Role of γδ T Cells in bronchial responsiveness and epithelial repair after chlorine gas exposure in mice

Hossein Koohsari

Department of Physiology
McGill University, Montreal
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Abstract

Role of γδ T Cells in Airway Epithelial Injury and Bronchial Responsiveness After Chlorine Gas Exposure in Mice H. Koohsari, H.R. Campbell, M. Tamaoka, J.G. Martin Meakins-Christie Laboratories, McGill University, Montreal , QC, Canada

Reactive airways dysfunction syndrome is a form of irritant-induced asthma that has been documented after acute exposure to chlorine (Cl_2) gas. Animal models of Cl_2 exposure indicate that the airway epithelium is a target. γδ T-Cells are present in the mucosal surface of the airways and may control the growth and differentiation of the airway epithelial cells. However, the role of these cells in the airway response to Cl_2 exposure has not been elucidated.

AIM: To study the role of γδ T-cells in the response to Cl_2 exposure with respect to inflammation and lung function.

METHODS: C57Bl/6J (wild type) and TCR-$\delta^{-/-}$ mice exposed to Cl_2 (400ppm) for 5 minutes were mechanically ventilated for measurement of responses to i.v. methacholine (MCh) at 1, 3, and 5 days after exposure. Bronchoalveolar lavage was performed to determine epithelial and leukocyte counts, and protein content. Tissues were harvested for PCNA immunoreactivity to evaluate the rate of repair of the epithelium.

RESULTS: Wild type mice developed a greater degree of airway hyperresponsiveness to MCh at 1 day post exposure to Cl_2 compared with TCR-$\delta^{-/-}$ mice. Epithelial cell counts in BAL after Cl_2 exposure were greater in TCR-$\delta^{-/-}$ mice, and the pattern of inflammation differed in wild type and TCR-$\delta^{-/-}$ mice; macrophages showed a later peak and granulocyte numbers were lower in TCR-$\delta^{-/-}$ than in wild type mice. Both groups of mice had increased levels of total protein content in BAL after Cl_2 exposure that resolved after 3 and 5 days, respectively. TCR-$\delta^{-/-}$ mice have a lower rate of epithelial regeneration as shown by PCNA immunoreactivity.

CONCLUSION: The severity of airway injury after Cl_2 appears to be greater in TCR-$\delta^{-/-}$ mice but the lack of TCR-δ seems to abrogate the changes in airway responsiveness to i.v. methacholine.

Résumé

Le rôle des cellules T γδ dans les dommages aux cellules épithéliales des voies respiratoires et dans la réactivité bronchique chez les souris exposées au chlore.

INTRODUCTION : Le syndrome des voies aériennes dysfonctionnelles est une forme d'asthme induit par des irritants qui a été documenté suite à l'exposition aigüe au chlore (Cl_2). Les études réalisées chez les modèles animaux indiquent que l'épithélium des voies aériennes constitue une cible vraisemblable lors d'exposition au Cl_2. Les cellules T γδ sont présentes au niveau de la surface mucosale des voies aériennes et pourraient être impliquées dans le contrôle du développement et de la différentiation des cellules épithéliales à ce niveau. Toutefois, le rôle de ces lymphocytes dans la dysfonction des voies respiratoires suite à l'exposition au Cl_2 n'a pas encore été élucidé.

OBJECTIF : Documenter le rôle des cellules T γδ suite à l'exposition au Cl_2 en étudiant les modifications inflammatoires et la mécanique des voies respiratoires.

MÉTHODES : Des souris C57Bl/6J wild-type et doubles négatives pour le TCR-δ (TCR-$\delta^{-/-}$) ont été exposées au Cl_2 (400 ppm) pour 5 minutes et ventilées mécaniquement après 1, 3 et 5 jours suivant l'exposition afin de mesurer la réponse à la métacholine I.V. Des lavages bronchoalvéolaires (LBA) ont été réalisés aux mêmes moments afin d'effectuer une quantification des protéine et un comptage des cellules épithéliales et des leucocytes. Des tissus pulmonaires ont aussi été prélevés pour évaluer l'immunoréactivité au PCNA.

RÉSULTATS : Une journée suivant l'exposition au Cl_2, les souris wild-type ont démontré une hyperréactivité bronchique supérieure à celle des souris TCR-$\delta^{-/-}$. Le LBA a révélé un compte de cellules épithéliales supérieur chez les souris TCR-$\delta^{-/-}$ ainsi qu'un pattern d'inflammation différent d'un groupe à l'autre; le nombre de macrophages a augmenté plus tardivement chez les souris TCR-$\delta^{-/-}$ et le nombre de granulocytes s'est avéré plus bas comparé au groupe wild-type. Les deux groupes ont démontré une augmentation similaire des protéines qui s'est résolue après 3 et 5 jours suivant l'insulte au Cl_2. Finalement, les cellules TCR-$\delta^{-/-}$ ont démontré un taux de regénération inférieur, tel que démontré par l'immunoréactivité au PCNA.

CONCLUSION : La sévérité des dommages induits par le Cl_2 est plus importante chez les souris déficientes en lymphocytes T γδ. Toutefois, l'absence du TCR-δ semble atténuer la réactivité des voies respiratoires, exposées au chlore, suite à l'administration de métacholine.

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ABBREVIATIONS

AHR	Airway hyperresponsiveness
AEC	Airway epithelial cell
BAL	Bronchoalveolar lavage
E_{RS}	Dynamic elastance
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony stimulating factor
i.v.	Intravenous
IFNγ	Interferon gamma
Ig-	Immunoglobulin
IL-	Interleukin
IrIA	Irritant induced Asthma
KGF	Keratinocyte growth factor
MCh	Methacholine
MHC	Major histocompatibility complex
MOM	Mouse on mouse kit
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Occupational asthma
P	Pressure
PCNA	Proliferating cell nuclear antigen
PEEP	Positive end expiratory pressure

PEFR	Peak expiratory flow rate
PGE_2	Prostaglandin E_2
ppm	parts per million
RADS	Reactive airways dysfunction syndrome
RANTES	Regulated upon activation in normal T cells expressed and secreted
R_{RS}	Respiratory system resistance
SPT	Skin prick test
T Cell	T lymphocyte
TCR	T cell receptor
Th-	T helper cell
V	Volume

Table of Contents

LIST OF FIGURES

1. Introduction / Background

Asthma is a chronic disease of the lung characterized by airway inflammation, which is a key feature of the disease, variable airflow limitation and extra-sensitive, twitchy, or hyper-responsive airways. Individuals who are affected by this disease have symptoms of wheezing, coughing, and shortness of breath or dyspnea. These symptoms can be triggered by a variety of stimuli including, but not limited to allergens, infection, exercise, cold air and pollution. This disease can be classified as having three general subtypes: allergic (extrinsic), non-allergic (intrinsic), and occupational asthma. It has been confirmed that airway inflammation is a feature common to all of these subtypes.[1,2]

1.1 Occupational Asthma

1.1.1 Epidemiology of OA

The term occupational asthma (OA) is used when asthma is induced because of stimuli that are encountered only in a particular work environment. Many agents to date have been reported to cause occupational asthma and the list grows annually. Table 1 shows a list of commonly responsible occupational agents categorized depending on their specific properties. Epidemiological surveys indicate that occupational factors are associated with 10-15% of cases of new onset adult asthma.[3-6] However, these values may be slightly underestimated, because estimates of asthma prevalence may be distorted by survivor effects, which may fail to include those who do not remain in the occupational setting that is causing symptoms. A relatively new

study by Johnson A.R. et al, conducted in six Canadian communities showed a 36.1% prevalence rate of OA among subjects with adult-onset asthma.[7]

Other aspects of OA that need to be addressed are the exposure/response relationship and the risk factors for developing OA. A number of studies have reported that a higher exposure (i.e. duration or dose) level leads to a higher incidence of asthma.[8-10] However, only a proportion of those exposed to an occupational agent develop OA. This suggests that there must be other risk factors besides the exposure to the occupational agent that lead to an increased probability of developing OA. For example, cigarette smoking, and atopic tendency, or both combined contribute to an increased risk of developing OA.[11] Therefore, an interaction between multiple genetic and environmental influences can determine whether an individual will develop occupational asthma or not.

1.1.2 Classification and Pathogenesis of OA

The precise mechanism(s) leading to occupational asthma is(are) still unknown. Immunological, pharmacological and genetic mechanisms, have been proposed to cause occupational asthma. Additional causes such as airway neurogenic inflammation have also been considered and indeed several mechanisms may be operational at the same time. For this reason occupational asthma has been divided into two main categories: (1) Immunological OA and (2) Non-immunological OA.[12]

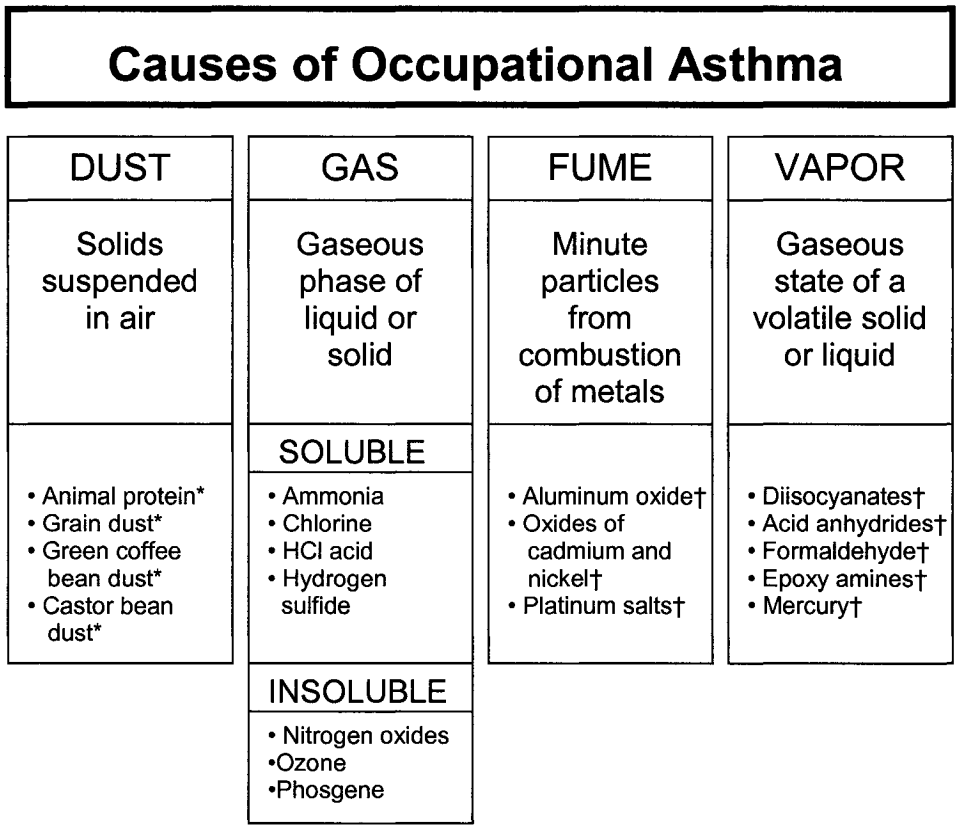

Table 1: Characteristics of inhaled occupational agents leading to OA. * and † indicate high and low molecular weight substances respectively, with the capacity to sensitize subjects who are exposed to them.[13]

1.1.2.1 Immunological OA

Immunological OA is usually triggered by exposure to high-molecular-weight agents (>5000 Da), usually proteins, it arises after a latency period of exposure that is necessary for becoming sensitized to an agent and asthma reactions occur on re-exposure to the causal agent. However, immunological mechanisms do not necessarily imply an immunoglobulin E (IgE) mediated immune response, because the immune system is divided into the antibody-mediated and cell-mediated immunity. Antibody mediated processes are orchestrated by B-lymphocytes that are

capable of producing and secreting specific antibodies, while cell-mediated processes depend on T-lymphocytes.

