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Expression of cytokines and chemokines in mouse skin treated with sulfur mustard

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ABSTRACT

Sulfur mustard (2,2′-dichlorodiethyl sulfide, SM) is a chemical warfare agent that generates an inflammatory response in the skin and causes severe tissue damage and blistering. In earlier studies, we identified cutaneous damage induced by SM in mouse ear skin including edema, erythema, epidermal hyperplasia and microblistering. The present work was focused on determining if SM-induced injury was associated with alterations in mRNA and protein expression of specific cytokines and chemokines in the ear skin. We found that SM caused an accumulation of macrophages and neutrophils in the tissue within one day which persisted for at least 7 days. This was associated with a 2–15 fold increase in expression of the proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor α at time points up to 7 days post-SM exposure. Marked increases (20–1000 fold) in expression of chemokines associated with recruitment and activation of macrophages were also noted in the tissue including growth-regulated oncogene α (GROα/CXCL1), monocyte chemoattractant protein 1 (MCP-1/CCL2), granulocyte-colony stimulating factor (GCSF/CSF3), macrophage inflammatory protein 1α (MIP1α/CCL3), and IFN-γ-inducible protein 10 (IP10/CXCL10). The pattern of cytokines/chemokine expression was coordinate with expression of macrophage elastase/MMP12 and neutrophil collagenase/MMP8 suggesting that macrophages and neutrophils were, at least in part, a source of cytokines and chemokines. These data support the idea that inflammatory cell-derived mediators contribute to the pathogenesis of SM induced skin damage. Modulating the infiltration of inflammatory cells and reducing the expression of inflammatory mediators in the skin may be an important strategy for mitigating SM-induced cutaneous injury.

1. Introduction

Sulfur mustard [bis (2-chloroethyl) sulfide; SM] is a bifunctional alkylating agent known to be highly toxic to skin. The extent of injury is dependent on the dose of SM and time following exposure. In humans, acute responses to SM include erythema and edema, and are often accompanied by the formation of fluid-filled blisters and necrosis of the tissue. This can lead to prolonged inflammation and delayed wound repair (Shakarjian et al., 2010; Graham and Schoneboom, 2013). The mouse ear vesicant model (MEVM) has been used to characterize SM-induced skin injury (Casillas et al., 1997; Smith et al., 1997; Casillas et al., 2000; Ricketts et al., 2000). Following exposure to SM, histopathological changes in mouse ear skin such as edema, epidermal necrosis, and microblister formation are observed beginning 2–24 h post exposure. Frequent formation of microvesicles with significant dermal-epidermal separation of the basement membrane zone is also routinely observed (Casillas et al., 1997; Monteiro-Riviere et al., 1999). The histopathology of SM induced skin injury also shows a marked inflammatory response, which involves the production or release of inflammatory mediators (Dannenberg et al., 1985; Casillas et al., 1997; Arroyo et al., 2000; Ricketts et al., 2000; Sabourin et al., 2002; Sabourin et al., 2003; Wormser et al., 2005; Gerecke et al., 2009). Excessive production of proinflammatory mediators including cytokines and chemokines have been shown to contribute to the pathogenesis of

Abbreviation list: CCL2/MCP-1, monocyte chemoattractant protein 1; CCL3/MIP1α, macrophage inflammatory protein 1α; CSF3/GCSF, granulocyte-colony stimulating factor; CXCL1/GROα, growth-regulated oncogene α; CXCL10/IP10, IFN-γ-inducible protein 10; MEVM, mouse ear vesicant model; MMP8, matrix metalloproteinase 8/neutrophil collagenase; MMP12, matrix metalloproteinase 12/macrophage elastase; MPO, myeloperoxidase; SM, Sulfur mustard (2,2′-dichlorodiethyl sulfide)

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inflammation and skin injury (Gillitzer and Goebeler, 2001; Behm et al., 2012). These mediators are secreted by both resident skin cells and infiltrating inflammatory cells. Some chemokines are chemoattractants for neutrophils (e.g., CXCL1), monocytes/macrophages (CCL2, CCL3), and T cells (CXCL10) (Zlotnik and Yoshie, 2000; Gillitzer and Goebeler, 2001; Roberts, 2005; Turner et al., 2014; Su and Richmond, 2015). In earlier reports, most in vivo studies showed increasing cytokines/chemokines gene expression in mouse or pig skin exposed to SM (Sabourin et al., 2000; Sabourin et al., 2002; Sabourin et al., 2004; Mouret et al., 2015). There are limited numbers of cytokines where the protein levels have been examined in skin exposed to SM. Increases in protein expression of IL1β, IL6, and TNFα have been reported 6–24 h following SM exposure in mouse skin (Ricketts et al., 2000). In the present studies, we examined expression of mRNA and protein for multiple cytokines and chemokines in the MEVM over periods of time up to seven days. We found an array of mediators that were expressed in the tissue likely to be important not only in inflammation, but also in wound repair. Identifying specific cytokines and chemokines as important mediators of skin toxicity may lead to the development of medical countermeasures against SM.

2. Materials and methods

2.1. Animals and exposures

All SM exposures were performed at Battelle Memorial Institute (Columbus, OH). Male CD1 mice (Charles River Laboratories, Portage, MI), 25–35 g, (n = 10 per group), were exposed to SM as previously described (Casillas et al., 1997; Shakarjian et al., 2006; Chang et al., 2009; Chang et al., 2013). Briefly, animals were treated topically with 0.49 μmoles of SM (5 μl of a 97.5 mM solution diluted in CH_2Cl_2) on the ventral (inner) side of the right ear. The left ear was treated with vehicle control. After increasing periods of time, animals were sacrificed, and ear punch biopsies collected for analysis. Animals and their body weights were monitored throughout the study and no systemic effects of SM were observed in any of the experimental animals. Additionally, no differences were noted between left ear vehicle controls and naïve, unexposed ear skin with respect to structural alterations in the tissue.

