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Organic Dust-Induced Lung Injury and Repair: Bi-directional regulation by TNFα and IL-10

T.A. Wyatt[1,2,3], **M. Nemecek**[1,2], **D. Chandra**[1], **J.M. DeVasure**[1], **A.J. Nelson**[4], **D.J. Romberger**[1,3], **J.A. Poole**[4]

[1]Department of Internal Medicine Pulmonary, Critical Care, and Sleep, University of Nebraska Medical Center, Omaha, NE

[2]Department of Environmental, Agricultural and Occupational Health, University of Nebraska Medical Center, Omaha, NE

[3]Department of Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, NE

[4]Department of Internal Medicine Allergy and Immunology Divisions, University of Nebraska Medical Center, Omaha, NE

Abstract

Exposure to organic dust increases chronic airway inflammatory disorders. Effective treatment strategies are lacking. It has been reported that hog barn dust extracts (HDE) induce TNFα through protein kinase C (PKC) activation and that lung inflammation is enhanced in scavenger receptor A (SRA/CD204) knockout (KO) mice following HDE. Because interleukin (IL)-10 production can limit excessive inflammation, it was hypothesized here that HDE-induced IL-10 would require CD204 to effect inflammatory responses. C57BL/6 wild-type (WT), SRA KO, and IL-10 KO mice were intranasally challenged daily for 8 d with HDE and subsequently rested for 3 d with/without recombinant IL-10 (rIL-10) treatment. Primary peritoneal macrophages (PM) and murine alveolar macrophages (MH-S cells) were treated *in vitro* with HDE, SRA ligand (fucoidan), rIL-10, and/or PKC isoform inhibitors. HDE induced *in vivo* lung IL-10 in WT, but not SRA KO mice, and similar trends were demonstrated in isolated PM from same treated mice. Lung lymphocyte aggregates and neutrophils were elevated in *in vivo* HDE-treated SRA and IL-10 KO mice after a 3-d recovery, and treatment during recovery with rIL-10 abrogated these responses. *In vitro* rIL-10 treatment reduced HDE-stimulated TNFα release in MH-S and WT PM. In SRA KO macrophages, there was reduced IL-10 and PKC zeta (ζ) activity and increased TNFα following *in vitro* HDE stimulation. Similarly, blocking SRA (24 hr fucoidan pre-treatment) resulted in enhanced HDE-stimulated macrophage TNFα and decreased IL-10 and PKCζ activation. PKCζ inhibitors blocked HDE-stimulated IL-10, but not TNFα. Collectively, HDE stimulates IL-10 by an SRA- and PKCζ-dependent mechanism to regulate TNFα. Enhancing resolution of dust-mediated lung inflammation through targeting IL-10 and/or SRA may represent new approaches to therapeutic interventions.

Please direct all correspondence to: Todd A. Wyatt, PhD, 985910 Nebraska Medical Center, Omaha, NE 68198-5910. (F) 402-559-4140 twyatt@unmc.edu.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Keywords

Hog barn dust; swine; lung; macrophage; TNFα; IL-10; CD204; PKCζ

Introduction

Agricultural workers exposed to organic dusts from concentrated animal feeding operations (CAFO) have increased risk of chronic inflammatory respiratory disease such as bronchitis, asthma, and chronic obstructive pulmonary disease (COPD) with disease persisting even after cessation of work exposure. Important inflammatory mediators and pattern recognition receptor pathways responsible for mediating airway injury/inflammation have been identified. The mechanisms regulating the transition from lung inflammation to repair and recovery, however, is a critical, but understudied, area that could lead to the development of therapeutic targeted interventions to alleviate disease burden in occupationally exposed persons.

Previously, an animal model of inflammatory lung injury was developed to identify functional roles/mechanistic insights on specific microbial components and signaling pathways regulating complex agricultural dust extract exposure consequences (Poole et al. 2009). When adapted for use by others (Robbe et al. 2014), this model was used to show that hog barn dust extract (HDE) consistently resulted in release of pro-inflammatory cytokines including tumor necrosis factor (TNF)α, interleukin (IL)-6, and IL-8, development of lymphoid aggre-gates, and key roles for lung macrophages and epithelial cells. Removal of the dust exposure for a recovery period allows for this model to be extended to studies of lung repair (Warren et al. 2017). Among mechanisms investigated to understand signal transduction pathways leading to inflammatory consequences, a temporal regulation pathway in airway epithelium governed by protein kinase C (PKC)-alpha (α) and -epsilon (ε) isoforms required for the sequential release of TNFα, IL-6, then IL-8 were demonstrated (Wyatt et al. 2010). PKC regulation of HDE-stimulated proinflammatory cytokines was also identified in monocytes and macrophages (Poole et al. 2007). Knocking down Toll-like receptor (TLR)-2, -4, -9 and MyD88 have led to reduced airway inflammation, yet in contrast, scavenger receptor A (SRA; CD204) knockout mice challenged with HDE displayed substantial increase in lymphoid aggregates and delayed resolution after cessation of dust exposure, suggesting a key role for SRA in both the magnitude and duration of dust-induced injury (Poole et al. 2015). The mechanisms by which SRA negatively-regulated inflammation and recovery is not clear, but a relationship between scavenger receptors differentially mediating IL-10 under various experimental conditions has been reported (Jozefowski et al. 2014).

As IL-10 is recognized as an anti-inflammatory cytokine that can limit tissue damage caused by excess host inflammation, the goal of this study was to delineate the mechanistic and functional role of IL-10 in agricultural organic dust-associated lung inflammation and repair. Because it has been reported that macrophage IL-10 release is dependent upon the action of PKC (PKC) ζ (Foey and Brennan 2004), it was hypothesized here that HDE recognition by SRA in the lung/lung macrophages stimulated IL-10 release via a PKCζ mechanism, and

moreover, that lung-directed therapy with IL-10 would facilitate post-inflammatory repair. These current studies define a potentially beneficial role of IL-10, acting through SRA and PKCζ, in moderating HDE-induced injury and leading to repair.

Materials and methods

Animals

WT C57BL/6 mice, SRA KO (Strain B6.Cg-Msr1<tm1Csk>/J), and IL-10 KO (Strain B6.129P2-Il10tm1Cgn/J) on C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for 1 week. Male mice (6–10 wk-of-age) were used for all studies. All mice were housed under 12 hour light/dark cycles and had *ad libitum* access to standard rodent chow and filtered water through the course of the studies. All animal procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were in accordance with the NIH guidelines for the use of rodents.

Hog barn dust extract

Aqueous extracts were prepared from settled dust collected from horizontal surfaces (~3 ft above floor) of local swine confinement feeding operations (housing ≈ 400–600 animals). These hog barn dust extracts (HDE) were batch prepared utilizing previously described methods (Poole et al. 2015). Coarse particles were removed by filtration (0.2 μm filter) and aliquots of HDE frozen until use in experimental assays. HDE was diluted to a final concentration of 12.5% (v/v) in sterile phosphate-buffered saline (PBS; pH 7.4) diluent as this was previously shown to elicit optimal lung inflammation in mice and is well-tolerated (Poole et al. 2009). Endotoxin concentrations in these samples ranged from 20–60 EU/ml as determined by limulus amebocyte lysate assay (Sigma, St. Louis, MO).

Hog barn dust extract instillation model

An established intranasal inhalation repetitive exposure animal model was utilized whereby mice were lightly sedated under isoflurane and received treatment once daily with 50 μl of PBS (pH 7.2) or 12.5% HDE daily for 8 d (Poole et al. 2009). For recovery experiments, HDE treatment was withdrawn and mice were allowed to recover for 3 d with daily nasal instillation of sterile PBS or 10 ng/ml mouse recombinant IL-10 (Biolegend, San Diego, CA). Animals were euthanized via isoflurane inhalation - followed by opening the thoracic cavity - 5 hr following the final exposure for experimental endpoint quantification. No respiratory distress, signs of stress, or weight loss throughout the treatment period was observed.

Bronchoalveolar lavage fluid and tissue homogenate analysis

At necropsy, bronchoalveolar lavage fluid (BALF) was accumulated using three 1-ml PBS washes taken using standard harvesting protocols (Poole et al. 2009). Total cell numbers for each mouse from their respective combined recovered lavages were enumerated and differential cell counts determined from cytospin-prepared slides (Cytopro cytocentrifuge, ELITech Group, Logan, UT) stained with DiffQuick (Siemens, Newark, DE). From the

cell-free supernatant of the first lavage fraction, TNF-α and IL-10 levels were quantitated using commercial ELISA kits (R&D Systems, Minneapolis, MN).