T-lymphocytes in addition to being capable of controlling B-lymphocyte function, have pro-inflammatory actions through the expression of various cytotoxic molecules and cytokines. An immune response is generated when an antigen presenting cell (e.g. dendritic cell, macrophage and B-lymphocyte) activates the T-lymphocytes through the appropriate receptors. CD8+ T cells recognize peptide presented in conjunction with the MHC class I molecules, whereas CD4+ T cells recognize peptide presentation in association with MHC class II molecules (figure 1). There are several types of CD4+ T cells based on the types of cytokines they produce and of these subtypes T-helper-1 and T-helper-2 (Th1, and Th2) cells are the most explored. Th1 cells mainly produce IL-2, interferon-γ and tumor necrosis factor-β, while Th2 cells secrete IL-4, IL-5 and IL-13. Other subtypes of regulatory T cells such as Th3 cells exist. But these cells have been difficult to study because of the difficulty in isolating clones and they are not well explored in the context of allergic airway disease. It is evident that an immunological reaction depends on the balance between the levels of cytokines produced by these two subtypes of T helper cells. Cytokines secreted by the Th2 subtype are responsible for the development of the immediate hypersensitivity reaction by promoting isotype switching of B-lymphocytes from mainly IgG & IgM isotypes to IgE synthesis, while Th1 cytokines inhibit B-lymphocyte activation and IgE synthesis and promote delayed type hypersensitivity reactions.[14-16]

High-molecular-weight agents act as antigens and responses to them are mediated through an IgE-dependent pathway, whereas low-molecular-weight agents do not seem to trigger this pathway and may be IgE-independent (Figure 1), Some low-molecular-weight agents such as acid anhydrides and isocyanates can also trigger an immunological response by acting as haptens in order to produce a specific IgE antibody by combining with an endogenous host protein.

IgE-dependent mechanisms

Occupational asthma induced by an IgE-dependent mechanism has the same characteristics as allergic asthma. B-lymphocytes secrete IgE antibody under the regulation by Th2 cells after exposure to antigen by antigen presenting cells. Chronic or repetitive exposure to an antigen leads to the sensitization of the subject and upon re-exposure to the sensitizing antigen (allergen) there is an increased production of IgE that can bind to high affinity receptors on the surface of mast cells, eosinophils, basophils, and possibly macrophages leading to an influx of inflammatory cells and further release of inflammatory mediators leading to asthma.[12,17] It has also been shown that mast cells and basophils can support B lymphocyte IgE synthesis suggesting that these cells can contribute to maintenance of allergic reactions through an IgE dependent, T-lymphocyte independent mechanism.[18] Pathologic changes in patients with OA are also similar to other forms of asthma. There is airway wall thickening, subepithelial fibrosis, smooth muscle hypertrophy, edema, and airway obstruction.

IgE-independent mechanisms

In contrast to IgE-dependent OA, IgE-independent OA seems to mainly lead to recruitment of large numbers of activated cytotoxic T lymphocytes to the airways, suggesting that these cells are the main mediators of airway inflammation in this setting. Studies of bronchial biopsies from these subjects suggest that CD8+ T cells play a central role in mediating airway inflammation in OA, independent from the production of IgE antibodies.[12,19]

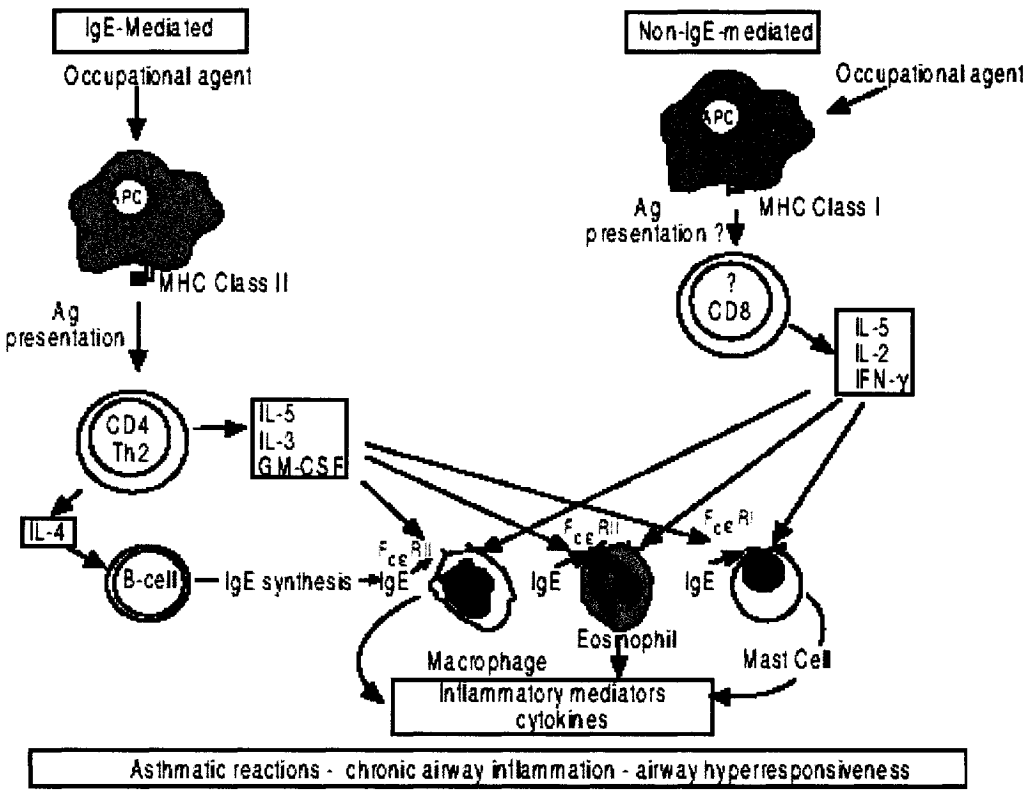

Figure 1. – Postulated immunological mechanisms in occupational asthma. IgE: immunoglobulin E; APC: antigen presenting cell; MHC: major histocompatibility complex; Ag: antigen; Th: T-helper; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN-g: interferon-g. [20]

1.1.2.2 Non-immunological OA

Non-immunologic OA results from short-term inhalational exposure to high levels of irritant materials in the workplace. In contrast to immunological OA, there is a rapid onset of disease without a latent period. The symptoms are slightly different from those of asthma that follows a latent period. The airflow obstruction or non-specific airways hyperreactivity persists for a variable period after the causal exposure and is provoked by a range of irritants or other provoking factors such as cold air and smoke. The term irritant-induced (IrIA) asthma or reactive airways dysfunction syndrome (RADS) was first coined by Brooks et al. in 1985 to refer to this condition.[21]

It is important to note that the risk factors that lead to the development of IrIA are not as yet determined. This condition can be caused by a variety of chemical irritants, but the severity or type of exposure does not determine whether an individual will develop IrIA or not. Some of the documented chemicals that can lead to IrIA after exposure include: ammonia, sulfur dioxide, nitrogen oxides, ozone, phosgene, and chlorine. Some of these chemicals are water soluble gases (Table 1) such as sulfur dioxide, and chlorine and can result in acid and/or oxidant injury. However, for the most part the exact mechanisms of their actions leading to injury, inflammation and bronchial hyperreactivity are still unclear.

It has been suggested that the extensive shedding of the airway epithelium results in airway inflammation.[22-24] This epithelial cell shedding can also lead to the

development of neurogenic inflammation by exposing nerve endings that release neuropeptides such as substance P and neurokinin A., Non-specific activation of macrophages and mast cells can lead to the release of inflammatory cytokines and chemotactic mediators. [2, 12] Airway remodeling is observed as a consequence of the epithelial damage. Bronchial biopsies show replacement of epithelium by fibrinohemorrhagic exudates, and subepithelial fibrosis, which may be responsible for the lesser degree of reversibility of airflow obstruction seen in this disease compared to usual asthma.[23-25] It has been observed that increased airways hyperresponsiveness follows the airway inflammation, suggesting that inflammation is the basis for the persistence of airway hyperresponsiveness.[12]

There are other forms of non-immunologic OA that can be triggered by agents such as insecticides that have direct pharmacologic actions on the respiratory mucosa. These agents can produce a transient bronchoconstriction in sufficient doses by inhibiting acetylcholinesterase, which potentiates the effect of acetylcholine released by the innervating nerves (parasympathetic) of the bronchial smooth muscle.[13]

Reflex bronchoconstriction is another form of non-immunologic OA. This is caused by chronic, low-moderate dose exposure to irritants. However, it is very unlikely that this can lead to new-onset adult asthma. Therefore, it is probably a phenomenon that occurs in individuals with atopy, subclinical asthma, or asymptomatic AHR.[13,26]

1.1.3 Clinical Aspects and Diagnosis of OA

The diagnosis of occupational asthma is a two step process. First the clinician must determine that the patient has asthma, and second determine if the asthma was caused as a result of the work environment. This has to be confirmed by objective testing, and a diagnosis based on history alone is generally considered unacceptable by compensation boards that are responsible for the evaluation of occupational disease. This is very critical since the diagnosis of OA can have significant social and financial consequences. Keeping an individual who has OA at work will lead to a worsening of his condition and the longer the subject remains exposed to the causal agent the greater the likelihood of the patient acquiring permanent asthma after removal from work. An erroneous diagnosis of OA could impose a financial burden on a worker not affected with OA.[12,27,28]

OA should be suspected in adults with new onset asthma. A good occupational history of recent and past exposures should be obtained. Material safety data sheets can be requested from the workplace to determine possible exposure to sensitizing or irritant chemicals. [12,27,29] There are various conventional diagnostic tools for OA, each with its own advantages and disadvantages. They are as follows:

Questionnaire:

Like many other medical conditions a questionnaire is a method that is widely and commonly used for the assessment of OA. The questions mainly focus on occupational history and possible substances or chemicals a patient may have been

exposed to. Questions like "do your symptoms improve while away from work?" should be asked. The advantage of this technique is that it is simple to perform and is a sensitive tool; however it has low specificity to the cause of the disease. Also, there are no universal or validated questionnaires that are available for use in the clinical setting.[28,29]

Immunological evaluation:

Immunologic testing is another simple and sensitive tool that can be used for diagnostic purposes. Skin-prick test (SPT) and presence of specific IgG or IgE antibodies are useful methods for establishing exposure and sensitization. However, the presence of specific IgE is mostly important for high molecular weight antigens and a negative SPT does not exclude OA. *Vice versa*, a positive test is not necessarily indicative of OA, since not all sensitizations lead to OA. Another limitation of immunologic testing is the availability of specific extracts for most occupational agents. Overall this technique is a good way to determine the atopic state of a patient. However, more objective testing is needed to confirm the presence or absence of OA.[13,28]

Pulmonary Function Testing:

There are several methods available for assessing lung function. Methacholine* or histamine challenge tests can be a useful method for measuring nonspecific bronchial hyperresponsiveness. However, a positive test result cannot

* Methacholine, definition: *n* : a parasympathomimetic drug ($C_8H_{19}NO_3$) administered in the form of its crystalline chloride or bromide

discriminate between usual asthma and OA. On the contrary, a negative test result does not rule out OA, and specific inhalation challenge tests are the only option for those individuals.[28,29] The latter technique may be a powerful diagnostic tool, however there is some cost, and risk associated with it, since specific bronchial challenges can lead to a severe asthma attack and require the presence of highly specialized personnel. Informed consent is necessary in order for this procedure to be performed. Other methods of lung function testing that are not as powerful include: measurements of peak expiratory flow rate (PEFR), and/or FEV_1 before, during, and after work.

Markers of airway inflammation:

There are three main diagnostic tools for assessing inflammation in OA. These include induced sputum, bronchoalveolar lavage (BAL), and bronchial biopsies. As with non-occupational asthma, airway inflammation consisting of neutrophil and eosinophil influx, is a main hallmark of occupational asthma and can be assessed by using these methods. Induced sputum is a relatively new technique in comparison to BAL and fiberoptic biopsies. Standardized techniques have been developed for inducing and processing sputum as a safe and informative diagnostic tool.[30,31] BAL can also be used to not only look at inflammatory cell profile in the airways, but also protein and cytokine content in the supernatant. The supernatant of BAL after an asthmatic reaction has increased levels of albumin compared to controls, which can be attributed to microvascular leakage and mucosal edema.[32]

For further in-depth analysis of the underlying disease process and characterization of disease bronchial biopsies can be obtained. The advantage of obtaining biopsies is that they can not only be used for clinical diagnosis, but they can also be used in scientific research to further understand the disease processes that occur in the airways. Some of the main histological findings in asthmatic patients include but are not limited to: denudation of the airway epithelium, thickening of the epithelial basement membrane, inflammatory cell infiltration, and smooth muscle hypertrophy/hyperplasia. Epithelial shedding is the main feature of occupational asthma. Epithelial cells are not only mechanical barriers between host and the environment and are also metabolically active tissues that can have a role in modulating inflammatory responses. These functions of epithelial cells will be covered later in this chapter.