2.2. RNA analysis

Punch biopsies were frozen in liquid nitrogen and RNA isolated and analyzed as previously described (Chang et al., 2009). For RT-PCR analysis, mRNA was transcribed into cDNA by standard protocols using a superscript first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA). RT-PCR was performed with a TaqMan Gene Expression Assay (Assay ID, Applied Biosystems, Foster City, CA) using probes for MMP8 (Mm00439509_m10), MMP12 (Mm00500554_m1), CCL2 (Mm00441242_m1), CCL3 (Mm00441259_g1), CSF3 (Mm00438335_g1), CXCL1 (Mm04207460_m1), CXCL10 (Mm00445235_m1), IL1β (Mm00434228_m1), IL6 (Mm01210733_m1), TNFα (Mm00443258_m1) and GAPDH (used as an endogenous control). RT-PCR was performed using an ABI (Applied Biosystems) ViiA 7 PCR system. Each gene expression assay was performed in triplicate. All values were normalized to GAPDH. The untreated control was arbitrarily assigned a value of 1 and treated samples were calculated relative to untreated controls. Expression of mRNA was reported in fold change (mean ± SEM, n = 10).

2.3. Cytokines/chemokines multiplex immunoassay studies

Frozen punch biopsies were incubated with lysis buffer containing protease inhibitor cocktail as previously described (Shakarjian et al., 2006). A Multiplex Immunoassay using a mouse cytokine/chemokine Milliplex kit (#MPXMCYTO-70KPMX22, Merck Millipore Inc., St. Charles, MO) was used to quantify cytokines and chemokines in tissue lysates. Cytokine assay plates consisted of eight standards in duplicate, three positive controls in triplicate, two blank wells in duplicate, and samples in triplicate. Pooled samples from ten animals per group were analyzed. Total protein concentration was determined by the Pierce BCA protein assay (ThermoFisher Scientific, Waltham, MA) with bovine serum albumin as the standard. Samples were immediately processed using a Bio-Plex Protein Array System and related Bio-Rad Bio-Plex Manager software (Bio-Rad; Hercules, CA). All signals were normalized by blank wells consisting of only tissue lysis buffer (for background fluorescence subtraction). Samples and positive controls exhibiting a coefficient of variation (CV) > 15% were omitted from final data analyses. Cytokine and chemokine concentrations in samples were then normalized to the total protein concentration determined for each sample.

2.4. Histology and immunohistochemistry

Tissue sections were analyzed for structural damage and immunohistochemical markers as previously described (Shakarjian et al., 2006; Chang et al., 2009). Briefly, ear punch biopsies were fixed in 10% neutral buffered formalin and then paraffin embedded. Seven-micron tissue sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). Skin sections from three mice were evaluated for edema, necrosis and inflammation after scanning on an Olympus VS120 Virtual Scanning Microscope (Olympus Co., Waltham, MA). For immunohistochemical staining, tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked for 10 min by incubating tissue sections in 3% hydrogen peroxide in methanol. Tissue sections were then treated with rat anti–-mouse BD Fc block™ (cat # 553141, BD Biosciences, San Jose, CA) diluted 1:100 (5 μg/ml) in PBS/0.05% Tween-20/5% normal goat serum (NGS); or CAS- Block (Invitrogen) for one hour to block non-specific binding. Samples were then incubated for one hr with rat monoclonal antibody to F4/80 (ab6640, diluted 1:500 (2 μg/ml) in PBS/0.05% Tween-20/1.5%, NGS, Abcam, Cambridge, MA), rabbit polyclonal antibody to myeloperoxidase (ab45977, Abcam, diluted 1:500 (2 μg/ml) in PBS/0.05% Tween-20/1.5%, NGS) or appropriate isotypic and secondary antibody controls (rat IgG2b, rabbit IgG, and normal goat serum). Tissue sections were then incubated for 30 min at room temperature with biotinylated goat anti-rat secondary antibody (diluted 1:200 in PBS/0.05% Tween-20/1.5% NGS), followed by avidin-biotin-horseradish peroxidase complex (diluted 1:50; Vector laboratories, Inc., Burlingame, CA) for 30 min at room temperature. Antibody binding was visualized using, 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO). Slides were then washed in water, counterstained with hematoxylin, dehydrated, and mounted in Permount (Fisher, Fair Lawn, NJ). To evaluate the distribution of macrophages in skin sections, $F4/80^+$ cells were enumerated. Macrophage cell counts per mm^2 (area of dermis per high magnification image) were reported as the mean ± SE (n = 8–10). Total macrophage counts per skin session include both ventral ear skin and dorsal ear skin.