TNF-α and IL-10 were also measured from total lung homogenates in lungs of some treated animals. In brief, after BALF collection, blood was removed from the pulmonary vasculature by infusion through right ventricle with 10 ml sterile PBS (containing 5 U heparin/ml). After the blanching, homogenates were prepared by homogenizing the total lungs in 500 μl sterile PBS. The resulting suspension was clarified by centrifugation (425 g, 10 min, 4°C). The levels of TNFα and IL-10 in the homogenate were then quantitated by ELISA.

Histopathology

Following lung lavage, the remaining lungs not homogenated were excised and inflated (at 15 cm H_2O pressure) with 10% formalin (Sigma) for 24 hr to preserve pulmonary architecture, as previously described (Poole et al. 2009). The fixed lungs were then processed, embedded in paraffin, and lung sections were cut (4–5 μm) and stained with hematoxylin and eosin (H&E). Each slide was then reviewed at scanning magnifications (using 2X, 4X, and 10X objectives) with an Eclipse Model E600 microscope (Nikon, Tokyo, Japan). The total number of lymphocyte aggregates were counted for multiple sections in each lung section per treatment condition (Poole et al. 2015).

Macrophage isolation and treatment

Resident peritoneal macrophages from wild-type, SRA KO, and IL-10 KO mice were collected using a previously described method (Zhang et al. 2009) from either in vivo exposed or unexposed mice where indicated. These cells were cultured in DMEM with 10% fetal bovine serum (FBS) for no longer than 5 d for all assays. Immortalized murine alveolar macrophages (MH-S cells) were obtained from ATCC (Manassas, VA) and plated in RPMI-glucose media containing 10% FBS and allowed to grow for 2 days. MH-S were passaged no more than 10 times for any experiment. All culture reagents were purchased from ThermoFisher (Waltham, MA). At confluency, cells is each experiment were treated in triplicate for each treatment condition and each timepoint to be tested. In all cases, both supernatant media and cells were collected. All experiments were repeated a minimum of three times.

After determining the optimal concentration of HDE for *in vitro* cell exposure to be 1–2%, cells were treated for up to 24 hr with 1% HDE diluted in tissue culture media. Other sets of cells were also treated with 10 μM lipopolysaccharide (LPS; Type O111:B4 from *Escherichia coli*), 1–10 μg fucoidan/ml, 10 μg fetuin/ml, or 10 ng recombinant IL-10/ml for 1 or 24 hr in the presence or absence of HDE. Some sets of cells were also pre-treated for 1 hr with myristolated PKCζ or Calphostin-C before HDE treatment, where indicated. All reagents, unless specifically stated elsewise, were obtained from Sigma (St. Louis, MO).

Protein kinase C (PKC)-ζ activity assay

PKCζ activity in the cells was tested via a radiolabeled ATP assay to confirm efficacy of PKC inhibitors using the method described previously (Wyatt et al. 2010). In brief, cells were pre-incubated for 1 hr with media, 1 μM Calphostin C, or 1 μM myristolated PKCζ

inhibitory peptide followed by a 5 hr exposure to 1% HDE. Cells were then fractionated in a cell lysis buffer, and cell extracts collected. After determining protein content using Bradford reagent (BioRad, Hercules, CA), an aliquot of extract (20 μl) was added to 40 μl of a Tris-HCl acid buffer (pH 7.5) solution containing 900 μmol/L PKCζ substrate peptide (Enzo Life Sciences, Farmington, NY), 30 mM dithiothreitol, 150 μM ATP, 24 mM magnesium acetate, and 10 μCi [$\gamma-^{32}$P]-ATP/ml (Perkin Elmer, Waltham, MA). Samples (50 μl) were then spotted onto P-81 paper (Jon S. Oakhill, St. Vincent's Institute of Medical Research, Fitzroy, Australia) and the reaction in each spot then halted by submerging each paper in 50 mM phosphoric acid solution. Radioactivity (in relation to total cell protein assayed) was then counted using a Beckman LSC (Beckman Coulter, Brea, CA) and a non-aqueous scintillant (National Diagnostics, Atlanta, GA). Kinase activity was calculated in relation to total cell protein assayed and expressed in terms of picomoles of incorporated phosphate/min/mg.

TACE inhibition assay

Cell extracts prepared for enzyme detection as above were also assayed for TACE activity using a commercially-available kit (BioVision, Milpitas CA).

Statistical analysis

Data are presented as means ± SEM. To detect significant changes between groups, a one-way analysis of variance (ANOVA) was utilized and a *post-hoc* test (Tukey/LSD) or non-parametric Mann-Whitney test was performed to account for multiple comparisons if the p-value was < 0.05. For all analyses, Prism software (v.8.31, GraphPad, La Jolla, CA) was used.

Results

HDE-induced IL-10 release is dependent upon SRA signaling pathway.

To understand the observation of enhanced HDE-associated inflammation and delayed resolution in SRA KO mice (Poole et al. 2015), levels of anti-inflammatory IL-10 were assessed in lung homogenates of WT and SRA KO mice repetitively-exposed for 8 d to HDE. HDE treatment significantly increased IL-10 in WT, but not SRA KO mice (Figure 1A). There was no difference in IL-10 levels between the WT or SRA KO mice following PBS treatment. Because macrophages are a primary source for IL-10 production, whether SRA-mediated HDE-induced IL-10 responses in macrophages was examined. Peritoneal macrophages (PM) were isolated from the same mice that had been instilled *in vivo* with PBS or HDE. These cells were then cultured and treated *in vitro* with or without 1% HDE for 24 hr. It was seen that HDE significantly stimulated IL-10 release in macrophages isolated from among the WT and SRA KO treatment groups as compared to saline control (Figure 1B). In WT conditions, there was an equivalent HDE-stimulated release of IL-10 from the PM, regardless of PBS or HDE *in vitro* treatment conditions. In comparison to WT mice, differences were demonstrated with IL-10 with the isolated SRA KO macrophages. The magnitude of the HDE-simulated IL-10 release was significantly reduced in the PM isolated from saline-treated SRA KO mice as compared to WT. This decreased *in vitro* HDE response by PM was even more pronounced in SRA KO vs. WT mice that had also been

in vivo HDE-treated. Thus, IL-10 responsiveness to HDE stimulation was reduced in lung homogenates and macrophages of SRA KO mice.

Lung delivered IL-10 hastens recovery from HDE-induced lung inflammation in SRA KO and IL-10 KO mice

To define a functional role for IL-10 in regulating post-inflammatory disease resolution, mice were challenged daily for 8 d with HDE and subsequently allowed to recover in the absence of HDE for 3 d, with treatment (nasal instillation) daily with either saline or recombinant murine IL-10 (rIL-10; 1 μg/mouse). Consistent with previous reports (Poole et al. 2015), SRA KO mice demonstrated a significant increased presence of lymphocyte aggregates as compared to WT mice. In further proof-of-concept studies, there was a robust and significant increase in the total number of lung lymphocyte aggregates in IL-10 KO mice as compared to both WT and SRA KO mice (Figure 2A), corroborating a central role for IL-10 in regulating post-inflammatory lung responses. Importantly, lung-delivered IL-10 therapy daily for 3 d post-HDE exposure resulted in significant reduction/resolution of lymphoid aggregates among WT, SRA KO and IL-10 KO animals.

Likewise, similar results were demonstrated with respect to lung lavage neutrophils (Figure 2B). Namely, the neutrophil percentage was increased in HDE-challenged SRA KO and IL-10 KO as compared to WT mice treated with saline control during the post-exposure interval and that lung-delivered rIL-10 treatment significantly reduced neutrophil burden. By microscopic review, the lung infiltrates were markedly greater in IL-10 KO > SRA KO > WT mice during the recovery period, and rIL-10 treatment in these mice resulted in a resolution equivalent to WT mice (Figure 2C).

HDE-induced TNFα and IL-10 is bi-directionally dependent in macrophages

HDE is known to enhance inflammatory cytokine production in mouse lung through a TNFα-dependent pathway (Wyatt et al. 2010); in the current studies, levels of IL-10 and TNFα formation in HDE-treated mouse lung macrophages were evaluated. To obtain adequate numbers, a murine alveolar macrophage cell line (MH-S) was used to investigate the dose- and time-dependency of cell TNFα and IL-10 responses to HDE. HDE stimulated a linear and significant increase in IL-10 release from MH-S cells beginning at 8 hr and reaching maximum detection levels by 24 hr (Figure 3A). The optimum concentration producing IL-10 release occurred at 1–2% HDE (Figure 3B), with higher concentrations (5%) resulting in decreased IL-10 release due to reduced cell viability. No cell death was observed with a 2% HDE treatment. Conversely, while similar HDE concentrations (1%–2%) produced the greatest TNFα release, TNFα was maximally elevated by 8 hr with significant decreases detected by 24 hr (Figures 3C and 3D). Pre-treatment of MH-S cells with mouse rIL-10 (10 ng/ml) prevented HDE-stimulated release of TNFα measured at 8 hr (Figure 3E). As a control, rIL-10 treatment was assayed along with HDE treatment at 24 hr (Figure 3F).

