb muscle. This differential response of diaphragm and limb muscle to *P. aeruginosa* lung infection is consistent with greater vulnerability of the diaphragm to contractile dysfunction under the same conditions, as we have shown in chapter 2. Therefore, we show that under condition of pulmonary infection with *P. aeruginosa*, muscle weakness was associated with higher expression of pro-inflammatory gene expression within the muscle, which emphasizes adverse effects of multiple pro-inflammatory cytokines on skeletal muscle function.

The adverse effects of cytokines on the intrinsic contractile properties of skeletal muscle can be either direct or indirect. Incubation of isolated skeletal muscle with TNF-α produces membrane depolarization (2) and a blunting of the response of myofilaments to calcium activation, which impairs muscle contractility *in vitro* (3). Cytokines also stimulate a complex of signaling pathways including reactive oxygen species (ROS) or reactive nitrogen species (RNS), which can both alter muscle contractile properties. Transgenic mice that overexpress TNF-α have shown elevated levels of cytosolic oxidants in diaphragm muscle as well as diaphragmatic dysfunction (4). More recently, the oxidation of some key proteins (e.g. actin), which can lead to contractile dysfunction, have been identified and localized inside of the diaphragm of septic animals with endotoxemia (5). Therefore, *P. aeruginosa* lung infection leads to increased exposure of the diaphragm muscle to pro-inflammatory cytokines that may either directly or indirectly, via activation of ROS/RNS pathways, induce diaphragmatic dysfunction. In this study, we have not evaluated the exact source of these cytokines; however, because neither histologic nor biochemical examination revealed any evidence of increased diaphragmatic inflammation in the infected mice, myocytes are probably the source of these cytokines. It is known that myocytes are capable of producing variety of cytokines *in vitro* (6) as well as *in vivo* (7) (8).

To address more precisely the role of diaphragmatic pro-inflammatory mediator gene expression in diaphragmatic contractile dysfunction, we have employed recombinant adenovirus (Ad) as a vehicle for the systemic delivery of the anti-inflammatory cytokine IL-10, with the intention to shift the balance between pro- and anti-inflammatory cytokines within the diaphragm. Thus, in the present thesis, we are the first to have reported the functional benefits of systemic delivery of IL-10 on the diaphragm, by suppressing pro-inflammatory gene expression and improving force generating capacity after *P. aeruginosa* infection. We also have revealed that the IL-10 receptor is expressed in the diaphragm, suggesting that the effects of IL-10 might be directly on the diaphragm muscle fibers. Although other anti-inflammatory treatment (anti-TNF-α and IL-1 receptor antagonist) failed to show beneficial effects in septic patients, this could be due to the fact that inhibition of only one cytokine may not have a significant impact, since there is redundancy between pro-inflammatory cytokines. We show that IL-10 is able to simultaneously suppress

multiple pro-inflammatory cytokines in the diaphragm.

Sepsis and its adverse effects on respiratory muscle function represent an important clinical problem, particularly in the Intensive Care Unit (ICU) setting. The etiology of muscle dysfunction in critically ill patients is multi-factorial. Systematic inflammation is a common phenomenon in patients in the ICU. Inflammation is initiated and regulated in large part by small biologically active mediators, cytokines. Cytokines like TNF-α have been shown to induce muscle contractile dysfunction and catabolism in different human inflammatory diseases, such as sepsis, cancer, congestive heart failure, and AIDS (5). However, our understanding of the key pathophysiologic cytokines involved in this process remains limited. We now show for the first time that cytokines are strongly up-regulated in the diaphragm during pulmonary *P. aeruginosa* infection and that anti-inflammatory cytokine (IL-10) treatment suppresses intra-diaphragmatic cytokine production and improves diaphragmatic force production. This study emphasizes the role of IL-10 as an anti-inflammatory cytokine and a beneficial immune modulator in excessive inflammatory diseases that can cause respiratory muscle failure. This is not only novel but potentially very important clinically, since it may offer an avenue for therapeutic intervention. Investigating the pattern of selected cytokine levels has the potential to provide unique biologic markers that identify patients at risk for muscle dysfunction. Moreover, a balance between pro- and anti-inflammatory cytokines is now shown to be important in preventing myocyte degradation/dysfunction.

P. aeruginosa lung infection is a major cause of morbidity and mortality among cystic fibrosis (CF) patients and many patients with CF have weak peripheral and respiratory muscles. CFTR expression has been reported in rat skeletal muscle (9). CFTR appears to functions in the autonomic control of smooth muscle cells (10) and also serves as a chloride channel in cardiac myocytes (11) (12), but its functional role in skeletal muscle has not been elucidated. Myotonia congenita is a disease that is caused by a mutation in the gene coding for the muscle chloride channel (CLC-1), which causes increased muscle stiffness due to an increase in membrane-excitability (13). In rested muscle, chloride is the most membrane permeant ion (14) and 85% of resting membrane ion conductance is attributed to chloride (15). A decreased concentration of extracellular chloride

favors fatigue in skeletal muscle from mice (16). The beneficial effect of chloride on skeletal muscle force is probably due to protection against excessive depolarization after a large stimulation, perhaps through some active transport of chloride (16;17). Interestingly, skeletal muscle weakness is observed in CF patients with normal lung function and good nutritional status (18), suggesting intrinsic abnormalities in the skeletal muscle. Therefore, the CFTR chloride channel play a role in skeletal muscle after a large stimulation such as intense exercise/lung infection by affecting cell membrane potential. Moreover, changes in the membrane potential may trigger signaling transduction events and subsequent gene expression (19;20). Future studies will be needed to determine the precise role of CFTR in skeletal muscle function.

The expression of pro-inflammatory mediators has not been studied in skeletal muscle lacking CFTR, in either animal models or human CF disease. In the present thesis, we have used a CF mouse model of *P. aeruginosa* lung infection to address several fundamental questions related to muscle function in CF. First, we demonstrate that diaphragmatic skeletal muscle cells *per se* express CFTR. Second, we determined that there is an intrinsic difference in terms of pro-inflammatory gene expression between CF and wild type muscle cells *in vitro*, which might differentially affect the regulation of pro-inflammatory mediators in the setting of infection/inflammation. Third, we reveal that absence of CFTR expression is associated with upregulated intra-diaphragmatic pro-inflammatory gene expression and impaired force-generating capacity of the diaphragm muscle, after *P. aeruginosa* lung infection *in vivo*. Therefore, in CF patients, increased circulating cytokines during the setting of symptomatic respiratory exacerbations may trigger respiratory muscles to overexpress pro-inflammatory cytokines/chemokines, which can contribute to muscle weakness through several mechanisms as mentioned earlier. These include (1) direct effects of pro-inflammatory cytokines on contractile muscle properties, (2) induction of muscle wasting, and (3) recruitment of inflammatory cells to the muscle which could in turn augment the production of pro-inflammatory mediators and muscle injury.

In conclusion, 40-45% of total body mass is composed of skeletal muscle, which is able to express a variety of immunologically relevant molecules, like cytokines, chemokines, adhesion

molecules, and both classes of major histocompatibility molecules. Remote inflammatory processes in different diseases, such as cancer, arthritis, sepsis, and cystic fibrosis might contribute to muscle wasting and weakness through circulating cytokines by increased exposure of the muscle fibers to force-inhibiting cytokines. This, in turn, may trigger further autocrine/paracrine effects due to pro-inflammatory mediator expression by the muscle fibers themselves. Thus, insight to the basic mechanisms of muscle weakness and interference or modulation of such a mechanism may have beneficial effects. In the end, we are fully aware of the gap that exist between translation of animal studies to human diseases. However, we hope the data in this thesis can serve as a foundation for the future studies for understanding of the mechanisms underlying respiratory muscle weakness in septic or CF patients.

Claims of originality

This thesis has provided several original contributions to the existing body of scientific knowledge, theses are as follows:

Chapter 1.

- Demonstration of preferential weakness of diaphragm compared to hindlim muscles in a clinically relevant animal model of pulmonary *Pseudomonas aeruginosa* infection.

Chapter 2.

- Demonstration of pro-inflammatory cytokines expression in the diaphragm but not hindlimb muscles after *Pseudomonas aeruginosa* lung infection.
- Demonstration of IL-10 receptor expression in the skeletal muscle both *in vivo* and *vitro*.
- Demonstration that systemic adenovirous IL-10 gene delivery suppresses the pro-inflammatory gene expression within the diaphragm and ameliorates the diaphragmatic force-generating capacity during *Pseudomonas aeruginosa* lung infection.

Chapter 4.

- Demonstration that CFTR is expressed at the mRNA level in mouse skeletal muscle both *in vivo* and *vitro*.
- Demonstration that absence of CFTR exaggerates cytokines/chemokines production in the diaphragm muscle both *in vitro* and *in vivo*.
- Demonstration that absence of CFTR affects diaphragmatic dysfunction after *Pseudomonas aeruginosa* lung infection.

Chapter 5.

- Demonstration that increased diaphragm muscle activity augments inflammatory gene expression within the diaphragm.

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APPENDIX

ıflict of Interest Statement: M.J.T. does not have a relationship with any me-
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ung Infection and the Diaphragm
lacing Basic Research in Clinical Perspective

ır understanding of respiratory muscle dysfunction induced sepsis has advanced markedly in the last 20 years: different echanisms have been proposed (alterations in calcium homeo-ısis, mitochondrial dysfunction, and sarcolemal injury) and the le of several mediators (proinflammatory cytokines, reactive ygen species, and nitric oxide) has been characterized (1). espite these advances, however, several unresolved questions main.

In this issue of the *Journal* (pp. 679–686), Divangahi and co-orkers show, for the first time, that *Pseudomonas aeruginosa* ng infection lasting for 7 days induces significant diaphragmatic eakness without change in the strength of hindlimb muscles). Because respiratory mechanics were unchanged during the ıtire experimental period, diaphragmatic fatigue seems an un-ely cause of the muscle weakness. This study sets the stage discuss two clinically relevant features of respiratory muscle ıthophysiology that are still relatively underinvestigated: the fects of infections arising in thoracic or abdominal organs; and e effects of chronic infections.

Respiratory failure is a common complication of severe infec-ous or inflammatory processes originating in organs or tissues the abdominal and thoracic cavities, such as peritonitis, pan-eatitis, or pneumonia. These pathological conditions can theo-tically affect the diaphragm directly by contiguity. Relatively w investigators have examined the effects of acute peritonitis ıd pancreatitis on the diaphragm in animals (3–6); they showed significant reduction in diaphragmatic strength, as observed ' Divangahi and coworkers (2) with lung infection. Preferential eakness of the respiratory muscle was also observed with necro-ing pancreatitis in rats (5), the only study investigating the fects of abdominal processes that compared the diaphragm and hindlimb muscles. In contrast with these data, systemic inoculation of two boluses of *Escherichia coli* endotoxin in hamsters produced equivalent decreases in the strength of the diaphragm and a fast-twitch peripheral muscle (7). Divangahi and coworkers (2) hypothesize that both muscle activity (sustained in the case of the diaphragm) and topographic proximity between the respiratory muscle and the inflamed lung could be responsible for its selective weakness. This is also probably true in the case of abdominal disorders. Diaphragmatic histology was normal and muscular levels of myeloperoxidase, a marker of neutrophil infiltration, did not increase in either the muscle of the *Pseudomonas*-infected animals (2) or in animals with pancreatitis and peritonitis (3, 5), thus excluding direct extension of the infectious/inflammatory process to the muscle. Anatomical proximity to the infectious site could, however, be responsible for direct exposure of the diaphragm to bacteria and/or inflammatory mediators synthesized in the infected organ via direct lymphatic spread (8). Another pathway might involve mediators synthesized by activated macrophages and/or mesothelial cells of the pleural or peritoneal surfaces of the muscle. These mediators could, in turn, act on the underlying diaphragm, as seen in the heart, where mediators released by the cardiac endothelium act on the underlying myocardium (9).