2.5. Statistical analysis

Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test or non-pair student *t*-test; a p value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of SM on mouse ear skin

In naïve, unexposed tissue, both the ventral and dorsal ear skin showed a thin epidermis (1–2 cells thick) with a prominent stratum corneum (Fig. 1A–C). Dermal appendages were apparent in both the ventral and dorsal skin and the dermal-epidermal junction (DEJ) was

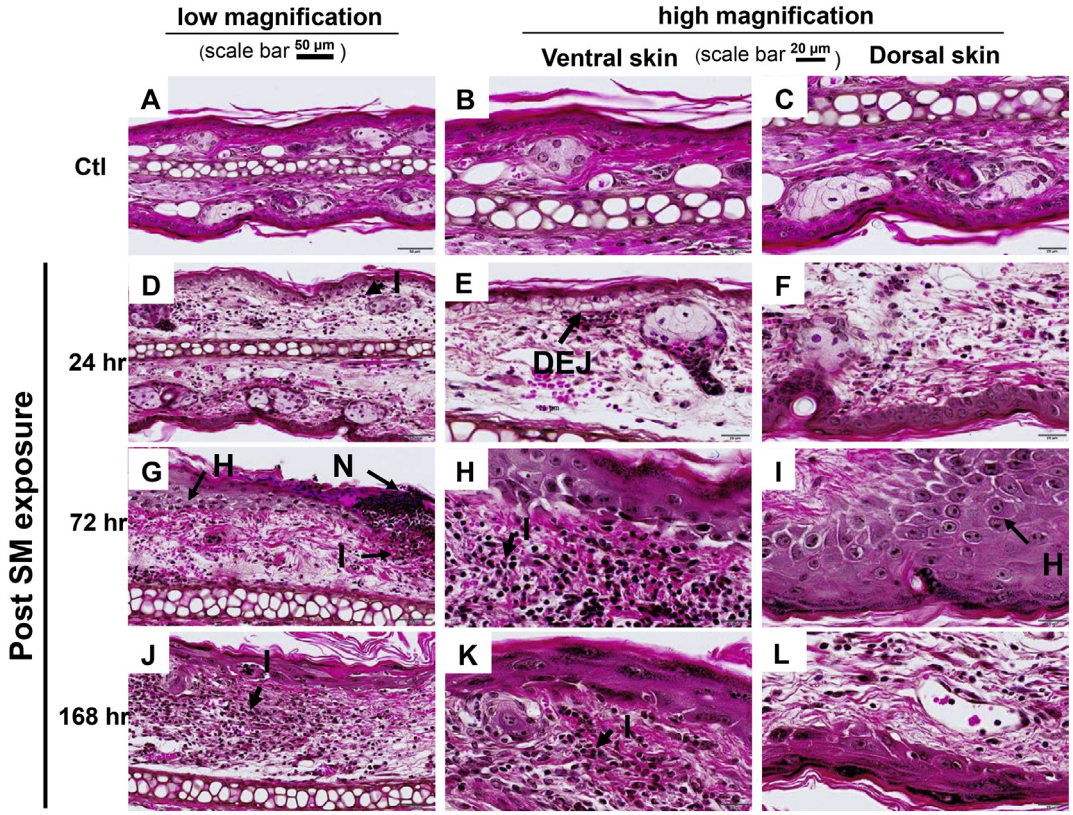

Fig. 1. Structural alterations in mouse ear skin following exposure to SM. Hematoxylin and Eosin (H&E) stained histological sections of control and SM-treated mouse ear skin. Panels A-C, control, naïve tissue (Ctl); panels D-F: mouse ear skin 24 h post-SM exposure; panels G-I: mouse ear skin 72 h post-SM exposure; panels J-L: 168 h post-SM exposure. Images shown in panels A, D, G, and J are lower magnifications (20× objective; scale bar = 50 μm); ventral side ear skin shown in panels B, E, H, and K are higher magnifications (40× objective; scale bar = 20 μm); dorsal side ear skin shown in panels C, F, I, and L (40× objective; scale bar = 20 μm). DEJ: dermal-epidermal junction; N: necrosis; H: hyperplasia; I: inflammatory cell infiltrate. Ventral is exposed side (panels B, E, H, and K) and dorsal unexposed side (panels C, F, I, and L).

intact. Twenty-four hr following treatment with SM, tissue edema was evident in both the ventral (SM exposed) and dorsal (unexposed) sides of the ear skin (Fig. 1D–F). Although the dorsal side of the mouse ear was not directly exposed to SM, an influx of inflammatory cells was still evident (arrow I in Fig. 1D). Separation of the DEJ on both the ventral and dorsal sides of the SM exposed ear was also noted (arrows, Fig. 1E and F). At 72 h post-SM, inflammation persisted with significant numbers of infiltrating cells observed in the dermis (arrows I in Fig. 1G and H). Epidermal hyperplasia (arrow H) and areas of necrosis (arrow N) were also evident (Fig. 1G–I). By 168 h post-SM exposure, necrosis and epidermal hyperplasia at the wound site was still visible throughout the ear skin (Fig. 1J–L). At this time point, numerous inflammatory cells appeared throughout the dermis (1J-1L).

3.2. Characterization of the inflammatory cell response to SM

Myeloperoxidase (MPO), an index of neutrophil activity, and F4/80 (a specific macrophage marker) were used to further characterize neutrophil and macrophage influx (Figs. 2, and 3). Very few neutrophils (MPO positive cells) were evident in unexposed naïve, control mouse skin (Fig. 2A). Conversely, numerous infiltrating neutrophils with intense MPO staining were evident near the DEJ and throughout the dermis 24 h post-SM (Fig. 2B). At 72 h post-SM, neutrophils were sparsely clustered in the dermis, but were abundant near necrotic areas (Fig. 2C, upper right). At 168 h post exposure, numerous neutrophils were found in the dermis of SM treated skin (Fig. 2D). Individual neutrophils could not be enumerated because they were often found in overlapping clusters, especially in necrotic areas. Minimal numbers of macrophages staining for F4/80 were observed in unexposed, naive control skin (Fig. 3A). Following SM exposure, a time related increase in the appearance of F4/80$^+$ macrophages were observed (Figs. 3 and 4). Macrophages were mainly evident in the dermis and were not found in either the epidermis or the cartilage of the ear skin.

