Published in final edited form as:

Toxicol Appl Pharmacol. 2016 August 15; 305: 1–11. doi:10.1016/j.taap.2016.05.014.

Mustard Vesicant-induced Lung Injury: Advances in Therapy

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Abstract

Most mortality and morbidity following exposure to vesicants such as sulfur mustard is due to pulmonary toxicity. Acute injury is characterized by epithelial detachment and necrosis in the pharynx, trachea and bronchioles, while long-term consequences include fibrosis and in some instances, cancer. Current therapies to treat mustard poisoning are primarily palliative and do not target underlying pathophysiologic mechanisms. New knowledge about vesicant-induced pulmonary disease pathogenesis has led to the identification of potentially efficacious strategies to reduce injury by targeting inflammatory cells and mediators including reactive oxygen and nitrogen species, proteases and proinflammatory/cytotoxic cytokines. Therapeutics under investigation include corticosteroids, N-acetyl cysteine, which has both mucolytic and antioxidant properties, inducible nitric oxide synthase inhibitors, liposomes containing superoxide dismutase, catalase, and/or tocopherols, protease inhibitors, and cytokine antagonists such as anti-tumor necrosis factor (TNF)- α antibody and pentoxifylline. Antifibrotic and fibrinolytic treatments may also prove beneficial in ameliorating airway obstruction and lung remodeling. More speculative approaches include inhibitors of transient receptor potential channels, which regulate pulmonary epithelial cell membrane permeability, non-coding RNAs and mesenchymal stem cells. As mustards represent high priority chemical threat agents, identification of effective therapeutics for mitigating toxicity is highly significant.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

Mustard gas; vesicant; lung injury; fibrosis; inflammatory mediators; therapeutic approaches

1. Introduction

Sulfur mustard (SM) is a vesicating chemical warfare agent that is relatively easy to synthesize, stockpile, and deliver. Therefore, the development of approaches to counter its acute and chronic respiratory toxicities is a high priority. SM was first used as a chemical weapon during World War I, where pulmonary, dermal and ocular toxicity was reported. While ocular and skin lesions in exposed subjects are debilitating, most mortality and morbidity following SM exposure is attributable to damage to the respiratory tract (Keyser et al., 2014). Early descriptions of acute lung injury included intense inflammation, epithelial necrosis, ulceration of the trachea and sloughing of the tracheal mucous membrane, and bronchioles filled with fibrin and pus (Figures 1 and 2). Pseudomembranes were also noted in the pharynx and trachea (Ghabili et al., 2010). These acute toxic effects have been confirmed more recently in Iranian soldiers and Kurdish civilians exposed to SM by Iraqi forces in the 1980's. In most victims, acute respiratory tract injury caused by SM results in shortness of breath and productive cough, with an obstructive pattern on spirometric responses (Ghasemi et al., 2013; Sohrabpour, 1984). Over time, this proceeds to airway hyperreactivity, chronic bronchitis, bronchiectasis, lung fibrosis, and in some individuals, to acute respiratory distress syndrome with high mortality (Emad and Rezaian, 1997; Saber et al., 2012). Lung disease pathogenesis in these individuals is often dominated by bronchiolitis obliterans, with a distinctive pattern of peribronchial collagen deposition and a constellation of symptoms including productive cough, difficult expectoration, and chronic dyspnea (Ghanei and Harandi, 2011; Saber et al., 2012). Cross-sectional studies have confirmed that respiratory complications increase with time following mustard exposure (Namazi et al., 2009). In a group of 197 Iranian veterans, 10 years of a single high dose SM exposure, pulmonary sequelae included bronchitis (58%), asthma (10%), bronchiectasis (8%), large airway narrowing (9%), and pulmonary fibrosis (12%) (Emad and Rezaian, 1997; Tang and Loke, 2012). Inflammatory mediators including interleukin (IL)-8 and profibrotic transforming growth factor (TGF)- β remained elevated in bronchoalveolar lavage (BAL) fluid years after exposure to SM, and these were correlated with the degree of chronic disability (Emad and Emad, 2007).

Increasing numbers of reports document long term effects of SM 20-25 years after exposure. Common lower respiratory diseases include chronic obstructive pulmonary disease (84%), bronchiectasis (44.1%), and lung fibrosis (7.7%) (Balali-Mood et al., 2011). High resolution lung CT scans in victims 18-23 years after exposure show significant air trapping and parenchymal attenuation patterns (Idani et al., 2012). Specific alterations in circulating T-lymphocyte subpopulations and immunoglobulin subclasses have also been reported (Ghazanfari et al., 2013a, b). Serum levels of matrix metalloproteinase (MMP)s, elastase and IL-6 are also elevated, with levels directly correlating with the severity of pulmonary disease (Attaran et al., 2010; Kiani et al., 2013; Pourfarzam et al., 2013). Expression of inflammatory mediators including tumor necrosis factor (TNF)- α and IL-1 β , as well as

specific isoforms of TGF- β , are also chronically elevated in the lungs of SM exposed victims and related to the severity of symptoms (Yaraee et al., 2013; Zarin et al., 2010). High pepsin levels in sputum of exposed subjects suggest gastro-esophageal reflux, potentially contributing to microaspiration and recurrent pneumonias, which can exacerbate pulmonary symptoms (Karbasi et al., 2013). SM exposure has also been associated with the development of cancer. In a Japanese cohort followed for decades after industrial exposure to SM and other toxins, the age range for the onset of lung cancer was accelerated by approximately 4 years (Doi et al., 2011). On autopsy of veterans dying approximately 20 years after SM exposure, the main pulmonary findings were fibrosis, infections, and malignant cellular infiltration (Taghaddosinejad et al., 2011). There was also a higher incidence of coronary artery disease and vascular disease in exposed veterans (Karbasi-Afshar et al., 2013). This may be related to decreased expression of serum paraoxonase-1 (PON1), which is a major anti-atherosclerotic component of high-density lipoprotein (HDL) (Taravati et al., 2012, 2013).

