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Suppression of Hyperactive Immune Responses Protects against Nitrogen Mustard Injury

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DNA alkylating agents like nitrogen mustard (NM) are easily absorbed through the skin and exposure to such agents manifest not only in direct cellular death but also in triggering inflammation. We show that toxicity resulting from topical mustard exposure is mediated in part by initiating exaggerated host innate immune responses. Using an experimental model of skin exposure to NM we observe activation of inflammatory dermal macrophages that exacerbate local tissue damage in an inducible nitric oxide synthase (iNOS)-dependent manner. Subsequently these activated dermal macrophages reappear in the bone marrow to aid in disruption of hematopoiesis and contribute ultimately to mortality in an experimental mouse model of topical NM exposure. Intervention with a single dose of 25-hydroxyvitamin D3 (25(OH)D) is capable of suppressing macrophage-mediated iNOS production resulting in mitigation of local skin destruction, enhanced tissue repair, protection from marrow depletion, and rescue from severe precipitous wasting. These protective effects are recapitulated experimentally using pharmacological inhibitors of iNOS or by compounds that locally deplete skin macrophages. Taken together, these data highlight a critical unappreciated role of the host innate immune system in exacerbating injury following exposure to NM and support the translation of 25(OH)D in the therapeutic use against these chemical agents.

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INTRODUCTION

Mustard gas and mustard-related compounds are vesicating agents that, on skin exposure, cause severe epithelial and deep tissue injury characterized by blistering, acute inflammation, induration, and edema (Requena *et al.*, 1988; Sharma *et al.*, 2010a; Sharma *et al.*, 2010b). Historically, these powerful vesicants were exploited as chemical warfare agents during World War I and later conflicts (Pearson, 2006). Through its action as a DNA alkylating agent, nitrogen mustard (NM) and related compounds like nitrosourea, chlorambucil, and estramustine phosphate generate DNA strand breaks with consequent cell death, a unique property that was exploited and adapted in medicine as effective therapy against rapidly proliferating cancer cells (DeVita and Chu, 2008). However, its clinical utility is limited by its dose-dependent toxicity (DeVita and Chu, 2008).

On exposure, NM is absorbed through skin and re-deposited in subcutaneous fat to inflict tissue destruction directly from the alkylating effects of NM. Injured tissue creates an inflammatory foci (Keramati *et al.*, 2013), (Gunnarsson *et al.*, 1991) to attract neutrophils, monocytes, and macrophages (Jain *et al.*, 2014). Persistence of the initial inflammatory phase can amplify an immune response and induce further tissue injury (Laskin *et al.*, 1996a; Laskin and Laskin, 1996; Laskin *et al.*, 1996b; Kondo and Ishida, 2010). NM-induced wounds generate oxidative and nitrosative stress to exacerbate tissue destruction (Yaren *et al.*, 2007; Zheng *et al.*, 2013). We and others have shown that inducible nitric oxide synthase (iNOS)-producing hyper-activated macrophages delay wound repair and exaggerate wound pathogenesis (Cash *et al.*, 2014; Das *et al.*, 2014). Therefore therapeutic intervention(s) targeting these inflammatory cells may be a suitable strategy to subdue inflammatory damage. The use of pharmacologic inhibitors of iNOS, though efficacious in experimental animal models, has limited translation clinically due to cytotoxicity and adverse off-target physiological effects on circulatory function (Laskin *et al.*, 1996b; Bogdan, 2001; Malaviya *et al.*, 2012). Consequently, we focused on

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Abbreviations: BM, bone marrow; CBC, complete blood count; NM, nitrogen mustard; 25(OH)D, 25-hydroxyvitamin D3; iNOS, inducible nitric oxide synthase

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Vitamin D3, a hormone that has acquired recognition as an immunomodulator through direct inhibition of NFκB activation and suppression of TNF-α and iNOS expression (Cohen-Lahav, 2006; #9; Holick, 1993, 2003; Chen *et al.*, 2011; Lagishetty *et al.*, 2011). Typically, the kidneys control the rate limiting step in converting circulating 25-hydroxyvitamin D3 (25(OH)D), the inactive form of vitamin D3, into calcitriol, the active form (1,25α$(OH)_2$D). The ability of macrophages to perform this conversion by virtue of its intracellular enzyme CYP27A1 (Mora *et al.*, 2008) allowed us to hypothesize that 25(OH)D should effectively block macrophage-mediated iNOS upregulation and confer protection from exacerbated local and systemic tissue injury that follows NM exposure.

This study investigates a NM skin wound model that demonstrates a critical role for activated cutaneous macrophages in delaying wound healing and causing disruption of hematopoiesis via iNOS production. The model emphasizes the therapeutic efficacy of 25(OH)D intervention to counteract an acute immune response that exacerbates NM-mediated pathology and enables repopulation of bone marrow (BM) cells. We determine that topical application of NM activates cutaneous macrophages to produce iNOS that traffic to the BM and cause further disruption of hematopoiesis. A single administration of 25(OH)D promotes survival by moderating the immune response and restoring blood cell loss and BM depletion.