The numbers of macrophages increased with time; F4/80 positive cells per mm^2 are presented as mean ± SE (n = 8–10) (Fig. 4). We found F4/80 positive cells in tissues following SM exposure increased from 20 ± 1 cells/mm^2 in the ventral ear skin, and 19 ± 2 cells/mm^2 in the dorsal ear skin at 24 h post exposure; to 185 ± 22 cells/mm^2 in ventral ear skin, and 210 ± 32 cells/mm^2 in the dorsal ear skin at 72 h; and 301 ± 23 cells/mm^2 in the ventral ear skin, and 181 ± 13 cells/mm^2 in the dorsal ear skin at 168 h. The total number of macrophages in the tissue post-SM increased from 39 ± 2 cells per mm^2 at 24 h to 394 ± 53 cells per mm^2 at 72 h and 482 ± 28 cells per mm^2 at 168 h.

3.3. Upregulation of neutrophil collagenase (MMP8) and macrophage elastase (MMP12) in SM skin wounds

We next analyzed expression of MMP8 and MMP12 as markers of neutrophil collagenase and macrophage elastase, respectively. MMP8 is primarily expressed by neutrophils that are recruited to inflamed tissues and is often associated with chronic wounds (Hasty et al., 1990). Figure 5 shows that MMP8 mRNA is biphasic and increased 20–90 fold 6–168 h post-SM. MMP12, also known as macrophage specific metalloelastase, is predominantly secreted by macrophages during inflammation. MMP12 mRNA was found to increase with time 15–600-fold 12 to 168 h post-SM.

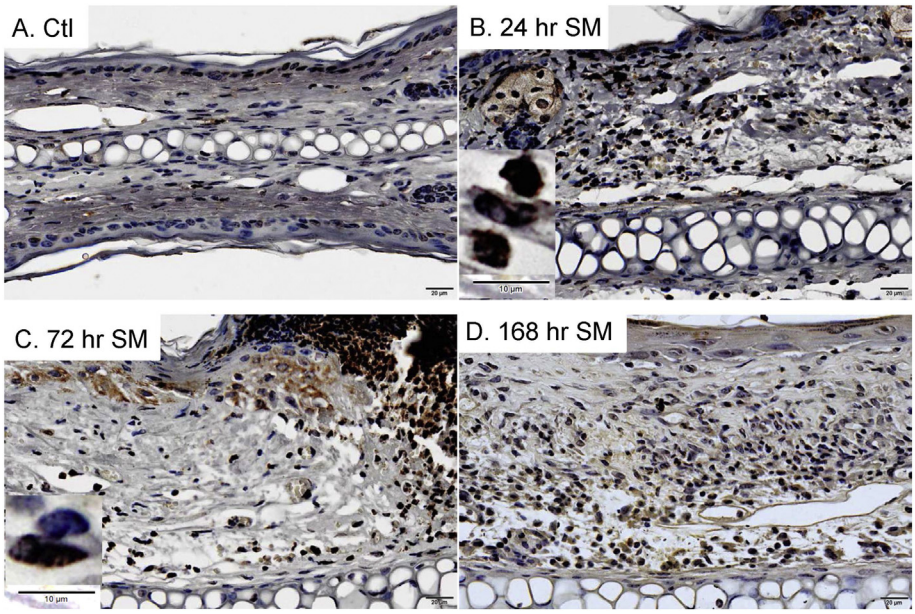

Fig. 2. Myeloperoxidase (MPO) staining of mouse ear skin post-SM exposure. Tissue sections were stained using an antibody to MPO. Panel A: Control, naive (Ctl); panel B: mouse ear skin 24 h post-SM exposure; panel C: 72 h mouse ear skin post-SM exposure; panel D: mouse ear skin 168 h post-SM exposure. Sections were stained with DAB and counterstained with hematoxylin (scale bar = 20 μm). Bottom left inserts of Fig. 2B and C show higher magnifications of MPO positive cells, dark staining indicates MPO expression in the cells (100× objective, scale bar = 10 μm).

3.4. Effects of SM on expression of inflammatory mediators in mouse skin

In further studies, we analyzed expression of mRNA and protein for cytokines and chemokines implicated in cutaneous injury and wound healing (Gillitzer and Goebeler, 2001). At 6 h post-SM, increased mRNA expression for CCL2, CCL3, CSF3, CXCL1, CXCL10, IL1β, IL6, and TNFα were observed (Fig. 6). The greatest increases were for CXCL1 (> 1000-fold, 6–168 h post-SM) followed by CSF3 (20–125 fold, 6–168 h post-SM), CXCL10 (10–30 fold, 6–168 h post-SM), and CCL2 (5–20 fold 6–168 h post-SM). CCL3, IL1β, IL6, and TNFα increased by about 3–10-fold at 6 h post-SM and remained upregulated at all-time points examined. Increases in mRNA corresponded to increased protein levels for these mediators in the tissue (Fig.7). We observed that the protein expression of cytokines/chemokines significantly upregulated in a time-dependent manner following SM exposure, compared to the unexposed controls (Fig. 7). IL6 protein levels (11 ± 1.2 pg/mg total protein) in skin were immediately elevated as early as 6 h post exposure. Expression of IL6 persisted for at least 168 h and spiked significantly at 168 h SM (300 ± 31.52 pg/mg total protein; a > 300-fold increases in SM exposed tissues compared to control). At 24 h post exposure, the protein levels of chemokines CXCL1 (34 ± 3.6 pg/mg total protein) and CCL2 (108 ± 11.5 pg/mg total protein) were greatly expressed; and continued to increase with time post-SM exposure. By 72–168 h post-SM, all examined cytokine/chemokine protein expressions were significantly upregulated; in particular, the chemokines/growth factors CCL3, CSF3, and CXCL10. Each appeared at substantially elevated levels (> 100 pg/mg total protein), compared to unexposed controls.

