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Translocation of Endogenous Danger Signal HMGB1 From Nucleus to Membrane Microvesicles in Macrophages

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Abstract

High mobility group box 1 (HMGB1) is a nuclear protein that can be released from activated or dead cells. Extracellular HMGB1 can serve as a “danger signal” and novel cytokine that mediates sterile inflammation. In addition to its soluble form, extracellular HMGB1 can also be carried by membrane microvesicles. However, the cellular mechanisms responsible for nuclear HMGB1 translocation to the plasma membrane and release onto membrane microvesicles have not been investigated. Tobacco smoking is a major cause of sterile inflammation in many diseases. Smoking also increases blood levels of HMGB1. In this study, we found that exposure of macrophages to tobacco smoke extract (TSE) stimulated HMGB1 expression, redistribution, and release into the extracellular milieu both as a soluble molecule and, surprisingly, as a microvesicle-associated form (TSE-MV). Inhibition of chromosome region maintenance-1 (CRM1), a nuclear exporter, attenuated TSE-induced HMGB1 redistribution from the nucleus to the cytoplasm, and then its release on TSE-MVs. Our study demonstrates a novel mechanism for the translocation of nuclear HMGB1 to the plasma membrane, and then its release in a microvesicle-associated form.

High mobility group box 1 (HMGB1) is a nuclear chromosomal protein with DNA binding domains that is important in maintaining DNA structure in the nucleus (Degryse et al.,

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2001). HMGB1 can also be released to the extracellular milieu in soluble form from activated monocyte/macrophages or dead cells (Andersson and Tracey, 2011). Once released, extracellular HMGB1 serves as an endogenous danger signal and a novel proinflammatory cytokine that initiates innate immune responses (Andersson and Tracey, 2011) and has chemotactic properties (Fiuza et al., 2003; Rouhiainen et al., 2004). HMGB1 is involved in broad range of human diseases that have either acute or chronic inflammatory responses (Tsung et al., 2014). In addition to its soluble form, a few recent studies from our and other groups reported that extracellular HMGB1 can also be carried by membrane microvesicles (MV) (Chen et al., 2012; Schiller et al., 2012; Pisetsky, 2014). MVs (also known as microparticles) are micron-scale bilayer membrane vesicles that not only harbor membrane surface molecules but also carry some intracellular molecules acquired from their parent cells during the membrane budding process (Morel et al., 2004; Ratajczak et al., 2006; Liu et al., 2007; Liu and Williams, 2012; Kim et al., 2015). Accumulating studies from our and other groups indicate that MVs carry various bioactive molecules that mediate intercellular communications, and play important roles in the development of various human diseases (Morel et al., 2004; Ratajczak et al., 2006; Liu et al., 2007; Liu and Williams, 2012; Kim et al., 2015). In contrast to soluble HMGB1, which might be quickly diluted by the large pool of circulating blood, MV-associated HMGB1 may exist longer with relatively higher concentrations (Liu and Williams, 2012) in the microenvironment of inflamed tissue, and thus might be particularly important in the pathogenesis of the inflammatory diseases (Liu and Williams, 2012; Buzas et al., 2014). However, the cellular mechanisms responsible for translocation of HMGB1 from the nucleus to the cell plasma membrane, and then subsequent release with microvesicles still remain unclear.