1.1.4 OA: Prevention & exposure limits

Inherent in the definition of occupational asthma is that it can be prevented. Therefore, strict prevention measures should be put in place for OA, especially in high-risk workplaces, and exposure limits set for all high-risk agents that can lead to OA. The most effective means is to prevent exposure altogether, either by not performing the task or by substituting the agent for a less harmful material. However, this is not always feasible. Other preventative measures such as good exhaust ventilation or fume hoods, protective clothing, and gas masks or in extreme cases airhoods that have personal supply of breathing air can be used.

1.2 Chlorine Gas

At room temperature and atmospheric pressure, chlorine (Cl_2) is a non-combustible greenish-yellow gas. Cl_2 is heavier than air and has a pungent odor. It can be transformed to liquid under high pressures or low temperatures. Cl_2 is mainly used as a bleaching agent in the pulp and paper, and cloth industries. Cl_2 is highly toxic and Cl_2 derivatives are used for their disinfectant properties.

A principal property of Cl_2 is that it is water soluble. When released into the air Cl_2 will react with water to form hypochlorous (HOCl) and hydrochloric acid (HCl) which are removed from the atmosphere by rainfall. The chemical reactions that occur by combining Cl_2 with water are outlined as follows:

1) $Cl_2 + H_2O \rightarrow HCl + HOCl$

2) $2Cl_2 + 2H_2O \rightarrow 4\,HCl + O_2$

2) $4HOCl \rightarrow 2HCl + O_2$

Since Cl_2 readily reacts with water, the general population is not at risk of chlorine gas exposure; however it is heavily used in industry as an ingredient for making other chemicals, and bleach or disinfectant. Workers employed in such occupations are at risk of accidental exposure and may inhale chlorine gas leading to occupational asthma

1.2.1 History

Commercial production of Cl_2 gas began before World War I. The German army first used gas cylinders as weapons in 1915 against the French army at Ypres.[33] French soldiers reported seeing yellow-green clouds drifting towards the allied trenches and reported a distinctive odor of pineapple and pepper. When the smoke cloud reached the front line soldiers began to complain of chest pain and burning sensation in the throat. This high dose of Cl_2 severely impaired lung function and led to respiratory failure or cardiac arrest due to pulmonary edema.[34] Soon masks containing sodium bicarbonate were developed to neutralize Cl_2 in case of a possible gas attack.[35]

Other cases of chlorine gas exposure have been documented. Nowadays most exposures occur accidentally in subjects during the course of work in industries such as pulp and paper mills,[36-38] in swimming pools due to accidental release of Cl_2 gas from chlorinators,[39-43] and at home where Cl_2 gas can be released by mixing bleach with other cleaning products.[44-46]

1.2.2 Clinical features of Cl_2 exposure and treatment

As mentioned earlier acute exposure to high concentrations of chlorine gas can lead to a form of occupational asthma called IrIA, or RADS. There are many reports of accidental exposure in the literature, and hence a wide array of symptoms that can persist for a variable period of time after Cl_2 gas inhalation injury.[47-50]

Immediate symptoms after exposure to sub-lethal doses of Cl_2 gas include upper airway irritation, cough, wheezing, shortness of breath, and difficulty in breathing.[36,39,51] Other clinical features of chlorine gas exposure include pulmonary edema, inflammation and non-specific bronchial responsiveness.[49] A study conducted by Shroff C.P. et al. has looked at the cytopathologic changes that occurred in a group of individuals exposed to Cl_2 gas. Some of the findings from bronchial brushings taken five days after exposure included: basal and goblet cell hyperplasia, airway inflammation, and chromatolysis of columnar epithelial cells. On day 15 post exposure there was evidence of epithelial regeneration and repair by fibrosis that could lead to chronic airway obstruction and RADS.[52] Other studies have looked at pulmonary function after exposure to Cl_2 gas and have documented obstructive or restrictive deficits immediately following exposure with resolution over time. However, some individuals develop chronic and persistent obstructive and restrictive deficits or AHR after exposure to high levels of chlorine gas.[34] A significant decrease in forced vital capacity (FVC), and forced expiratory volume in one second (FEV_1), and peak expiratory flow rate are seen.[38,53] Changes in the FEV_1/FVC ratio can discern between restrictive and obstructive lung disease where a decrease in the ratio (less than 0.7) is indicative of obstructive lung disease, while restrictive diseases tend to have either a slight increase or same ratio due to equal decreases in both values. Increases in airway resistance and elastance have been also demonstrated.[53,54]

Current treatment of Cl_2 gas inhalation injury is symptomatic. Nebulized sodium bicarbonate, corticosteroids, and oxygen therapy have been used to treat

symptoms of injury in patients.[41,45,55] However, there are no prospective clinical studies that have been performed to assess the safety and efficacy of these therapies for Cl_2 gas inhalation, and further research into the mechanisms of injury is required in order to develop specific treatments for this type of injury. Some animal studies have shown that treatment of lung injury caused by acute chlorine gas exposure with aerosolized terbutaline (beta-2 adrenergic agonist) followed by aerosolized budesonide (corticosteroid) can help improve lung function in pigs exposed to 400 ppm Cl_2 for 20 minutes.[56,57] Similarly, another study by Demnati et al. has shown that dexamethasone (glucocortiocosteroid) treated rats have improved lung function and AHR, and reduced inflammation after exposure to chlorine gas.[58]

1.2.3 Animal models of Cl_2 exposure

Despite the many studies that have described the pathophysiology in individuals who have been exposed to chlorine, there is not much insight into the mechanisms leading to airway dysfunction following chlorine injury, or possible treatment modalities. For this reason animal models have been developed to further explore these mechanisms of injury and to explore ways of treating those who are exposed to Cl_2. These models range from mice, rats, and rabbits to pigs and humans, and include *ex vivo* and *in vivo* studies.

Animal models of Cl_2 gas exposure show that at a concentration of 2000 ppm, immediate death occurs, and there is 50% mortality rate at doses of 800-1000 ppm. Bronchoconstriction is observed at 200 ppm and function of cilia is impaired in doses

as low as 18 ppm. Chemical pneumonitis and bronchiolitis obliterans have been seen in acute exposures to doses between 9-50 ppm. Irritation of nose and trachea occurs at a dose of 2 ppm without any respiratory effects.[59]

Detailed studies have looked at the *in vivo* effects of acute chlorine gas inhalation in order to develop rodent models.[58,60-62] In the model developed by Demnati et al. a Cl_2 concentration of 500ppm did not lead to any significant histological changes to the airways in Sprague-Dawley rats. However, at the exposure level of 1500 ppm for 2 minutes, perivascular edema and focal mild inflammation were present, while exposure to the same concentration for 10 min caused profound histological changes. In a 72 hour experiment where lungs were harvested at 1, 3, 6, 12, 24, and 72 h after exposure to 1500ppm Cl_2 for 10 minutes the following histological changes were observed: 1 h: airspace and interstitial edema associated with bronchial epithelial sloughing; 6 to 24 h: decreased edema and the appearance of mucosal polymorphonuclear leukocytes, maximal at 12 h; 72 h: epithelial regeneration, manifested by hyperplasia and goblet cell metaplasia.[60] Having determined that acute exposure to a Cl_2 gas concentration of 1500 ppm was optimal for a model of IrIA in the Sprague-Dawley rats a second study was conducted by Demnati et al. to further examine the time course of functional and pathological changes after exposure.[61] Histological findings included epithelial necrosis and detachment, increased smooth muscle fractional area of the airway wall, epithelial regeneration, and mucous cell hyperplasia. Most of these abnormalities resolved after a mean interval of 90 days. Increased lung resistance (R_L) and/or bronchial responsiveness to inhaled methacholine were observed. The functional changes were

related to the overall abnormal airway epithelial damage and there was a significant correlation between R_L and BAL neutrophilia.[61] The extent of epithelial damage including epithelial shedding and necrosis peaked at 24 hours post exposure and correlated with the time point for maximal airway hyperresponsiveness. Also, R_L values were highest during the time point of peak neutrophilia in bronchoalveolar lavage fluid.

A murine (A/J strain) model of Cl_2 induced IrIA was recently developed by Martin et al. to look at possible mechanisms leading to injury. [62] Similar to Demnati et al. animals were acutely exposed to different concentrations of Cl_2 (100, 200, 400, and 800ppm), and were studied over a period of several days to find the most informative dose and time point for the model. Significant increase in airway responsiveness and inflammation were observed with 400-800ppm Cl_2 at 24 hours post exposure, and correlated with increased airway epithelial damage as observed in histology sections and increased epithelial cell counts in BAL. Furthermore, this study suggests that the mechanism of injury leading to functional and pathological changes is through oxidant stress, and affects mainly the airway epithelial cells and alveolar macrophages.[62]

Other studies looking at a porcine and rabbit model of Cl_2 injury have described similar findings, where increases in resistance and elastance, edema, sloughing of bronchial epithelium, and inflammatory cell influx were observed.[54,63,64]

1.3 Airway Epithelium

Airway epithelial cells are the largest mucosal barrier separating the host from the environment, and damage to the lung by the environment occurs first in this tissue. During inflammatory respiratory disorders, extensive injury of airway epithelium may occur, with shedding of a large sheet of damaged cells in the bronchial and alveolar lumen that could lead to inflammation and altered lung function. As we have seen Cl_2 injury by inhalation and subsequent development of RADS, have similar characteristics of airway epithelial injury and shedding. However, the epithelial surface of the airways besides acting as a physical and functional barrier to external agents is capable of producing and releasing pro-inflammatory cytokines and chemokines, and working with immune cells to contribute to airway repair.

This section will focus on the physiology and pathophysiology of airway epithelial cells with respect to their immunomodulatory roles in recruiting inflammatory cells, and their role in modulating airway muscle tone and responsiveness to contractile agents.

1.3.1 Airway Epithelium Anatomy & Physiology

The airway epithelium consists of several different types of distinct epithelial cell types including ciliated cells, goblet cells, serous cells, basal cells, and Clara cells. Other cell types including immune cells, inflammatory cells, and phagocytic cells migrate from the blood and reside within the airway epithelium as well.[65,66]

Ciliated cells are the predominant cells of the airways. The primary function of these cells is the unidirectional transport of mucus from the lung to the throat. Special barb like structures on the tip of cilia grab the mucus during the active stroke phase of ciliary beating but not during the relaxation phase thereby propelling the mucus towards the throat.[65,66] The primary role of goblet cells is to produce mucus. These cells have large granules containing mucins and sulphomucins. The release of correct amount of mucus is essential for proper mucociliary clearance function. Mucous cell hyperplasia and metaplasia are common pathological findings in chronic respiratory diseases such as asthma and are thought to contribute to the persistent cough present in these patients.[65,66] Serous cells are morphologically similar to goblet cells, however, their granules contain substances other than mucin that are not very well characterized. The basal cells are thought to be the primary stem cell that can give rise to mucous and ciliated cells. These small flattened cells underlie the ciliated and goblet cells and together they give rise to a unique pseudostratified appearance to the airway epithelium. Beside their progenitor roles basal cells can also secrete biologically active molecules such as cytokines.[65,66] Clara cells are capable of producing pulmonary surfactant and have antiprotease activity.

The epithelium, in addition to these more common cells contains other cells known as neuroendocrine cells. The location of these cells near the basement membrane, and the content of their granules would suggests that these cells might be involved in regulating secretion, smooth muscle function and cell growth modulation.[65,66]

Lastly, the airway epithelium contains many types of immune cells including mast cells, intraepithelial lymphocytes (eg. γδ T cells), macrophages and dendritic cells. These cells can provide some level of protection by providing an innate immune response.[65]

1.3.2 Host defense function

On a daily basis a vast volume of air moves into and out of the lungs carrying with it not only air, but also much foreign material. The nose and the upper airways filter large particulate matter and humidify the air that enters the lungs. However, many agents such as fumes, gases, and biological matter still enter the airways with the ability to cause harm. The epithelium has several functions that help protect the airspaces and maintain functionality as outlined in table 2.