The results of Divangahi and coworkers (2), showing diaphragmatic impairment 7 days after the bacterial inoculum, are of clinical importance because long-lasting infections are common. Few investigators have examined the effects of chronic or semichronic infections on the respiratory muscles. Drew and associates (10) studied the effects of chronic visceral leishmaniasis on the diaphragm and hindlimb muscles of hamsters. They noted atrophy of all muscles and a selective loss in force of a

hindlimb fast-twitch muscle, even after correction for the loss of muscle mass. The effects of long lasting infections are probably close to these of semistarvation, with progressive catabolism and degradation of contractile proteins possibly playing a greater role than in acute sepsis (1). Data from the study of Divangahi and coworkers (2) support the progressive nature of muscle impairment in that the *Pseudomonas* inoculum, which induced diaphragmatic weakness at 7 days, did not impair strength generation at 2 days. What is the role of known mediators of acute diaphragmatic dysfunction in chronic infectious impairment? Both tumor necrosis factor-α and reactive oxygen species induce acute contractile dysfunction and stimulate muscle catabolism by increasing ubiquitin conjugation to muscle proteins (11, 12), a persistent response that accelerates the targeting of muscle proteins for degradation by the 26S proteasome. These mediators act probably on different steps of the ubiquitin–proteasome pathway: tumor necrosis factor upregulates the UbcH2 gene via nuclear factor-κB activation (11), whereas reactive oxygen species upregulates the polyubiquitin gene and genes for key E2 and E3 proteins, independently of nuclear factor-κB activation (12). To date, there are no data on the interplay between these biochemical events and respiratory muscle dysfunction in chronic infections.

The role of diaphragmatic weakness as a predisposing factor for ventilatory failure and/or to difficulties in weaning from mechanical ventilation raises important questions in the context of altered lung function secondary to chronic lung infection. Placing the muscle at rest protects the rat diaphragm against contractile failure induced by acute sepsis (13), although the effects of prolonged mechanical ventilation on a muscle with an already curtailed function because of prolonged infection are unknown. Controlled mechanical ventilation in rabbits decreases the generation of diaphragmatic strength per se at 24 hours, and this phenomenon is accentuated at 3 days in conjunction with apparent myofibril damage (14). An increased protease activity and augmented oxidative stress is observed in the rat diaphragm as early as 18 hours after controlled mechanical ventilation (15). If these biochemical alterations are prolonged or accentuated with time, as suggested by sequential analysis of gene expression patterns in immobilized soleus muscle in rats (16), they could mimic those induced by chronic infection. Therefore a deleterious additive and/or synergistic effect on the diaphragm between long-lasting infections and mechanical ventilation cannot be excluded. A time-dependent evaluation of diaphragmatic strength, mass, fiber-type composition, protein metabolism, and gene expression profile could help us to understand the molecular basis of the effects of chronic infections on the muscle and their interactions with mechanical ventilation. The experimental model of lung infection utilized by Divangahi and coworkers (2) could be a useful and interesting experimental tool in this setting. This model may help us find strategies to prevent or minimize the effects of chronic infections on the respiratory muscles.

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Sickle Cell Disease—Pulmonary Complications and a Proinflammatory State?

Pulmonary complications frequently lead to mortality and morbidity in patients with sickle cell disease. In a multicenter study, more than 20% of the adults suffered fatal pulmonary complications. (1). Lung function abnormalities are present in young children with sickle cell disease (2). Restrictive abnormalities become more prominent with increasing age (2) and even young adults with sickle chronic lung disease have restrictive lung disease with abnormal diffusing capacity and hypoxemia. The development of pulmonary hypertension increases the mortality up to seven fold (3). A major risk factor for sickle chronic lung

Preferential Diaphragmatic Weakness during Sustained *Pseudomonas aeruginosa* Lung Infection

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Infection with *Pseudomonas aeruginosa* plays a major role in the pulmonary inflammation and injury associated with cystic fibrosis. Lung inflammation may also lead to more widespread systemic effects on other organs. We tested the following hypotheses: (*1*) ongoing *P. aeruginosa* lung infection produces diaphragmatic and limb muscle weakness and (*2*) such muscle dysfunction is directly correlated with the level of pulmonary inflammation. Chronic bronchopulmonary infection with mucoid *P. aeruginosa* was induced in C57BL/6 mice. At Day 2 after infection, diaphragmatic force was decreased (37%) only in mice infected with a high dose of 1×10^6 cfu, whereas by Day 7 after infection, diaphragmatic force was similarly reduced (36%) even at a fivefold lower inoculating dose. No significant correlations were found between diaphragmatic weakness and pulmonary inflammation, as assessed by the number of neutrophils, macrophages, and lymphocytes in bronchoalveolar lavage fluid. Moreover, in marked contrast to the diaphragm, no effects of *P. aeruginosa* infection on contractile function were observed in prototypical slow- and fast-twitch hindlimb muscles. We conclude that sustained lung infection with *P. aeruginosa* induces preferential weakness of the diaphragm, which is not directly correlated with the degree of pulmonary inflammation induced under these conditions.

Keywords: respiratory muscles; sepsis; cystic fibrosis; lung inflammation; chronic Pseudomonas pneumonia

Cystic fibrosis (CF) is the most frequent autosomal recessive disorder in the white population, affecting approximately 1 in 2,500 live births. Exercise capacity is significantly reduced in patients with CF, and this is associated with a worsened prognosis (1). Interestingly, muscle weakness and a diminished capacity for performing work have been reported in patients with CF having essentially normal spirometry and nutritional status (2). This is also in keeping with the fact that abnormalities of muscle function not readily attributable to muscle atrophy have been observed (3). Therefore, additional factors beyond diminished lung function or malnutrition and muscle atrophy are likely to be involved in producing skeletal muscle weakness in patients with CF.

Patients with CF are particularly prone to chronic or recurrent pulmonary infections with the mucoid strain of *Pseudomonas aeruginosa*. This pathogen plays a central role in the vicious cycle of lung infection and inflammation, which ultimately culminates in irreparable lung damage, respiratory failure, and death (*see* Reference 4 for review). Although the role of local pulmonary inflammation in the pathogenesis of CF lung disease is well established, it is unknown whether this also contributes to skeletal muscle dysfunction. However, there is increasing recognition that lung injury and pulmonary inflammation may trigger a systemic inflammatory response (5–7). In addition, several investigators have reported that serum levels of tumor necrosis factor-α, a known inducer of muscle wasting and weakness (8, 9), are significantly elevated in patients with CF (10–12).

In this study, we hypothesized that pulmonary inflammation triggered by *P. aeruginosa* infection could be an important cause of diaphragmatic as well as peripheral limb muscle dysfunction, thereby contributing to the global muscle weakness found in patients with CF. To mimic the scenario found in CF, we employed a previously characterized murine model of chronic *P. aeruginosa* infection (13, 14). In this model, bacteria are encapsulated within agar beads to impede pulmonary clearance of the organisms, which allows for the establishment of an ongoing but clinically tolerable infection (13, 14). In this study, our specific objectives were threefold: (*1*) to determine the effects of a sustained pulmonary infection with *P. aeruginosa* on the function of the diaphragm, as well as prototypical slow-twitch (soleus) and fast-twitch (extensor digitorum longus [EDL]) hindlimb muscles; (*2*) to examine the relationship between alterations in respiratory or limb muscle contractile function and pulmonary mechanics, pulmonary bacterial burden, and the level of lung inflammation induced by *P. aeruginosa* infection; and (*3*) to ascertain the extent to which these responses might differ at different stages of the infection process. Some of the results of this study have been reported previously in the form of an abstract (15).

METHODS

Animal Model of Sustained *P. aeruginosa* Infection

Studies were performed in 8–10-week-old C57BL/6 male mice weighing 20 to 25 g (Charles River Laboratories, Saint Constant, PQ, Canada), which were used in accordance with the guidelines established by the Canadian Council on Animal Care. Under anesthesia, the trachea was intubated with a sterile cannula to deliver either *Pseudomonas*-laden or sterile agar bead suspension to mouse lungs. The model of chronic pulmonary infection with *P. aeruginosa* was performed essentially as described by Starke and coworkers (13), using a mucoid strain of the bacteria originally isolated from a patient with CF (16).

Bronchoalveolar Lavage

The trachea was cannulated with a 22-gauge catheter connected to two separate syringes via a three-way stopcock. One syringe was used to instill 5 ml of cation-free Hank's balanced salt solution (GIBCO, Burlington, ON, Canada) into the lungs, whereas the second syringe allowed the fluid to be collected by gentle aspiration. Differential cell counts were performed on cytospin preparations stained with Diff-Quick (American

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entific Products, McGaw Park, IL). A total of 300 to 400 cells were ınted on each cytospin preparation, and the cells were classified as crophages, lymphocytes, and polymorphonuclear leukocytes using ndard morphologic criteria (16).

/eloperoxidase Assay

/eloperoxidase activity in lung and muscle was measured as described Koike and coworkers (17), with minor modifications.

ng Bacterial Colony Assay

rial dilutions (1:10) of homogenized lungs were plated on Petri dishes ıtaining trypticase soy agar. The number of *P. aeruginosa* cfu was ınted after overnight incubation at 37°C (16).

easurements of Respiratory Mechanics

e trachea was cannulated with a snug-fitting metal needle and con- cted to a computer-controlled small animal ventilator (flexiVent; IREQ, Montreal, PQ, Canada) for measurement of respiratory sys- n mechanics as described previously (18). The mice were paralyzed th pancuronium chloride (0.07 mg/kg intraperitoneally) and venti- ed in a quasisinusoidal fashion. Respiratory system resistance was rived from the relationship between airway opening pressure and flow, and quasistatic deflation pressure–volume curves were collected evaluate potential alterations in compliance.

ntractile Function of Diaphragm and Limb Muscles

aphragm, soleus, and EDL muscles were surgically excised for *in vitro* ntractility measurements under isometric conditions, as described eviously in detail (19). The excised diaphragm strip and limb muscles re each mounted simultaneously into separate jacketed tissue bath ambers filled with equilibrated Krebs solution. The muscles were pramaximally stimulated using square wave pulses (Model S88; Grass struments, West Warwick, RI). The force–frequency relationship was termined by sequentially stimulating the muscles for 1 second at 5, , 20, 30, 50, 100, 120, and 150 Hz, with 2 minutes between each mulation train. Fatigability of the muscles was assessed by measuring e loss of force in response to repeated stimulations over a 3-minute riod (30 Hz, 330 millisecond duration).

atistical Analysis

ll data are presented as mean values ± SE. Group mean differences re determined by analysis of variance, with *post hoc* application of e Tukey test where appropriate. Linear regression was performed ing the least-squares method. A statistics software package was used r all analyses (SigmaStat V2.0; Jandel Scientific, San Rafael, CA). atistical difference was defined as p value less than 0.05.

RESULTS

Clinical Status

Mice were killed at either 2 or 7 days after infection with *P. aeruginosa*–laden agar beads. Two different doses of inoculating bacteria (2×10^5 and 1×10^6 cfu) were studied at 2 days after infection, whereas only the lower inoculating dose was used for the 7-day time point due to unacceptable signs of ill health at the higher dose in the 7-day group. As has been reported previously (20), body weight was slightly reduced in infected mice at Day 2 (−7 and −11% for 2×10^5 and 1×10^6 cfu, respectively) and to a lesser extent at Day 7 (−6%) compared with animals injected with sterile beads at the same time points.