Similar results found in the alveolar macrophages were demonstrated using cultured primary peritoneal macrophages. HDE (1%) significantly stimulated a later (8–24 hr) release of IL-10 subsequent to the earlier HDE-mediated stimulation of TNFα (Figure 4A). Consistent

with *in vivo* observations (Figure 1), primary PM stimulated with HDE demonstrated significantly increased TNFα (Figure 4B) and decreased IL-10 (Figure 4C) levels at all timepoints in cells from the SRA KO mice compared to those from the WT mice.

Blocking SRA binding results in increased TNFα and decreased IL-10 production following HDE stimulation in macrophages

Whether SRA present on alveolar macrophages could be regulated to change HDE-mediated TNFα and IL-10 responses was next evaluated. Mouse alveolar macrophages (MH-S) were treated with the SRA ligand, fucoidan (1–10 μg/ml), in the presence or absence of 1% HDE for 8 hr (TNFα assay) and 24 hr (IL-10 assay). Fucoidan pre-treatment for 24 hr significantly enhanced HDE-stimulated TNFα production (Figure 5A), but dose-dependently decreased HDE-stimulated IL-10 release (Figure 5B). By itself, fucoidan did not stimulate any detectable levels of TNFα or IL-10. The negative control for fucoidan (i.e., fetuin) did not affect HDE-stimulated TNFα or IL-10 formation/release (data not shown).

HDE-induced PKCζ is key to mediating IL-10 release

Whereas protein kinase C (PKC)-α and -ε are involved in signal transduction of HDE-mediated inflammatory cytokine release (Wyatt et al. 2010), PKCζ has been implicated in IL-10 production (Foey and Brennan 2004) in other experimental settings. Here, PKCζ activity in response to HDE treatment was examined in mouse primary PM from WT, SRA KO, and IL-10 KO mice. PKCζ was activated 3-fold in response to 1% HDE at 6 hr treatment in PM from WT mice (Figure 6A). Fucoidan (10 μg/ml) also stimulated PKCζ at 1 hr, but did not additively change HDE-stimulated PKCζ activity when PM were exposed for an additional 6 hr. Pre-treatment with fucoidan for 24 hr, followed by 6 hr HDE, however, significantly prevented the activation of PKCζ. Moreover, pretreatment of PM for 1 hr with 1 μM of a cell-permeable inhibitor of PKCζ, i.e., myristolated PKCζ inhibitory peptide (myrZ), blocked HDE-stimulated PKCζ activity. Treating PM with rIL-10 either in the presence or absence of HDE did not change the observed PKCζ responses. Similarly, PM from IL-10 KO mice evidenced similar PKCζ activity responses to myrZ pre-treatment, 1 hr fucoidan stimulation, or 6 hr HDE treatment as seen in WT PM (Figure 6B). In contrast, no PKCζ activity increases were observed in primary PM from SRA KO mice stimulated with HDE for 6 hr or fucoidan for 1 hr compared to WT mice (Figure 6C).

To confirm that PKCζ could be activated in alveolar macrophages - and to connect this activation to HDE-stimulated production of IL-10, the role of PKCζ in cultured MH-S alveolar macrophages was investigated. The data show that HDE (1%) significantly and maximally stimulated PKCζ activity in MH-S cells after 1 hr (Figure 7A). While 1 hr pre-treatment with 1 μM of the pan-PKC isoform-inhibitor Calphostin C inhibited HDE-stimulated TNFα release, specific inhibition of PKCζ by myrZ had no effect on HDE-stimulated TNFα release (Figure 7B). Conversely, HDE stimulation of IL-10 release was significantly blocked by either the non-specific (Calphostin C) or specific (myrZ) inhibitors of PKCζ (Figure 7C).

IL-10 inhibits HDE-stimulated TACE activity in MH-S cells

It is established that HDE causes a release of TNFα through the activity of the TNFα converting enzyme (TACE; ADAM-17). Accordingly, this study also examined whether IL-10 regulated TACE activity in MH-S cells. Pre-treatment of these cells for 1 hr with 10 ng rIL-10/ml followed by 1% HDE treatment for 8 hr resulted in a 50% inhibition of TACE activity as compared to HDE treatment alone (Figure 8). Media control or rIL-10 alone produced no inhibition of TACE. Collectively, these data suggest that IL-10 can reduce HDE-mediated inflammation through the inhibition of TACE activity as regulated by SRA via PKCζ in the macrophage (Figure 9).

Discussion

Chronic inflammatory respiratory diseases including rhinosinusitis, asthma, bronchitis, and COPD affect approximately two-thirds of agriculture workers (Dosman et al. 2004; Merchant et al. 2005). Progressive symptoms of dyspnea, wheezing, cough, and airway flow limitation greatly add to the morbidity associated with farming (May et al. 2012). The numbers of animals in outdoor and enclosed spaces has risen to meet increased worldwide consumer demands (May et al. 2012). Despite modernization of facilities and improvements in outreach efforts, efficacious therapeutic options for reducing symptoms of airway disease are currently limited (Poole et al. 2012). Therapies such as inhaled corticosteroids with long-acting beta agonists have only been shown to have partial benefit when applied to exposed agricultural workers (Ek et al. 2005; Strandberg et al. 2008). An immediate need exists to understand the pathologic mechanism(s) responsible for lung disease in order to develop novel therapies to improve adverse respiratory consequences in exposed workers.

The study here identified four novel observations supporting a key role of IL-10 in the recovery response to organic dust-mediated lung inflammation. These included: 1) utilizing an established animal model (Poole et al. 2009), it was found that repetitive daily exposure to HDE for 8 d resulted in a significant accumulation of lung IL-10 as compared to control mice instilled with sterile saline; 2) HDE-induced IL-10 production was abrogated in SRA KO mice, strongly supporting a direct role for SRA in regulating IL-10 release in response to HDE; 3) because SRA is highly expressed on macrophages, analyses of isolated primary macrophages revealed that HDE-stimulated IL-10 production in macrophages from WT mice was significantly reduced in SRA KO macrophages [furthermore, macrophages isolated from SRA KO animals following 8 d of *in vivo* dust-treatment demonstrated a significant reduction in IL-10 production following *ex vivo* challenge as compared to control mice]; and, 4) these results were replicated in a murine alveolar macrophage cell line (MH-S) where HDE-stimulated release of TNFα could be significantly reduced by rIL-10 treatment. The inability of SRA KO animals to generate an anti-inflammatory and pro-resolving IL-10 signal to HDE treatment helps to explain prior published findings of a significant increase in adverse lung pathology in SRA KO mice as compared to WT mice following HDE exposure. Collectively, these data strongly support an essential role for SRA in regulating the IL-10 response to inhalant HDE treatment.

The IL-10 pathway is a clinically relevant pathway to modulate and target for enhancing repair and recovery consequences following organic dust-induced injury. IL-10 may

represent a “master switch” in regulating and promoting repair and recovery of agricultural dust-induced disease. It has been previously shown that repetitive agricultural organic dust exposure induces lung infiltrates composed of T-helper (T_H)-1/T_H17 cells and exudative/activated macrophages (Poole et al. 2012a,b). IL-10 is recognized to inhibit T_H17 cells and activated macrophages and a cytokine milieu rich in TNFα (Oft 2014), all of which are responses central to organic dust-induced airway disease. IL-10 production acts in a negative feedback manner to inhibit the expression and signaling of inflammatory cytokines and signaling of pattern recognition receptors (Hsiao et al. 2014).

In the mouse model here, it was shown that low levels of IL-10 remain present up to 1 wk post-repetitive organic dust exposure, and lung-delivered treatment with recombinant IL-10 accelerates recovery in WT, SRA KO and IL-10 KO mice. This response to IL-10 was most pronounced in mice (i.e. SRA KO and IL-10 KO) that had the greater degree of lung pathology. Indeed, treatment with exogenous IL-10 has been shown to be protective in a mouse model of endotoxemia of vascular shock, and human IL-10 has been used to benefit patients with psoriasis, hepatitis, Crohn’s disease, and potentially, cancer (Yamano et al. 2001; Asadullah et al. 2003; Loebbermann et al. 2012; Williams et al. 2015). Other studies have provided evidence that IL-10 is important in endotoxin-induced lung disease. Namely, treatment with chronic, high concentration-endotoxin (6–7 μg/m^3) in IL-10-deficient mice resulted in increased adverse airway injury outcomes (Quinn et al. 2000; Garantziotis et al. 2006). Furthermore, airway injury was reduced when human IL-10 expression was delivered by adenoviral vector treatment in the mouse lung (Garantziotis et al. 2006). These new studies here support the concept that lung-targeted therapy with IL-10 following an inflammatory-inducing exposure could represent a novel potential therapeutic approach.