4. Discussion

Skin exposure to SM produces a marked inflammatory response in humans and animals; prolonged inflammation contributes to tissue damage and can lead to delayed wound repair. In the present studies, we examined inflammation in the mouse ear vesicant model to better understand SM-induced tissue injury and repair. We found that SM induced an array of inflammatory mediators (i.e., cytokines and

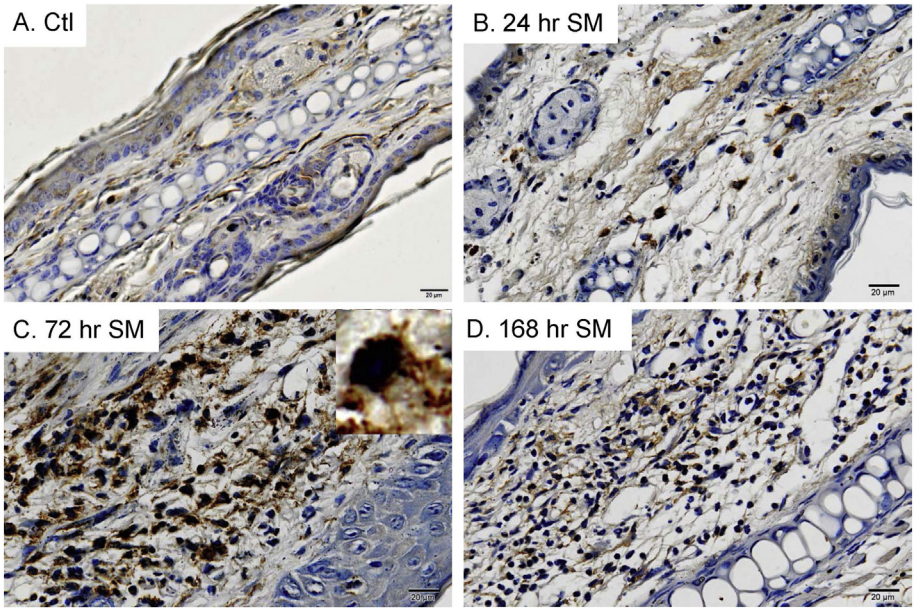

Fig. 3. Accumulation of macrophages in mouse ear skin post-SM exposure. Tissue sections were stained for macrophages using antibody to F4/80. Panel A, Control, naive (Ctl); panel B: mouse ear skin 24 h post-SM exposure; panel C: mouse ear skin 72 h post-SM exposure (insert showed enlarged image of an F4/80+ cell); panel D: mouse ear skin 168 h post-SM exposure. Sections were stained with DAB and counterstained with hematoxylin (Scale bar = 20 μm).

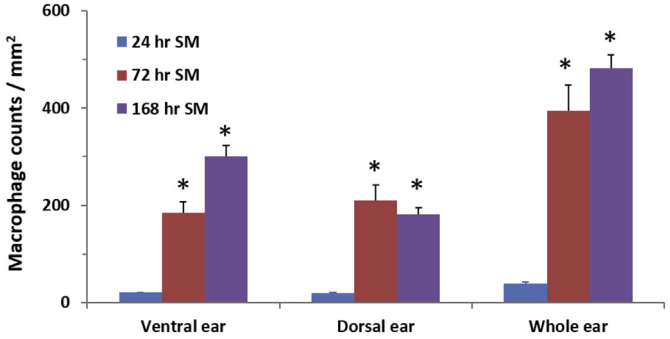

Fig. 4. Effect of SM on accumulation of macrophages in mouse ear skin. Tissue sections were stained for macrophages using antibody to F4/80. Total macrophage counts for the ventral (inner, exposed side) and dorsal side (outer, unexposed side) of the ear mouse skin, and the whole ear were determined. At least three animals and three different slides per group were analyzed for F4/80+ cells. Data are presented as mean ± S.E. (macrophage counts per mm^2). All data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test or non-pair student *t*-test. Macrophages counts at 72 h (red bar) and 168 h (purple bar) post-SM in ventral and dorsal ear skin are significantly increased when compared to macrophages counts in the tissues in naïve unexposed control and at 24 h (blue bar) post-SM (*$p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

