Carbon monoxide–based therapy ameliorates acute pancreatitis via TLR4 inhibition

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The protective role of hemeoxygenase-1 (HO-1) in various inflammatory conditions is mediated in part by its products, carbon monoxide (CO) and biliverdin. Here we investigated a therapeutic role for CO and CO-primed cells in acute pancreatitis (AP). In a mouse model of AP, treatment with CO-releasing molecule–2 (CORM-2) decreased mortality, pancreatic damage, and lung injury. CORM-2 decreased systemic inflammatory cytokines, suppressed systemic and pancreatic macrophage TNF-α secretion, and inhibited macrophage TLR4 receptor complex expression. In both human and mouse cells, CORM-2 inhibited endogenous and exogenous ligand-dependent TLR4 activation, which indicates that CORM-2 could be therapeutic for both early and late stages of AP, which involve sterile- and endotoxin-mediated inflammation, respectively. Mice engrafted with TLR4-deficient hematopoietic cells were protected against caerulein-induced AP. In the absence of leukocyte TLR4 expression, CORM-2 did not confer additional protection, which indicates that CORM-2–dependent effects are mediated via suppression of macrophage TLR4 activation. We determined that CO was directly responsible for the protective effects of CORM-2 in AP, as inactive forms of CORM-2 were ineffective. Importantly, adoptive transfer of CORM-2–primed cells reduced AP. Such a therapeutic approach would translate the beneficial effects of CO-based therapies, avoiding CO- or CO-RM–mediated toxicities in AP and a wide range of diseases.

Introduction

Carbon monoxide (CO) is increasingly recognized as a cytoprotective and homeostatic molecule with important signaling capabilities in physiologic and pathophysiologic situations (1). CO is 1 of 3 products generated from heme degradation by the rate-limiting enzyme heme oxygenase–1 (HO-1) (2). The antiinflammatory and protective properties of CO are supported by accumulating evidence in animal models of cardiovascular disease, inflammatory disorders, and organ transplantation (3–6).

Transition metal carbonyls, termed CO-releasing molecules (CO-RMs), have been used in biological systems to deliver CO in a controlled manner while keeping carboxyhemoglobin levels stable. The first compound to corroborate the feasibility of this technology was the lipid-soluble metal carbonyl complex tricarbonyl dichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$; also known as CORM-2). CORM-2 is able to transfer CO spontaneously and can exert typical CO-mediated pharmacologic effects (7). CO is released rapidly from CORM-2 once in solution with a half-life of about 20 minutes, and each mole of CORM-2 liberates approximately 0.7 mol CO (7).

One of the most feared complications of acute pancreatitis (AP) is infection and bacterial colonization of the necrotic pancreas, which leads to a significant rise in mortality. TLR4 activation is one of the mechanisms by which bacterial translocation may account for the development of severe experimental AP (8). Classically, the danger-associated molecules sensed by TLRs are highly conserved pathogen-associated molecular patters expressed by bacteria and other pathogens that are not present in mammalian cells. However, numerous more recent reports have suggested that diverse molecules of host-cell origin may also serve as endogenous ligands for TLRs. In the case of TLR4, endogenous ligands such as heat shock proteins (HSPs), high-mobility group box protein 1 (HMGB1), and molecules from necrotic or dying cells have been proposed (9–11). Since AP progression and severity is associated with significant cell death and necrosis, such endogenous ligands are likely to play an important role in TLR4 activation (8, 12). In the present study, we investigated the therapeutic role of CO in experimental AP and its effect on TLR4 activation by exogenous and endogenous ligands using mouse and human cells. Using BM chimeras, we determined the contribution of immune cell TLR4 and the effect of CO therapy in AP. In addition, as a way of avoiding toxicities associated with systemic CO therapy, we tested the therapeutic utility of ex vivo CORM-2–primed cells.