IL-10 production is dependent on multiple pattern recognition receptor signaling pathways (e.g. TLR), with studies demonstrating that injections of bacterial LPS induce IL-10 (Murphey and Traber 2001). Once IL-10 is secreted, it binds to IL-10 receptors on both lung macrophages and epithelial cells, resulting in an “off switch” to reduce further inflammation (Asadullah et al. 2003). Studies in our laboratory have found, however, that HDE-induced IL-10 production in lung tissues was not dependent upon TLR4 signaling pathway as LPS-free preparations of HDE-stimulated macrophage IL-10 with equal magnitude (data not shown). This has led to investigations to determine whether other pattern recognition receptors might be involved in modifying the IL-10 response to organic dust. SRA (CD204), which has been implicated in facilitating a return to immune homeostasis after tissue inflammatory injury (Canton et al. 2003), has been shown by others to stimulate IL-10 production following ligand engagement (Jozefowski et al. 2014). SRA can also recognize and respond to a wide variety of bacterial ligands; moreover, SRA has been found to both positively and negatively modify TLR4 signaling events (Areschoug and Gordon 2009; Yu et al. 2011, 2012). It was previously shown that SRA is pivotal in the resolution of HDE-induced lung inflammation (Poole et al. 2015); the current study now shows that SRA exerts this influence through the regulation of IL-10. By blocking further binding to SRA using a 24 hr “saturation” with the SRA ligand, fucoidan, we observed reduced HDE-stimulated IL-10 release, but no decrease in TNFα production.

Downstream of SRA, the current data show that HDE stimulates PKCζ activation and IL-10 release is blocked by PKCζ Inhibitors. The PKC family is an important family of serine/threonine kinases of 11 isoforms based on substrate specificity, which are responsible for intracellular activation of diverse signaling pathways (Mellor and Parker 1998). Others have shown that the atypical isoform PKCζ regulates IL-10 production (Foey and Brennan 2004); this led the current study to examine its role in mediating HDE-induced IL-10. Indeed, it was found here that non-selective pan-inhibitors of PKC (such as Calphostin C) inhibited HDE-stimulated IL-10 release, but also blocked pro-inflammatory cytokine release as expected (Wyatt et al. 2010). However, using the PKCζ isoform-specific myristolated PKCζ inhibitory peptide, HDE-stimulated IL-10 was blocked, with no effect on stimulated TNFα release. Likewise, rapid fucoidan activation of PKCζ was also inhibited by myristolated PKCζ inhibitory peptide. As an atypical PKC, PKCζ lacks the phorbol ester binding region. It has been shown that phorbol esters such as PMA do not stimulate the release of IL-10 in macrophages (Boehringer et al. 1999). Similar to the current findings, evidence exists that endotoxin stimulates production of TNFα and IL-10 via separate pathways (Boehringer et al. 1999). A limitation of the current study is that PKCζ KO mice could not be used to complement the *in vivo* organic dust-induced TNFα findings; these animals have a known impairment in NF-κB activity (Leitges et al. 2001).

It was also previously demonstrated that decreasing release of TNFα reduces subsequent and sequential activation of inflammatory mediators underlying HDE-stimulated neutrophil influx and airway inflammation (Wyatt et al. 2010). Thus, it was not unexpected here that IL-10 production in response to HDE reduced TNFα production through inhibition of TACE (ADAM-17). This outcome was supported by previous findings showing that IL-10 binding to its receptor resulted in rapid inhibition of TACE as well as later induction of tissue inhibitor metallo-proteinase-3 (TIMP-3), a natural endogenous inhibitor of TACE (Brennan et al. 2008). Functional enzyme catalytic activity assays require a significant number of cells. A limitation of this study is that it was not possible to isolate primary lung macrophages at numbers necessary for either measures of TACE or PKC activity. As a result, the studies for now must rely on using primary PM and alveolar macrophage cell lines. Importantly, these cells yielded similar results.

The present findings justify an approach for the potential use of recombinant human IL-10 applications, exploitation of the SRA/PKCζ pathway, and identification of key human *IL10* genetic polymorphisms to target dust-induced lung diseases. Such studies could ultimately lead to improved approaches to impact on respiratory disease burdens in affected workers.

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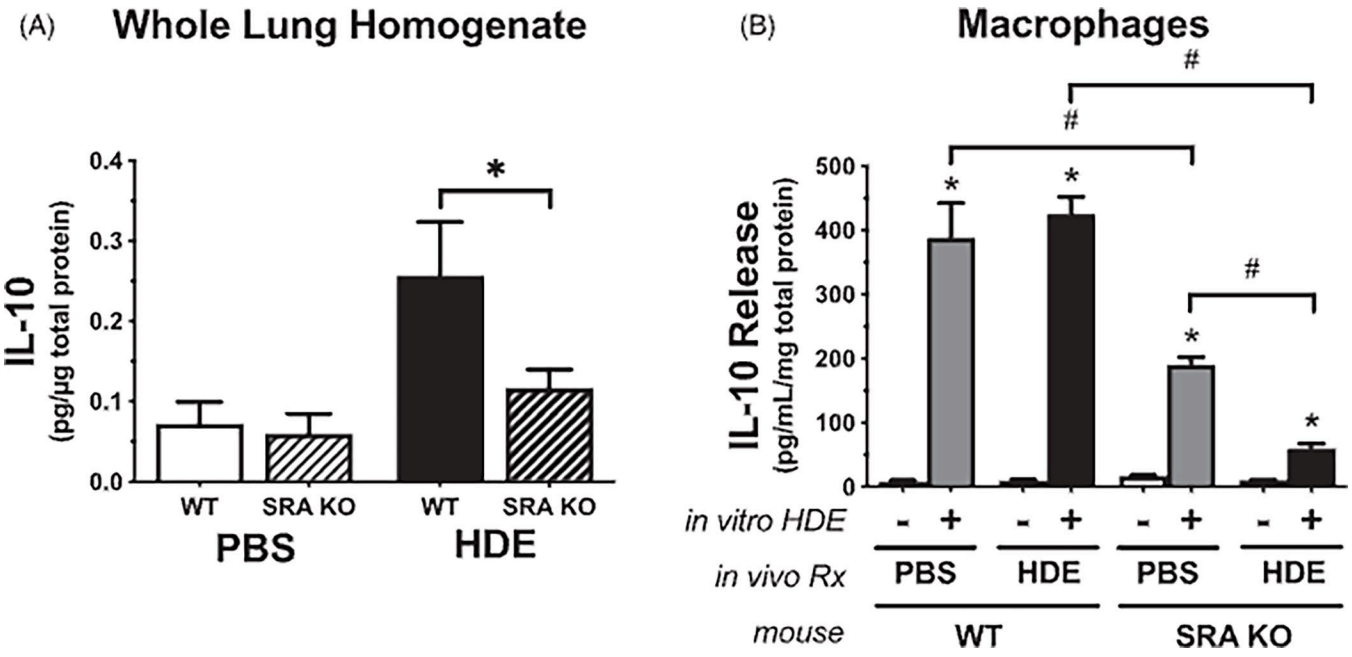

Figure 1. Repetitive daily exposure to organic dust extract (HDE) results in IL-10 release mediated through scavenger receptor A (SRA) signaling.

(A) Whole lung homogenate and **(B)** peritoneal macrophages were measured from mice nasally instilled for 8 d with 12.5% HDE or sterile saline (PBS) and IL-10 measured by ELISA. SRA KO mice demonstrate reduced HDE-stimulated IL-10 release from total lung as well as isolated macrophages treated with 1% HDE *in vitro*. *$p < 0.02$ WT vs SRA KO **(A)**. *$p < 0.001$ HDE vs no HDE. $^{\#}p < 0.05$ WT vs KO and PBS vs HDE KO macrophages **(B)**. N = 10 mice/treatment condition.