The acute and chronic phases of SM-induced lung injury have been defined at the cellular and molecular level in animal models using SM, the related bifunctional alkylating agent, nitrogen mustard (NM), or the half mustard, 2-chloroethyl ethyl sulfide (CEES). In general, acute and chronic pulmonary complications following exposure of rodents to mustards are similar to those observed in humans (Table 1). Within 6 h of exposure of rodents to SM, histopathological changes in the upper airways including epithelial cell detachment, deposition of fibrin-containing cellular debris, and edema of the submucosal lining are observed (Calvet et al., 1994, 1996, 1999a; Guignabert et al., 2005; Malaviya et al., 2010; van Helden et al., 2004). Lower airway effects, characterized by thickening of alveolar septal walls and perivascular edema, lung parenchymal congestion, hemorrhage, and epithelial cell apoptosis have also been reported (Guignabert et al., 2005; Malaviya et al., 2010, 2012; van Helden et al., 2004; Veress et al., 2010). This is accompanied by increased microvascular permeability and pulmonary resistance. By 24 h post SM exposure, alterations in tidal volume, respiratory frequency, peak inspiratory and expiratory pressures, and airway hyperreactivity are also noted (Anderson et al., 2009; Calvet et al., 1994, 1999a; van Helden et al., 2004). Similar structural and functional changes are observed in the respiratory tract after NM exposure; these include necrotizing bronchiolitis and thickening of alveolar septa accompanied by decreases in static compliance and end tidal lung volumes within 24 h (Sunil et al., 2011b). This is associated with inflammatory cell accumulation in the airways and lung, and production of reactive oxygen species, reactive nitrogen species, eicosanoids, TNF α , IL-1, IL-6, IL-8, IL-12, IL-13, MCP-1 (CCL-2) and interferon (IFN)- γ , as well as connective tissue growth factor (Anderson et al., 2009; Malaviya et al., 2010, 2012; Mishra et al., 2010; Sawale et al., 2013; Tang and Loke, 2012; Venosa et al., 2015; Wigenstam et al., 2009). Markers of oxidative stress (i.e., malondialdehyde, nitrite-nitrates, reduced glutathione and iNOS), proteolysis (i.e., MMP-9, gelatinase), DNA damage and repair (i.e., PARP-1, phospho-H2A.X), apoptosis (i.e., activated caspase-3 and caspase-9), and autophagy (i.e., LC3B) are also markedly elevated, and there is biochemical evidence of alveolar epithelial damage (i.e., increased bronchoalveolar lavage protein and cell content) (Allon et al., 2009; Calvet et al., 1999b; Das et al., 2003; Guignabert et al., 2005; Malaviya

et al., 2010; Sawale et al., 2013; Shohrati et al., 2010; Sunil et al., 2011b; Venosa et al., 2015).

As observed in humans, lung injury and inflammation continue to progress following acute exposure of animals to mustards. Thus, 14 days after exposure, the tracheal epithelium in guinea pigs is disorganized with decreased cell density (Calvet et al., 1994), while in non-human primates, mucosal bronchial epithelium is replaced by stratified squamous epithelium (Mishra et al., 2012). Airway hyperreactivity to substance P and histamine are noted, consistent with the onset of asthma-like symptoms in victims of SM exposure (Calvet et al., 1994; Emad and Rezaian, 1997; Hefazi et al., 2005). Macrophages remain present in the lung for at least 28 days after acute exposure of rodents to NM (Hoesel et al., 2008; Malaviya et al., 2012; Mukherjee et al., 2009; Venosa et al., 2015). Evidence suggests that these macrophages consist of distinct subpopulations that sequentially accumulate in the lung. Whereas initially these cells exhibit a proinflammatory/cytotoxic phenotype, contributing to tissue injury, subsequently, during the chronic phase, they become profibrotic playing a role in mustard induced fibrosis (Malaviya et al., 2012; Venosa et al., 2015). The chronic phase of vesicant-induced respiratory injury (> 2-4 weeks in rodents), is characterized by a predominance of enlarged foamy macrophages, lymphocytes and IL-17⁺ cells, with marked increases in TGF- β , and myofibroblast proliferation, (Malaviya et al., 2015b; Mishra et al., 2012; Venosa et al., 2015). Fibrin and collagen deposition also increase in the lung leading to a collapse of alveolar structures, and the appearance of honeycombing (Malaviya et al., 2012; Mukherjee et al., 2009; Veress et al., 2010). The symptoms of chemical burn in the lung (edema, congestion, hemorrhage, and inflammation) also increase progressively over 28 days (Das et al., 2003; Malaviya et al., 2012, 2015a; Venosa et al., 2015).

2. Limitations of current therapeutic strategies

In humans exposed to SM, chest X-rays remain normal for a period of days to weeks after exposure, potentially providing a window for therapy (Ghasemi et al., 2013; Kehe et al., 2009). Nevertheless, to date, there are no FDA approved medications for treating mustard-induced pulmonary injury. Life-saving interventions during the acute phase, including oxygen supplementation, endotracheal intubation, tracheostomy, humidification, hydration, respiratory physical therapy, and bronchodilators, are primarily palliative. Bronchoscopy with lavage of isotonic saline to remove debris that accumulates from necrosis and epithelial sloughing has also been reported to be beneficial in the early stages of respiratory management (Graham and Schoneboom, 2013). Similarly, during the chronic phase, symptomatic interventions such as therapeutic bronchoscopy, laser therapy, and respiratory tract stents have been used, but with only limited effectiveness (Razavi et al., 2013). Inhaled corticosteroids and long-term β 2 adrenergic agonists minimize scarring leading to reduced airway stenosis and obstruction (Ghasemi et al., 2009). Frequent follow-up and aggressive treatment of infections have also been shown to slow the progression of symptoms. However, these approaches are non-specific and do not adequately ameliorate morbidity and mortality. For example, in a case report of an exposed child, the progression from chemical pneumonitis to severe deterioration of pulmonary function over 10 years was not ameliorated by either corticosteroids or bronchodilators (Dompeling et al., 2004). Advances

in understanding of mechanisms underlying mustard-induced pulmonary toxicity are clearly essential for the development of improved strategies, which target reactive oxygen and nitrogen species, proteases, inflammatory cells and mediators, and fibrogenesis. In this review, emerging approaches to treating pulmonary injury induced by mustard vesicants are discussed.