Damage-associated molecular patterns (DAMPs), also known as endogenous danger signals, provide a major stimulus for helpful or harmful immune responses (Seong and Matzinger, 2004; Liu et al., 2012) to sterile tissue insults, such as injury, tobacco smoking, or UVB exposure (Tsung et al., 2007; Chen et al., 2012; Gallo and Bernard, 2014; Heijink et al., 2015). The danger signals from damaged or dead cells guide leukocytes to the sites of sterile inflammation, and contribute to many pathologic conditions or human diseases (Chen and Nunez, 2010; Shen et al., 2013). Tobacco smoking is a major cause of sterile inflammation (You et al., 2015) in various inflammatory diseases in humans (Johannsen et al., 2014; Yuan et al., 2015; Perricone et al., 2016). As a DAMP molecule, HMGB1 is elevated in the blood in humans and animals that are exposed to tobacco smoke (Saiwichai et al., 2010; Krakowiak et al., 2015). Tobacco smoke exposure induces HMGB1 expression in macrophages both in humans and animals (Ferhani et al., 2010; Bezerra et al., 2011). Given that over a billion people smoke worldwide, and even more are exposed to secondhand smoke, tobacco smoking plays a significant, deadly role in many human diseases, not only respiratory and cardiovascular diseases, and cancer, but also metabolic and autoimmune diseases (Domagala-Kulawik, 2008; Arnson et al., 2010; Chen et al., 2012; Liu et al., 2014; Li et al., 2015).

In the current study, we established a cell-culture model to explore the effects of tobacco smoke extract (TSE) exposure on the redistribution and release of nuclear HMGB1 from macrophages, into the extracellular milieu in both soluble and microvesicle-associated form. Inhibition of CRM1 interfered with the translocation of HMGB1 induced by TSE from the

nucleus to the cytoplasm, and the subsequent release from the cell membrane onto TSE-MVs.

Materials and Methods

Reagents and antibodies

Monoclonal antibodies (mAb) against human HMGB1 (R&D Systems), phycoerythrin (PE)-labeled annexin-V (BD Pharmingen), and CRM1 inhibitor III (Calbiochem) were purchased. Tobacco smoke extract (TSE, 100%) was prepared by extracting mainstream smoke from lighted research cigarettes through phenol red-free RPMI-1640 medium containing 0.2% BSA (RPMI/BSA), then filtered (0.22- μ m) and standardized according to its absorbance at 320 nm as described (Li et al., 2010, 2013).

Cell culture, differentiation, and treatment

Macrophages were differentiated from human THP-1 monocytes by exposure to 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 h, followed by a 48-h wash-out period in fresh RPMI-1640/10% FBS, without PMA, to minimize ongoing effects of PMA (Li et al., 2013). At the beginning of each experiment, macrophages were transferred to serum-free RPMI/BSA supplemented with 0% (control) to 2.5% TSE (v:v, approximately equivalent to a 60-kg person smoking two-packs of cigarettes) similar to our previous publications (Li et al., 2010, 2013; Folkesson et al., 2015), for 0–24 h at 37°C. In experiments using inhibitors, the compounds were added to cells 1 h before the addition of buffer or TSE and remained until the end of the study, at concentrations of 150 μ M for glycyrrhizin and 2.5 μ M for CRM1 inhibitor III. TSE (100%) with water-soluble components was prepared by using a Kontes gas-washing bottle to bubble mainstream smoke from research cigarettes through serum-free, phenol red free RPMI media containing 0.2% BSA (RPMI/BSA), then filtered (0.22 μ m) and standardized according to their absorbance at 320 nm as in our published protocols (Li et al., 2010, 2013; Folkesson et al., 2015).

Precipitation of soluble HMGB1 in conditioned medium

Soluble HMGB1 from the conditioned media of TSE-treated macrophages was concentrated by precipitation with PTA/TCA as described before with modification (Caldwell and Lattemann, 2004; Tsuchiya et al., 2010). In brief, one volume of PTA (1%), two volume of TCA (20%), and seven volume of conditioned medium (1:2:7 ratio for total 1 ml) were mixed, and incubated on ice for 30 min. Then the mixture was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was carefully removed. The pellet was washed for twice with 300 μ l cold acetone following by the above vortex/spin process. To dissolve the pellet at the bottom of tube completely in acetone, the tube was vortexed thoroughly. Finally, the pellet was dried by heating at 95°C for 10 sec to evaporate the acetone. For immunoblotting, the pellet was dissolved in loading buffer (including 50 mM Tris-HCl, pH 8.8, 2% SDS, 100 mM DTT, 10% Glycerol, and 0.01% bromophenol blue).