Lung Bacteriology

Pulmonary bacterial counts at Days 2 and 7 after infection are shown in Figure 1, together with values obtained from the lungs of control mice. The pulmonary bacterial burden did not differ significantly between the two inoculating doses evaluated at Day 2 after infection, although there was a trend toward increased cfu values with the higher dose (Figure 1A). In addition, there was no significant change in pulmonary bacterial load between Days 2 and 7 after infection at the lower inoculating dose of 2×10^5 cfu, indicating an inability to clear the bacteria-laden beads and persistent ongoing infection (Figure 1B). Control (CTL) mice in which no previous intervention had been made, as well as mice that had been instilled with sterile agar beads (CTL-beads), were culture negative at both time points.

Bronchoalveolar Lavage

The total number of inflammatory cells contained within bronchoalveolar lavage (BAL) fluid was markedly increased at Day 2 after infection, particularly in mice inoculated with the higher dose of 1×10^6 cfu (*see* Figure 2A). This was due to increases in both polymorphonuclear leukocytes and macrophages. In contrast, by Day 7 after infection, the total number of cells found in infected mice was not significantly greater than those found in the CTL-beads group, although higher numbers of polymorphonuclear leukocytes and lymphocytes were found in infected mice (Figure 2B). There was no significant difference in BAL cell numbers between the CTL and CTL-beads groups at either time point.

Respiratory Mechanics

To ascertain whether the instillation of agar beads (either sterile or bacteria laden) into the lungs produced changes in resistance

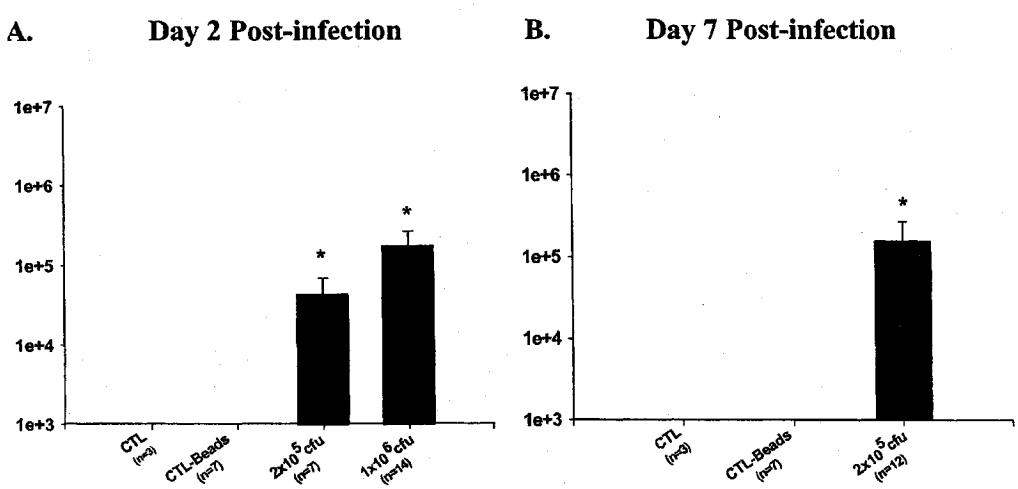

Figure 1. Pulmonary bacterial burden. Mice were killed to quantify numbers of bacteria in the lung at (*A*) Day 2 and (*B*) Day 7 after infection. Values are group means ± SE. *p Value less than 0.05 compared with control (CTL) mice in which no previous intervention had been made and mice that had been instilled with sterile agar beads (CTL-beads) groups.

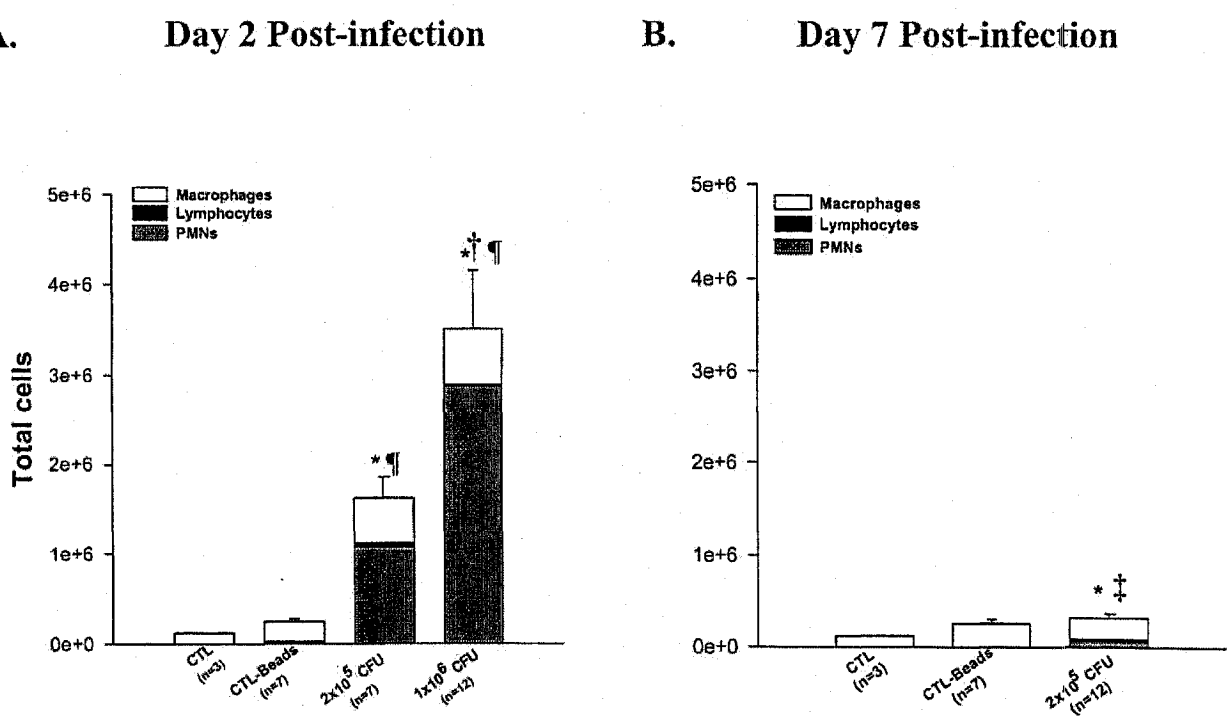

Figure 2. Pulmonary inflammatory response to *Pseudomonas* infection. Inflammatory cells in bronchoalveolar lavage (BAL) fluid at (*A*) Day 2 and (*B*) Day 7 after infection. Values are group means ± SE. p Value less than 0.05 compared with CTL and CTL-beads groups: *Polymorphonuclear leukocyte (PMN); †macrophages; ‡lymphocytes; ¶total inflammatory cells.

or compliance, respiratory mechanics were measured in a subset of mice from the different experimental groups. As can be seen from Figure 3, there were no significant differences among the various experimental groups in either the resistance or the pressure–volume relationship.

Diaphragmatic Contractile Function

Figure 4 illustrates the effects of *P. aeruginosa* infection on the diaphragmatic force–frequency relationship at both time points. With the inoculating dose of 2×10^5 cfu, the force–frequency relationship of the diaphragm was unaltered at Day 2 after infection. However, the higher dose of 1×10^6 cfu greatly depressed the force-generating capacity of the diaphragm in comparison with the other three groups (CTL, CTL-beads, and 2×10^5 cfu). Moreover, in contrast to its lack of effect at Day 2, the lower inoculating dose of 2×10^5 cfu was associated with a marked decrease in diaphragmatic force production at Day 7 after infection (Figure 4B). On the other hand, the ability of the diaphragm to resist fatigue *in vitro* was not significantly affected by *P. aeruginosa* infection under any of the conditions studied (Figure 5).

Relationship between Contractile Dysfunction and Inflammation

To determine whether there was any direct relationship between the level or nature of pulmonary inflammation and the observed impairment in diaphragmatic force production after *P. aerugi-*

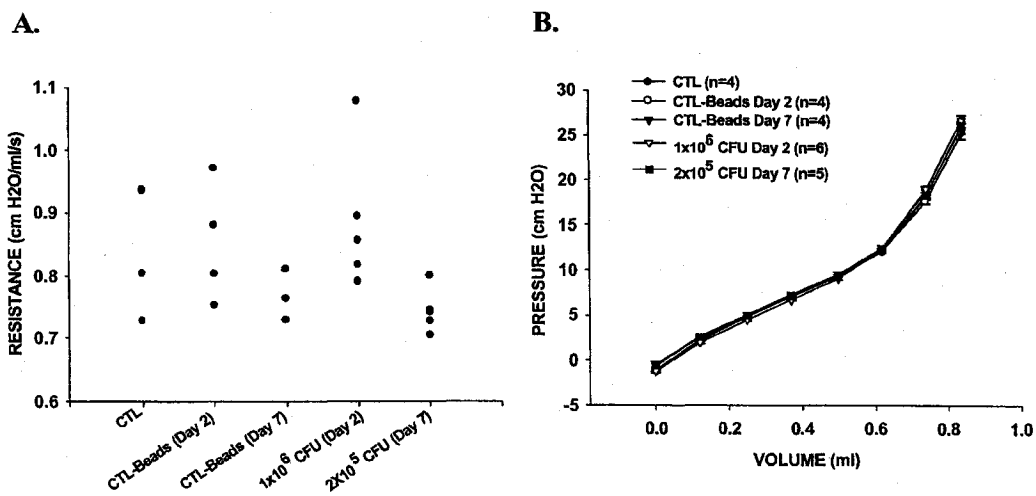

Figure 3. Respiratory system mechanics. (*A*) Individual values for resistance of the respiratory system at the indicated time points. (*B*) Group mean values (± SE) for quasistatic pressure–volume curves are shown. There were no significant differences among groups.

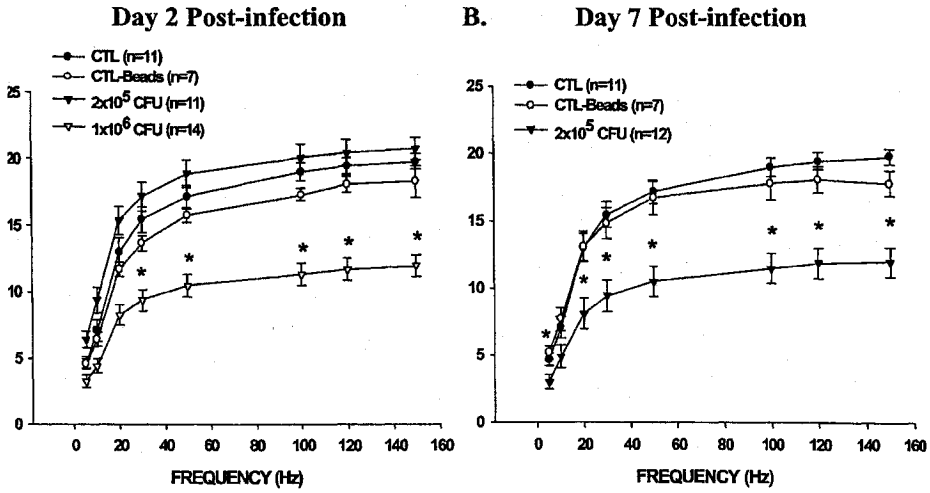

Figure 4. Effects of *Pseudomonas* lung infection on the diaphragmatic force–frequency relationship. (*A*) At Day 2 after infection, diaphragmatic force was significantly decreased at a dose of 1×10^6 but not with the lower dose of 2×10^5 cfu. (*B*) At Day 7 after infection, diaphragmatic force was also decreased at the lower infecting dose of 2×10^5 cfu. Values are group means ± SE. *p Value less than 0.05 compared with CTL and CTL-beads groups.

sa infection, correlation analysis was performed (*see* Table 1). Day 2 after infection, total BAL cell number showed the ongest correlation with diaphragmatic force impairment, although this did not achieve statistical significance (p = 0.11). At ıy 7 after infection, the best correlation with diaphragmatic ce impairment was obtained for total lymphocytes in BAL, t once again this failed to reach statistical significance (p = 1). There was also no significant correlation between diaragmatic weakness and bacterial burden in the lungs at either ıy 2 (p = 0.51) or Day 7 (p = 0.26) after infection.