3. Targeting reactive oxygen and nitrogen species

Mustard toxicity in both humans and experimental animals is associated with oxidative stress, caused by an imbalance between the generation of oxidants and antioxidants (Korkmaz et al., 2006; Laskin et al., 2010). Sulfonium ions derived from SM contribute to the production of carbon-based free radicals; these reduce molecular oxygen to form superoxide anion and subsequently peroxy and hydroxyl radicals (Brimfield et al., 2012). *In vitro*, the half mustard CEES has been reported to inhibit cytochrome P450 reductase, an enzyme important in the detoxification of mustards in the lung (Gray et al., 2010). Mustards also modify thioredoxin and thioredoxin reductase in lung epithelial cells, potentially exacerbating oxidative stress and tissue injury (Jan et al., 2010, 2015). Consequently, antioxidant therapies have been investigated as a means of ameliorating vesicant-induced lung injury with some moderate success. N-acetyl cysteine (NAC) has both mucolytic and antioxidant properties, making it an appealing therapy for both the acute and chronic phases of vesicant-induced pathology (Shohrati et al., 2014). In humans, NAC administered in conjunction with supportive measures, including humidified oxygen, bronchodilators, antibiotics, and chest physiotherapy, during the acute phase, reduces symptoms associated with vesicant exposure (Razavi et al., 2013). In the chronic phase, NAC improves pulmonary function and reduces cough and dyspnea caused by mustards; this is associated with decreased production of reactive oxygen species (Ghanei et al., 2008a; Razavi et al., 2013; Shohrati et al., 2008a). Oral NAC, alone or in combination with clarithromycin, has also been reported to contribute to improved lung function in patients with bronchiolitis obliterans due to SM inhalation (Shohrati et al., 2014). These beneficial effects of NAC are likely related to its antioxidant and anti-inflammatory activity. In cultured macrophages, NAC inhibits the generation of reactive oxygen and nitrogen species induced by CEES and upregulates antioxidants (Sagar et al., 2014). It may also facilitate tissue growth and repair following SM exposure by improving vascularization (Steinritz et al., 2010). Administration of NAC as late as 90 min after CEES exposure significantly reduces acute lung injury in rats (Das et al., 2003; McClintock et al., 2002). Similarly, treatment of rats with NAC-containing liposomes immediately or up to one hour after CEES reduces both lung permeability and BAL levels of pro-inflammatory cytokines including IL-1, IL-6, TNF α , and CXCL1 (Hoesel et al., 2008; McClintock et al., 2006; Mukherjee et al., 2009); this was associated with down regulation of the AP-1 transcription factor, which is important in regulating inflammatory gene expression (Mukhopadhyay et al., 2009). In a more recent study in pigs, treatment with multiple doses of NAC after SM exposure led to significant improvements in key physiologic measures, including oxygenation, pH, and shunt fraction, as well as decreased neutrophil infiltration and protein leakage in the lung (Jugg et al., 2013).

Other antioxidants have also been examined as potential countermeasures against mustard-induced lung injury, but their activity is limited. For example, Trolox, a water-soluble analog

of α -tocopherol (vitamin E) has been reported to reduce markers of oxidative damage induced by SM in rodents (Kumar et al., 2001), although inflammatory markers were not affected (Anderson et al., 2009). Liposomes containing tocopherols, alone or in combination with NAC, were also found to suppress CEES-induced lung inflammation and injury (Hoesel et al., 2008; Mukherjee et al., 2009; Mukhopadhyay et al., 2009). Liposomes containing both NAC and tocopherols appear to be more effective in reducing collagen deposition in the chronic injury phase induced by CEES than those containing either agent alone (Hoesel et al., 2008; Mukherjee et al., 2009).

Superoxide dismutase (SOD) is a key antioxidant enzyme that catalyzes the degradation of superoxide anion (Allon et al., 2009; Mukhopadhyay et al., 2006). Whereas expression of CuZn-SOD and MnSOD is increased in the lung after acute exposure of rodents to CEES (Sunil et al., 2011a) or NM (Malaviya et al., 2012; Ucar et al., 2007), during the chronic phase of SM-induced lung injury, expression of SOD protein is decreased in airway walls (Jafari and Ghanei, 2010; Mirbagheri et al., 2013; Shahriary et al., 2015a). Administration of liposomes containing SOD and/or catalase was found to decrease acute lung injury and inflammation induced by CEES (McClintock et al., 2002, 2006; Mukhopadhyay et al., 2010). Oxidant and inflammatory markers in the lung are also reduced by subcutaneous administration of the metalloporphyrin catalytic antioxidant AEOL 10150 following exposure of rodents to CEES (O'Neill et al., 2010).