Results

CORM-2 ameliorates established experimental AP. To determine the therapeutic effects of CORM-2, we used 2 independent mouse models of AP: caerulein hyperstimulation, which causes mild to moderate AP, and choline-deficient diet supplemented with DL-ethionine (CDE) feeding, which causes severe hemorrhagic AP associated with significant mortality. Mice were given CORM-2 or vehicle (VE) at 24 hours after starting CDE feeding, and serum and tissues (pancreas and lung) were harvested at 72 hours (Figure 1A). CORM-2 recipients had significantly lower mortality than VE recipients (Figure 1B). Single-dose CORM-2 dramatically suppressed serum amylase and lipase elevations (Figure 1, C and D). Pancreatic injury, as determined by histology scores and trypsin activity, was significantly lower in CORM-2–treated mice (Figure 1, E–G). CORM-2 additionally protected against pancreatitis-associated distant lung injury, as shown by decreased lung myeloperoxidase (MPO) levels (Figure 1H). Similarly, CORM-2 was also effective in treating caerulein-induced AP (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI71362DS1). We revealed the protective effect of CORM-2 in AP to be mediated by CO release, as 2 of its inactive

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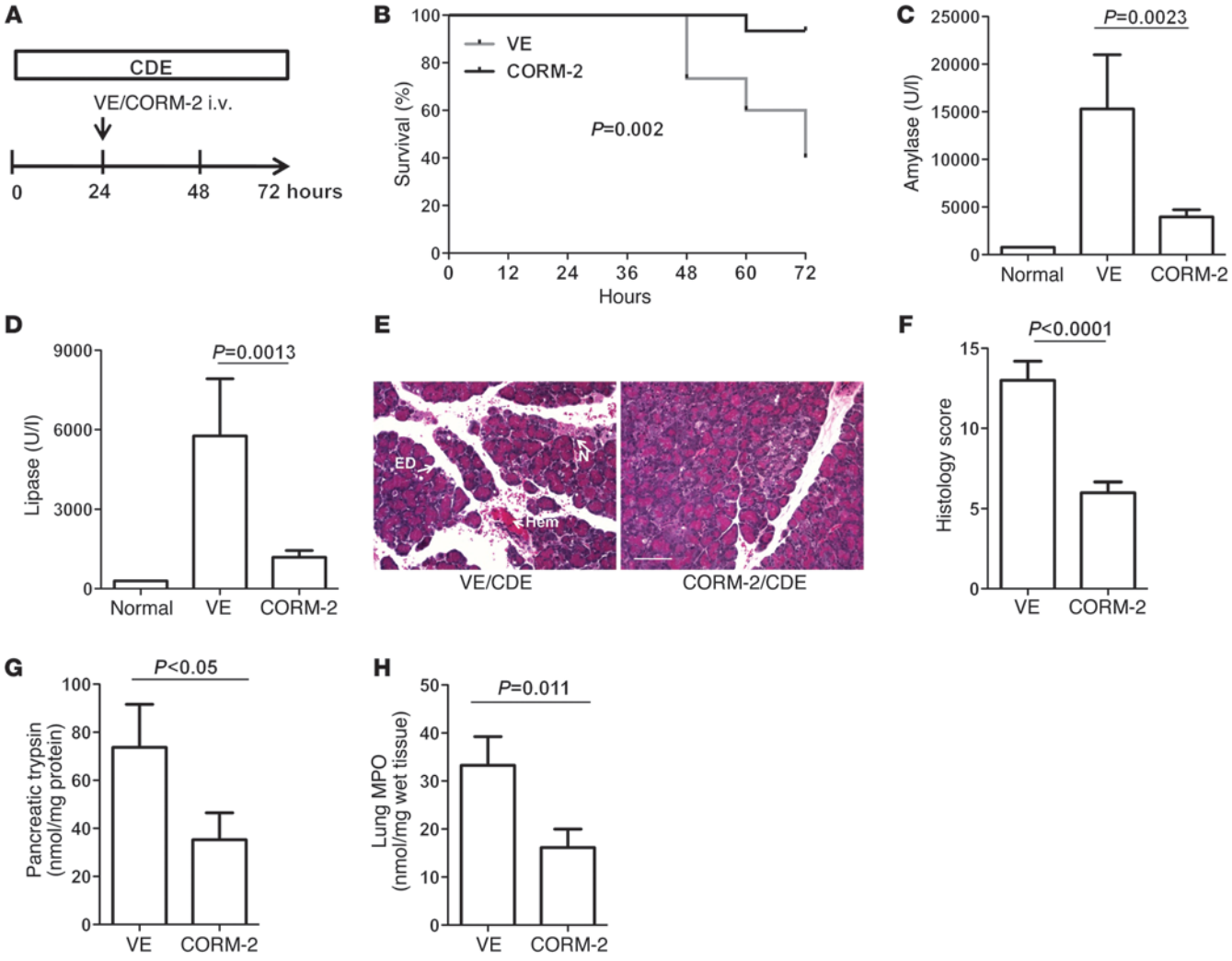