We next evaluated whether *P. aeruginosa* infection of the lungs ıs associated with inflammatory cell infiltration of the diaragm. Diaphragm muscle sections stained with hematoxylin and sin (not shown) did not reveal inflammatory cell infiltration at her Day 2 or Day 7 after infection. In addition, to address is issue in a more quantitative manner, we also performed yeloperoxidase assays (marker of neutrophil content) on the sues of infected mice. In keeping with the BAL data, Figure ı shows a large increase in myeloperoxidase activity within e lung tissue of infected mice at Day 2 after infection, with a bsequent decline toward control values by Day 7. In contrast, gure 6B shows that myeloperoxidase activity within the diaragm was negligible under control conditions and remained at both Days 2 and 7 after infection.

Hindlimb Muscle Contractile Function

Figure 7 shows the effects of instilling *P. aeruginosa*–laden beads into the lungs on the force–frequency relationship of the soleus muscle at Days 2 and 7 after infection. In marked contrast to results obtained in the diaphragm, there was no significant effect of either 1×10^6 cfu (Day 2) or 2×10^5 cfu (Day 7) on specific force production by the soleus. Similarly, the endurance properties of the soleus muscle were not significantly altered by *P. aeruginosa* infection (*see* online supplement). Because the diaphragm contains a higher proportion of fast-twitch fibers than the soleus, we also determined the response of a fast-twitch limb muscle (the EDL) under the same conditions. Essentially identical results were obtained for the fast-twitch EDL, i.e., no significant effects of *P. aeruginosa* infection on either the force–frequency relationship or endurance properties of the muscle were found (*see* online supplement).

DISCUSSION

This study demonstrates that persistent pulmonary infection with *P. aeruginosa* produces significant contractile dysfunction of the diaphragm. The major findings of our study can be summarized as follows: (*1*) the diaphragm was preferentially susceptible to the adverse effects of *P. aeruginosa* infection on skeletal muscle

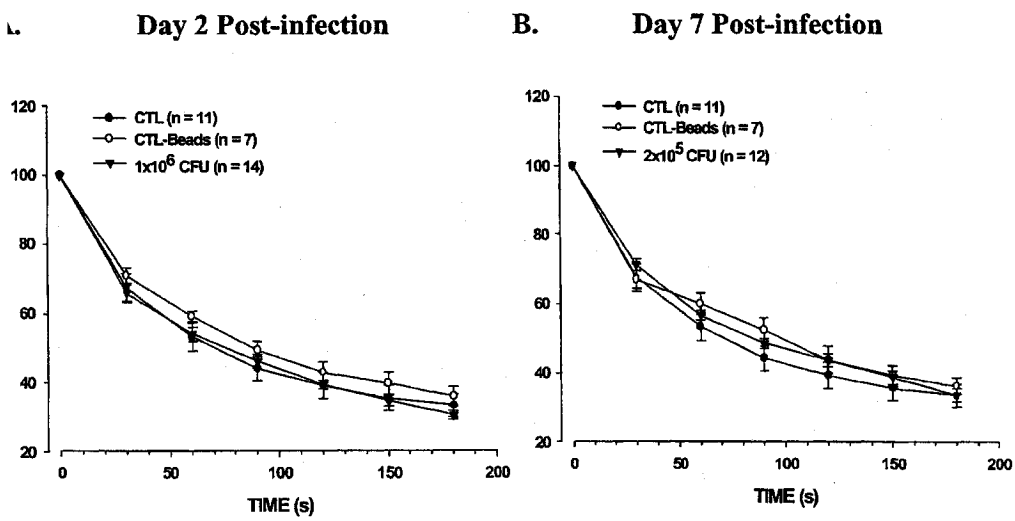

Figure 5. Effects of *Pseudomonas* lung infection on diaphragmatic endurance properties. Values are group means ± SE and are expressed as a percentage of the initial force values obtained at the onset of the fatigue protocol. There were no significant differences among groups at either (*A*) Day 2 or (*B*) Day 7 after infection.

TABLE 1. RELATIONSHIP BETWEEN MAXIMAL FORCE PRODUCTION BY THE DIAPHRAGM AND BRONCHOALVEOLAR LAVAGE INFLAMMATORY CELLS

	Total BAL Cells	Macrophages	Lymphocytes	PMNs
Maximum diaphragm force, Day 2 after infection				
p Value	0.11	0.87	0.77	0.17
r Value	−0.36	−0.03	−0.11	−0.34
Maximum diaphragm force, Day 7 after infection				
p Value	0.38	0.85	0.21	0.93
r Value	−0.12	−0.05	−0.37	−0.06

Definition of abbreviations: BAL = bronchoalveolar lavage; PMN = polymorphonuclear leukocytes.

Data sets used for the calculations consisted of individual values obtained from the control (CTL)-beads and infected animal groups at the indicated time points.

function because hindlimb muscles displayed normal function under the same conditions, (*2*) the process was exacerbated by a more prolonged duration of infection, and (*3*) no statistically significant relationships were found between the level of diaphragmatic weakness after infection and either pulmonary bacterial burden or lung inflammatory cell counts within BAL fluid.

Pseudomonas Lung Infection Model

The *Pseudomonas* lung infection model used in this study offers several advantages. First, it may be more clinically relevant than the high doses of LPS typically used to induce diaphragmatic dysfunction in most studies. Second, it involves inclusion of additional virulence factors within the bacteria other than LPS (e.g., *Pseudomonas* exoenzyme S, a potent inducer of cytokine expression; *see* Reference 21), such that the full range of microbe versus host interactions can be expressed. Third, it produces a predominately neutrophilic inflammatory infiltrate within the lungs and an associated tissue damage, which are both similar to changes observed in the infected CF lung (14). Finally, by virtue of its more sustained nature, the chronic lung infection model offers the ability to study responses at different stages of the infection process. On the other hand, the model used in our study also suffers from certain limitations. In particular, it does not precisely mimic the natural history of *P. aeruginosa* infection in CF from airway colonization to lung injury. In addition, the mice employed in our study lack impaired pulmonary defense mechanisms and other aspects of the multiorgan dysfunction found in patients with CF.

To date, the vast majority of studies aimed at investigating the effects of sepsis on respiratory muscle function have employed LPS to produce a state of acute endotoxemia. Under these conditions, diaphragmatic dysfunction appears not to be caused by LPS itself but rather by its ability to induce the release of endogenous free radical species (22, 23) and other proinflammatory mediators (9, 24). To our knowledge, the only study examining the effects of a chronic infection on diaphragmatic function was performed by Drew and coworkers (25), who found reduced specific force generation by the fast-twitch plantaris muscle, but not the diaphragm or soleus, at 7 to 12 weeks after infecting hamsters with the protozoan parasite *Leishmania donovani*. In addition, despite the high frequency of pneumonia as a clinical problem, few studies have examined the effects of pulmonary infection on diaphragmatic function. Desmecht and coworkers (26) performed intratracheal instillation of *Pasteurella haemolytica* in calves and reported that a subset of animals displayed evidence of diaphragmatic dysfunction over a 10-hour period. Boczkowski and coworkers (27) also reported a significant reduction in diaphragmatic force production 3 days after subcutaneous inoculation of rats with *Streptococcus pneumoniae*, although there was no histologic evidence of pneumonia in their model.

In immunocompetent mice, direct intratracheal inoculation or aerosolization of *P. aeruginosa* produces only transient infection, with essentially complete bacterial clearance from the lungs within 24 to 48 hours (13, 14). To induce a more sustained infection, we employed a model in which *P. aeruginosa* bacteria are first embedded in agar before intrapulmonary instillation. The ability of this method to achieve a chronic *Pseudomonas* lung infection has been validated in several animal species (14). However, because instillation of sterile agar beads alone can

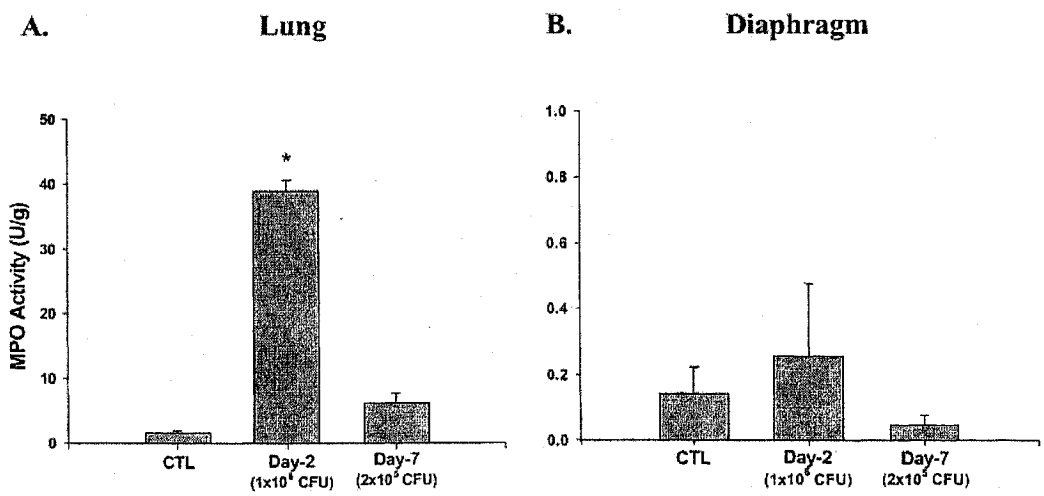

Figure 6. Myeloperoxidase (MPO) activity in lung and diaphragm after *Pseudomonas* infection. (*A*) In the lung, there was a large increase in MPO activity at Day 2 after infection, which then declined toward CTL values by Day 7. (*B*) In the diaphragm, there was no significant effect of *Pseudomonas* lung infection on MPO activity, which remained extremely low (note the difference in *y-axis* scale as compared with the lung). Values are group means ± SE (n = 6 per group). *p Value less than 0.05 compared with CTL.