Reactive nitrogen species have also been shown to be also important in the pathogenesis of mustard-induced injury, providing another potential target for therapeutic intervention. Nitric oxide is generated in the lung by macrophages and epithelial cells via an inducible form of the enzyme nitric oxide synthase (iNOS). Expression of iNOS is upregulated in the lung following exposure of rodents to mustards (Gao et al., 2008; Malaviya et al., 2010, 2012, 2015b; Venosa et al., 2015). Moreover, transgenic mice with a targeted disruption of the iNOS gene are protected from acute pulmonary toxicity, oxidative stress and altered lung functioning induced by CEES, a response associated with down regulation of cyclooxygenase (COX)-2 (Sunil et al., 2012). Reactive nitrogen species including nitric oxide and peroxynitrite activate redox sensitive transcription factors in macrophages including NF- κ B and AP-1, leading to pro-inflammatory gene expression (Zouki et al., 2001). Concomitantly, reactive nitrogen species induce DNA damage by cross-linking, leading to activation of poly (ADP-ribose) polymerase (PARP). PARP consumes the respiratory coenzyme NAD⁺, causing energy depletion that can lead directly to apoptosis and necrosis (Korkmaz et al., 2008; Tang and Loke, 2012). Thus, early intervention to prevent the accumulation of reactive nitrogen species in the respiratory tract after vesicant exposure may be effective in ameliorating toxicity. Ebselen (a peroxynitrite scavenger) and melatonin (an antioxidant that scavenges both reactive oxygen and reactive nitrogen species) have been reported to reduce lung injury and oxidative stress induced by NM in rodents (Ucar et al., 2007; Yaren et al., 2007); melatonin also decreases pulmonary expression of IL-1 β and TNF- α . Similarly, transient inhibition of iNOS using aminoguanidine attenuates NM-induced oxidative stress and inflammation in rats during the acute phase of lung injury (1-3 day post-exposure) (Malaviya et al., 2012; Yaren et al., 2007). Aminoguanidine also reduces NM-induced alterations in lung architecture and increases in BAL fluid protein;

conversely, it does not prevent the progression to lung fibrosis during the chronic phase (Malaviya et al., 2012).

4. Targeting matrix metalloproteinases

MMPs are zinc-dependent endopeptidases that degrade extracellular matrix proteins, contributing to inflammatory cell recruitment, tissue injury, and fibrosis (Chakrabarti and Patel, 2005; Corbel et al., 2002; Lagente et al., 2005). Airway remodeling following mustard exposure is thought to be due to an imbalance between these proteases and antiproteases (i.e., tissue inhibitor of MMPs or TIMPs) (Lagente et al., 2005). MMPs increase in the respiratory tract within 6-24 h of SM exposure, with particularly high expression levels in bronchiolar epithelium and alveolar macrophages (Calvet et al., 1999b; Malaviya et al., 2010). Dose-dependent and persistent increases in MMP-9 expression in epithelial and immune cells have also been described in the respiratory tract after exposure of rodents to CEES or NM, and this appears to contribute to alveolar epithelial barrier disruption (Sunil et al., 2011b, 2012). Therefore, anti-protease therapies have been investigated as treatment for mustard-induced pulmonary toxicity (Corbitt et al., 2007). One candidate is doxycycline, a semi-synthetic tetracycline that exhibits non-specific MMP inhibitory activity. Treatment of guinea pigs with doxycycline prior to SM resulted in reduced activity of MMP-2 and MMP-9 and decreased lung inflammation and injury (Guignabert et al., 2005). Doxycycline and other tetracyclines are known to down regulate iNOS expression and nitric oxide production, reduce inflammatory cytokine release, and scavenge reactive oxygen species (Amin et al., 1996; Raza et al., 2006; Wasil et al., 1988). It is likely that the diverse anti-inflammatory actions of doxycycline enhance their potential efficacy as a therapeutic for mustard poisoning. This is supported by findings that the serine protease inhibitor, aprotinin, ameliorates mustard-induced lung injury, in part, by decreasing inflammatory cytokine production (Anderson et al., 2009). The effects of post-exposure treatment with protease inhibitors on vesicant-induced lung toxicity remain to be investigated.

5. Targeting inflammatory cells and TNF α

Lung injury induced by mustards is associated with a rapid and persistent inflammatory response (Malaviya et al., 2015a; Tang and Loke, 2012). Cytotoxic/proinflammatory proteins including TNF- α , IL-1, and COX-2, as well as reactive oxygen and nitrogen species are also elevated in the lung after pulmonary exposure to mustards (Malaviya et al., 2010, 2012; Sunil et al., 2012; Tang and Loke, 2012; Venosa et al., 2015; Xiaoji et al., 2014). These findings have prompted investigations on the use of anti-inflammatory agents to mitigate pulmonary injury induced by mustard vesicants. In rodents, administration of betamethasone, a moderately potent anti-inflammatory glucocorticoid, between 7 and 14 days post SM exposure, decreased airway injury (Calvet et al., 1996). Similarly, dexamethasone, a more potent glucocorticoid analog, reduced airway inflammation, lymphocyte activity, and collagen deposition when administered 1 h after exposure of mice to NM (Wigenstam et al., 2009). Dexamethasone also reduced airway leukocytosis, inflammatory cytokine production, and tracheal reactivity, when administered intraperitoneally to guinea pigs, just prior to SM exposure, effects augmented by the presence of vitamin E (Boskabady et al., 2011). This appeared to be due to suppression of

SM-induced activation of alveolar macrophages (Amir et al., 2000). In humans with chronic bronchiolitis as a result of SM exposure, inhaled corticosteroids improve pulmonary function, a response enhanced by inhaled β_2 agonist bronchodilators (Ghanei et al., 2007). Similarly, approximately 50% of patients with SM-induced chronic bronchitis demonstrate improved pulmonary function after receiving corticosteroids. Steroid responsiveness does not, however, appear to be sensitive to the dose or route of administration, since pulse intravenous and oral dosing are both efficacious (Ghanei et al., 2005). Another less potent class of anti-inflammatory agents are plant-derived curcuminoids (turmeric). Oral curcuminoids significantly decrease serum levels of inflammatory mediators and improve lung function in chronically affected survivors of SM inhalation (Panahi et al., 2015). Macrolide antibiotics including erythromycin, azithromycin, roxithromycin, clarithromycin have been shown to possess anti-inflammatory activity. In cultured human airway epithelial cells and monocytes, macrolides suppress mustard-induced expression of pro-inflammatory cytokines and iNOS (Gao et al., 2007, 2008, 2010). Roxithromycin treatment of animals before and after SM inhalation also reduces upper airway pathology (Gao et al., 2011). Moreover, administration of clarithromycin in combination with NAC to humans with chronic SM-induced lung injury improves pulmonary function and decreases cough and sputum production (Ghanei et al., 2004). Taken together, these data suggest that targeting inflammation is likely to be a useful approach to mitigating pulmonary injury induced by mustards.