RESULTS

25(OH)D prevents NM-mediated tissue destruction by antagonizing macrophage-derived iNOS

We established a NM-skin contact model characterized by topical (percutaneous) application of NM to an 8 mm diameter (50 mm^2) circular template on the dorsal skin of C57BL/6J mice, herein referred to as wound area. Working on the hypothesis that elevated macrophage-derived iNOS is the stimulus for exacerbated tissue injury following NM exposure led us to explore whether 25(OH)D can effectively counteract NM-induced iNOS. One hour following NM exposure, an intraperitoneal (i.p.) bolus of 5 ng 25(OH)D was administered. We show that NM-induced wound appears on the first day and increases progressively over time. Treatment with 25(OH)D or a specific pharmacological inhibitor of iNOS (compound 1400W, 10 mg kg^{-1}) delays hemorrhagic crust formation and eventually resolves wound by day 19 (data not shown) (Figure 1a). In both treatment intervention groups, the surrounding skin appeared healthy with full recovery of hair regrowth and a small residual scar by day 40. Gross wound images correlated with a percentage wound area size relative to the initial 50 mm^2 template (Figure 1b). Histological examination of skin at the corresponding time point in mice not treated with either 25(OH)D or 1400W intervention reveal full-thickness necrosis, robust inflammation, and edema following NM contact. Skin from NM+25(OH)D mice displayed a milder histological phenotype with diminished inflammatory infiltrates, skin necrosis limited to the epidermis and superficial dermis with preservation of deep skin structures including hair follicles, subcutaneous fat, and panniculus carnosus (Figure 1c). Furthermore, a similar protection from exacerbated skin damage was also observed with iNOS inhibition using compound 1400W (Figure 1a–c). The protective effect of 25(OH)D is not strain specific, as similar results were observed using BALB/c mice (Supplementary Figure S1a–c online). Exacerbation of skin necrosis was associated with elevated levels of skin specific iNOS and TNFα mRNA 48 hours post NM exposure, that was significantly reduced by intervention with 25(OH)D (Figure 1d, Supplementary Figure S1d online). This was consistent with results using *nos2*$^{-/-}$ mice, which exhibit mild inflammatory response to NM with preservation of the skin layers, deep skin structures and minimal tissue destruction (Supplementary Figure S1e online). Since iNOS is primarily produced by inflammatory macrophages and monocytes, confocal microscopy was performed to co-localize F4/80+/iNOS+ macrophages infiltrating the wound bed that were significantly diminished with 25(OH)D intervention (Figure 1e).

To demonstrate a role for dermal macrophages in delaying wound healing, the latter were depleted by intradermal injection with liposomal clodronate 1 hour after NM exposure (Ward *et al.*, 2011). We observed dramatic reduction of skin wound with sparse inflammation and edema (Figure 1f) corresponding to accelerated skin wound healing. Consistent with reduced skin wound area, clodronate treatment protected animals from disruption of skin architecture (Figure 1g) and mice exhibited significantly diminished iNOS (Figure 1h) and TNFα (Supplementary Figure S1f online) mRNA expression, supporting the hypothesis that hyperactive dermal macrophages may be the source of exacerbated cutaneous destruction.

25(OH)D rescues mice from systemic effects of NM

Injury from NM exposure is known to cause systemic damage especially to adipose-rich tissue such as the BM leading to morbidity and mortality (Schein *et al.*, 1987). Our experimental model of NM exposure (26.6 mg kg^{-1}) was developed based on a dose response (Supplementary Figure S2a online). Daily evaluation of animal well-being showed that by day 4, NM caused severe morbidity including hunched posture and statistically significant precipitous loss of body weight by almost 30% (Figure 2a). At this exposure dose, mortality (either observed or meeting weight loss criteria for compassionate euthanasia) was observed in 90% of mice between days 4 and 13 in contrast to mice that received 25(OH)D intervention (Kaplan–Meier survival plot), (Figures 2b, $P<0.001$, log-rank test). Examination of whole blood by complete blood count (CBC) analysis (Table 1) shows acute anemia and lymphopenia with visible loss of cells on peripheral blood smears (Figure 2c). Intervention with 25(OH)D or 1400 W restored blood cell counts comparable to healthy controls (Figure 2c, Table 1). Disruption of the hematopoietic compartment was characterized by acute loss of cellularity selectively in the BM (Figure 2d) with no observed overt histologic abnormalities in the visceral organs (Supplementary Figure S2b online), suggesting compartmental specificity of NM-mediated effects in our experimental model.