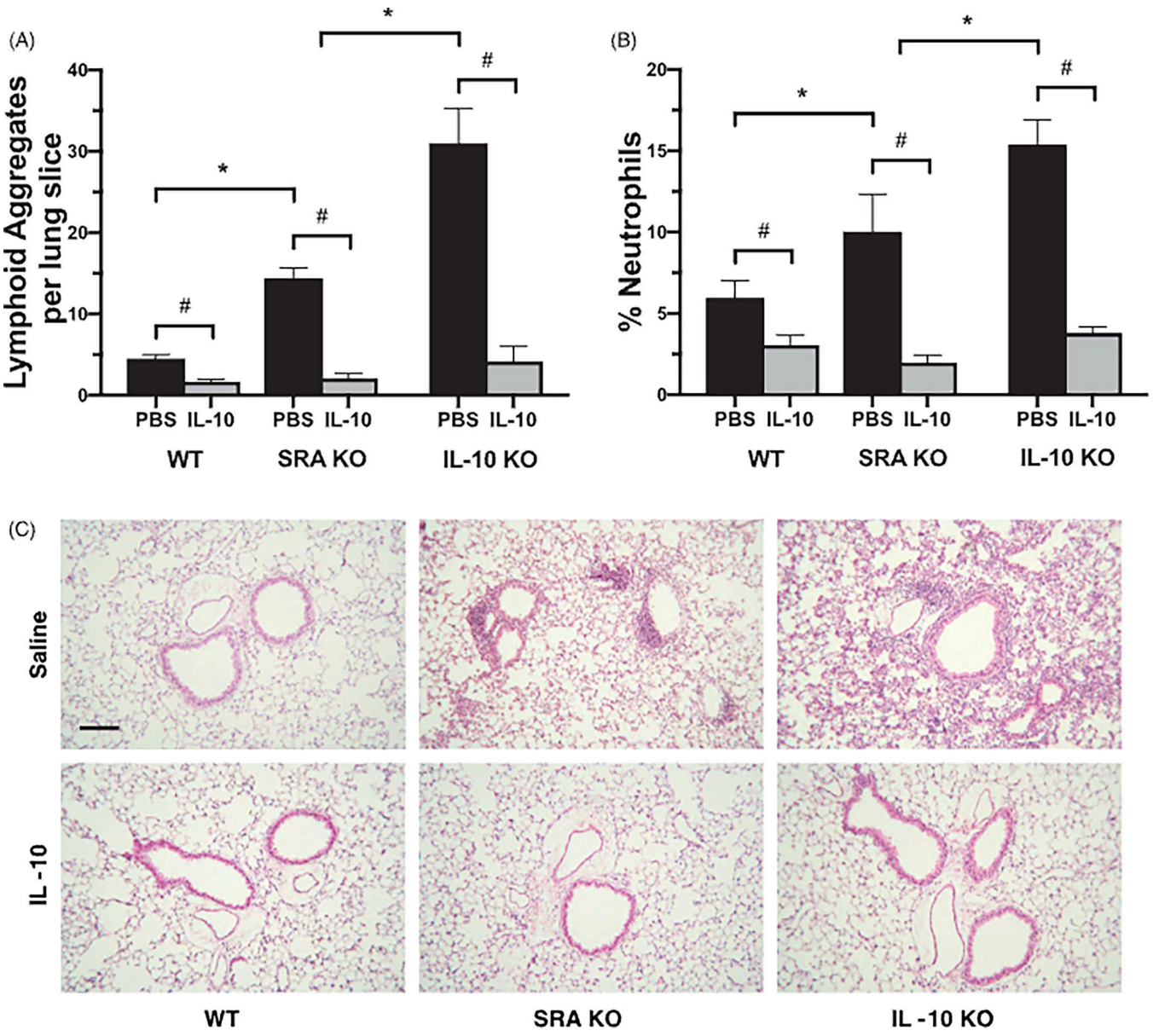

Figure 2. IL-10 treatment hastens recovery from HDE-induced lung inflammation in SRA KO and IL-10 KO mice.

All mice were exposed daily for 8 d with 12.5% HDE, then treated with intranasal PBS or IL-10 for 3 d. **(A)** Lung lymphoid aggregates and **(B)** lavage neutrophils were enumerated. SRA KO and IL-10 KO impaired recovery following HDE exposure is reversed by IL-10 treatment. Representative H&E stained lung sections corresponding to treatment conditions as defined **(C)**. *$p < 0.001$ WT vs SRA KO; SRA KO vs IL-10 KO and $^{\#}p < 0.05$ PBS vs IL-10 **(A)**. *$p < 0.05$ WT vs SRA KO; SRA KO vs IL-10 KO and $^{\#}p < 0.002$ PBS vs IL-10 **(B)**. Bar = 100 μm (C). N = 10 mice/treatment condition.

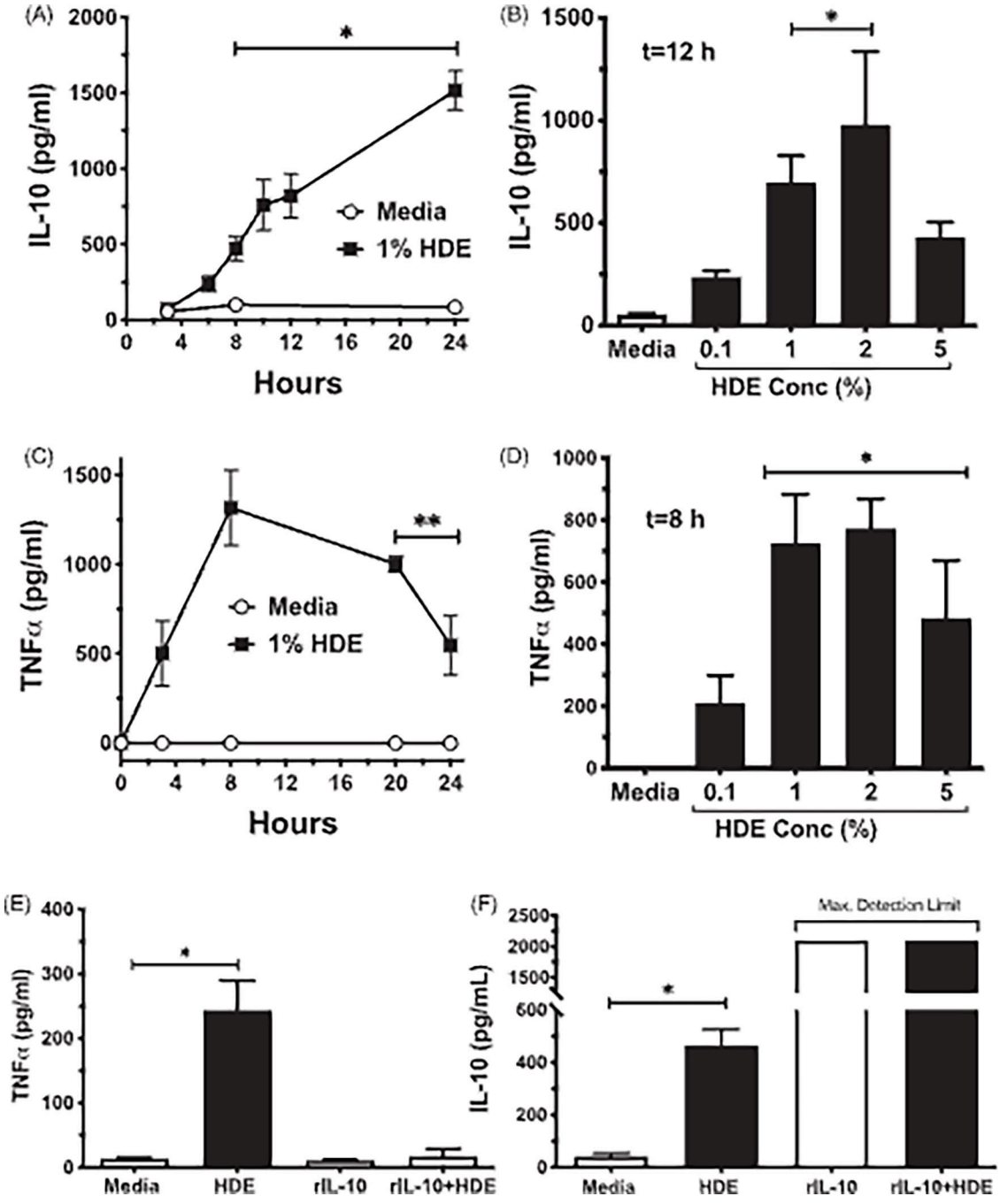

Figure 3. HDE stimulated release of IL-10 and TNFα in murine alveolar macrophages.