TNF α is a proinflammatory early response cytokine generated mainly by activated macrophages in response to tissue injury, and evidence suggests that it plays a role in mustard-induced pulmonary injury and disease pathogenesis. Thus, during the acute injury phase, TNF α promotes inflammation and the release of cytotoxic/proinflammatory mediators, while during the chronic phase, it promotes fibrosis by upregulating scavenger receptors and stimulating the development of profibrotic foamy macrophages (Malaviya et al., 2015b; Oikonomou et al., 2006; Venosa et al., 2015). These findings suggest that inhibition of TNF α will effectively reduce both acute lung injury and fibrogenesis. In wild type mice, COX-2 and MCP-1 are elevated in the lung within 14 days of CEES administration; this response is abrogated in mice lacking the p55 receptor for TNF α (TNFR1 $^{-/-}$) (Sunil et al., 2011a). TNFR1 $^{-/-}$ mice are also protected from CEES-induced alterations in lung mechanics and oxidative stress. Pharmacological antagonists of TNF α are also effective in reducing lung injury induced by mustards. Pentoxifylline is a nonselective phosphodiesterase inhibitor known to block TNF α synthesis. Treatment of rats with pentoxifylline 30 min after NM reduces histopathological changes in the lung (Sunil et al., 2014). This is associated with decreased expression of inflammatory proteins and markers of oxidative stress, as well as improved pulmonary mechanics. Similarly, monoclonal anti-TNF α antibody is highly effective in mitigating NM-induced histopathologic alterations in the lung, including edema, interstitial thickening, and leukocytic infiltration (Malaviya et al., 2015a, b). Of particular interest are findings that anti-TNF α antibody treatment of rats downregulates NM-induced TGF β expression, a response associated with decreases in collagen deposition and the development of fibrosis (Malaviya et al., 2015b). These findings suggest that the TNF α pathway is a promising target for the development of therapeutics for mustard toxicity.

6. Targeting TGF β and other profibrotic proteins

TGF β plays a pivotal role in airway remodeling and the development of fibrosis, and it has been suggested that it is key to mustard-induced pulmonary pathologies (Shahriary et al., 2015b). A number of inflammatory proteins have been identified that stimulate TGF β production including MMPs and reactive oxygen species, which are increased in the lung after mustard exposure (Shahriary et al., 2015b). TGF β and its downstream signaling molecules, Smad3 and Smad 4, are increased in BAL fluid and pulmonary tissue from SM exposed victims (Adelipour et al., 2011; Aghanouri et al., 2004; Mirzamani et al., 2013; Zarin et al., 2010). Similarly, TGF β gene and/or protein expression is upregulated in lung epithelial cells and macrophages after exposure of rodents to mustard vesicants (Ghane Zadeh et al., 2015; Malaviya et al., 2015b; Mishra et al., 2012). This is associated with myofibroblast proliferation, collagen deposition, and the presence of enlarged foamy macrophages and IL-17⁺ cells in the lung, which are thought to play a role in fibrogenesis (Malaviya et al., 2012; Mishra et al., 2012). Although treatment of chronic SM-induced lung injury is generally symptomatic, some of the therapies utilized appear to act by interfering with TGF β signaling. For example, oral curcuminoids significantly decrease serum levels of TGF β , as well as some measures of lung function, in long term survivors of SM inhalation (Panahi et al., 2014). Administration of IFN γ to these individuals similarly decreases expression of TGF β and improves respiratory symptoms (Panahi et al., 2014). These findings suggest that interventions directed at blunting TGF β may have utility for mitigating chronic injury and fibrosis induced by mustards. This is supported by findings that the antifibrotic agent pirfenidone reduces the risk of disease progression in victims of SM poisoning (Noble et al., 2011; Zamani, 2013). Recent studies have shown that profibrotic macrophages (YM-1⁺ and galectin-3) accumulate in the lung within 1 day of exposure of rats to NM (Malaviya et al., 2012). Thus, it is possible that treatment with antifibrotic agents during the early acute phase of vesicant-induced injury may also be effective in ameliorating lung remodeling associated with chronic injury and fibrosis.

7. Targeting fibrin and coagulation

Exposure of rats to the half mustard CEES results in an accumulation of a fibrin-rich proteinaceous exudate in the airways within 4 h; this is followed by the appearance of obstructive fibrin-containing casts (Veress et al., 2010). At the same time, functionally active reservoirs of plasminogen activator-inhibitor-1, thrombin-activated fibrinolysis inhibitor, and α 2 anti-plasmin appear in the airways, which inhibit thrombin-mediated fibrinolysis (Rancourt et al., 2014). SM exposure has been reported to lead to plastic bronchitis, characterized by the formation of branching bronchial casts which can obstruct the airway and lead to segmental lung collapse, patchy infiltrates, and significant respiratory distress. Long-term respiratory pathologies, such as bronchiolitis obliterans, tracheal/bronchial stenosis, and chronic bronchitis are generally attributable to conducting airway lesions that persist months to years after SM exposure (Ghanei et al., 2008b). It is possible that there is a therapeutic window during which treatment with exogenous fibrinolytic agents can ameliorate the progression to airway disease. Consistent with this idea are findings that intratracheal administration of tissue plasminogen activator (tPA) to rats 6 h after exposure to CEES or SM normalizes oxygenation, ventilation, and symptoms of respiratory distress

(Veress et al., 2013, 2015). Of note, tPA also markedly decreases airway fibrin casts and prevents mortality.