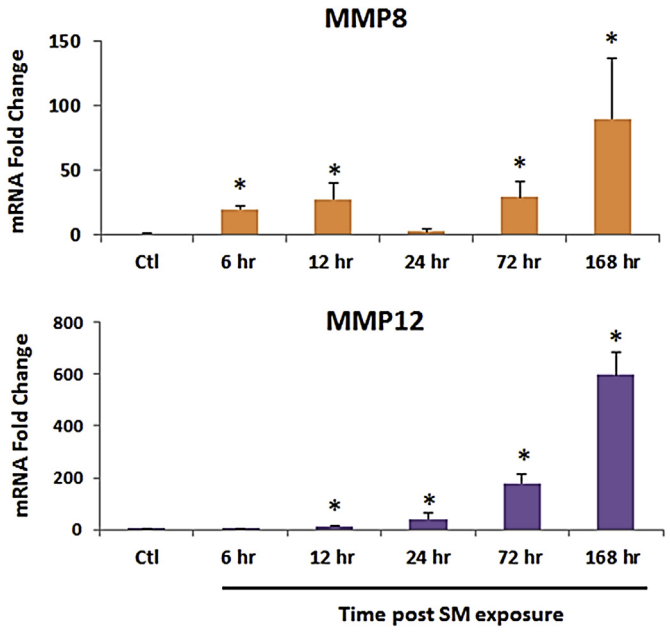

Fig. 5. Expression of mRNA for MMP8 (neutrophil collagenase) and MMP12 (macrophage elastase) in mouse ear skin post-SM exposure. Fold changes were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test or non-pair student *t*-test. Data are expressed as fold changes over time and are presented as the mean ± SE (n = 10); a p value of < 0.05 was considered statistically significant and marked with *, when compared to unexposed, naïve, control samples.

chemokines) in the tissue. The temporal expression of these inflammatory mediators appeared to be time-dependent and was coordinate with accumulation of macrophages and neutrophils in the skin; these cells are known to be an important source of cytokines/chemokines as well as growth factors that mediate inflammation and wound healing (Gillitzer and Goebeler, 2001; Barrientos et al., 2008; Turner et al., 2014). Persistent accumulation of macrophages and neutrophils in SM injured skin is likely to play a role in the pathogenic response to SM.

Inflammatory mediators are largely synthesized de novo by activated cells in response to injury. In our study, following SM exposure, we observed significantly elevated tissue production of inflammatory cytokines (i.e., IL1, IL6, TNFα, CCL2, and CCL3), as well as chemokines (i.e., CSF3, CXCL1, and CXCL10). These are proinflammatory mediators important in signaling inflammatory cell communication and migration to sites of tissue injury. Cytokines/chemokines regulate the induction and termination of immune responses; they also control the function and migration of resident and infiltrating inflammatory cells (Martin, 1997; Gillitzer and Goebeler, 2001). The infiltrating inflammatory cells (e.g., neutrophils, macrophages, mast cells, and lymphocytes) sequentially migrate to the wound site and serve as sources of inflammatory and growth-promoting cytokines/chemokines (Werner and Grose, 2003). In turn, these infiltrating inflammatory cells are also tightly regulated by chemokines during tissue inflammation (Turner et al., 2014; Su and Richmond, 2015). For example, IL1 and TNFα are stimulated during the early cellular inflammatory response. CXCL1 is chemoattractant for neutrophils while CCL2, CCL3, and CXCL10 are chemoattactants for monocytes/macrophages, and T cells, respectively (Zlotnik and Yoshie, 2000; Gillitzer and Goebeler, 2001; Turner et al., 2014). SM-induced skin injury is closely associated with inflammatory cell infiltration (Vogt et al., 1984; Dannenberg et al., 1985; Smith et al., 1995; Levitt et al., 2004; Ruff and Dillman, 2007; Shakarjian et al., 2010; Joseph et al., 2011; Mouret et al., 2015). Excessive, uncontrolled, and prolonged production of these inflammatory mediators, by the inflammatory cells that infiltrate the tissue may exacerbate skin injury induced by SM.

In our time course studies, we observed that gene expression of many inflammatory mediators (i.e., IL1, IL6, TNFα, CCL2, CCL3, CSF3, CXCL1, and CXCL10) were significantly increased as early as 6 h in the SM treated tissues and remained elevated up to 168 h post exposure. Our data are consistent with earlier studies showing upregulation of mRNA expression for IL1β, IL6, and TNFα in the MEVM 24 h following SM exposure (Sabourin et al., 2000). Upregulation of CCL2, CCL3, CXCL1, IL6, IL1β, and TNFα mRNA expression was also reported in the dorsal skin of hairless mice 1–7 days post-SM exposure (Sabourin et al., 2003; Mouret et al., 2015). In addition, increased mRNA levels for IL1β, IL6, and TNFα have also been observed in weanling pig skin 72 h post-SM exposure (Sabourin et al., 2000; Sabourin et al., 2003; Sabourin et al., 2004). In contrast to mRNA expression, only limited in vivo studies reported protein expression in skin wounds following SM exposure. In one study, protein expression of IL6, but not TNFα or IL1α, was significantly elevated in wounded skin by 24 h post-SM in both mouse ear and dorsal skin (Ricketts et al., 2000).

Using immune-multiplex analysis of an inflammatory profile panel, we determined that there was a coordinated temporal increase in protein expression for proinflammatory cytokines and chemokines; including IL6, IL1β, TNFα, CCL2, CCL3, CSF3, CXCL1, and CXCL10. In particular, we found IL6 upregulated in both mRNA and protein levels as early as 6 h post exposure. The expression of IL6 continued to be elevated for all time points (6–168 h) in mouse skin following SM exposure. This observation is consistent with the suggestion that IL6 is an early (1–3 day post exposure) inflammatory marker for SM injury in both mouse and weanling pig skin (Ricketts et al., 2000; Sabourin et al., 2000; Sabourin et al., 2002; Sabourin et al., 2003; Mouret et al., 2015). Furthermore, overexpression of IL6 has been described in chronic wounds and autoimmune skin diseases such as psoriasis (Turksen et al., 1992; Lin et al., 2003). The persistence of IL6 expression (1–7 days post exposure) in our model may indicate a potential contribution to chronic injury in SM induced wounds. In addition, IL6, secreted by macrophages and skin cells during inflammation, is known to play a critical role in cutaneous wound healing due to its effects on neutrophil chemotaxis to the wound edge (Gallucci et al., 2004; Finnerty et al., 2006). In our study, we observed elevated IL6 protein expression (> 300-fold) at 168 h post exposure. The substantial protein levels of IL6 in the tissue at the later time points of our study may result from increased release