Immunoblotting

After treatment of macrophages without or with TSE, conditioned medium was harvested and centrifuged twice at 1,500g for 10 min to eliminate cells and large debris, then

ultracentrifuged at 100,000*g* for 30 min at 4°C to pellet MVs (Li et al., 2013). Immunoblotting was conducted as previously described with modifications (Li et al., 2010, 2013). In brief, cells, isolated MVs, or soluble HMGB1 precipitated from conditioned medium was lysed or homogenized in a buffer containing 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM of protein tyrosine phosphatase inhibitor Na₃VO₄ (an inhibitor of protein tyrosine phosphatases), and a commercial protease inhibitor cocktail (Roche) for 30 min on ice. Samples (50–80 mg protein/lane) were electrophoresed through a 4–15% precast polyacrylamide gradient gel (BioRad), followed by the transfer of proteins to nitrocellulose membranes, and then immunoblotting as described before (Li et al., 2010, 2013) with anti-human HMGB1 primary antibody (R&D). Bands were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, MA). Signals were quantified using ImageJ version 1.45 densitometry software and normalized in each independent experiment to values from control cells (Li et al., 2010, 2013).

Flow cytometry of cells and membrane microvesicles

Flow cytometry of MVs and cells was performed according to our published methods (Liu et al., 2007; Li et al., 2010, 2013). In brief, culture supernatants were fixed in filtered 1% paraformaldehyde (PFA), and supplemented with counting beads. The number of MVs in each sample was quantified using a FACSCalibur flow cytometer, with gating criteria based on particle size and surface exposure of PS, detected by PE-labeled annexin-V (BD Pharmingen) staining (Liu et al., 2007; Li et al., 2010, 2013). The portion of PS-positive MVs that were also HMGB1-positive was quantified by simultaneous staining with mouse anti-human HMGB1 antibody (R&D), followed by FITC-labeled goat anti-mouse IgG secondary antibody (Abcam). After treatment without or with TSE, adherent monolayers of macrophages were detached and fixed by suspension in 1% PFA for detection of cell-surface HMGB1 with flow cytometry (Li et al., 2013).

Regular fluorescent and confocal fluorescent microscopic analysis

For regular fluorescence or confocal fluorescence microscopy, cultured adherent macrophages were incubated without (control) or with 2.5% TSE for 24 h at 37°C, and then fixed on the plates with 4% paraformaldehyde in PBS on ice for 10 min. The fixed cells were then washed twice with PBS, followed by a blocking solution (1% BSA and 0.1% Triton X-100 in PBS) for 60 min at room temperature for staining of the intracellular molecules. For the purpose of staining the cell surface HMGB1 only, the fixed cells were blocked without permeabilization by using blocking solution (1% BSA in PBS) that does not contain detergent. Thereafter, the cells were incubated simultaneously with primary anti-human HMGB1 antibody (R&D), and PE-labeled annexin V in the presence of 1 mM CaCl₂, in the dark at 4°C overnight. The cells were washed 3 times with 0.2% BSA in PBS to remove unbound primary antibody and incubated with a FITC-labeled goat anti-mouse IgG secondary antibody (1:2,000) in PBS with 1% of BSA for 1 h. The stained cells were analyzed with a Leica TCS SP5 fluorescent confocal microscope (Li et al., 2013, 2015).

Statistical analysis

Normally distributed data are shown as means $\pm$ SEM (n $\geq$ 4–6). Comparisons among three or more groups were performed using one-way analysis of variance (ANOVA), followed by pairwise comparisons using the Student–Newman–Keuls (SNK) test, with $P < 0.05$ considered significant. Comparisons between two groups used the Student's unpaired, two-tailed t -test. Comparisons between two groups of non-normally distributed data used the Mann–Whitney rank-sum test.