Another approach to ameliorating the formation of fibrinous casts in the airway after vesicant exposure may be to prevent coagulation. Tissue factor (TF, factor III, thromboplastin, CD142) is a cell surface glycoprotein expressed by epithelial cells, subendothelial tissues, and leukocytes following mechanical or chemical injury to the lung or airways. It is a high-affinity receptor for the coagulation factor VII which triggers the coagulation cascade, leading to the generation of thrombin from prothrombin via activated factor X. *In vitro*, the TF pathway inhibitor (TFPI) decreases the procoagulant activity of BAL fluid obtained from CEES-exposed rats (Rancourt et al., 2012). Intratracheal instillation of TFPI one hour after CEES exposure decreases casts, reduces hypoxemia and mortality, and limits biochemical evidence of thrombin activation in the airways (Rancourt et al., 2013). Both anticoagulation and the promotion of fibrinolysis appear to be promising avenues for further investigation on their potential efficacy in reducing acute and chronic injury following exposure to mustards.

8. Transient receptor potential (TRP) channels

TRP channels constitute a large cation channel superfamily widely expressed in the respiratory tract. Six families of TRP isoforms (TRP-C, -V, -M, -ML, -P, and -A) have been identified based on sequence homology, function, and distribution (Schaefer, 2005). TRP channels play important roles in sensing and responding to oxidative and mechanical stress, inflammation, hypoxia, and chemicals (Abbott-Banner et al., 2013; Villalta and Townsley, 2013). In the lung, TRP channels have been identified in pulmonary epithelial cells, vascular endothelium and macrophages; they regulate endothelial permeability and calcium uptake and have been implicated in alveolar epithelial barrier dysfunction, pulmonary edema and altered vascular tone following acute lung injury, as well as in the pathogenesis of chronic obstructive pulmonary disease and asthma (Balakrishna et al., 2014; Goldenberg et al., 2015). In the airways, TRP channels including TRPV1 (vanilloid1), TRPV4 (vanilloid4), TRPA1 (ankyrin 1), and TRPM8 (melastatin 8) are implicated in tissue injury induced by inhaled irritants (Buch et al., 2013a).

TRPA1 channels in the airway were initially identified as sensor channels, expressed by neuronal cells that mediate cough, mucous secretion, and hyperreactivity in response to respiratory irritants and air pollutants (Buch et al., 2013b; Shigetomi et al., 2013). More recently, TRPA1 channels have been identified in lung epithelial cells, and they are thought to play a role in mediating physiologic responses to noxious stimuli (Andre et al., 2008; Buch et al., 2013b; Fernandes et al., 2012; Grace et al., 2014; Rech et al., 2010; Shapiro et al., 2013). For example, airway fibroblasts, epithelial cells, and smooth muscle cells release IL-8 after stimulation of TRPA1 channels with cigarette smoke extract (Nassini et al., 2012). As TRPA1 channels are in general activated by electrophilic compounds (Bessac and Jordt, 2010), they represent a plausible target for mustards in the airway. In this regard, Stenger et al., (2015) reported that CEES causes transient calcium influx into lung epithelial cells, and that this response is significantly attenuated by the TRPA1-channel blocker AP18. Interestingly, TRPV1 is also expressed in epithelial cells and human bronchial epithelial

cells and stimulation with capsaicin has been shown to induce production of IL-6. Moreover, this response is attenuated by co-treatment with a TRPV1 antagonist (Seki et al. 2007). TRPA1 and TRPV1 are also important in regulating cough responses induced by pulmonary irritants (Bessac and Jordt, 2008; Birrell et al., 2009; Buch et al., 2013b; Geppetti et al., 2010). Evidence suggests that tussive responses are mediated by prostaglandin E₂ and bradykinin, which also activate TRPA1 and TRPV1 in human, guinea pig and mouse airways (Grace et al., 2012a, b). These findings indicate that antagonists of TRPA1 and TRPV1 may be used to suppress cough induced by vesicants.

TRPV4 channels are also being considered as potential targets for ameliorating lung diseases associated with maladaptive responses to stressors. Treatment of mice with synthetic TRPV4 inhibitors after exposure to intratracheal acid or inhaled chlorine gas suppresses markers of pulmonary inflammation and improves clinical and histologic indices (Thorneloe et al., 2012). Leukocyte infiltration, inflammatory cytokines, vascular leakage, and airway hyperreactivity are also reduced by TRPV4 blockade, as well as in TRPV4-deficient animals (Balakrishna et al., 2014). These findings suggest a potential therapeutic approach for mitigating mustard induced lung injury.

The notion that TRP channels mediate various aspects of acute SM toxicity in the airway is reasonable because they are known to contribute to characteristic symptoms of mustard injury including inflammation, bronchoconstriction, hyper-reactivity, mucus production, and pulmonary edema. The rapidly increasing understanding of the molecular biology of these transmembrane proteins, and the growing number of inhibitors makes TRP channels a very promising area for research to identify new countermeasures to ameliorate acute and/or chronic mustard toxicity in the lung.