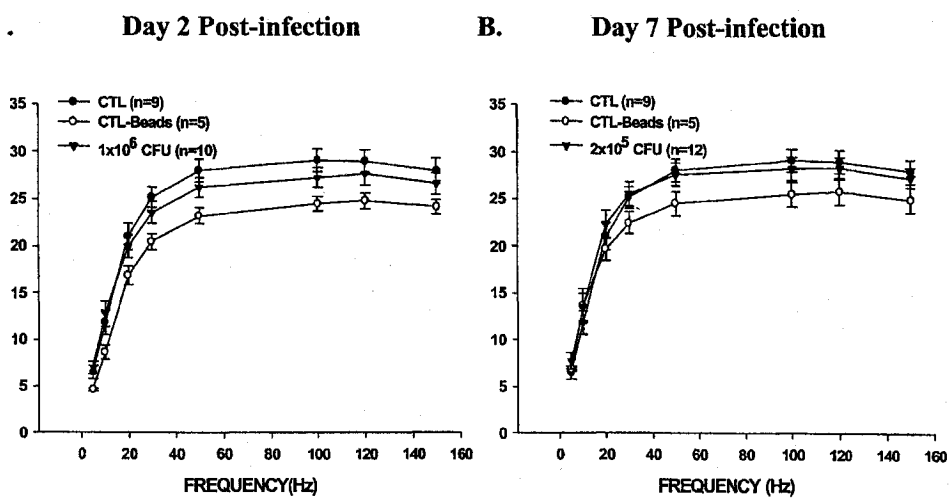

Figure 7. Effect of *Pseudomonas* lung infection on the limb muscle (soleus) force–frequency relationship. Values are group means ± SE. *Pseudomonas* lung infection had no significant effects on soleus muscle force production at either (*A*) Day 2 or (*B*) Day 7 after infection.

use mild and transient mononuclear cell infiltration in the ngs (14), we also ascertained the effects of this intervention ı BAL cell counts and skeletal muscle function. Importantly, ı significant effects of sterile agar beads on these parameters ere observed. In addition, we ascertained that intrapulmonary stillation of agar beads (either alone or combined with bacte- ı) had no significant effects on respiratory mechanics, thus nfirming a previous report (20). Therefore, we believe that e changes found in our study can be attributed to *P. aeruginosa* fection rather than to any nonspecific effects related to the perimental procedure.

ıle of Pulmonary Inflammation

ıere is a large body of literature implicating local pulmonary flammation in the deterioration of lung function observed in ıtients with CF (*see* Reference 4 for review). Recently, there ıs also been increased interest in the idea that pulmonary flammation and lung injury may trigger more widespread sysmic inflammatory effects (5, 7). Increased peripheral blood vels of various markers of inflammation have been documented patients with CF as well as other forms of chronic obstructive ılmonary disease (6, 10–12, 28). In addition, circulating tumor crosis factor-α and C-reactive protein levels in CF are further creased in the setting of symptomatic respiratory exacerbations 1, 12). Such findings have led to the suggestion that systemic anifestations of disease, including muscle wasting and weak- ss, may be caused by ongoing pulmonary inflammation.

In this study, we sought to determine whether there is a direct lationship between either the number or type of inflammatory lls present within the lung and *P. aeruginosa*–induced diaphragatic dysfunction. Previous studies have reported a significant rrelation between BAL fluid neutrophils and infection-related eight loss in wild-type mice, as well as in genetically altered CF ice, after intrapulmonary instillation of *Pseudomonas*-laden agar ads (20, 29). In our study, although there were trends relating tal BAL cell count at Day 2 after infection and BAL lymphote count at Day 7 after infection with diaphragmatic weakness, ne of the relationships examined was statistically significant. addition, at the lower inoculating dose of 2×10^5 cfu, severe aphragmatic dysfunction developed between Days 2 and 7 ter infection despite a fall in BAL inflammatory cell counts er the same time period.

There are several possible explanations for these findings. or example, a better correlation may have existed between aphragmatic weakness and the levels of certain cytokines produced by pulmonary inflammation, rather than the numbers of inflammatory cells present within the lungs of infected animals. Although we cannot exclude this possibility, in the same model, van Heeckeren and coworkers (20) reported that the correlations between infection-induced weight loss and either proinflammatory cytokine levels or absolute neutrophil counts within BAL fluid were of similar statistical strength. Another possibility is that inflammatory cells within the lung interstitium were not accurately reflected by the cells retrieved in BAL fluid and that it is the former that are most involved in the systemic inflammatory response induced by *P. aeruginosa* lung infection. However, BAL fluid cell counts were found previously to be significantly correlated with infection-related weight loss as mentioned earlier (20, 29). In addition, previous studies have generally reported a good relationship between BAL and whole-lung inflammatory cell characteristics in this model (30–32).

We believe that differential regulation of the inflammatory response in the pulmonary and extrapulmonary compartments is the most likely explanation for our findings. In support of this proposition, it has recently been shown that in patients with chronic obstructive pulmonary disease, there is no direct correlation between sputum and serum levels of individual markers of inflammation, despite the fact that both sputum and serum show elevated levels of these markers compared with control subjects (6). This suggests that although there is no doubt cross talk between the two compartments, the extrapulmonary systemic inflammatory response does not simply reflect spillover from the lung but is instead an independently regulated process. Moreover, it is important to note that proinflammatory mediators can be expressed by diaphragm muscle fibers themselves (9, 22, 33) and that the timing of such an expression may differ from that found in neighboring inflammatory cells (22). Therefore, differences between the pulmonary and extrapulmonary compartments in the specific mediators involved and/or the timing of their expression likely accounts for the fact that certain aspects of the systemic response, such as contractile dysfunction of the diaphragm, do not correlate well with local pulmonary inflammation.

Preferential Weakness of the Diaphragm

A particularly interesting finding in our study was the presence of muscle-specific contractile impairment, i.e., in the diaphragm but not in limb muscles (EDL and soleus) of infected animals. The EDL is adapted for relatively infrequent bursts of phasic activity, whereas the soleus is tonically activated to maintain posture. The diaphragm is essentially always active except for

very short pauses, even during sleep. However, differences in fiber type composition among these muscles are unlikely to have played a role in our findings because the diaphragm is intermediate in this respect between the fast-twitch, glycolytic EDL and the slower-twitch, more oxidative soleus. In addition, our data do not support inflammatory cell infiltration into the muscle as a cause for the preferential diaphragmatic impairment because neither histologic nor biochemical (myeloperoxidase activity) examination revealed any evidence of increased diaphragmatic inflammation in the infected mice.

Several previous studies (22, 34, 35) have reported a greater susceptibility of the diaphragm to the effects of endotoxemia in comparison with limb muscles. On the other hand, Supinski and coworkers (36) found equivalent reductions in force production by the diaphragm and flexor halluces longus muscle after LPS injection. The precise reasons for these apparent discrepancies are not clear but could relate to variations in the route, timing, and dosage of LPS administration as well as species differences. In a transgenic mouse model of heart failure in which cardiac and serum (but not diaphragmatic) tumor necrosis factor-α levels are elevated, Li and coworkers (37) reported a major loss of force-generating capacity in the diaphragm, whereas the EDL and soleus muscles were unaffected. However, this same group also found no differences in the intrinsic susceptibility of isolated diaphragm and limb muscle fibers to tetanic force depression by tumor necrosis factor-α administered *ex vivo* (38).

We speculate that the greater activity level of the diaphragm *in vivo* may have contributed to its increased vulnerability to *P. aeruginosa* infection in our study. Muscle activity can potentially exacerbate diaphragmatic injury and weakness during sepsis through several mechanisms. These include (*1*) an exaggerated generation of free radical species by contracting muscle fibers (23), (*2*) imposition of contraction-induced mechanical stress on muscle fiber membranes made hyperfragile by exposure to free radicals (39), and (*3*) increased exposure of muscle fibers to force-inhibiting cytokines, either through increased endogenous production of such molecules by the muscle fibers themselves (9, 22, 33) or via augmented flow of blood-borne molecules to working muscles (40). Regarding the latter, fever and increased respiratory rates associated with sepsis, although not directly documented in our study, would be expected to further increase blood flow to the diaphragm. In addition, although our data do not indicate an increased susceptibility to *in vitro* diaphragmatic fatigue after infection, this may not be the case *in vivo*. This is because the propensity to develop fatigue is inversely related to the maximal force-generating capacity of the muscle, as reflected by an increase in the tension–time index of the diaphragm (41). Therefore, diaphragmatic weakness *per se* favors the onset of diaphragmatic fatigue under conditions of spontaneous breathing *in vivo*.

It is also possible that the close proximity between the infected lung and the diaphragm contributed to the preferential impairment of diaphragmatic contractility. The peritoneal and pleural surfaces of the diaphragm are both lined by mesothelial cells, and beneath this layer lies a network of lymphatics (42–44). On the peritoneal side, small openings (stomata) connect the peritoneal cavity with these diaphragmatic lymphatics, and tracer studies have revealed that substances injected intraperitoneally are capable of attaining the lymphatics as well as connective tissue spaces of the diaphragm (42). Similar but less frequent stomata have also been reported on the pleural surface of the diaphragm (43). Accordingly, it is conceivable that proximity and indeed direct communication between the diaphragmatic interstitial compartment and proinflammatory mediators induced within the pleural space by lung infection (45) might be involved in the loss of diaphragmatic force-generating capacity observed in our study.

Conclusions

In summary, we have shown that sustained lung infection with *P. aeruginosa* results in significant weakness of the diaphragm. Interestingly, even relatively mild respiratory tract infections have been found to cause further decreases in respiratory muscle strength, together with attendant hypercapnia, in patients with underlying respiratory muscle impairment (46). By impairing diaphragmatic function, chronic lung infection may similarly contribute to ventilatory insufficiency in patients with underlying lung disease from various causes, such as CF and chronic obstructive pulmonary disease. To the extent that patients with CF have a greatly reduced ability to clear *P. aeruginosa* from the lungs, this phenomenon could be particularly exaggerated in patients with CF. Application of the *P. aeruginosa* infection model in genetically altered CF mice (16, 29) could provide valuable insights into these questions.

Conflict of Interest Statement: M.D. has no declared conflict of interest; S.M. has no declared conflict of interest; R.W.R.D. has no declared conflict of interest; S.A.T. has no declared conflict of interest; W.B. has no declared conflict of interest; D.R. has no declared conflict of interest; A.S.C. has no declared conflict of interest; B.J.P. has no declared conflict of interest.

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Differential Cytokine Gene Expression in the Diaphragm in Response to Strenuous Resistive Breathing

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Strenuous resistive breathing induces plasma cytokines that do not originate from circulating monocytes. We hypothesized that cytokine production is induced inside the diaphragm in response to resistive loading. Anesthetized, tracheostomized, spontaneously breathing Sprague-Dawley rats were subjected to 1, 3, or 6 hours of inspiratory resistive loading, corresponding to 45–50% of the maximum inspiratory pressure. Unloaded sham-operated rats breathing spontaneously served as control animals. The diaphragm and the gastrocnemius muscles were excised at the end of the loading period, and messenger ribonucleic acid expression of tumor necrosis factor-α, tumor necrosis factor-β, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IFN-γ, and two housekeeping genes was analyzed using multiprobe RNase protection assay. IL-6, IL-1β, and, to lesser extents, tumor necrosis factor-α, IL-10, IFN-γ, and IL-4 were significantly increased in a time-dependent fashion in the diaphragms but not the gastrocnemius of loaded animals or in the diaphragm of control animals. Elevation of protein levels of IL-6 and IL-1β in the diaphragm of loaded animals was confirmed with immunoblotting. Immunostaining revealed IL-6 protein localization inside diaphragmatic muscle fibers. We conclude that increased ventilatory muscle activity during resistive loading induces differential elevation of proinflammatory and antiinflammatory cytokine gene expression in the ventilatory muscles.