Table 2: Functions of the airway epithelium

- Protection from the external environment
- Regulation of fluid and ion transport across the airway surface into the lumen
- Mucus secretion and ciliary transport
- Recruitment and regulation of inflammatory cells
- Antimicrobial activities
- Protection against oxidants and proteases
- Modulation of airway smooth muscle tone
- Modulation of repair responses

Adopted from Sacco O et al. Paediatr Respir Rev. 2004

The mucociliary layer and the junctional complexes provide a morphological and functional barrier between the host and the environment. Junctional complexes are characterized by three main components: zonula adherens, desmosomes, and tight junctions. Tight junctions are located between the apices of adjacent cells and

provide a physical barrier function. Zonula adherens and desmosomes contribute to cellular adhesion and contain numerous proteins that help maintain structural integrity of the epithelium. The lining of the airways by this continuous epithelium along with junctional complexes forms an impermeable membrane to macromolecules and infectious agents, and limits electrolyte movement from the plasma to the lumen of the airways.[65,66]

This barrier function is paramount in protecting the host from invasion by microbes, and the cells underlying the epithelium including the neural plexus, mast cells, and smooth muscle cells from irritant materials. The stimulation of the neural plexus or mast cells by irritants can lead to the secretion of various mediators that are associated with airway obstruction and airway hyperresponsiveness.[66]

1.3.3 Regulation of inflammation

The pulmonary epithelium produces a wide array of proteins including chemokines, cytokines, growth factors, and bronchoconstricting peptides. Table 3 outlines factors expressed or regulated by airway epithelium in inflammatory diseases such as asthma.[67]

GM-CSF, IL-6 and IL-8 are cytokines that are produced by the epithelium under basal conditions, and have been shown to increase in bronchial epithelial cells in asthmatics.[68] IL-6 is a pro-inflammatory cytokine that induces growth and differentiation of B cells, T cells, and macrophages. IL-8 is a strong neutrophil

chemoattractant. GM-CSF has an important role in prolonging the life span of neutrophils, and eosinophils in the airways. Epithelial cells can also release IL-1 that in turn can activate other immune cells such as macrophages to release IL-6. RANTES has also been shown to be produced by airway epithelial cells and may have a role in asthmatic inflammation, by recruiting eosinophils, basophils, and T cells.[67,69]

There are many other examples of inflammatory cell activation and immunoregulation by epithelial cells such as antigen presentation. However, these are beyond the scope of this review and will not be reviewed here.

1.3.4 Regulation of bronchial tone and responsiveness

Following epithelial damage where the barrier function of this organ is diminished, inhaled allergens and irritants can reach the underlying smooth muscle, and intra-epithelial nerves more easily causing reflex bronchoconstriction. Also, the epithelial layer itself is capable of synthesizing histamine and acetylcholine that can be released from the cells under various stressful conditions.[70] The epithelium is the source of other important molecules involved in the regulation of bronchial tone, known as epithelium-derived relaxing factors. Damage to the epithelium can result in lower levels of these factors being released into the surroundings thus leading to bronchoconstriction.

Table 3: Factors released by epithelium during inflammatory diseases

"Factors"	Triggering Agent	Possible Consequence
Mediator		
PGE_2, $PGF_{2\alpha}$, PGI_2, TxB_2 LTB_4 LTC_4, LTD_4, LTE_4 15-HETE, HHT	O_3, oxidants	Bronchospasm, mucus secretion, microvascular leakage, chemotaxis
PGE_2 NO_2 LTC_4 12-HETE, 15-HETE	NO_2	Vasodilation, bronchospasm
Endothelin	Asthma	Bronchospasm, smooth muscle proliferation
Cytokines Chemokine		
GM-CSF	IL-1, histamine, NO_2, inflammation, asthma	Prolong eosinophil/neutrophil survival, increase eosinophil activation
IL-1	TDI	Augment inflammation
IL-6	IL-1, histamine, TNF-α, TGF-β asthma	Augment inflammation
IL-8	RSV, TNF, IL-1, NO_2	Neutrophil recruitment
TNF-α	NO_2	Augment inflammation
RANTE	IL-1,TNF	Eo, mono, basophil, T-cell recruitment
MIP-2	TNF	Inflammatory cell recruitment
Adhesion		
ICAM-1	IFN-γ, TNF-α, IL-1, PIV, asthma Phorbol ester	Inflammatory cells recruitment
Growth		
Fibronectin	Histamine	Increased matrix formation
PDGF	Injury	Fibroblast/smooth muscle proliferation
Immune		
MHC-II	Inflammation	
HLA-DR	Asthma	Increased antigen presentation Increased antigen presentation

Eo: eosinophil; GM-CSF: granulocyte-macrophage colony-stimulating factor; HETE: hydroxyeicosatetraenoic acid; HHT:hydroxyheptadecatrienoic acid; HLA-DR: human leucocyte antigen-DR; ICAM: intracellular adhesion molecule; IFN-g: interferong; IL: interleukin; LT: leukotriene; MHC-II: major histocompatibility complex II; MIP-2: macrophage inflammatory protein-2; mono: monocyte; neut: neutrophil; PDGF: platelet-derived growth factor; PG: prostaglandin; PIV:parainfluenza virus; RANTES: regulated on activation, normal T-cells, expressed and secreted; RSV: respiratorysyncytial virus; TxB2: thromboxane B2; TDI: toluene diisocyanate; TGF-b: transforming growth factor-b; TNF-a: tumour necrosis factor-a.

It has been observed in asthmatic patients and in models for airway hyperresponsiveness that epithelial shedding is correlated with bronchial responsiveness[71] and the degree of bronchial hyperresponsiveness is proportional to the extent of denudation of the basement membrane.[72] Soon after, it was realized that epithelium contains the enzymes iNOS and eNOS, and has the ability to produce and release an epithelium-derived relaxing factor (NO) into the surroundings.[73] Another relaxing factor that is produced by epithelial cells is PGE_2 and it has been shown to have a direct relaxant effect on bronchial tone and responsiveness to spasmogens.[74-76]

Looking at the role of the epithelium in bronchial inflammation and responsiveness it can be concluded that it is vital to maintain a healthy epithelial lining. Damage to this barrier from environment exposures such as Cl_2 gas would be expected to have many unfavorable outcomes such as airways hyperresponsiveness, and inflammation that could persist for a long period of time.

1.4 γδ T Cells

Ever since their discovery in the 1980s the role of γδ T cells in the immune response has been of much interest. Even now, the specific role and mechanism of action of these cells in the immune response to inflammation due to pathogenic and non-pathogenic insults remains unclear. One immediate characteristic of these cells is their tendency to be located in the body's mucosal surfaces including the intrapulmonary airways. Studies have demonstrated that γδ T cells can control the growth and differentiation of airway epithelial cells (AECs), and may be essential in the regulation of repair and removal of necrotic AECs.[77] However, the underlying mechanisms of the relationship between γδ T cells and the epithelial cells of the airways remain to be elucidated.

Previously, γδ T cells have been implicated in the regulation of αβ T cell responses to allergen in both the respiratory and gastrointestinal tracts. However, Lahn et al. in their recent publication have shown that γδ T cells have a role as regulators of airway responses that are independent of αβ T cells. This leaves AECs as a very attractive target for regulatory γδ T cells.[78] King et al. have shown that γδ T cells are indeed able to recognize and respond to self-tissue damage due to pathogenic (bacteria) and non-pathogenic (ozone) insults.[79] Other studies have shown that γδ T cells express the epithelial cell mitogen keratinocyte growth factor (KGF) which again suggests that these cells are involved in preventing damage or repairing damaged epithelial cells.[80]

1.4.1 γδ T cells and inflammation

The specific role of γδ T cells in the immune response is still not resolved. However, in trying to identify the ligands that are recognized by γδ T cells in human and mice, many features of γδ T cells have been characterized. It is also important to note that these cells have the ability to produce different cytokine profiles depending on the inflammatory environment further adding to the enigma of the function of these cells in inflammation.

The physical location of these cells in mucosal tissues or other areas of continuous exposure to antigens and irritant substances, suggests that these cells may be involved in the innate immune response, by contributing to immunosurveillence. γδ T cells have the ability to produce high amounts of IFNγ, and to enhance IFNγ production in other cells of the innate immune system such as natural killer cells.[81] The dual role of these γδ T cells implicates them in the adaptive immune response as well where they have the ability to modulate the B cell and αβ T cell response. In the absence of γδ T cells these cells seem to have higher functionality as evidenced by their ability to release various cytokines and immunoglobulins, suggesting that γδ T cells may have a negative regulatory effect on the adaptive immune response.[82,83]

The response of γδ T cells varies with the infecting agent. For example a *Listeria* infection model in mice has shown that γδ T cells acquire Th1 type properties, while they show Th2 type properties after infection with *Nippostrongylus braziliensis*.[84] The development of Th2 properties following exposure to antigens by

γδ T cells could lead to eosinophillic inflammation and subsequently asthma. Thus, it is important to identify stimuli and further explore the mechanisms that steer the γδ T cells towards having either Th1 or Th2 properties.

1.4.2 γδ T cells and airway hyperresponsiveness

Airway responsiveness is the outcome of many complex interactions between the cells of the airways and their responses to environmental agents. A handful of studies have shown that γδ T cells are implicated in the regulation of processes leading to airway responsiveness. As mentioned earlier γδ T cells exhibit different immune properties depending on the type of stimulus and inflammation, thus may have differing roles in the context of the regulation of airway responsiveness.

Mouse models of ovalbumin-induced allergic asthma have been used extensively to study the mechanisms leading to increased airway responsiveness in asthma. Also, knockout mice that lack γδ T cells have been developed to look at the role of these cells in responsiveness. In the context of immunologic / allergic asthma there have been studies to indicate that γδ T cells act as negative regulators of airway responsiveness by contributing to the maintenance of normal lung function.[78] However, other studies again have looked at the dual functionality of these cells and have shown that different subsets of γδ T cells and different experimental designs, either have an enhancing or dampening effect on AHR.[85] Thus it would be difficult to predict what their role would be in Cl_2 gas induced inflammation and AHR.

1.4.3 δ T-cell receptor deficient mice

In this study, mice homozygous for the Tcrδ deletion mutation were used. Mice homozygous for this mutation are viable and fertile. There is lack of expression of the γδ T-cell receptors in tissues especially in lymphoid and epithelial organs. The development of the αβ T cells is not interrupted, and patterns of CD4+ and CD8+ αβ T cells are normal. In contradiction to some hypotheses these mice do not develop inflammatory bowel disease.[86] It has been suggested that these mice may be susceptible to infection due to an inadequate immune response. A deficiency in epidermal wound repair has been documented in these mice.[87,88]

1.5 AIMS / HYPOTHESIS

The main focus of this project was to study the role of γδ T-cells in the response to Cl_2 exposure with respect to inflammation and lung function. In order to specifically look at the function of these cells, a line of TCR $\delta^{-/-}$ (γδ T cell deficient) mice on the C57BL/6J background was studied. The basis for the choice of TCR $\delta^{-/-}$ mice on the C57BL/6J background and not others was that they are commercially available, and in addition it has been suggested that these mice characteristically have higher numbers of γδ T-cells. Therefore a deficiency of these cells in C57BL/6J might be expected to show larger effects of the deficiency compared to the wild type animals than in other strains. Additionally, C57BL/6J mice are known to have lower airway responsiveness to bronchoconstrictive agents than other strains of mice at baseline and in models of allergic asthma, thus any increases in AHR after Cl_2 exposure might be more easily discerned.

This project had three main objectives: (1) to evaluate the time-course of airway response to Cl_2 gas exposure, (2) to evaluate the time-course of inflammatory response to Cl_2 gas exposure, and (3) to contrast injury and repair phenomena in γδ T-cell-deficient and wild-type mice. Specifically we hypothesized that γδ T-cells were involved in modulating airway responses to methacholine and the repair of airway epithelial cells after acute chlorine gas exposure.

2. Materials and Methods

2.1 Animals

Male C57BL/6J and TCR $\delta^{-/-}$ (B6.129P2-Tcrd ^{tm1Mom}) mice 8 to 10 weeks of age were purchased from Jackson Laboratories and housed under specific pathogen-free conditions in a conventional animal care facility at McGill University. Only male mice were studied in order to exclude sex effects and the animals are larger and more robust for these experiments. All the experiments were approved by the Animal Care Committee of McGill University.

2.2 Experimental Protocol

The respiratory system resistance (Rrs) and elastance (Edyn, rs) obtained under baseline conditions and during methacholine-induced constriction in a murine model of acute lung injury to chlorine (Cl_2) gas inhalation were compared with those obtained in control mice. In mice exposed to chlorine gas, physiological evaluation was performed 1, 3, and 5 days after exposure. The animals were tested for airway responsiveness (n=8) on each of the 3 test days, and BAL analysis and immunohistochemical staining were performed on 7 pairs of lungs (n=7) at each time interval.

2.3 Chlorine Gas Exposure

Chlorine gas (Matheson Gas Products, Ottawa, Canada) was mixed with room air in a standard 3 L rebreathing bag to make a concentration of 400 ppm Cl_2. The intake port of an exposure chamber was connected to the re-breathing bag while the

outlet port was connected to a flow meter and vacuum. Animals were restrained to receive nose-only exposure for 5 minutes.