Results

Exposure of macrophages to tobacco smoke extract stimulates HMGB1 expression and increases its release into the extracellular milieu

We found that exposure of macrophages to TSE gradually upregulated HMGB1 content in the cells in the first 8 h (Fig. 1A). In parallel, the amount of extracellular release of HMGB1 in the conditioned medium also showed a trend of increase in the same period of time (Fig. 1B). By 24 h after TSE exposure, the amount of HMGB1 in the conditioned medium was substantially increased (Fig. 1B), while the cellular content of HMGB1, however, returned to baseline levels (Fig. 1A), suggesting the extracellular release of all HMGB1 stimulated by TSE.

Analysis of the distribution of soluble and microvesicle-associated forms of extracellular HMGB1 in the conditioned medium of TSE-stimulated macrophages

Interestingly, we found that MVs pelleted from conditioned medium contains a significant amount of HMGB1 (Fig. 2). These results indicate that HMGB1 released from TSE-stimulated macrophages exists as both soluble and MV-associated forms (Fig. 2). To analyze the distribution of the two forms of extracellular HMGB1 released from macrophages, we developed a methodology by using immunoblot analysis of: (i) protein precipitated from pre-ultracentrifugation conditioned medium (total HMGB1); (ii) protein precipitated from post-ultracentrifugation conditioned medium (soluble HMGB1); (iii) MV lysates (MV-associated HMGB1) (Fig. 2). When we performed immunoblot analysis (Fig. 2), the amount of protein from each fraction of the above-mentioned extracellular HMGB1 was applied into the electrophoresis proportionally, to allow us to calculate the distribution comparably (Chen et al., 2012, 2013). Our results showed that the major portion of extracellular HMGB1 released from TSE-exposed macrophages is in a soluble form, and a minor portion of it is carried by membrane microvesicles (Fig. 2).

Exposure of macrophages to TSE induces HMGB1 translocation from the nucleus to the cytosol and plasma membrane, and then budding with cell membrane microvesicles

HMGB1 is a nuclear protein that can be released to the extracellular milieu as a soluble form from activated monocyte/ macrophages or dead cells (Andersson and Tracey, 2011). To understand how nuclear HMGB1 is translocated and released, we conducted a time course to determine the effects of TSE on HMGB1 redistribution. We analyzed permeabilized macrophages under fluorescence microscopy and found that intracellular HMGB1 was predominately located in the nuclei of resting macrophages (Fig. 3A, 0 h of TSE exposure).

After 4 h of exposure to TSE, HMGB1 had been gradually translocated from the nucleus to the cytoplasm and plasma membrane. After 8 h of TSE exposure the majority of HMGB1 was out of the nucleus and translocated to the cytoplasm and cell membrane. By 24 h a portion of the HMGB1 was released with MVs from budding plasma membrane (Fig. 3A). To further determine the relationship between HMGB1 and the plasma membrane, we confirmed the presence of HMGB1 on the plasma membrane surface when we analyzed non-permeabilized, TSE-treated macrophages by visualization with both ordinary and confocal fluorescent microscopy (Fig. 3B), and by flow cytometry (Fig. 3C). In addition, confocal microscopic analysis showed that HMGB1 and exteriorized phosphatidylserine (PS) were colocalized and concentrated in small, circumscribed domains (arrows in Fig. 3D) on the surface of fixed, TSE-treated macrophages. These domains appear to be budding MVs that are about to be, or already released, from the cell surface (Fig. 3B,D). These results indicate that exposure of macrophages to TSE induces the translocation of nuclear HMGB1 into the cytosol and plasma membrane, and then this HMGB1 is released either as soluble molecules or as a MV-associated form into the extracellular milieu (Fig. 2).