9. Non-Coding RNAs

Non-coding RNAs are functional RNA molecules that regulate gene expression. The most extensively characterized are microRNA (miRNA)s and small interfering RNA (siRNA)s, each approximately 20 nucleotides in length. miRNAs bind to target mRNA transcripts, negatively regulating their translation or causing mRNA degradation. A single miRNA may have numerous targets, resulting in down regulation of many genes to varying degrees (Lim et al., 2005; Selbach et al., 2008). siRNAs also generally suppress gene expression, but with greater sequence and gene specificity. Non-coding RNAs have been implicated in a number of disease processes and pathologies. For example, the let-7 miRNA family has tumor-suppressive functions and in its absence lung cancer is increased (Mestdagh et al., 2015). Similarly, pulmonary fibrosis is associated with down regulation of let-7, miR-29, miR-30, and miR-200. These miRNAs are thought to inhibit TGF- β 1 expression and activity, resulting in decreased epithelial to mesenchymal cell transition (Pandit et al., 2011; Yang et al., 2012). Non-coding RNA-based therapeutics are currently under investigation for treatment of lung diseases and pathologies including injury caused by mustards. Keyser et al. (2013) analyzed the effects of siRNA targeting the Fas receptor (FasR) on the toxicity of SM in human bronchial/tracheal epithelial cells. FasR siRNA was found to inhibit SM-induced caspase 3 activation via the extrinsic pathway; this was associated with significant decreases in both apoptosis and necrosis. A related approach that warrants further

investigation is the use of synthetic short nucleotide sequences (“antagomirs”), which are complementary to and sequester specific miRNA species, preventing them from repressing target protein expression (Kishore et al., 2014). Manipulation of non-coding RNAs may become an important modality in the prevention of mustard toxicity in the lung.

10. Mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells (MSC)s exert anti-inflammatory and reparative effects via the release of mRNA and miRNA-containing microvesicles and exosomes. These are lipid bilayer enclosed particles ranging in size from 30-100 nm and 100-1000 nm, respectively. In animal models, MSCs have been reported to improve acute lung injury induced by endotoxin, bacteria and sepsis, an effect that can largely be reproduced by treatment with microvesicles alone (Lee et al., 2013; Mei et al., 2010; Xu et al., 2007). Intratracheal administration of MSC-derived microvesicles also reduces edema, protein leakage, neutrophil infiltration and other inflammatory markers in the lung after endotoxin-induced injury (Zhu et al., 2014). Similarly, intravenous injection of MSC-derived exosomes prevents hypoxia-induced inflammatory alveolar infiltrates and pulmonary hypertension in mice (Akyurekli et al., 2015). There is also some evidence in humans that MSCs may be therapeutic in chronic lung diseases (Antunes et al., 2014). Our emerging understanding of the central role of MSCs in tissue regeneration and repair raises the possibility that they may be useful in the early stages of mustard toxicity in the lung, however, this has not yet been systematically investigated. It is known that bone marrow-derived MSCs are highly resistant to SM *in vitro*, although their migratory capacity is impaired (Schmidt et al., 2013). This suggests that administration of MSCs after exposure to SM could potentially ameliorate pulmonary toxicity by facilitating the delivery of MSC-derived microvesicles/exosomes. Alternatively, microvesicles/exosomes could be purified and/or synthesized to exploit their efficacy as delivery vehicles, an approach that has already shown some promise in immunotherapy (Delcayre and Le Pecq, 2006).

11. Summary and conclusions

Mustard-induced lung injury is a major determinant of morbidity and mortality in exposed individuals. Acute respiratory tract injury clinically characterized by shortness of breath and productive cough, with an obstructive pattern, leads to airway hyperreactivity, chronic bronchitis, bronchiectasis and over time, lung fibrosis, and acute respiratory distress syndrome. Currently, there are no FDA approved guidelines or medications to treat victims of mustard gas poisoning. Animal models of mustard-induced lung injury have been developed to delineate mechanisms of toxicity and to test potential therapeutics. Thus far, these have focused mainly on targeting reactive oxygen and nitrogen species, TNF α , proteases, TGF β , and fibrin. Findings that therapeutics targeting just one of these inflammatory proteins are only partly effective in mitigating mustard-induced injury suggest that either a combination of agents or an agent affecting several signaling pathways are required. A close review of the therapies discussed in this article may help to develop successful clinical strategies to treat acute and chronic manifestations of vesicant injury in human.

Acknowledgements

This work was supported by National Institute of Health Grants U54AR055073, R01ES004738, and P30ES005022.

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Figure 1. Example of lung pathology from an individual exposed to mustard gas

In response to battlefield use of SM, a short report (“An Atlas of Gas Poisoning”, Medical Research Committee, British Expeditionary Forces, 1918), consisting of drawn-to-life images of individuals exposed to chemical warfare gasses and resultant lung pathology, was issued to familiarize Officers with key features of chemical warfare gas injuries of the lung. In cooperation with the American Red Cross Society, this report was made available to the American Army Medical Service.

The figure is a reproduction of ‘plate no. IX’ showing ulceration of the trachea post mustard gas exposure. The original description of the pathology is described below:

“The characteristic feature is the sloughing of the tracheal mucous membrane. The reddening of the base of the tongue and of the pharynx, with a sharp delimitation where the oesophagus has refused ingress to the toxic vapor, is seen also with chlorine and other irritant gasses. But the pharyngeal inflammation with mustard gas may proceed further to a local ulceration that will cause dysphagia for many days.”

“The mucous membrane of the trachea and bronchi is affected by the di-chlor-ethyl-sulphide in much the same way as the skin. It reacts with an intense

inflammation, and death of the surface layers soon results. The mass of necrotic tissue, exuded fibrin, and pus cells may form a yellowish-grey slough in which all manner of organisms flourish. Subsequently this false membrane comes away in patches or in entire casts from the raw surface of the bronchial wall.”

“Meantime the infected debris and secretions tend to accumulate in the bronchial ramifications at the bases of the lungs, and infection may spread from them into the lung tissues and alveoli. Septic broncho-pneumonia, localised abscesses, superficial pleurisy, and even empyema or pyopneumothorax then develop and cause death.”

“The drawing is of a trachea at the twelfth day after gassing. The base of the tongue and the pharynx show characteristic inflammation. Yellow necrotic sloughs lie on the larynx and at the bifurcation of the trachea. Between these the trachea is red and glistening, because it is now completely denuded of both mucous membrane and of slough. The dotted line points to a little group of ulcers on the posterior wall from which bleeding has occurred. The trachea and bronchi contained an abundance of this yellow pus.”