2.4 Evaluation of Airway Responsiveness

Mice were sedated with an intraperitoneal injection of 8mg/kg xylazine hydrochloride and anaesthetized with pentobarbital (30mg/kg i.p.). Upon disappearance of the corneal and toe pinch reflex, a cannula was placed in the left jugular vein for drug administration. Subsequently, the animal was tracheostomized and a tracheal tube was inserted in the trachea. The tracheal tube was connected to a small animal ventilator (Flexivent, Scireq, Montreal, Canada). Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg i.v.). The mice were ventilated at 150 breaths/min in a quasi-sinusoidal fashion with PEEP=2.0-3.0 cm H_2O, and V_T=0.18 ml. Methacholine (MCh) was administered via the jugular cannula in doubling doses from 10 to 640 ug/kg. Respiratory system resistance (R_{RS}) and elastance (E_{RS}) were measured during oscillations equal to those used during mechanical ventilation before challenge and repeated after each dose of MCh, with peak responses being reported.

R_{RS} and E_{RS} are parameters reflecting dynamic resistive and elastic properties of the respiratory system respectively that can be obtained using the forced oscillation technique. This technique involves applying a sinusoidal pattern of ventilation to the lungs of anesthetized mice while measuring the flow and pressure generated in the trachea using appropriate software. The software calculates parameters R_{RS} and E_{RS} by fitting the equation of motion to the tracheal pressure: ($Ptr = R_{RS} \times Flow + E_{RS} \times Volume + K$), K is a constant for positive end-expiratory pressure (PEEP); R is the

resistance to flow in the respiratory system and E is the elastance which is simply the inverse of compliance. Elastance more specifically reflects the ability of the lung to recoil after a volume change or the change in pressure for given change in volume ($E = \Delta P/\Delta V$). Resistance is inversely proportional to the radius of the airways ($R \propto 1/r^4$), therefore a small decrease of airway size due to contraction of smooth muscles can lead to large increases in airways resistance.

2.5 Bronchoalveolar Lavage

Following measurements of respiratory system mechanics the animals were sacrificed using an overdose of sodium pentobarbital (0.25 ml) and exsanguination. A tube was placed in the trachea (blunt Luer adapter) and the lungs were lavaged with 0.6 ml of sterile saline, followed by four lavages of 1 ml each. The first aliquot of 0.6 ml BAL fluid was centrifuged at 1600 rpm for 5 minutes at 4°C and the supernatant was retained for measurements of protein by Bradford assay. The cell pellet was pooled with the remaining lavage samples and total cell numbers were counted with a hemacytometer. The cytospin slides of BAL cells were stained with Dip Quick (Jorgensen Labs Inc., Loveland, CO). Differential cell counts were based on a count of 300 cells. Absolute cell numbers were also calculated as the product of the total and differential cell counts.

2.6 Histology and Immunohistochemistry

Mice were sacrificed by using a lethal dose of pentobarbital and lungs were collected following exsanguination and perfusion of the lung vasculature with saline.

Subsequently lung tissues were fixed overnight with 10% formalin at a pressure of 25 cm of H_2O. Formalin-fixed tissues were then placed in a tissue processor that automatically passes the tissue through a series of ethanol and xylene washes and finally were embedded in paraffin blocks. The blocks were cut into 5 μm sections and placed on Superfrosst slides. To evaluate the repair response of the airway epithelial cells in the various groups a specific mouse anti-PCNA monoclonal antibody was used. Antibody is a protein secreted by B lymphocytes in response to an antigenic stimulus. Monoclonal antibodies are produced in large quantities in the laboratory in pure form by using a single population or clone of cells. PCNA antigen is a widely used marker for proliferation, it is only present in proliferating cells and an increase in the number of cells with positive staining would give an indication of the repair response and the ability of the host to respond to Cl_2 induced injury to the epithelial layer.

For immunohistochemical detection of PCNA, slides were deparaffinized in three changes of xylene and dehydrated with ethanol. For proper detection of PCNA antigen, a high temperature antigen unmasking technique was necessary. Slides were placed in Antigen Unmasking Solution (Vector Laboratories, CA) and heated in a pressure cooker for 15 minutes, and cooled under running tap water for 20 minutes. Since this is a nuclear stain, the membrane was permeabilized using 0.2% Triton X-100 detergent. Use of a mouse-on-mouse (MOM) kit was necessary since mouse anti-PCNA antibody was being used on mouse tissues. Prior to applying the primary mouse anti-PCNA antibody tissues were blocked using MOM mouse IgG blocking

reagent to reduce non-specific binding. The tissues were then treated with anti-PCNA antibody and isotype control antibody (negative control) for 30 minutes at 37°C, rinsed with TBS and treated with MOM biotinylated anti-mouse IgG reagent. To develop the stain an avidin-biotin complex alkaline phosphatase (ABC-AP, Vectastain) kit was used, followed by alkaline phosphatase substrate that resulted in a distinctive red stain in cells with positive staining. Finally, tissues were counterstained with methyl green, and cover-slipped. Mouse intestinal tissue was used for positive control, due to a high turnover rate of epithelial cells.

Duplicates of the slides were used in histology. Slides were stained with hematoxylin and eosin to detect any morphological differences in tissue and airways.

2.7 Morphometry

For quantitative analysis of different groups of PCNA stained cells the following morphometric technique was used. Intact airways on tissue sections were traced at 20X magnification and a blinded investigator counted positively stained epithelial cells for each airway. The traces were then scanned into a computer using a scanner (Canon, Lake success, NY). The airways were then digitized using a digitizing tablet (Wacom, Vancouver, WA) and commercial software (Sigma Scan, Leesburg, VA) to calculate airway perimeter length. All pictures were calibrated at the same resolution with a tracing of a calibration slide. Results were then depicted as number of PCNA positive cells / mm of basement membrane squared. Squaring the basement membrane would allow correcting for variables that vary as a function

of area. Airway smooth muscle for example varies as a function of airway area and not circumference.

2.8 Statistical analysis

Comparison among several means was done by analysis of variance and post hoc testing was done using Fisher least significant difference test. P-values less than 0.05 were considered significant.

3. Results

3.1 Changes in Bronchoalveolar lavage composition after chlorine gas exposure

Bronchoalveolar lavage was performed on 7 mice per group at different time points over a 5 day period. The fluid recovered from lavage was 85% of amount infused and did not differ significantly among the groups. Figures 1-5 show the results of the cell counts and differentials. Total cell counts increased by 24 hours after exposure to chlorine and returned to baseline values after 3 and 5 days in wild type and knockout mice respectively (figure 2). A marked difference in trypan blue uptake in cells of different groups was noticeable. The numbers of the necrotic cells, with positive trypan blue staining, showed significant differences between wild type and knockout animals (figure 3). Twenty four hours following exposure to chlorine, 49% of the cells in wild type and 59% of the cells in knockout mice were necrotic ($p< 0.05$) and consisted of mainly epithelial cells. These values returned to baseline at 3 days in wild type and 5 days in knockout mice. The increase in total cell counts was mostly attributable to increases in macrophage numbers (figure 4A), however there were also significant increases in epithelial cells and neutrophils.

A delayed and lesser macrophage and neutrophil response was observed in $\gamma\delta$ T cell deficient mice as evidenced by differential counting of cells obtained in BAL. Macrophage numbers increased significantly in wild type compared to control mice 24hrs after exposure; while a significant but transient increase in knockouts was observed at 3 days post exposure (figure 4A). At the 5-day time point wild type mice still had significantly large number of macrophages in BAL compared to knockouts.

Same pattern of recruitment was observed for neutrophil numbers (figure 4B), however the significant increase in neutrophil numbers was at 3 and 5 days for wild type and knockout mice respectively.

To assess the extent of damage caused by inhalation of Cl_2 gas, epithelial cell counts and BAL protein contents were obtained. Cl_2 inhalation caused extensive shedding of the airway epithelial cells (figure 5). A significant increase in number of epithelial cells obtained in BAL was observed 24hrs after Cl_2 exposure in both groups. However, knockout mice appeared to be more susceptible to epithelial damage or shedding as evidenced by epithelial cell counts in BAL. It is important to also note that the damaged epithelial cells were cleared rapidly in wild type mice while knockout mice still had slightly elevated epithelial counts even after 3 days post exposure. Total protein content, depicted as ug/ml BAL supernatant, was elevated and sustained up to 3 days after exposure. However, differences between wild type and knockout are difficult to interpret because differences in baseline protein content were also present (figure 6).

3.2 Histologic and immunohistochemical findings after chlorine gas exposure

Qualitative examination of the airways exposed to Cl_2 gas showed major epithelial loss in the airways and replacement of cuboidal ciliated epithelium with flat cells. Knockout mice exposed to Cl_2 also had sustained damage to the tissue around the airways at the 24hour time point. Accumulation of inflammatory cells into alveolar walls was also observed. Baseline differences between wild type and

knockouts were not obvious. However, a few airways with abnormal epithelial lining were present in the knockouts.

Quantitative analysis of the number of positively stained epithelial cells showed no difference between wild type and knockout control animals (Figure 8). At the 24hour time point following exposure to 400 ppm Cl_2 for 5 minutes there was a significant increase in epithelial cell proliferation in both groups (Figure 7), while, the knockouts seemed to be impaired in their ability to regenerate epithelium compared to the wild type animals. This regenerative response was maintained in wild type animals for up to 3 days while it was diminished in knockouts after the 24hour time point compared to the wild type mice. Both groups returned to baseline numbers of PCNA positive cells by five days after initial Cl_2 injury.

3.3 Effects of chlorine exposure on bronchial responsiveness

The time course of methacholine responsiveness in the mice exposed to 400 ppm Cl_2 at 1, 3, and 5 days after exposure was examined. There were no baseline differences in Rrs and Ers between wild type and knock out mice and between sham-exposed and Cl_2-exposed groups. Wild type mice had significant increase in methacholine responsiveness compared at 1 day after exposure to 400 ppm Cl_2. This responsiveness decreased slightly by day five, however it was still significantly elevated compared to sham-exposed controls (figure 9). Knockout mice did not develop AHR under the same conditions. However, there was a transient increase in responsiveness to methacholine measured by changes in elastance (Ers) 1 day after

exposure (figure 10). Comparing the responsiveness of the wild type to knockout mice it is clear that there was a significantly reduced change in responsiveness in γδ T cell deficient mice (figure 11).

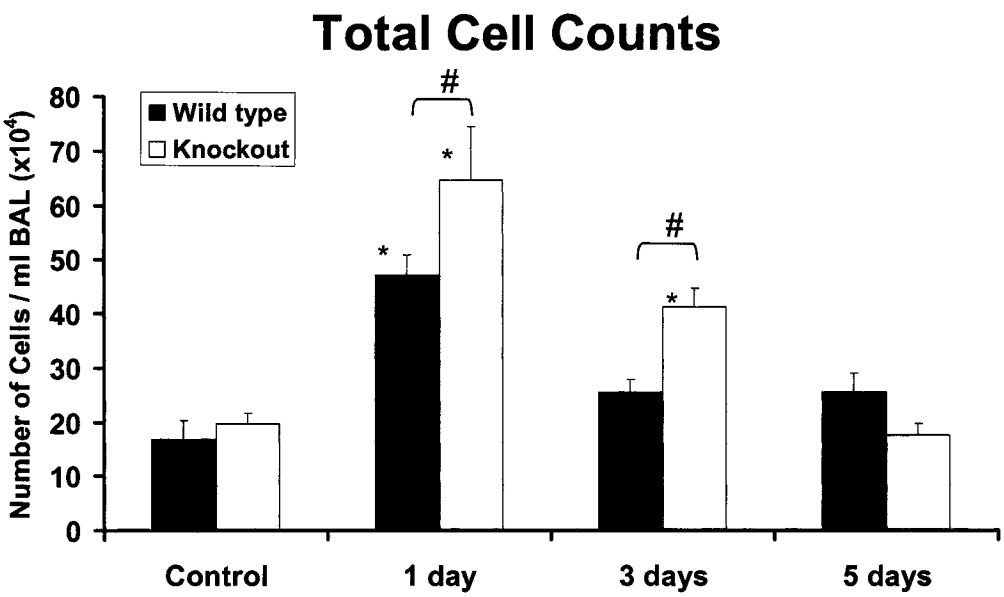

Figure 2: Total cells recovered from bronchoalveolar lavage. * P<0.05 compared to 0 ppm control. # P < 0.05