The nuclear exporter CRM1 is required for HMGB1 translocation from the nucleus to the cytoplasm, and release as soluble and MV-associated forms from macrophages exposed to TSE

We next investigated the potential mechanisms that regulate HMGB1 translocation from the nucleus to the cytoplasm and plasma membrane. Chromosome region maintenance 1 (CRM1, also known as exportin-1, XPO1) is a nuclear exporter receptor involved in the nuclear export of cargo proteins (Turner et al., 2012). HMGB1 is a nuclear chromosomal protein. CRM1 has been shown to mediate nuclear HMGB1 redistribution to the cytoplasm, and secretion to the extracellular space in TNF α -stimulated macrophages (Tang et al., 2007). In the current study, we found that both HMGB1 and CRM1 were concentrated in the nuclei of the resting control cells (left side of Fig. 4A), while exposure to TSE for 8 h provoked the translocation of both HMGB1 and CRM1 into the cytoplasm (right side of Fig. 4A). To examine their functional relationship, we found that pretreatment of macrophages with CRM1 inhibitor III attenuated TSE-induced translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 4B) and inhibited the release of HMGB1 into the extracellular milieu as either soluble HMGB1 or MV-associated HMGB1 (Fig. 4C).

Discussion

HMGB1 is abundantly present in the nuclei of almost all eukaryotic cells and serves as a nonhistone chromatin-binding protein for stabilization of chromatin structure and modulation of gene transcription (Tsong et al., 2014; Yanai and Taniguchi, 2014). In addition to its role in the nucleus, HMGB1 can be translocated to the cytosol and secreted into the extracellular space from activated monocyte/macrophages or dead cells (Andersson and Tracey, 2011). Extracellular HMGB1 has drawn increasing attention because it has been shown to activate multiple proinflammatory receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR) 2, TLR4, and TLR9 (Tsong et al., 2014; Yanai and Taniguchi, 2014), which are involved in immune responses and the

development of various inflammatory diseases (Sims et al., 2010; Andersson and Tracey, 2011; Tsung et al., 2014).

In line with our study, published work has shown that tobacco smoke exposure induces HMGB1 expression in macrophages from humans and animals (Ferhani et al., 2010; Bezerra et al., 2011) and also causes an elevation of blood levels of HMGB1 in humans and animals (Saiwichai et al., 2010; Krakowiak et al., 2015). In addition to the soluble form of HMGB1 that has been studied in most published work (Nativel et al., 2013; Tsung et al., 2014), a few recent studies from our and other groups reported that extracellular HMGB1 can also be carried by membrane MVs (Chen et al., 2012; Schiller et al., 2012; Pisetsky, 2014). In the current study, we established a cell-culture model to analyze the process of translocation of nuclear HMGB1 to the cytosol and plasma membrane, and then its release into the extracellular space either as soluble or as MV-associated forms. We found that TSE exposure not only increased HMGB1 expression in whole cells (Fig. 1A), but also enhanced its translocation from the nucleus to the cytosol and plasma membrane in the first 8 h after TSE treatment (Fig. 3A). By the 24-h time point, the cytosolic or membrane-associated HMGB1 was released into the extracellular space as either soluble molecules (Fig. 2) or a MV-associated form (Figs. 2 and 3D) during TSE-induced membrane vesiculation (Li et al., 2010, 2013; Liu and Williams, 2012; Wang et al., 2014). Yeung et al. (2008) reported that negatively charged cell-membrane PS regulates the cellular localization of positively charged proteins. MVs are known for their enrichment of PS on their surface (Liu and Williams, 2007; Liu et al., 2012). HMGB1 contains two positively charged domains, the A- and B-Box, in the N-terminus, and a negatively charged C-terminus (Gougeon et al., 2012). Studies have reported that PS can bind HMGB1 (Liu et al., 2008). We infer that negative charged PS on MVs can bind the positively charged A and B boxes of HMGB1, leading to the formation of MV-associated HMGB1 seen in the current study (Fig. 3).