Mouse alveolar macrophage cell line (MH-S) was treated with 0.1–5% HDE for up to 24 hr and media release of IL-10 and TNFα measured by ELISA. **(A)** Significant increases in IL-10 were observed from 8–24 hr treatment with 1% HDE. **(B)** Optimal dose of HDE for IL-10 release was determined to be 1–2%. **(C)** While maximal release of TNFα occurred at 8 hr with 1–5% HDE treatment **(D)**, a significant decrease in release was observed at 20–24 hr, coincident in time with maximal IL-10 release. Pre-treatment with recombinant mouse IL-10 (rIL-10; 10 ng/ml; 1 hr) completely blocked HDE-stimulated TNFα release **(E)** when

used at above endogenous cell levels **(F)**. *p < 0.001 vs Media control. **p < 0.05 vs HDE at 8 hr. N = 9 independent measurements/treatment condition.

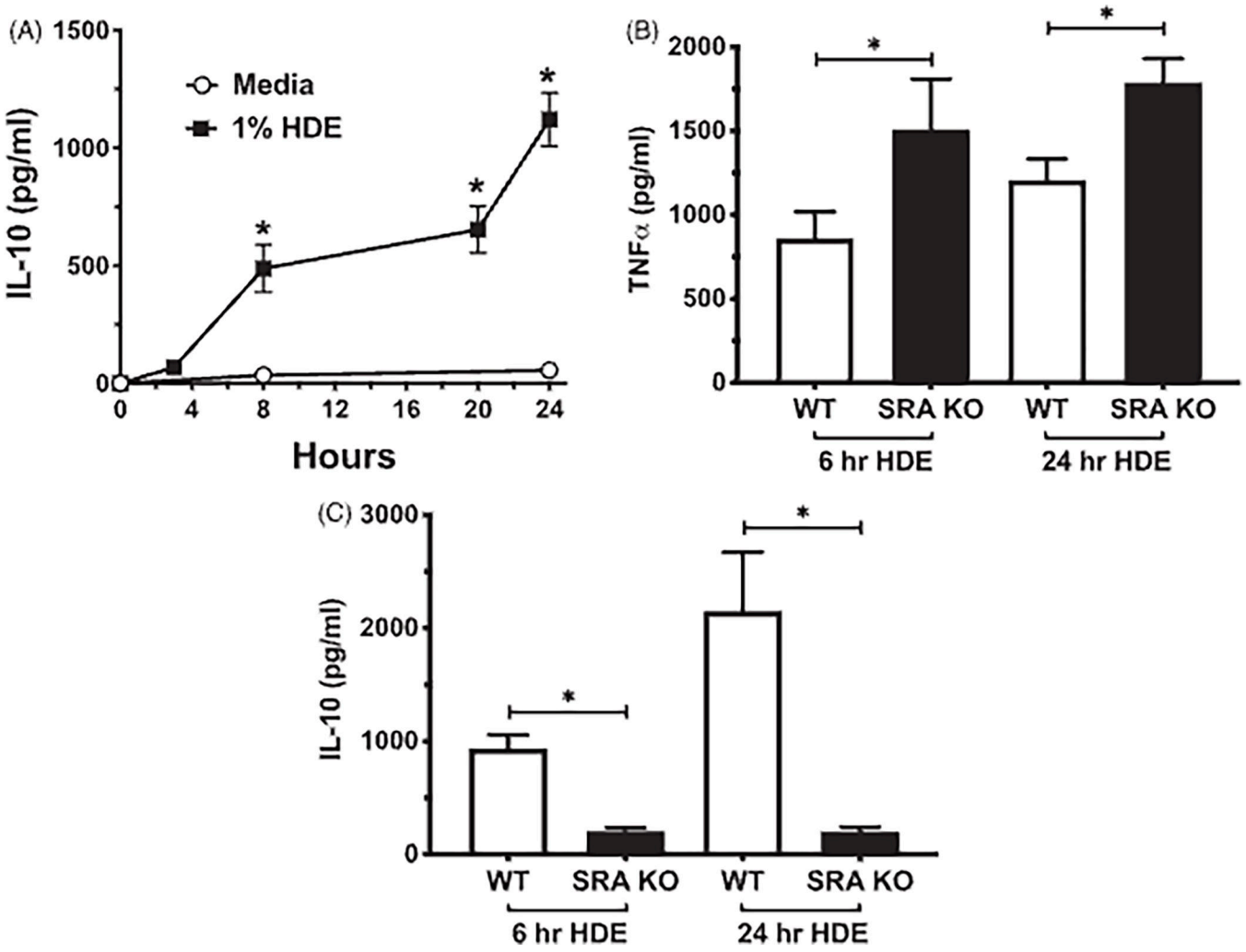

Figure 4. HDE-induced TNFα and IL-10 release in mouse primary peritoneal macrophages (PM) is dependent on SRA signaling with increased TNFα and decreased IL-10.

Significant increases in IL-10 were observed from 8–24 hr *in vitro* treatment with 1% HDE in PM from wild-type mice **(A)**. HDE-stimulated TNFα release was significantly elevated **(B)** and IL-10 significantly decreased **(C)** in *in vitro* treated PM from SRA KO vs wild type PM. *$p < 0.01$ vs Media control **(A)**. *$p < 0.05$ WT vs SRA KO **(B,C)**. N = 9 independent measurements/treatment condition.

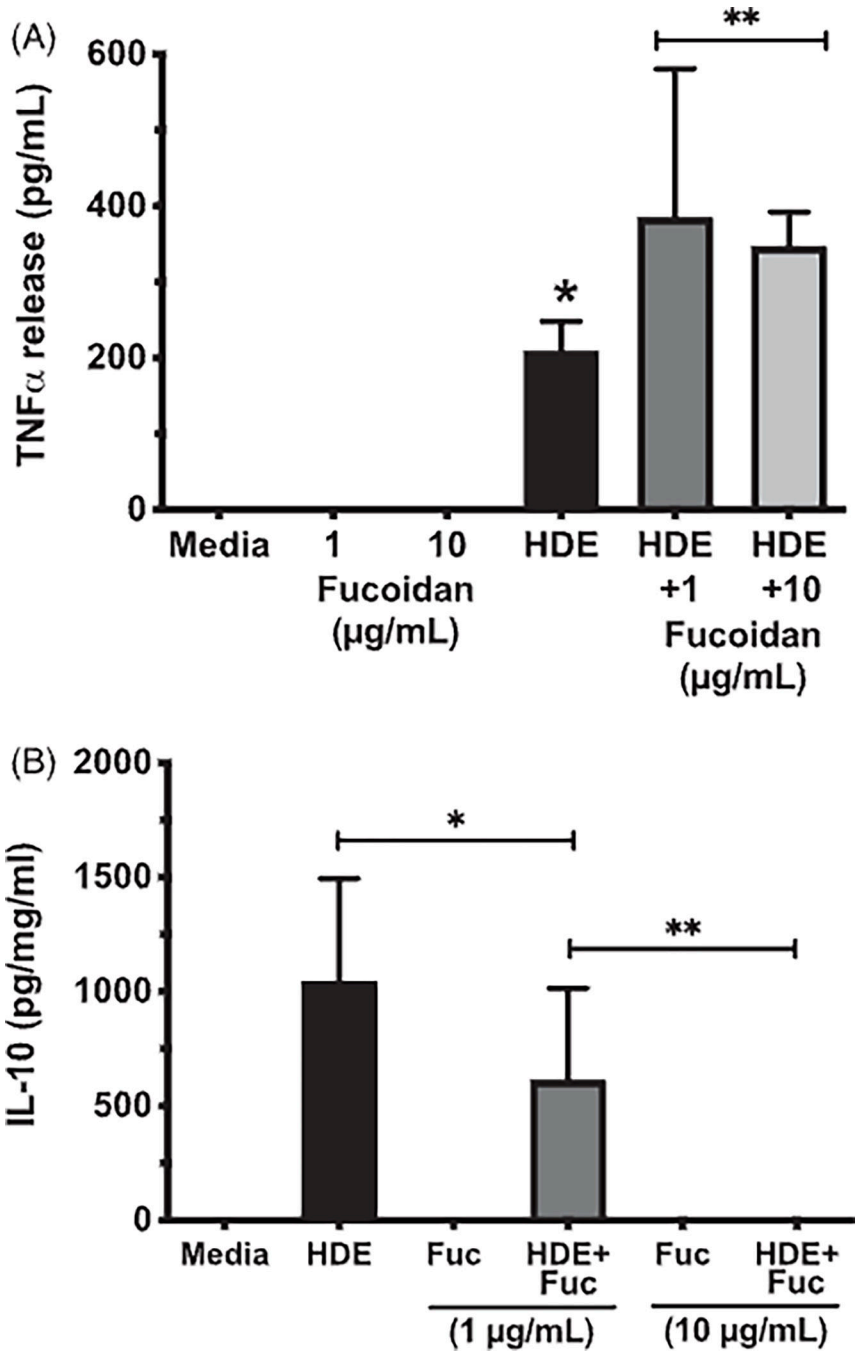

Figure 5. Blockade of SRA binding via fucoidan pre-treatment results in increased TNFα and decreased IL-10 release following HDE stimulation in murine alveolar macrophages.