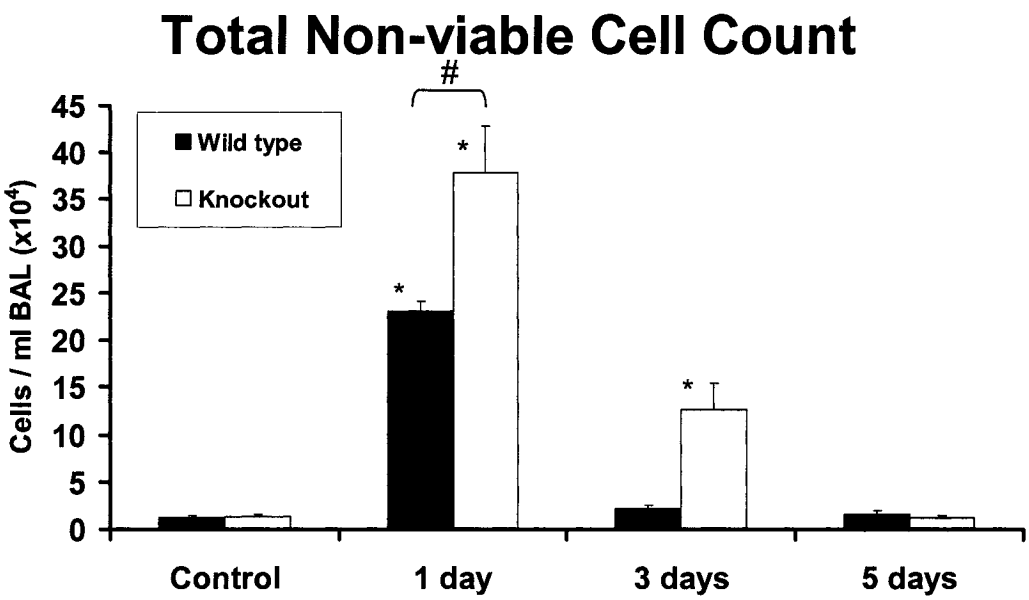

Figure 3: Total dead cells (trypan blue exclusion) recovered from bronchoalveolar lavage. Knockout mice have a greater number of necrotic cells after Cl_2 gas exposure. * P<0.05 compared to 0 ppm control. # P < 0.05

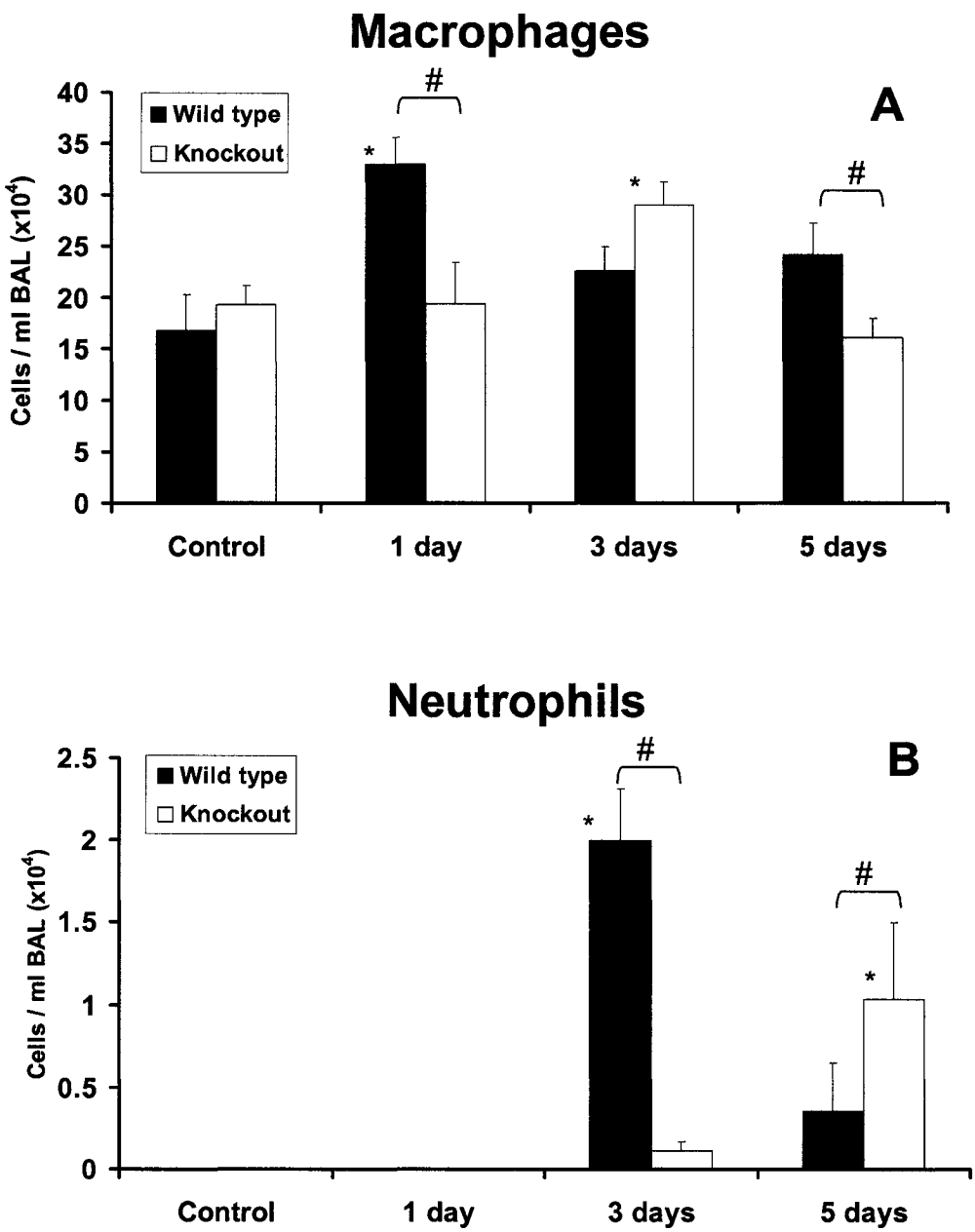

Figure 4: Inflammatory cell recruitment into the airways after Cl_2 gas exposure. Knockout mice have a delay in macrophage (A) and neutrophil (B) recruitment. * P<0.05 compared to 0 ppm control. # P < 0.05

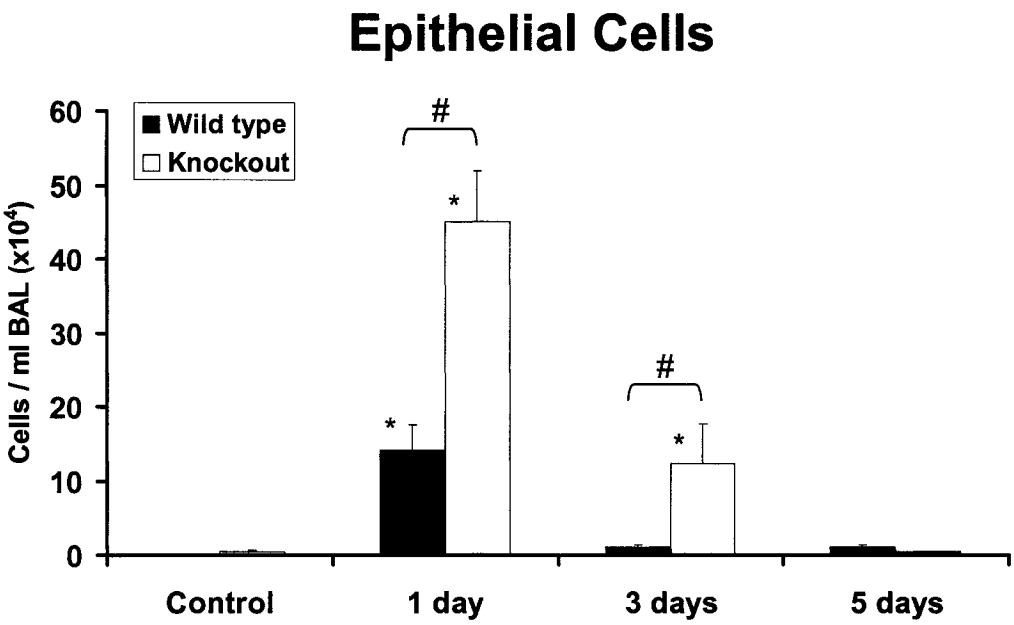

Figure 5: Exposure to Cl_2 gas leads to epithelial shedding in the airways. Knockout mice are more susceptible to injury. * P<0.05 compared to 0 ppm control. # P < 0.05

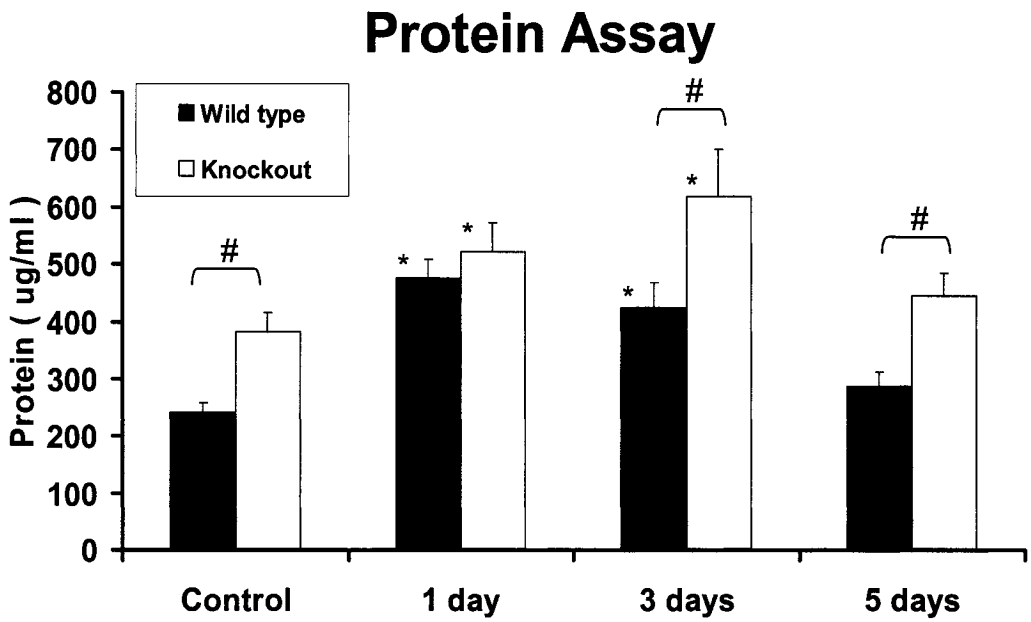

Figure 6: Exposure to Cl_2 gas leads to protein leak into the airways. Knockout mice are more susceptible to injury. * P<0.05 compared to 0 ppm control. # P < 0.05

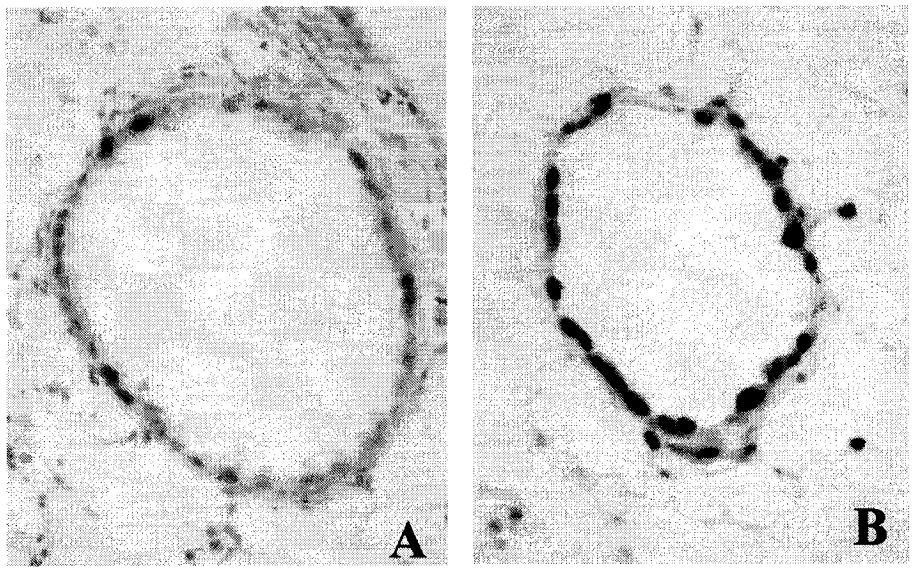

Figure 7: Representative pictures showing PCNA immunostaining in airway epithelial cells before (A) and 1 day after exposure to 400 ppm Cl_2 gas (B) in wild type mice.