In addition to HMGB1, the receptor activator of nuclear factor- κ B ligand (RANKL) (Deng et al., 2015) and IL-15 (Bulanova et al., 2009) also exist as both soluble and MV-associated forms. Studies indicate that membrane-bound cytokine RANKL is more biologically active than its soluble form (Nakashima et al., 2000), and membrane-bound IL-15 is the major physiologic form of the cytokine as compared to its soluble form (Giron-Michel et al., 2005). In the body, soluble extracellular HMGB1 in tissues may quickly diffuse into the systemic circulation and become diluted in a large pool of circulating blood. In contrast, MV-associated extracellular HMGB1 may exist longer, and thus accumulate to relatively higher concentrations locally, in the microenvironment of diseased or inflamed tissues or organs. This effect may be particularly important in conditions with impaired clearance of apoptotic cells or PS-positive microvesicles by macrophage phagocytosis in the context of smoking (Richens et al., 2009), or in human diseases, like lupus (Cohen et al., 2002), atherosclerosis (Li et al., 2009), obesity and T2DM (Lecube et al., 2011). Liu et al. (2008) reported that HMGB1 inhibits macrophage phagocytosis through binding to PS. All of these suggested that MV-associated HMGB1 might be participate in the development of several human diseases.

The nuclear protein HMGB1 functions as an endogenous danger signal with proinflammatory effects only when it is

released into the extracellular space (Rouhiainen et al., 2004; Andersson and Tracey, 2011). The redistribution of HMGB1 from the nucleus to the cytoplasm is tightly regulated by the nuclear importin protein karyopherin- α 1 and the nuclear exportin chromosomal maintenance 1 (CRM1) (Bonaldi et al., 2003; Youn and Shin, 2006). CRM1 has been shown to regulate HMGB1 translocation from the nucleus to the cytoplasm in TNF α -stimulated macrophages (Tang et al., 2007). In the current study, we found that CRM1 regulates the translocation of nuclear HMGB1 to the cytoplasm and plasma membrane in TSE-treated macrophages (Fig. 4A,B), and then the export of HMGB1 onto TSE-MVs (Fig. 4C). Inhibition of CRM1 during the formation of TSE-MVs attenuates the export of HMGB1 onto membrane MVs (Fig. 4C). These results suggested an essential role of CRM1 not only in nucleo-cytoplasmic shuttling of HMGB1, but also in exporting the molecule into the extracellular space either as soluble or membrane MV-associated HMGB1.

In summary, our findings indicated that the endogenous danger signal, HMGB1, is translocated from the nucleus to the cytoplasm and cell membrane, and released from tobacco smoke-exposed macrophages, either as a soluble form or as a microvesicle-associated form into the extracellular space. Microvesicle-associated HMGB1 might serve as an important mediator in normal and maladaptive inflammatory responses and the development of autoimmune diseases.

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Fig. 1.

Exposure of macrophages to tobacco smoke extract stimulates HMGB1 expression and increases its release into the extracellular milieu. Part A: quantification and representative immunoblots of HMGB1 content of macrophages after exposure to 2.5% TSE for 0–24 h. Part B: quantifications and representative immunoblots of the extracellular HMGB1 precipitated from the conditioned medium of cultured macrophages after exposure to 2.5% TSE for 0 to 24 h. Parts A, B, display means $\pm$ SEM, $n = 3-5$. In these parts, $P < 0.01$ by ANOVA; displayed are significance levels for individual pairwise comparisons by the Student–Newman Keuls test.

Fig. 2.

Procedure for analysis of soluble and microvesicle-associated forms of extracellular HMGB1 in the conditioned medium of TSE-stimulated macrophages. Our procedure for analysis of soluble and microvesicle-associated forms of extracellular HMGB1 in the conditioned medium of the TSE-stimulated macrophages is shown schematically. The summary and the representative immunoblots of at least three independent experiments indicate extracellular HMGB1 precipitated from either pre-ultracentrifugation conditioned medium (Pre-ultra Sup, total HMGB1), post-ultracentrifugation conditioned medium (Post-ultra Sup, soluble HMGB1), or HMGB1 pelleted with MVs by ultracentrifugation (Pelleted MVs, MV-associated HMGB1) from macrophages treated with 2.5% TSE for 24 h.