Pre-treatment of MH-S cells for 24 hr with 1–10 μg/ml fucoidan (Fuc) prior to 1% HDE stimulation resulted in a significant increase in TNFα release at 8 hr **(A)** and significant decrease in IL-10 at 24 hr **(B)**. *$p < 0.001$ vs Media control and **$p < 0.05$ vs HDE only at 8 hr **(A)**. *$p < 0.05$ HDE vs 1 μg/ml Fuc+HDE and **$p < 0.01$ 1 vs 10 μg/ml Fuc+HDE **(B)**. N = 9 independent measurements/treatment condition.

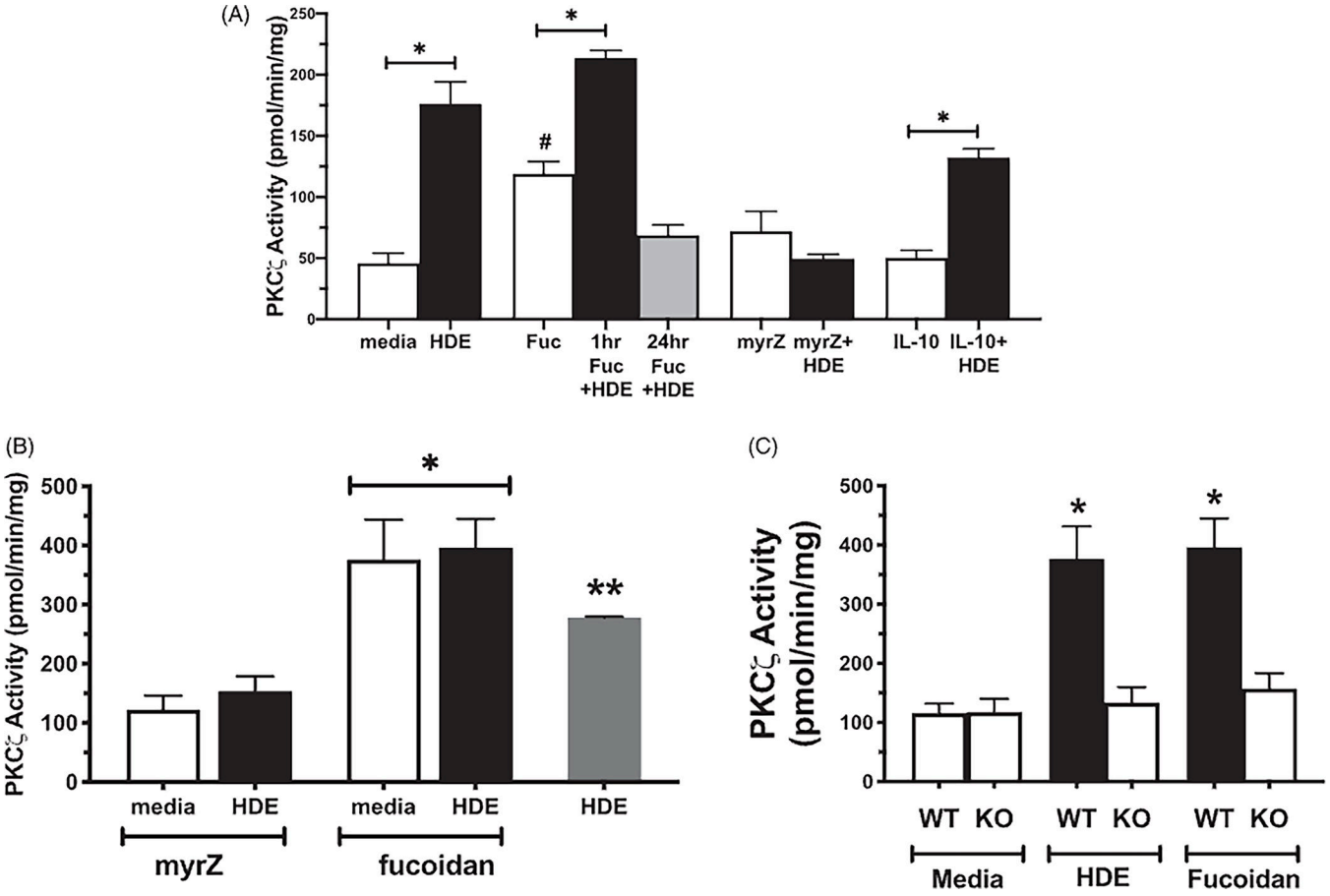

Figure 6. HDE stimulates protein kinase C (PKC)-ζ activation in mouse primary peritoneal macrophages (PM).

PKCζ was activated by 1% HDE at 6 hr and 10 μg/ml fucoidan (Fuc) at 1 hr treatment **(A)**. Combination of Fuc and HDE produced no additive effect. Pretreatment of PM with Fuc for 24 hr or 1 μM myristolated PKCζ inhibitory peptide (myrZ) blocked HDE-stimulated PKCζ activity. rIL-10 had no significant effect on HDE-stimulated increase in PKCζ. PM from IL-10 KO mice demonstrated similar PKCζ activity responses to myrZ pretreatment, 1 hr fucoidan stimulation, or 6 hr HDE treatment **(B)**. HDE did not stimulate PKCζ activity in PM from SRA KO mice *in vitro* stimulated with 6 hr HDE or 1 hr fucoidan vs wild-type mice (C). *$p < 0.01$ vs matched control and $^{\#}p < 0.05$ vs media **(A)**. *$p < 0.05$ fucoidan vs myrZ and **$p < 0.01$ HDE vs myrZ+HDE **(B)**. *$p < 0.01$ WT vs SRA KO under same treatment **(C)**. N = 9 independent measurements/treatment condition.

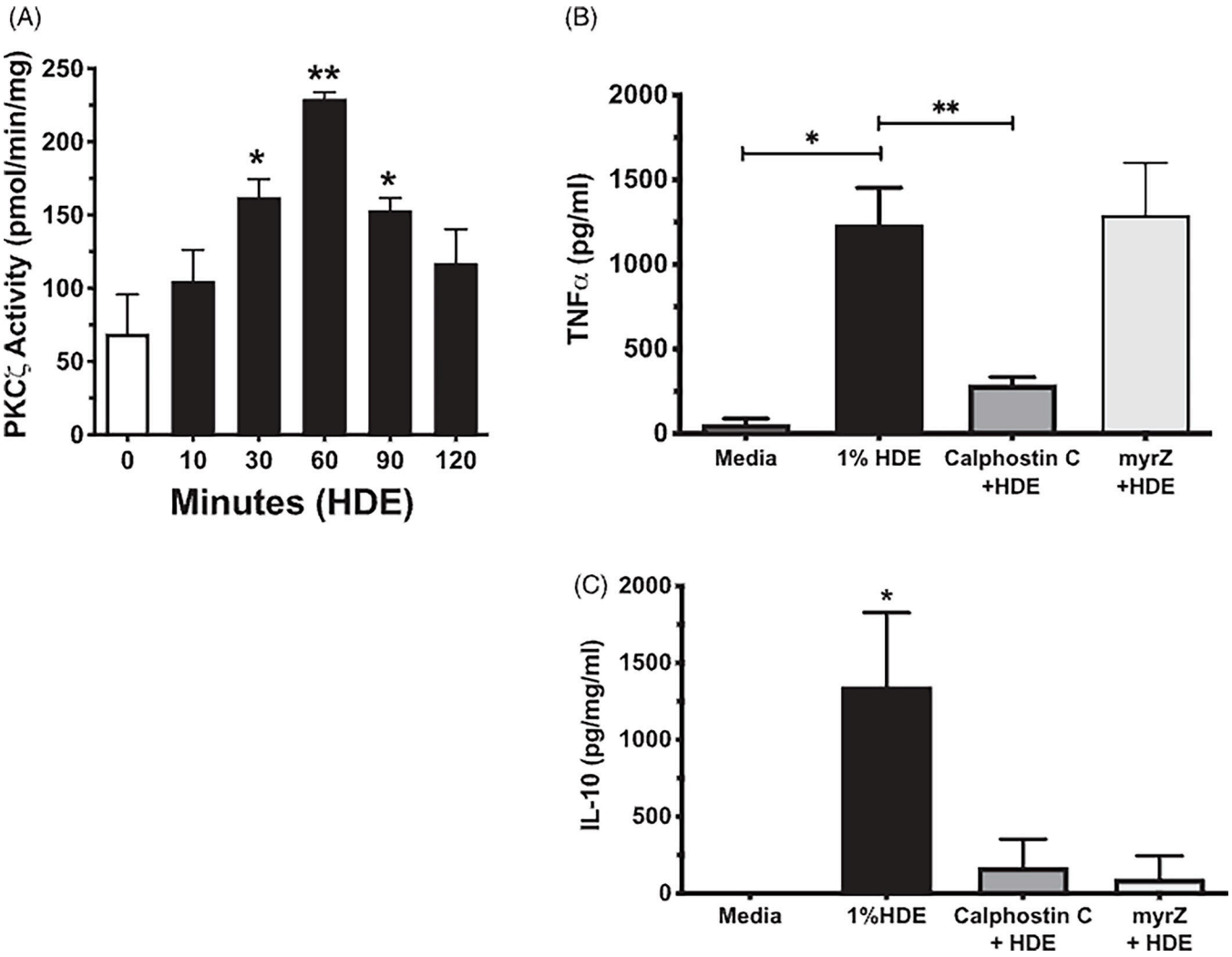

Figure 7. Inhibition of PKCζ reduces IL-10 release.