Keywords: interleukin; loaded breathing; respiratory muscles; ribonuclease protection assay

Strenuous resistive breathing has been recently shown to lead to elevation of the plasma levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (1, 2). Resistive breathing–induced plasma cytokines might serve several functions: They stimulate the hypothalamic pituitary adrenal axis (3) leading to β-endorphin release (1) and alterations in breathing pattern (4). They affect brain functions, including sleep (5) and sensation of fatigue (6, 7). IL-6 has a hormone-like glucoregulatory role (6), whereas TNF-α depresses muscle and especially diaphragm contractility (8) and induces insulin resistance (9). IL-6, IL-1β, and TNF-α also enhance protein degradation and have been implicated in muscle wasting (10) of chronic diseases such as chronic obstructive pulmonary disease (11–13). Whole body exercise has also been shown to induce an increase in plasma levels of cytokines such as IL-6, IL-1β, TNF-α, IL-1 receptor antagonist, and IL-10 (14).

The cellular origin of these cytokines remains unknown. Monocytes, a major source of immunoinflammatory mediators (15), have been excluded as sources of the resistive breathing–induced or whole-body exercise–induced elevation of plasma cytokines (2, 16–19). Myocytes have been suggested as a potential source of the exercise-induced cytokines. Indeed, muscle contraction during marathon running or knee extension increases IL-6 but not TNF-α gene expression within the exercising muscles (20–24), secondary to increased transcriptional activity (22), and leads to IL-6 protein release into the circulation (21). However, these results were not confirmed by other investigators who could not detect intramuscular cytokine upregulation secondary to treadmill running (24, 25) or electrical stimulation (24). These conflicting results suggest that activation-induced intramuscular cytokine expression might be exercise- and muscle-type specific, given that different types of exercise activate different transcription factors in a manner specific to the type of muscle (26, 27). Furthermore, the cells of origin of the exercise-induced muscle-derived cytokines are not known, and both resident and blood-derived invading cells are potential candidates.

Because resistive breathing is a form of exercise for the respiratory muscles associated with plasma cytokine elevation and some forms of skeletal muscle activation lead to intramuscular IL-6 production (20) and release into the circulation (21), we hypothesized that the expressions of proinflammatory and antiinflammatory cytokines are upregulated in the respiratory muscles secondary to resistive loading and that this upregulation is dependent on the duration of muscle activation. We evaluated in this study the nature and the time course of cytokine expression within the ventilatory muscles in response to increased activation secondary to inspiratory resistive loading. We have also identified the cellular sources of cytokines produced during strenuous ventilatory muscle contraction. We propose that myocytes are the main source of cytokine production in response to ventilatory muscle activation. Some of the results of these studies have been previously reported in the form of an abstract (28).

METHODS

Animal Preparation

Male Sprague-Dawley rats (300–325 g) were anesthetized with pentobarbital sodium and tracheostomized with polyethylene tubing connected to a two-way nonrebreathing valve. The inspiratory line delivered 100% O_2 to prevent hypoxemia. After a short stabilization period, animals (n = 8 in each group) were randomly assigned to periods of 1, 3, or 6 hours of moderate inspiratory resistive loading (peak inspiratory tracheal pressure of approximately 50% of maximum). Other animals (n = 6 per group) were exposed to either inspiratory loading for 1 hour followed by 2 hours of unloaded breathing or intermittent loading (20 minutes of loading followed by a 30-minute recovery repeated three times). Sham-operated animals breathing against no load for 1, 3, and 6 hours served as control animals (n = 8). Animals were killed at the end of

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the experiment, and the diaphragm and gastrocnemius muscles were quickly excised and frozen either in liquid nitrogen or cold isopentane (20 seconds) before liquid nitrogen.

RNase Protection Assay

Total RNA was isolated with proteinase K and DNase I treatments (RNeasy kit; Qiagen Mississauga, Ontario, Canada), and mRNA expression of IL-1α , IL-1β, TNF-α, TNF-β, IL-3, IL-4, IL-5, IL-6, IL-10, IL-2, IFN-γ, and two housekeeping genes (*L32* and *GADPH*) was measured by Multi-Probe RNase Protection Assay System (RiboQuant; PharMingen, San Jose, CA). Briefly, the multiprobe set was hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNA were digested with RNases. The remaining RNAase-protected probes were purified, resolved on a denaturing polyacrylamide gel, and detected by autoradiography. Optical densities of various mRNAs in the scanned autoradiography films were quantified with ImagePro Plus software (Media Cyberetics Inc., San Diego, CA).

Immunohistochemistry

Frozen tissue sections (5 μm in thickness) were incubated overnight at 4°C with primary goat anti-rat IL-6 or rabbit anti-rat IL-6 antibodies. After three rinses with phosphate-buffered saline, sections were incubated with biotin-conjugated anti-goat or anti-rabbit secondary antibodies followed by Cy3-labeled streptavidin. Sections were then examined under fluorescence microscopy and photographed with a digital camera.

Immunoblotting

Frozen muscle samples were homogenized in a homogenization buffer and centrifuged at 1,000 × *g* for 10 minutes, and supernatants (crude muscle homogenates, 80-μg total protein per sample) were separated onto tris-glycine sodium dodecyl sulfate-polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes and probed overnight with rabbit anti-rat IL-6 and IL-1β antibodies. Specific proteins were detected with horseradish peroxidase–conjugated anti-rabbit secondary antibody and an enhanced chemiluminescence kit and quantified with ImageProPlus software (Media Cybernetics Inc.).

Myeloperoxidase Activity Assay

Crude muscle homogenates (in 0.5% hexadecyltrimethylammonium bromide) were mixed with 50-mM potassium phosphate buffer (pH 6.0) containing o-dianisidine dihydrochloride and H_2O_2 (29). Absorbance was measured at 460 nm for 60 minutes. Myeloperoxidase activity was calculated in units: change in absorbance/minute/g protein.

Statistical Analysis

Values reported are means ± SEM. Comparisons were made using Friedman analysis of variance followed by Wilcoxon Matched Pairs Tests for post hoc comparisons. A p value of 0.05 was initially considered as statistically significant and was accordingly adjusted using a Bonferroni-type procedure for multiple comparisons (30).

RESULTS

RNase protection assay detected weak expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ, and IL-4, (highest to lowest mRNA concentration) in the diaphragm of quietly breathing (unloaded) rats. Different periods of unloaded breathing (1, 3, or 6 hours) did not change the expression of these cytokines. IL-6 mRNA was three times more abundant ($p < 0.05$) than the mRNAs of IL-1β, IL-10, TNF-α, and IFN-γ, which were equally abundant, whereas the expression level of IL-4 was one order of magnitude less than the other cytokines ($p < 0.05$). A very weak expression for these cytokines was detected in the gastrocnemius, which did not change at any time point in the unloaded animals. Expression of TNF-β, IL-1α, IL-2, IL-3, and IL-5 mRNAs could not be detected at any time point in the diaphragm and gastrocnemius of quietly breathing rats.

Maximum peak tracheal pressure measured before resistive loading averaged 75.2 ± 11.7 cm H_2O. Peak inspiratory tracheal airway pressure developed by the animals during loading averaged 35.5 ± 1.96 cm H_2O (46 ± 8% of maximum peak tracheal pressure). Loaded breathing resulted in worsening hypercapnia and acidosis in a time-dependent fashion, without concomitant hypoxemia, which was prevented because of the enriched inspired oxygen used (*see* the online supplement).

Loaded breathing resulted in a significant differential upregulation of the expression of IL-6, IL-1β, IL-10, TNF-α, IFN-γ, and IL-4 in the diaphragm but not the gastrocnemius (Figure 1). The increase in the cytokine mRNA expression (expressed as the fold increase above the respective value of equal duration unloaded breathing) in the diaphragms of loaded animals is presented in Figure 2. With the exception of IL-1β, which exhibited a nearly constant upregulation at different time points, the other cytokines were upregulated in a time-dependent manner, exhibiting the greatest increase after 6 hours of loaded breathing (Figures 2 and 3). IL-6 exhibited the greatest fold increase both at 3 and at 6 hours of loaded breathing. At each time point of loaded breathing, IL-6 mRNA was the most abundant (expressed as a percentage of the housekeeping gene L32 or glyceraldehyde 3-phosphate dehydrogenase), whereas the mRNA for IL-4 exhibited the weakest expression (Figure 4).

To evaluate the time course of cytokine gene expression after termination of muscle activation, a group of animals (n = 6) completed 1 hour of loaded breathing followed by 2 hours of recovery before tissue collection (Figure 5). With the exception of IL-10, all other cytokines were further upregulated after the termination of 1 hour of resistive loading ($p < 0.05$), suggesting that once initiated, contraction-induced diaphragmatic cytokine upregulation is a long-lasting process (see the online supplement). To evaluate the influence of total duration of muscle activation on cytokine gene expression, another group of animals (n = 6) underwent intermittent inspiratory resistive loading for 3 periods of 20 minutes separated by 30-minute periods of unloaded breathing for a total duration of muscle activation of 1 hour. This intermittent activation pattern resulted in marked upregulation of cytokine expression (Figure 5).

Figure 6 illustrates representative examples and mean values

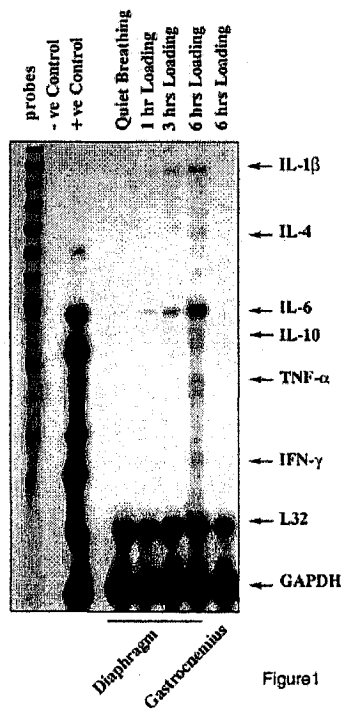
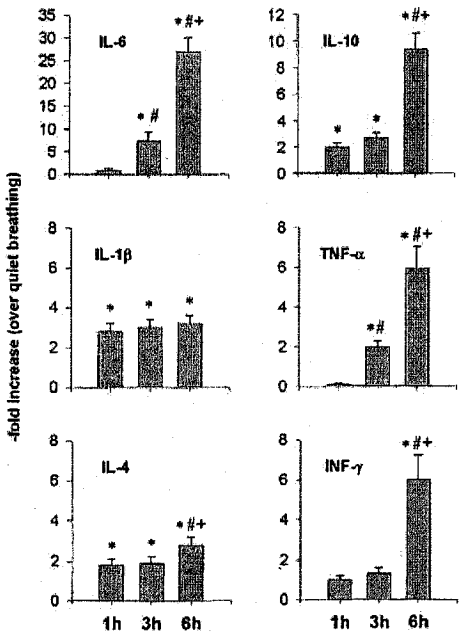


Figure 1. Representative autoradiograph of RNase protection assay showing the time course of cytokine gene expression in the diaphragm and gastrocnemius muscles. *Lanes 1–3*: probe, the negative (−ve) and positive (+ve) control, respectively. *Lane 4*: diaphragm sample from control rat (quiet breathing). *Lanes 5–7*: diaphragm samples obtained from animals exposed to 1, 3, and 6 hours of resistive loading, respectively. *Lane 8*: gastrocnemius sample obtained from rats exposed to 6 hours of inspiratory resistive loading. A total of 10 μg RNA was used in each lane. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL = interleukin; TNF = tumor necrosis factor.



ure 2. Time course of differential cytokine gene expression in the phragm secondary to inspiratory resistive loading. Data are expressed fold increase over equal duration of unloaded (quiet) breathing, rmalized to L32 mRNA. *p < 0.05 compared with quiet breathing.