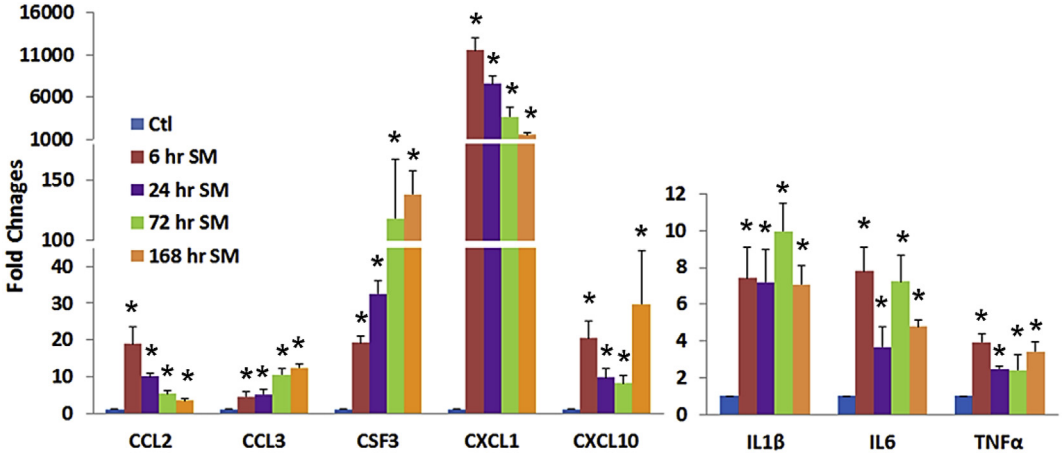

Fig. 6. Cytokine/chemokine mRNA expression in mouse ear skin post-SM exposure. Real-time PCR with Taqman gene expression assays was used to quantify mRNA in tissue sections. Data are expressed as fold changes over time and presented as the mean ± SE (n = 10). Fold changes were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test or non-pair student *t*-test; a p value of < 0.05 was considered statistically significant and marked with *, when compared to unexposed, naïve, control samples. Control skin (blue bars), mouse ear skin 6 h post-SM (red bars), mouse ear skin 24 h post-SM exposure (green bars), mouse ear skin 72 h post-SM (purple bars); mouse ear skin168 h post-SM (light blue bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by newly recruited macrophages in the SM injured tissues. This suggests that continual synthesis of IL6 is closely associated with infiltration of inflammatory cells to the SM skin wounds. In this regard, we observed that increased macrophage counts in the tissue were maximum at 168 h post-SM. Although SM was exposed only to the ventral side of mouse ear skin, there was no significant difference between macrophage counts per area from the ventral and the dorsal ear skin for each time point, indicating that SM can induce tissue inflammation throughout the whole ear skin. In addition, the inflammatory cell counts were coordinate with the gene expression of the inflammatory cell markers (using MMP12 as a macrophage marker and MMP8 as a neutrophil marker in this study). At 168 h post-SM exposure, the increases in macrophage counts in skin corresponded to the temporal upregulation of MMP12 (macrophage elastase) mRNA expression which was significantly greater than expression at 6 h. A similar increase in the mRNA expression of MMP8 (neutrophil collagenase) correlated with the increase in neutrophils noted in 168 h post-SM injured skin. Further studies will help to elucidate the mechanism for the inflammatory cell-derived IL6 and its contribution to the pathological effect on SM induced skin inflammation.

The infiltrating inflammatory cells, acting as sources of inflammatory mediators, are closely associated and regulated by chemokines during skin wound repair. Chemokines, secreted by macrophages, are known to be chemoattractant to neutrophils. In our study, we observed substantially increased expression of chemokines in skin following SM exposure. The mRNA and protein expression of CXCL1 and CXCL10 was highly upregulated 72–168 h post-SM. This expression pattern was also noted for CSF3, a growth factor, secreted by macrophages, also reported to attract neutrophils (Roberts, 2005). These observations coincided with increased neutrophils over time after exposure (as measured by MPO staining) at 72–168 h post-SM exposure (Fig. 2). These findings are in accord with earlier studies demonstrating increased numbers of MPO^+ neutrophils by 7 days in mouse skin exposed to mustards (Milatovic et al., 2003; Tewari-Singh et al., 2009; Jain et al., 2014; Tewari-Singh et al., 2014a; Tewari-Singh et al., 2014b; Mouret et al., 2015). However, this is contrary to normal healthy incisional skin wounds which have proper wound closure by 7–10 days. In normal incisional wounds, the neutrophil influx peaks at day 1 and subsides by day 3 (Witte and Barbul, 1997; Werner and Grose, 2003). Unlike, healthy skin wound progression, SM induced skin blisters often are accompanied by prolonged inflammation and delayed wound healing. We found neutrophils apparently clustered adjacent to necrotic