PKCζ was transiently activated by 1% HDE beginning at 0.5 hr in MH-S cells **(A)**. Pre-treatment with 1 μM Calphostin C for 1 hr inhibited HDE-stimulated TNFα release, but specific inhibition of PKCζ by 1 μM myrZ for 1 hr did not **(B)**. Inhibition of all PKC isoforms (Calphostin C) or just PKCζ (myrZ) blocked HDE-stimulated IL-10 release **(C)**. *$p < 0.05$ vs 0 min and **$p < 0.001$ vs 0 min (A). *$p < 0.01$ vs Media control and **$p< 0.05$ vs Calphostin C+HDE **(B)**. *$p < 0.01$ vs all treatments (C). N = 9 independent measurements/treatment condition.

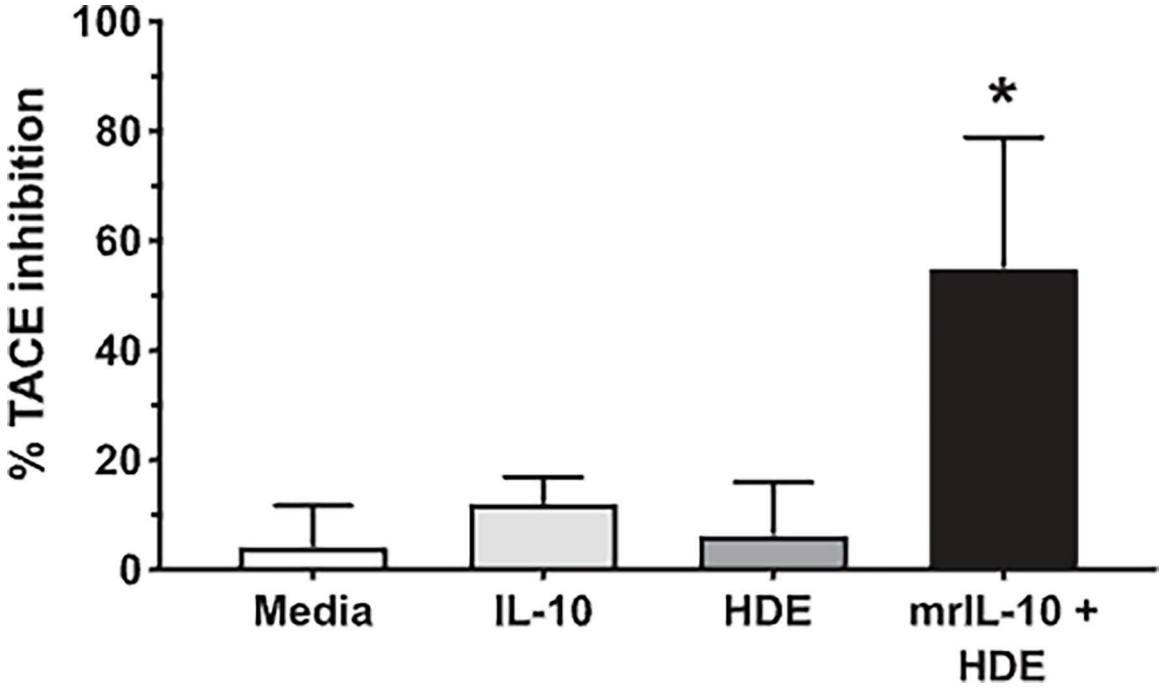

Figure 8. IL-10 inhibits HDE-stimulated TACE activity in MH-S cells.

Pretreatment of cells for 1 hr with 10 ng/ml rIL-10 followed by 1% HDE treatment for 8 hr significantly (*$p < 0.01$ vs HDE) inhibited TACE activity. N = 9 independent measurements/ treatment condition.

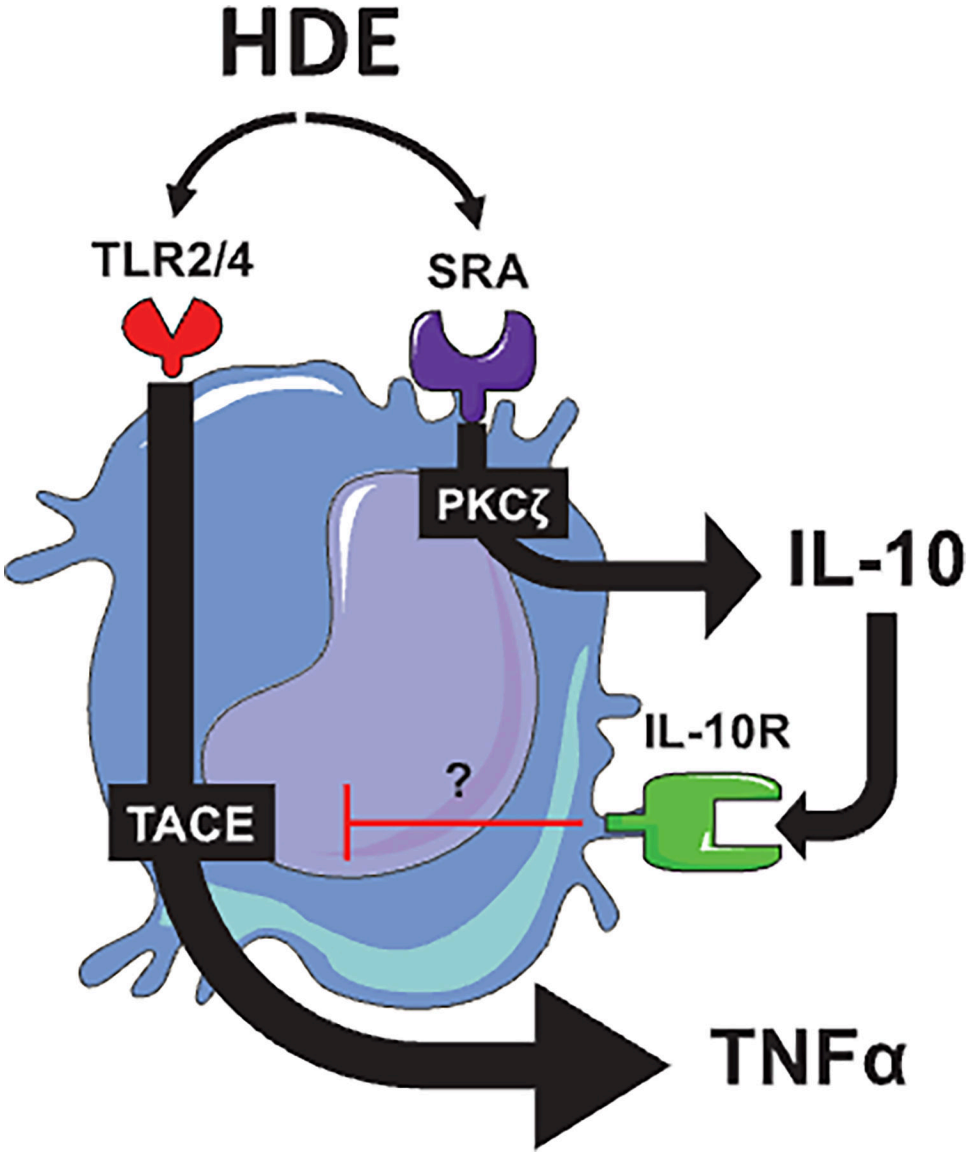

Figure 9.
Model diagram of HDE-mediated IL-10 regulation of TNFα release in macrophages.