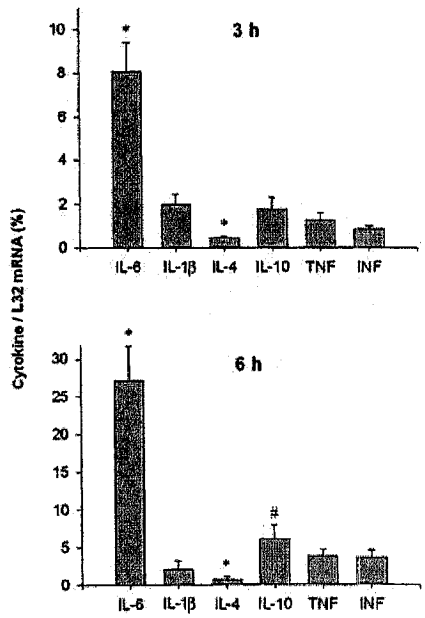
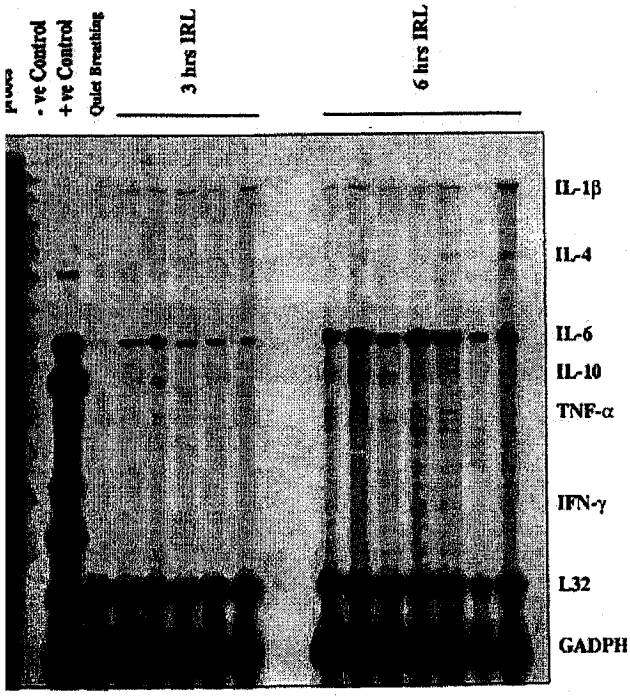


Figure 4. Relative abundance of cytokine mRNAs in the diaphragm after 3 (*upper panel*) and 6 (*lower panel*) hours of inspiratory resistive loading (data normalized to L32 mRNA expression). *p < 0.05. Please note that the scale of the *upper panel* is triple (0–10) that of the *lower panel* (0–30).



ure 3. Representative autoradiograph of RNase protection assay per- rmed on diaphragm muscle samples obtained after 3 (*lanes 5–9*) and hours (*lanes 10–16*) of inspiratory resistive loading. *Lanes 1–3*: probe, e negative (−ve) and positive (+ve) control, respectively. *Lane 4*: aphragm of a quietly breathing rat. A total of 10 μg RNA was used each lane. IRL = inspiratory resistive loading.

(n = 5) of the changes in IL-6 and IL-1β protein expression in the diaphragm of rats exposed to 3 and 6 hours of severe inspiratory resistive loading. No detectable IL-6 and IL-1β proteins were found in the diaphragms of animals breathing against no load. Inspiratory resistive loading for 3 hours elicited a significant rise in diaphragm protein expression of these cytokines (Figure 6). Six hours of inspiratory resistive loading elicited an even greater rise in protein expression of IL-6 and IL-1β, which averaged approximately 10-fold higher than that observed after 3 hours of inspiratory resistive loading (Figure 6). No detectable protein expression of these cytokines was found in the gastrocnemius muscle samples in the three groups of animals (results not shown).

Figure 7 illustrates localization of IL-6 protein expression in rat diaphragms. Both goat anti-rat IL-6 (Figure 7A) and rabbit anti-rat IL-6 antibody (Figure 7B) detected positive IL-6 protein staining in the diaphragms of rats exposed to 6 hours of inspiratory resistive loading. Both punctuate cytosolic and membrane-associated positive IL-6 staining (white arrows in Figures 7A and 7B) was evident inside small muscle fibers, whereas large muscle fibers showed no IL-6 staining. Blood vessels were negative for IL-6 protein (white arrow in Figure 7C). Very weak IL-6 staining was detectable in the diaphragm of quietly breathing rats (Figure 7D). The replacement of primary antibodies with nonspecific antibodies completely eliminated positive IL-6 staining (data not shown).

Inspiratory resistive loading elicited no change in the myeloperoxidase activity in the diaphragms, which averaged 72.9 ± 6.2 U in animals breathing against no load, 91.3 ± 18.0 U after 3 hours of inspiratory resistive loading, and 80.1 ± 9.7 U after 6 hours of inspiratory resistive loading (p = NS).

DISCUSSION

The major finding of this study is that IL-6 and to a lesser extent IL-1β, TNF-α, IL-10, IL-4, and IFN-γ were significantly increased in a time-dependent manner in the diaphragms of

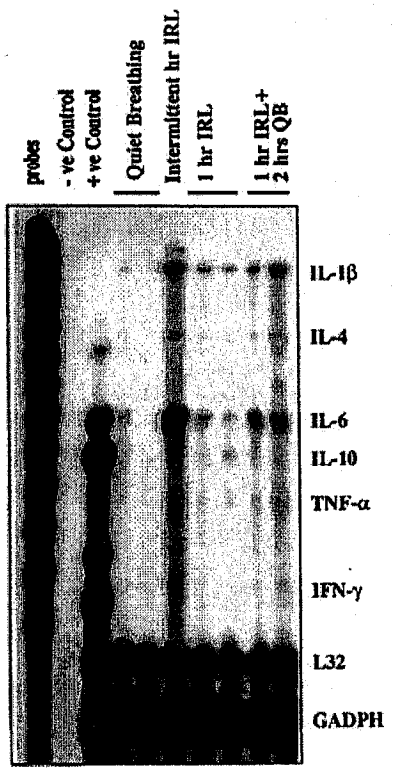


Figure 5. The influence of muscle activation pattern on diaphragmatic cytokine gene expression. *Lanes 1–3*: probe, the negative (−ve) and positive (+ve) control animals, respectively. *Lanes 4* and *5*: diaphragms of quietly breathing rats. *Lane 6*: diaphragm sample obtained after intermittent resistive loading (20 minutes of loading followed by 30 minutes of quiet breathing, repeated three times with a total of 1 hour of inspiratory resistive loading). *Lanes 7* and *8*: diaphragm samples obtained immediately after 1 hour of inspiratory resistive loading. *Lanes 9* and *10*: diaphragm samples obtained from rats exposed to 1 hour resistive loading followed by 2 hours of quiet breathing.

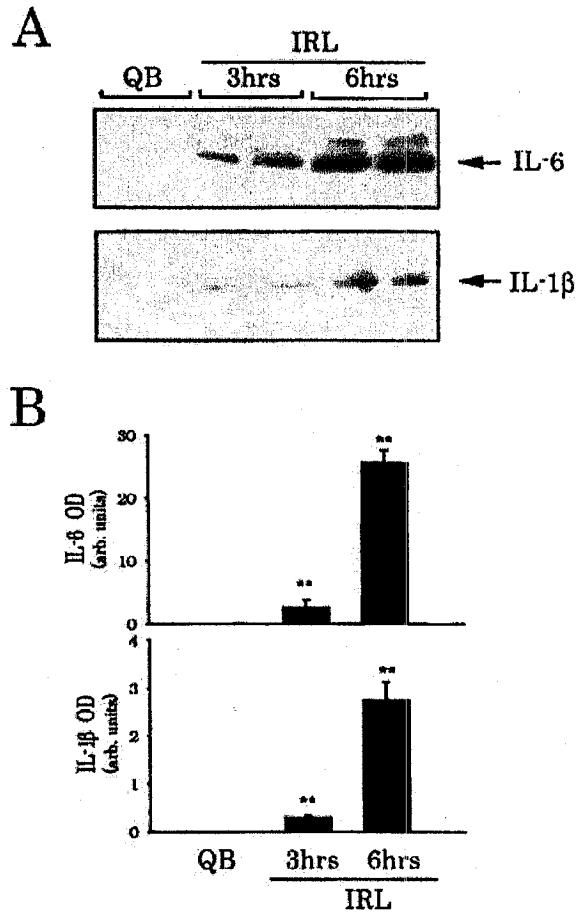


Figure 6. Representative examples of immunoblotting (*upper panel, A*) and mean optical density values (*lower panel, B*) of IL-6 and IL-1β protein expression in the diaphragm of rats exposed to 3 and 6 hours of inspiratory resistive loading. No detectable IL-6 and IL-1β proteins were found in the diaphragms of animals breathing against no load (*A, lanes 1–2*). Inspiratory resistive loading for 3 hours elicited a significant rise in diaphragm protein expression of these cytokines (*A, lanes 3–4*). Six hours of inspiratory resistive loading elicited even greater rise in protein expression of IL-6 and IL-1β (*A, lanes 5–6*), which averaged approximately 10-fold higher than that observed after 3 hours of IRL (*B*). OD = optical density; QB = quiet (unloaded) breathing.

animals subjected to inspiratory resistive loading. Immunohistochemical analysis and absence of any change in myeloperoxidase activity during resistive loading suggest that cytokines are produced inside muscle fibers and are not derived from infiltrating inflammatory cells up to 6 hours after inspiratory resistive loading.

To our knowledge, this is the first study showing that proinflammatory and antiinflammatory cytokines exhibit a low level of constitutive expression within the respiratory muscles under conditions of quiet-unloaded breathing, similar to what is observed in peripheral skeletal muscles (9, 21, 31, 32). More importantly, strenuous contraction of the respiratory muscles resulted in significant upregulation of IL-6 expression and to a lesser extent expressions of IL-1β, TNF-α, IL-10, IL-4, and IFN-γ. The upregulation of intradiaphragmatic cytokine expression was not due a generalized increase in transcription because no upregulation was observed in the noncontracting gastrocnemius. Furthermore, it was not due to surgical manipulation (as previously demonstrated for the soleus) (24), because no increase was observed in the diaphragms of the animals that were subjected to the same surgical procedures without inspiratory loading. Thus, the intradiaphragmatic cytokine upregulation was a specific response to increased activation of the diaphragm secondary to resistive loading.

It should be emphasized that we detected that the messenger RNA expression of cytokines using a multiprobe RNase protection assay, which does not amplify the RNA signal, is less prone to variability and errors and is significantly less sensitive from the usually used reverse transcription-polymerase chain reaction. The RNase protection assay requires 10^4 to 10^5 larger quantities of RNA to be present in the tissues for positive signal detection (33) compared with the reverse transcription-polymerase chain reaction that has been used for RNA detection in peripheral skeletal muscles (20, 21, 25). Because RNase protection assay is less sensitive than reverse transcription-polymerase chain reaction, some cytokine expression that was below the detection limit of the method might have been missed. On the other hand, this secures that the upregulation of cytokine expression within the diaphragm secondary to resistive loading that we observed represents relatively abundant tissue messenger RNA levels.

The mRNA upregulation was accompanied by commensurate increases in the cytokine protein levels, at least for the IL-6 and IL-1β. Although we have not detected the rest of the cytokines at the protein level (which is a limitation of our study), there is no reason to expect a different response for these cytokines, because whenever cytokine messenger RNA levels change within muscles, similar changes of protein levels occur (34–39).