Figure 2. Pathology of human lung following exposure to mustard gas

Reproduction of 'plate no. X' in "An Atlas of Gas Poisoning" showing a section of human lung 40 h post mustard gas exposure. The original description of the pathology is described below:

"The bronchiole is filled with fibrin and pus cells, and its lining epithelium has been completely destroyed. The inflammation has caused a characteristic ring of haemorrhage in the tissues around the bronchial tube, and infection is beginning to appear in the alveoli nearest to these inflamed tissues. But there is no generalized pulmonary oedema and no disruptive emphysema."

"Di-chlor-ethyl-sulphide [SM] may cause some catarrhal desquamation of the pulmonary endothelial cells, but it rarely excites an outpouring of oedema fluid from the pulmonary vessels. The pathological changes in the bronchioles and in the alveoli are therefore in the sharpest contrast with those caused by phosgene. As infection spreads into the lung tissues, patches of septic bronchopneumonia and small abscesses develop, and these often excite an inflammatory oedema around them."

"If the patient lives, his bronchial mucous membrane is slowly regenerated; and during this time he is naturally subject to reflex spasms of coughing or even protracted bronchitis."

Table 1

Comparison of Mustard-induced Pathological Changes in the Lungs of Rodents and Human

Pathology	Mouse	Rat	Guinea Pig	Human	References
Broncho-alveolar lavage					
Increased protein content	x	x	x	x	Anderson et al., 2000; Anderson et al., 2009; Calvet et al., 1999b; Ekstrand-Hammarstrom et al., 2011; Malaviya et al., 2012; Mehrani et al., 2009
Increase in cell content	x	x	x	x	Anderson et al., 2000; Anderson et al., 2009; Beheshti et al., 2006; Calvet et al., 1999b; Ekstrand-Hammarstrom et al., 2011; Emad and Rezaian, 1997; Malaviya et al., 2015b; Malaviya et al., 2012; Mehrani et al., 2009; Sunil et al., 2014; Wigenstam et al., 2012; Wigenstam et al., 2009
Lung – acute effects					
Necrosis of mucosa in trachea and bronchi		x	x	x	Allon et al., 2009; Anderson et al., 2009; Calvet et al., 1994; Gao et al., 2011; Gholamnezhad et al., 2015; Guignabert et al., 2005; Malaviya et al., 2010; Taghaddosinejad et al., 2011; Tang and Loke, 2012
Goblet cell hyperplasia		x		x	Beheshti et al., 2006; Malaviya et al., 2012
Pulmonary edema	x	x	x	x	Aasted et al., 1987; Anderson et al., 2009; Calvet et al., 1994; Das et al., 2003; Ekstrand-Hammarstrom et al., 2011; Graham and Schoneboom, 2013; Macit et al., 2013; Malaviya et al., 2012; Mukherjee et al., 2009; van Helden et al., 2004; Wigenstam et al., 2009
Pulmonary/Alveolar hemorrhage	x	x	x	x	Allon et al., 2009; Anderson et al., 2009; Balali-Mood and Navaeian, 1986; Das et al., 2003; Gholamnezhad et al., 2015; Macit et al., 2013; Mukherjee et al., 2009; Pant and Vijayaraghavan, 1999; Tang and Loke, 2012; Veress et al., 2015
Sloughing/Necroses of epithelial cells		x		x	Graham and Schoneboom, 2013; Malaviya et al., 2010; Xiaoji et al., 2014
Lung congestion/obstruction of airways	x	x	x	x	Dacre and Goldman, 1996; Gholamnezhad et al., 2015; Mukherjee et al., 2009; Pant and Vijayaraghavan, 1999; Rancourt et al., 2014; Tang and Loke, 2012; Veress et al., 2015; Wang and Xia, 2007; Xiaoji et al., 2014
Airway hyper-responsiveness	x	x	x	x	Boskabady et al., 2011; Calvet et al., 1994; Hefazi et al., 2005; Mirsadraee et al., 2005; Sunil et al., 2011b; Wigenstam et al., 2012
Lung – long term effects					
Alteration in pulmonary functions		x	x	x	Anderson et al., 2009; Calvet et al., 1994; Graham and Schoneboom, 2013; Khateri et al., 2003; Shohrati et al., 2008b; Sunil et al., 2011b
Bronchiectasis		x		x	Beheshti et al., 2006; Brown, 1949; Graham and Schoneboom, 2013; Hefazi et al., 2005; Malaviya et al., 2012; Sunil et al., 2014
Emphysema		x	x	x	Brown, 1949; Gholamnezhad et al., 2015; Gilchrist and Matz, 1933; Malaviya et al., 2012; Sunil et al., 2014
Bronchitis				x	Gilchrist and Matz, 1933; Graham and Schoneboom, 2013
Pulmonary fibrosis	x	x	x	x	Beheshti et al., 2006; Ekstrand-Hammarstrom et al., 2011; Emad and Rezaian, 1997; Graham and Schoneboom, 2013; Malaviya et al., 2012; McClintock et al., 2006; Mishra et al., 2012; Tang and Loke, 2012; Wigenstam et al., 2009
Fibrin deposits		x	x	x	Beheshti et al., 2006; Gao et al., 2011; Malaviya et al., 2012; Mukherjee et al., 2009; Sunil et al., 2014

Pathology	Mouse	Rat	Guinea Pig	Human	References
Collagen deposition	x	x	x	x	Aghanouri et al., 2004; Beheshti et al., 2006; Ekstrand-Hammarstrom et al., 2011; Gustafsson et al., 2014; Malaviya et al., 2012, 2015b; Mukherjee et al., 2009; Wigenstam et al., 2012; Wigenstam et al., 2009

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