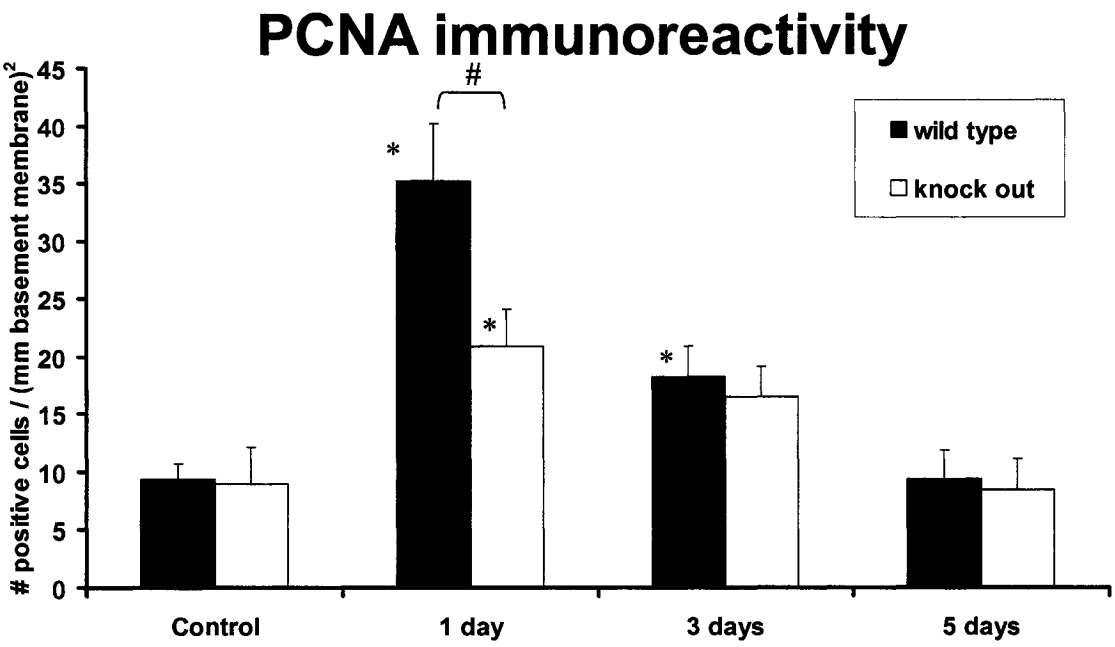

Figure 8: Numbers of epithelial cells with positive staining for PCNA per mm^2 of basement membrane. Knockout mice have impaired epithelial cell regeneration following Cl_2 gas injury. The vertical bars indicate one SEM. * $P<0.05$ compared to 0 ppm control. # $P < 0.05$

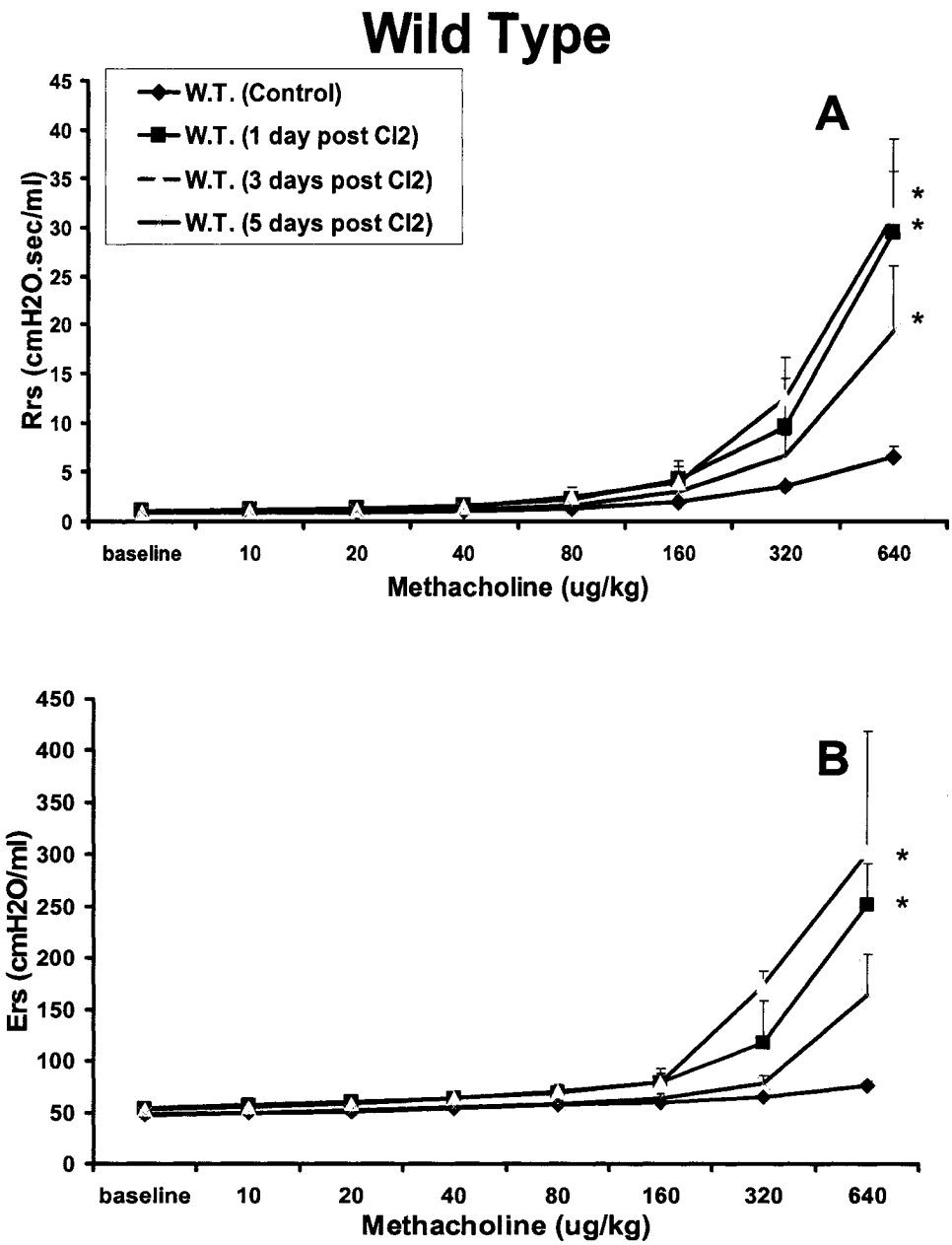

Figure 9: The values of respiratory system resistance (A; R_{RS}) and respiratory system dynamic elastance (B; E_{RS}) in response to intravenous injection of methacholine in wildtype C57Bl/6 mice are shown. The vertical bars indicate one SEM. * P<0.05 compared to 0 ppm control.

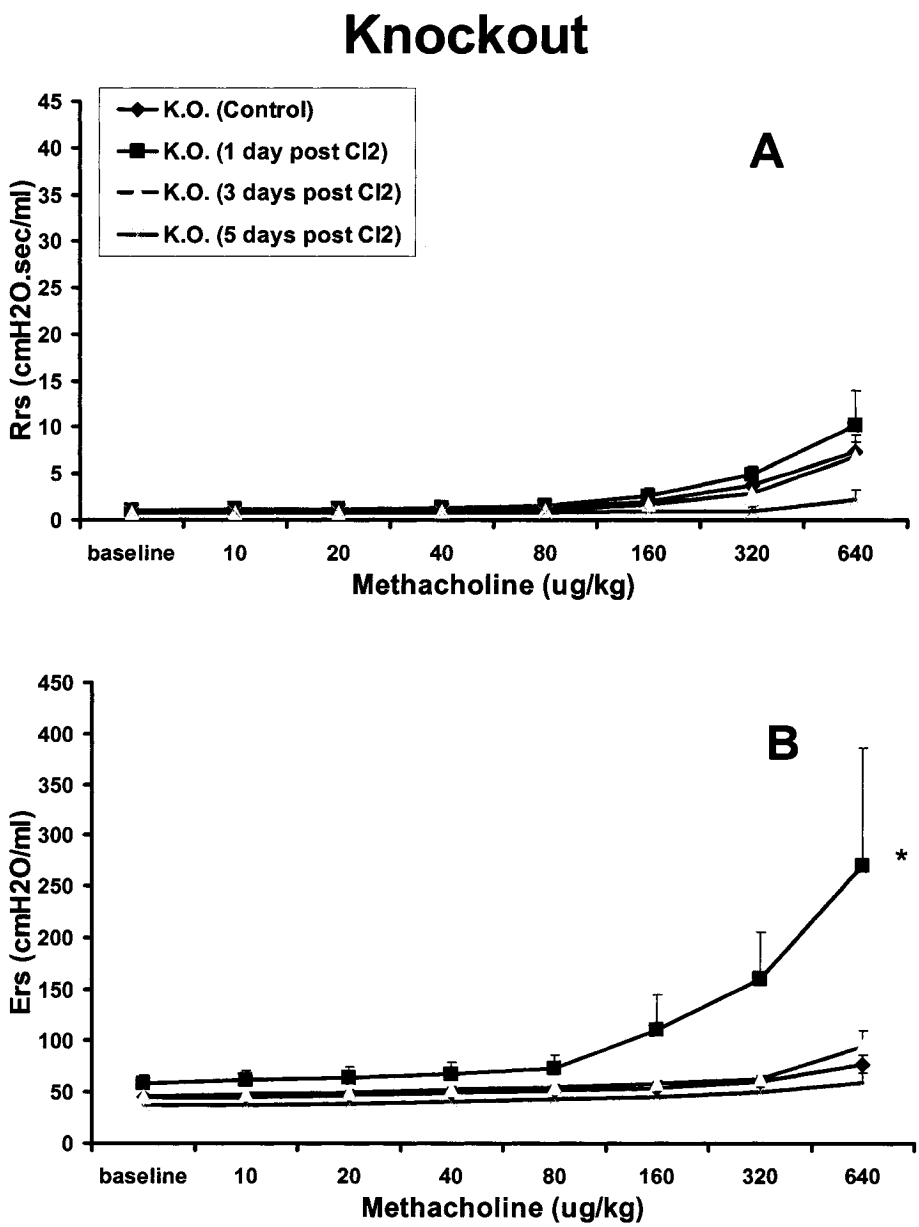

Figure 10: The values of respiratory system resistance (A; R_{RS}) and respiratory system dynamic elastance (B; E_{RS}) in response to intravenous injection of methacholine in TCR δ knockout mice are shown. The vertical bars indicate one SEM. * $P<0.05$ compared to 0 ppm control.