Cellular origins of muscle activation-induced cytokine expression are not yet established. Our results show that IL-6, the most abundantly expressed and upregulated cytokine secondary to increased muscle activation, originates from the myocytes themselves. In fact, IL-6 exhibited both a cytoplasmic and a perisarcolemmal staining pattern, which is characteristic of a

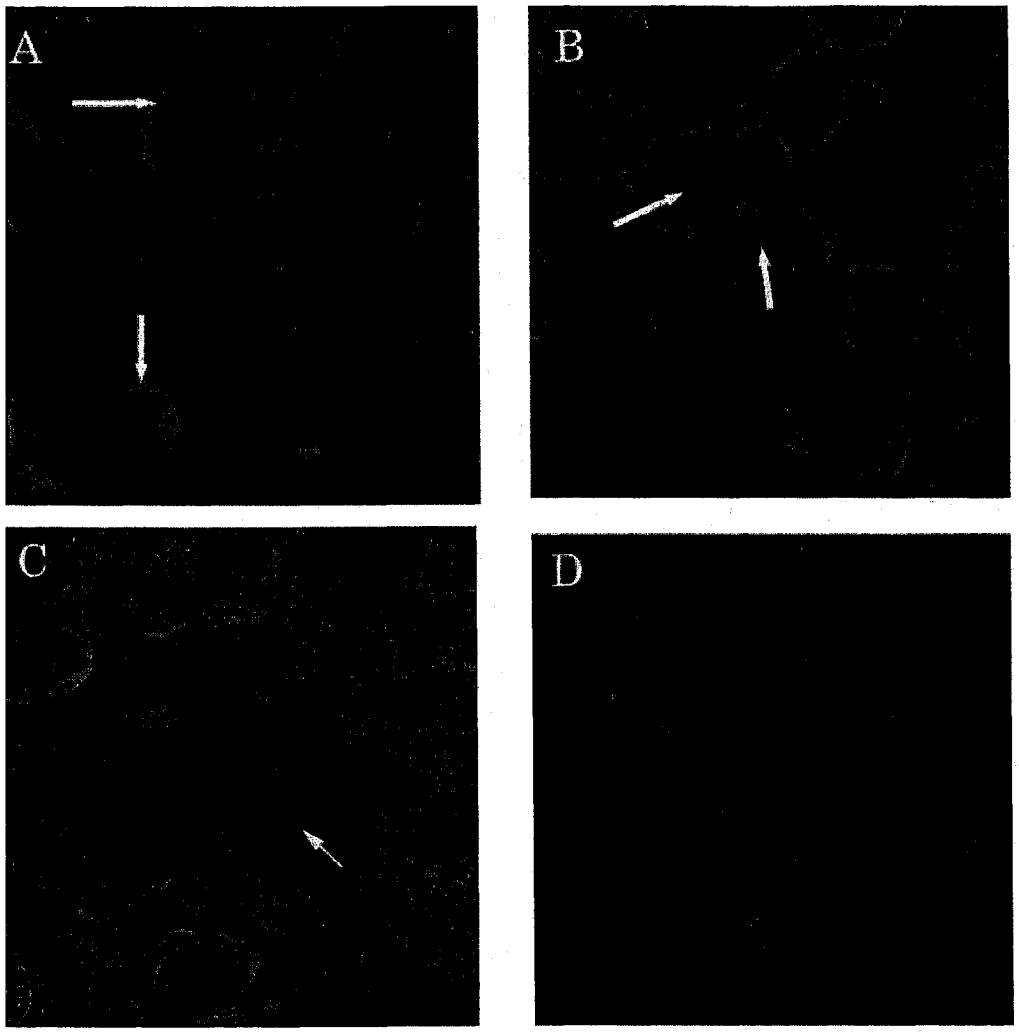


Figure 7. Localization of IL-6 protein expression in rat diaphragms. Both goat anti-rat IL-6 (*A*) and rabbit anti-rat IL-6 antibody (*B*) detected positive IL-6 staining in the diaphragms of rats exposed to 6 hours of inspiratory resistive loading. Both membrane-associated (*white arrows* in *A*) and punctuate cytosolic positive IL-6 staining (*white arrows* in *B*) was evident inside small muscle fibers, whereas large muscle fibers showed no IL-6 staining (*gray arrows*). Blood vessels were negative for IL-6 protein (*white arrow* in *C*). Very weak IL-6 staining was detectable in the diaphragm of quietly breathing rats (*D*).

creted protein. This finding is in keeping with *in vitro* results owing that myocytes are capable of producing IL-6 (38, 40, .) secondary to stimuli relevant for exercise, such as exposure reactive oxygen species (40) and increased intracellular Ca^{++} 1). Similar to what we found in the diaphragm, cytokines are pregulated within cardiac myocytes secondary to loading (35, 42), hich suggest that IL-6 upregulation is a general response of yocytes to increased muscle activation. We have not evaluated ie cellular origin of the rest of the cytokines; however, because yocytes are capable of producing a variety of cytokines *in vitro* 8), it is likely that myocytes are the sources of the augmented tokine expression within the diaphragm, although other cells uld not be excluded.

The stimulus for the upregulation of cytokine expression durg diaphragmatic activation is not known. We speculate that active oxygen species are important modulators of muscle tokine production, as indicated by the blunting by antioxidants f the elevation in plasma IL-6, IL-1β, and TNF-α (2, 19) induced y either resistive loading (2) or whole-body exercise (19) and y the induction of IL-6 production from cultured myocytes xposed to reactive oxygen species (40). Depletion of glycogen uscle stores during muscle activation could also regulate cytoine production as indicated by augmentation of muscle IL-6 xpression after glycogen depletion (22, 23). Finally, preliminary ata suggest that the rise in intracellular Ca^{2+} can also lead to -6 secretion by myocytes (41).

Implications

Resistive breathing–induced intradiaphragmatic cytokine production may serve several local and systemic functions, which could be both adaptive and maladaptive. For instance, cytokines may play an important role at the local level by promoting muscle fiber injury. Resistive loading achieved in our study was of such magnitude that likely produces diaphragmatic injury (43–47). Our results raise the interesting possibility that intradiaphragmatic cytokine induction could be involved in mediating the injurious process by upregulating the expression of adhesion molecules on the surface of endothelial cells (48) and by enhancing transendothelial migration of blood-derived inflammatory cells (49), responses that would augment infiltration of neutrophils and promotion of muscle fiber injury. Although myeloperoxidase activity—an index of tissue infiltration by neutrophils—was not increased in the diaphragms of animals up to 6 hours of resistive loading, this might be due to inadequate time (neutrophilic influx taking place later) or to inadequate power of our study to document a statistically significant response (a 25% increase in myeloperoxidase activity observed would require 70 animals per group). Proinflammatory cytokines such as TNF-α may also promote fiber injury by augmenting muscle reactive oxygen species production (10). These species are well known players in ventilatory muscle injury (50). The majority of evidence suggests that TNF-α also suppresses diaphragmatic contractility (8, 51, 52),

although earlier studies had suggested that TNF-α has either no effect (53) or affects diaphragmatic contractility only at high doses (54), which might explain the observation that force decline after resistive loading is proportionally greater than the observed muscle injury (44).

We should emphasize that not only proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ were induced inside the diaphragm during resistive loading but antiinflammatory cytokines such as IL-4, IL-10, and IL-6 (which has some proinflammatory but mainly antiinflammatory properties) (55) were also upregulated, suggesting that few of these cytokines may serve to oppose local muscle inflammation (55). Cytokines are also essential in orchestrating muscle recovery after injury. Cytokines such as TNF-α, IL-6, leukemia inhibitory factor, and IL-1β (31, 56–58) and their cognate receptors (59) are upregulated in skeletal muscle after injury. These cytokines enhance proteolytic removal of damaged proteins (60, 61) and damaged cells (through recruitment and activation of phagocytes). TNF-α and leukemia inhibitory factor are important signaling molecules for the regeneration of muscle fibers after injury (57, 62). TNF-α receptor double knockout mice or mice receiving TNF-α–neutralizing antibodies exhibit a reduced muscle strength recovery after injury compared with wild-type mice, associated with a reduced expression of the myogenic transcription factor MyoD (57). This is in concert with data suggesting that TNF-α promotes differentiation of myoblasts by increasing nuclear factor-κB activity (63) and both activates satellite cells to enter the cell cycle from the normally quiescent state and enhances their proliferation once it has been initiated (64). Nevertheless, it has to be acknowledged that the differentiation promoting effect of TNF-α has been debated (65–67). More studies are needed to elucidate the exact role of cytokines in skeletal muscle injury and recovery.

The significantly greater induction of IL-6 within the diaphragm compared with other cytokines suggests that IL-6 might be involved in physiologic muscle signaling (68). Diaphragmatic contraction leads to glycogen depletion, which greatly augments IL-6 production from skeletal muscles (22, 23). IL-6 has an hormone-like role, signaling that glycogen stores are reaching critically low levels in the contracting muscles and stimulating hepatic glucose output to maintain glucose homeostasis and muscle glucose supply (6, 69). IL-6 also mobilizes free fatty acids from triglycerides stored in fat tissue, thus increasing the energy that is available to the muscle.

We also speculate that diaphragm-derived cytokines might spill into the circulation leading to elevation of plasma cytokine levels. Ventilatory muscle production of cytokines could have been the source of elevated plasma cytokines observed after resistive loading in normal humans (1, 2) or in diseases of increased respiratory load, such as chronic obstructive pulmonary disease (70, 71) and sleep apnea (72). Elevation of circulating cytokines derived from the ventilatory muscles might have systemic effects, including changes in breathing pattern (1) and sensation of fatigue (6, 7). Muscle-derived cytokines may also contribute to the cachexia observed in some chronic obstructive pulmonary disease patients (11–13). Further studies are needed to elucidate these interesting possibilities.

In conclusion, we have shown that inspiratory resistive loading results in differential cytokine expression in the diaphragm. Both proinflammatory and antiinflammatory cytokines are expressed in a time-dependent manner, which might have both local and systemic effects.

Conflict of Interest Statement: T.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; O.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.N.A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

t should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the ɔhazards Committee, one month before starting new projects or expiry of a previously approved application.

NCIPAL INVESTIGATOR: Basil J. Petrof, MD

)RESS: Royal Victoria Hospital TELEPHONE: 934-1934 ext. 35946 or 35650

1 FAX NUMBER: 843-1695

'ARTMENT: Medicine - Respiratory E-MAIL: basil.petrof@muhc.mcgill.ca

)JECT TITLE: Respiratory and Peripheral Muscle Dysfunction in Pseudomonas

ɹung Infection.

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Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date March 31 2002

New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

FICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with icant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" d by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

ment Level (circle 1): 1 (2) 3 4

al Investigator or course director: date: 25 03 02
SIGNATURE day month year

ɛrson, Biohazards Committee: DR. DALIUS J. BRIEDIS Dept. of Microbiology & Immunology McGill University 3775 University Street Montreal, Quebec H3A 2B4 Canada date: 04-04-02
SIGNATURE day month year

'ed period: beginning 04 04 02 ending 31-03-03
day month year day month year

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I, Maziar Divangahi, am in the process of submitting my Ph.D. thesis at McGil. University. I would like to have copy right for my two publications (Vol 169. pp 679-686, 2004 & Vol 170. pp 154-161, 2004) and an editorial comment (Vol 169. pp 662-663, 2004) to include them in my Ph.D. thesis. I would appreciate if you could fax it to me as soon as possible, since the deadline for submitting is very close.

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My Fax number is (514) 398-7483

Sincerely,
Maziar Divangahi
Department of Medicine, McGill University

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