Fig. 3.

Tobacco smoke exposure induces translocation of HMGB1 from the nucleus to the cytosol and cell membrane, and then budding with membrane microvesicles. Part A: representative fluorescent images and their corresponding phase-contrast images of macrophages exposed to 2.5% TSE for 0–24 h, examined for HMGB1 intracellular distribution in the cell membrane of permeabilized cells stained with anti-human HMGB1 primary antibody (detected with *green* FITC-labeled secondary antibodies) and DAPI (*blue*) for the nucleus, as well as the merged images (*Overlap*) demonstrating the translocation of HMGB1 from the nucleus to the cytosol, plasma membrane, and microvesicles (arrows), and their phase-contrast images. Scale bar, 2 mm. Parts B, C: representative analyses of the fluorescent microscopic images by regular fluorescent or confocal microscopy (B), and the histograms of flow cytometry (C), for the non-permeabilized cells that were treated without (control) or with 2.5% TSE for 24 h, and then stained with anti-human HMGB1 primary antibody and FITC-labeled secondary antibodies. The localization of HMGB1 on the plasma membrane and microvesicles (arrows) was visualized by diametrically sliced spheroid cells with

confocal microscopy (B). Scale bar, 2 mm. Part D: Confocal fluorescent micrographs of representative macrophages incubated without (*Control*) or with 2.5% TSE for 24 h, and then stained simultaneously with anti-human HMGB1 antibodies (detected with *green* FITC-labeled secondary antibodies) and PE-labeled annexin V (*red*, which preferentially binds to exteriorized phosphatidylserine). Confocal slices were taken to visualize the upper surface of the cells. The merged images (*Merge*) demonstrates co-localization of the two labeled colors. Arrows indicate examples of the small, circumscribed, cell-surface domains that stain intensely for both HMGB1 and exteriorized PS and exhibit the same size as TSE-induced MVs, consistent with enrichment of HMGB1 in nascent membrane blebs. Scale bar, 1 mm.

Fig. 4.

The nuclear exporter CRM1 is required for HMGB1 translocation, secretion in soluble form, and export on MVs from macrophages exposed to tobacco smoke extract. Part A: representative fluorescent images and their corresponding phase-contrast images of the macrophages without or with 2.5% TSE exposure for 8 h. These examined the intracellular distribution and colocalization of HMGB1 and CRM1 in permeabilized cells stained with anti-human HMGB1 primary Ab (detected with *green* FITC-labeled secondary antibodies), antihuman CRM1 primary Ab (detected with *red* PE-labeled secondary antibodies), and DAPI (*blue*) for the nucleus as indicated. Scale bar, 2 mm. Part B: representative fluorescent images and their corresponding phase-contrast images of macrophages pretreated for 1 h without or with 2.5 mM CRM1 inhibitor III (CRM1i), following by 2.5% TSE exposure for 8 h. The HMGB1 intracellular distribution in permeabilized cells is stained with anti-human HMGB1 primary Ab (detected with *green* FITC-labeled secondary antibodies) and DAPI (*blue*) for nucleus, as well as the merged images (*Overlap*). These images demonstrate the redistribution of HMGB1 from the nucleus to the cytoplasm, and the phase contrast images of the cells. Scale bar, 2 mm. In parts A, B, solid arrows indicate the nuclear localization of HMGB1 or CRM1, while empty arrows demonstrate the empty nucleus whose nuclear HMGB1 or CRM1 is exported to the cytosol. Part C: representative immunoblots showed the effects of CRM1 inhibitor III on TSE-induced HMGB1 intracellular expression, release

into extracellular supernatant, and export to membrane MVs from macrophages after 24 h TSE exposure without or with CRM1 inhibitor III pretreatment.

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