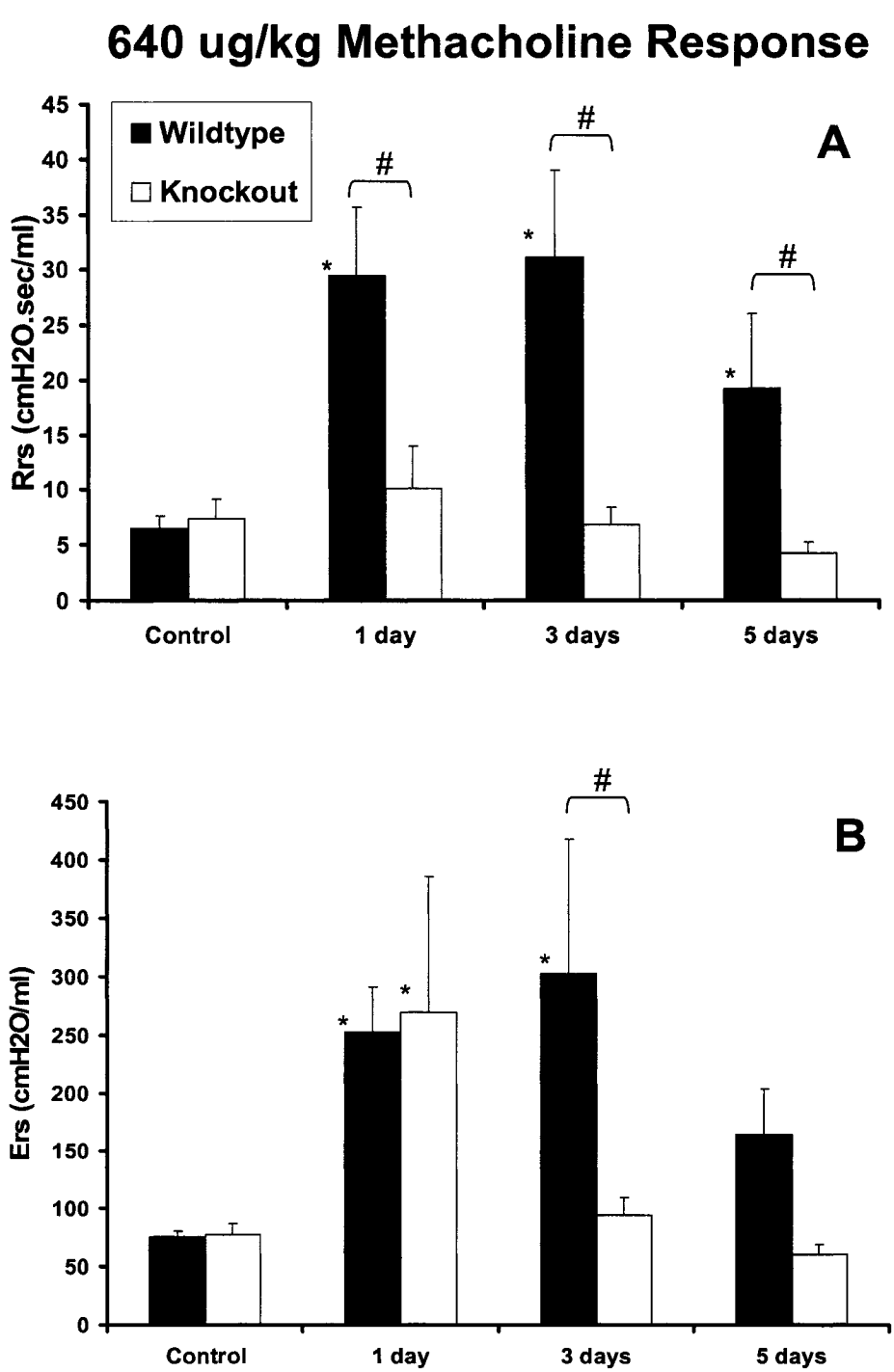

Figure 11: The values of respiratory system resistance (A; R_{RS}) and respiratory system dynamic elastance (B; E_{RS}) in response to intravenous injection of 640 ug/kg of methacholine are shown. * P<0.05 compared to 0 ppm control. # P < 0.05

4. Discussion and Conclusion

4.1 Discussion

In this study we examined injury and repair response of mice to acute exposure of 400 ppm Cl_2 gas, a highly toxic substance implicated in the causation of irritant induced asthma. We were specifically interested in the role of $\gamma\delta$ T-cells in response to this type of injury, and the possible role that these cells might play in regulating the repair response and bronchial responsiveness observed with this type of inhalational injury. To address this issue we developed a murine model of Cl_2 gas induced occupational asthma, by exposing wild type (C57BL/6J) mice to 400 ppm Cl_2 mixed with fresh air for 5 minutes (nose only exposure). For control, mice were sham exposed to air only for 5 minutes.

Twenty four hours following exposure to Cl_2, mice developed bronchial hyperresponsiveness to methacholine and inflammation that coincided with a great degree of epithelial damage and shedding. The rapid onset of AHR without latency is described in individuals with this type of injury leading to reactive airways dysfunction syndrome and the rapid changes we observed are consistent with reports in humans. Also, the changes in responsiveness seem to be transient and recovery occurs in a variable period of time.

This model is in agreement with previous studies on A/J mice [62] and rats [58,60,61] that show increases in airway responsiveness to methacholine and inflammation following exposure to high doses of Cl_2 gas. In our model we observed

an increase in airway responsiveness to methacholine at 24 hours following exposure that was maintained even at the 5day time point. Bronchoalveolar lavage fluid analysis showed increased protein content in airways, concomitant with increases in macrophages, and epithelial cell counts 24 hours following exposure to Cl_2. There was a significant increase in neutrophil numbers at 3 days post exposure. Histological changes included epithelial necrosis and detachment and also epithelial cell regeneration as shown by PCNA staining. Most of these abnormalities returned to baseline values after 5 days with the exception of AHR where, although there was a slight trend for decreased responsiveness, compared to the 24 hr time point, the levels remained elevated compared to air-exposed controls. It is important to note here that although there was clear evidence of AHR to methacholine; baseline values of R_{RS} and E_{RS} were not affected, similar to the study by Martin et al.[62] but, unlike the rat model where increased R_{RS} for up to 30 days was observed.[61] This suggests that in our model the mechanisms leading to altered baseline lung function and airway responsiveness to methacholine are different. Also, further examination of the study by Demnati et al. shows that although both increased R_{RS} and AHR are present in rats after exposure their duration or time course are indeed different. The anatomy of rat lungs and mice lungs are slightly different with fewer bronchial vessels associated with the airways in mouse lungs. Thus it is possible that rats could have greater degrees of microvascular leak leading to baseline increases in R_{RS} by either physically decreasing airway diameter, or altering the surfactant properties in the alveoli and possibly in small airways.

Other models of irritant induced asthma have been explored. For example, it is known that Cl_2 and ozone (O_3) injure the airways through the same oxidative mechanisms. These gases can combine with reactive oxygen species to form a variety of highly reactive compounds thus leading to the oxidative injury to the epithelial cells. Some of the reactive oxygen species present in the airways include hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-), superoxide anion radical (O_2^-), and hypochlorous acid (HOCl). Reactive nitrogen species such as peroxynitrate ($ONOO^-$) have also been shown to cause injury through oxidation (nitration) of proteins and lipids. Oxidants can lead to cellular injury through several mechanisms including, direct damage to DNA, lipid peroxidation with formation of pro-inflammatory molecules such as thromboxane, oxidation of proteins thus altering their activity, inactivation of antioxidants and antiproteases, and alteration of transcription factor activity leading to enhanced expression of proinflammatory genes.[89] While the effects of ozone inhalation on the lung have been extensively studied, few studies have been conducted on chlorine inhalation. Since ozone and Cl_2 share same mechanisms of injury it is likely that the results of studies on models of ozone injury may also apply to Cl_2.

Our results are confirmed by the observation that inhalation of toxic levels of ozone causes pulmonary edema, airway hyperresponsiveness, and alveolar epithelial damage.[90] These findings are further correlated with an accumulation of macrophages and neutrophils in the airways, that also seem to be activated following ozone inhalation and subsequently release mediators that contribute to toxicity.[91,92]

Some of the mediators that have been shown to be released from macrophages and epithelial cells after ozone exposure include TNFα, and NO. It has been shown that these mediators act as central mediators of airway hyperresponsiveness and inflammation in rodent airways following ozone exposure.[93-95]

To deduce the role of γδ T cells in regulating the pathological and functional changes observed in lungs of Cl_2 exposed mice, a line of γδ T cell deficient mice with the C57Bl/6J background was studied. γδ T cell deficient mice did not have altered baseline Rrs and Ers values in relation to their wild type controls, before and after exposure to Cl_2 gas, suggesting that the γδ T cells do not play a major role in determining baseline mechanics. Other parameters such as baseline cell counts in BAL were also similar to the wild types. However, there were significantly higher levels of total protein in the BAL supernatants without Cl_2 exposure as detected by the Bradford assay. Protein leak from blood vessels associated with the airways or from necrotic epithelial cells could be two possible sources for the increased protein content in the airways. As mentioned in the background section, γδ T cells have a major role in maintaining the epithelial barrier, and thus their absence could have adverse consequences in the development and maintenance of a healthy epithelium. Indeed examination of our histological tissue with H&E staining shows slight differences in proportion of "healthy" and "non-healthy" airways. γδ T cell deficient mice have more airways that seem to have deformed epithelial cells that may lack

tight junctions and are shed more easily, which could facilitate micro vascular leak leading to increased protein content at baseline levels.

It was evident only after exposure to Cl_2 gas that the lack of γδ T cells could have divergent effects on regulation of the inflammatory cell recruitment into the airways, and bronchial responsiveness to methacholine. By obtaining BAL fluid at 1, 3, and 5 days after Cl_2 exposure we were able to obtain an adequate idea of the epithelial cell damage and inflammatory cell influx in γδ T cell deficient mice compared to wild types. Following injury the γδ T cell deficient mice mounted a lower and delayed acute inflammatory response unlike wild type controls that had a response rich in macrophages and neutrophils at the 24 hour time point. This possibly led to a decreased clearance of necrotic epithelial cells thus resulting in a significant increase in epithelial cell counts in BAL, or simply the lack of γδ T cells renders the epithelium more susceptible to damage. These findings are similar to those by King et al. [79] where mice were exposed to ozone. They suggest that γδ T cells are able to recognize and respond to self-tissue damage in acute phases of epithelial injury.

Another interesting aspect of γδ T cell function in response to acute injury lies within the results obtained by immunohistochemistry. It is evident by the number of cells staining positive for PCNA following Cl_2 exposure that γδ T cell deficient mice regenerate the epithelial surface at a slower rate than the wild type counterpart.

Again this confirms the role of γδ T cells in maintaining and repairing the epithelial barrier.

The role of γδ T cells in epithelial repair has been explored in other models of injury. For example, a study by Chen et al. has looked at the role of these cells in a murine model of dextran sodium sulphate (DSS) induced colitis. In this model, lack of γδ T cells, or of KGF, an important growth factor produced by γδ T cells, but not αβ T Cells renders the epithelium of mice with colitis more susceptible to injury, and show delayed repair response following termination of DSS treatment. Thus concluding that γδ T cells help preserve the integrity of damaged epithelial surfaces by providing the localized delivery of an epithelial cell growth factor (KGF). Similar roles for these cells in wound repair have been shown.[96] Thus it is highly likely that the same mechanisms are involved in the repair of the bronchial epithelium.

In contrast to complementary results obtained from BAL and immunohistochemistry, results for bronchial responsiveness in γδ T cell deficient mice were contradictory. Unlike other studies that have shown a protective role of γδ T cells on AHR we found that the lack of γδ T cells protected the mice from developing AHR after exposure to Cl_2 gas. This could be explained by the multifunctionality of γδ T cells in response to different environmental stimuli as discussed earlier. The role of γδ T cells as negative regulators of airway responsiveness have been extensively described in models of allergic asthma. However, it is not unlikely that under our experimental conditions and type of injury these cells could provoke an increase in AHR. As we have seen in models of ozone

exposure where increased macrophage influx and activation are present, it is possible that the γδ T cell deficient mice are not able to recruit activated macrophages with the ability to release mediators such as TNFα and NO. However, this hypothesis remains to be explored.

As we have seen γδ T cell deficient mice lack the ability to mount a proper immune response to injury, and to effect repair of damaged epithelial tissue. Thus it is possible that the AHR in wild type mice is an unwanted consequence of a proper response to injury. It is not unlikely that the early recruitment neutrophils and macrophages into the airways, and proper regeneration of the epithelial cells could lead to a milieu that is favourable for increased responsiveness of the airway smooth muscle to methacholine. Indeed, with the example of ozone induced lung injury, neutrophilia and hyperresponsiveness have been documented in several species including mice, rats, and dogs.[97]

It should be noted that despite the decrease in resistance there was a transient increase in dynamic elastance in γδ T cell deficient mice in responsiveness to methacholine. Also changes in dynamic elastance in wild type mice were more prominent than changes in resistance. The differences in changes of resistance and elastance may be indicative of different patterns of injury and repair between central and peripheral airways. Changes in resistance reflect larger central airway function, whereas changes in dynamic elastance are more sensitive to peripheral airways and parenchymal tissue mechanics. Also, factors such as inhomogeneous ventilation

caused by mucus plugs or variable small airway narrowing can lead to large increases in dynamic elastance.

4.2 Summary

In summary we have seen that γδ T cell deficient mice have high numbers of epithelial cells in bronchoalveolar lavage fluid, and slower epithelial cell regeneration after exposure to Cl_2 gas. They seem to have an attenuated inflammatory response compared to wild type mice. Finally, lack of γδ T cells abrogates the changes in responsiveness to methacholine.

4.3 Conclusion

The mouse model we have developed exhibits many features of chlorine exposure in human subjects and may be of value in the evaluation of treatments. These results suggest that the severity of injury is greater in γδ T cell deficient mice and an adequate inflammatory and repair response is associated with increased responsiveness to methacholine.

Further examination of the specific mechanism by which γδ T cells modulate bronchial responsiveness is necessary to develop treatment for those with Cl_2 induced RADS. However, it is possible that treatment with anti-TCR δ antibody along with KGF administration into the lung could prove beneficial to patients, by lowering the inflammatory response while maintaining proper epithelial repair.

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