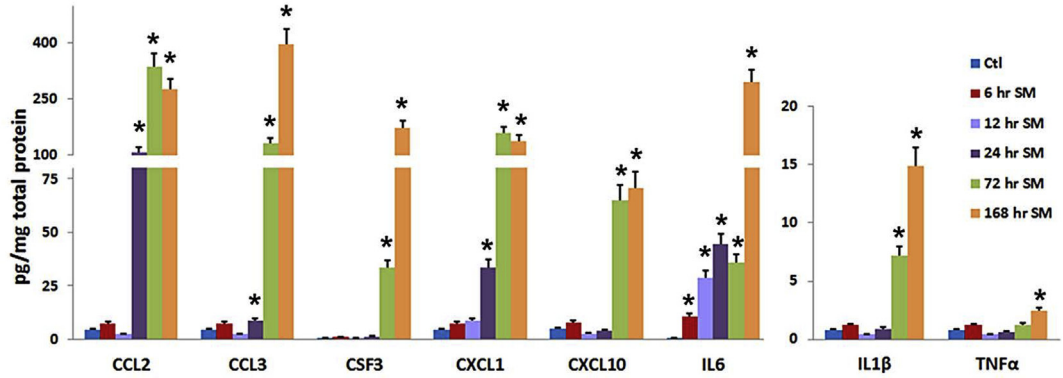

Fig. 7. Cytokine/chemokine protein expression in mouse ear skin post-SM exposure. Lysates from pooled samples prepared from mouse ears were analyzed using a mouse cytokine/chemokine Milliplex kit (Millipore Inc., Billerica, MA). Data are expressed as pg of cytokines/chemokines per mg total lysate protein and are presented as the mean ± SE, (CV $< 15\%$), n = 3. Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test or non-pair student *t*-test when compared to control samples; a p value of < 0.05 was considered statistically significant and marked with *. Control (blue bars), mouse ear skin 6 h post-SM (red bars), mouse ear skin 12 h post-SM (green bars); mouse ear skin 24 h post-SM (purple bars), mouse ear skin 72 h post-SM (light blue bars), mouse ear skin 168 h post-SM (orange bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

wound sites by 72–168 h post-SM. Studies showed that necrotic cells in the area of skin injury contribute to neutrophil chemoattraction by inducing CXCR2, a binding receptor for CXCL1 and a mediator of neutrophil chemotaxis in skin wounds (Gillitzer and Goebeler, 2001; De Filippo et al., 2013; Su and Richmond, 2015). The necrosis induced by SM may augment the expression of CXCL1, increasing the recruitment of circulating neutrophils and macrophages so that they can phagocytose the debris in the skin wound by 72–168 h post exposure. Tissue resident macrophages have been shown to also play a role in the release of CXCL1 via TLR signaling in mouse inflamed skin (De Filippo et al., 2008; De Filippo et al., 2013). In this study, we used F4/80, a surface marker of mature macrophages to identify activated macrophages in the skin. We found substantial amounts of F4/80 positive macrophages accumulating in the injured skin wounds 72–168 h post-SM exposure. We speculate that these activated macrophages also contribute to the secretion of CXCL1 in SM skin wounds by 168 h post exposure. Further studies using extended time points will help to clarify when wound repair actually begins and how long inflammation persists. The continuous non-resolved inflammatory response involving other inflammatory cells (e.g., activated macrophages) further exacerbates skin injury induced by SM. The overexpression of chemokines observed in mouse skin following SM exposure includes both macrophage derived chemokines (CCL2, CSF3, and CXCL1) and neutrophil derived chemokines (CCL3, and CXCL10). They may serve as a reservoir to further recruit other inflammatory cells and lead to prolonged inflammation and delayed wound repair observed in SM induced skin injury.

Cutaneous wound healing after injury is a complex process involving a host of inflammatory mediators produced by many cell types (Martin, 1997; Singer and Clark, 1999; Bielefeld et al., 2013). Various cytokines, chemokines, and growth factors contribute to inflammation and tissue injury as well as the regulation of epithelialization, tissue remodeling, and angiogenesis during wound repair (Gillitzer and Goebeler, 2001; Raja et al., 2007). The fact that anti-inflammatory agents can suppress skin injury supports the idea that inflammatory mediators are important in the activation of mustards (Casillas et al., 2000; Sabourin et al., 2003; Chang et al., 2014; Tewari-Singh et al., 2014a; Achanta et al., 2018). Of note, our studies demonstrate characteristic inflammatory signatures in SM-induced skin toxicity. We observed collateral damage on both the ventral and dorsal sides of the mouse ear skin when SM was only treated on the ventral side of the ear. Similar collateral damage in the MEVM has been reported earlier (Casillas et al., 2000; Dachir et al., 2002). SM may penetrate through the auricular cartilage that separates the ventral and the dorsal ear skin to damage both sides of the ear skin. Nevertheless, we may speculate that this damage on both the ventral and dorsal sides of mouse ear skin may be mediated by infiltrating inflammatory cells, and the release of inflammatory mediators that diffuse across the tissue. In this study, we detected that the macrophages and neutrophils infiltrate and persist in both the treated (ventral) and the untreated (dorsal) sides of the ear skin for at least 7 days. Inflammation is tightly regulated by the infiltrating inflammatory cells and their cytokines/chemokines network. The excess expression of cytokines/chemokines in SM induced skin injury may be associated with the macrophages and neutrophils that persistent in SM damaged tissue, which may further contribute to the pathogenesis of SM induced skin injury. Modulating these infiltrating inflammatory cells and/or cytokines and chemokines in skin following exposure to SM may provide a therapeutic strategy to mitigate tissue injury and/or accelerate wound healing.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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