Figure 1
CORM-2 treats CDE-induced AP. (**A**) Balb/c mice were treated with 8 mg/kg CORM-2 or VE control i.v. 24 hours after CDE feeding, then sacrificed at 72 hours. (**B**) Mouse survival over time. (**C** and **D**) Amylase (**C**) and lipase (**D**) results from sera collected at 72 hours. (**E**) Representative H&E-stained pancreatic sections. ED, edema; N, necrosis; Hem, hemorrhage. Scale bar: 100 μm. (**F**) Pancreas histology scores. (**G** and **H**) Pancreatic trypsin activity (**G**) and lung MPO measurements (**H**). Data are mean ± SEM of at least 3 independent experiments ($n \geq 5$ per group).

forms — inactivated CORM-2 (iCORM-2), in which CO is depleted, and $RuCl_3$, which does not contain CO and is thus unable to release it — were ineffective (Supplemental Figure 2).

CORM-2 suppresses systemic proinflammatory cytokines and TNF-α production by spleen and pancreatic macrophages. AP is not only a localized disease, but is also associated with systemic inflammatory response. Thus, we assessed the effect of CORM-2 on circulating cytokines and found that CORM-2 treatment significantly decreased the serum proinflammatory cytokines TNF-α, IL-1α, IL-6, and IL-12p40 (Figure 2A). Activated leukocytes during the early phase of pancreatitis release cytokines, which in turn mediate and enhance the inflammatory cascade observed in AP. In particular, TNF-α plays a critical and central role in the pathogenesis of AP and recruitment of inflammatory cells. Since monocyte/macrophages are major source for TNF-α, we isolated spleen and pancreas leukocytes from VE- versus CORM-2–treated mice and tested their ability to respond to TLR4 activation using LPS. Consistent with the systemic and local inflammatory-suppressive effects, CORM-2 treatment significantly reduced TNF-α production by the lineage-negative (Lin^-; i.e., $CD3^-CD19^-NK1.1^-$) $CD11b^+CD11c^-F4/80^+$ macrophage populations in the spleen and pancreas (Figure 2, B and C).

CORM-2 inhibits TLR4-mediated TNF-α production in mouse and human monocyte/macrophages. To further investigate the effect of CORM-2 on TLR4 signaling, we examined TNF-α production by spleen macrophages isolated from unmanipulated WT and TLR4-deficient (TLR4 KO) mice. CORM-2 inhibited both basal and LPS-induced TNF-α production by WT macrophages, but, as expected, it had no effect on TLR4 KO macrophages (Figure 3A). To determine whether the observed effects of CORM-2 are applicable to endogenous TLR4 ligands, we used S100 calcium-binding protein A8 (S100A8) and HMGB1 to activate TLR4 (13). Previous reports have shown direct binding of these endogenous ligands to the TLR4/myeloid differentiation 2 (TLR4/MD2) com-

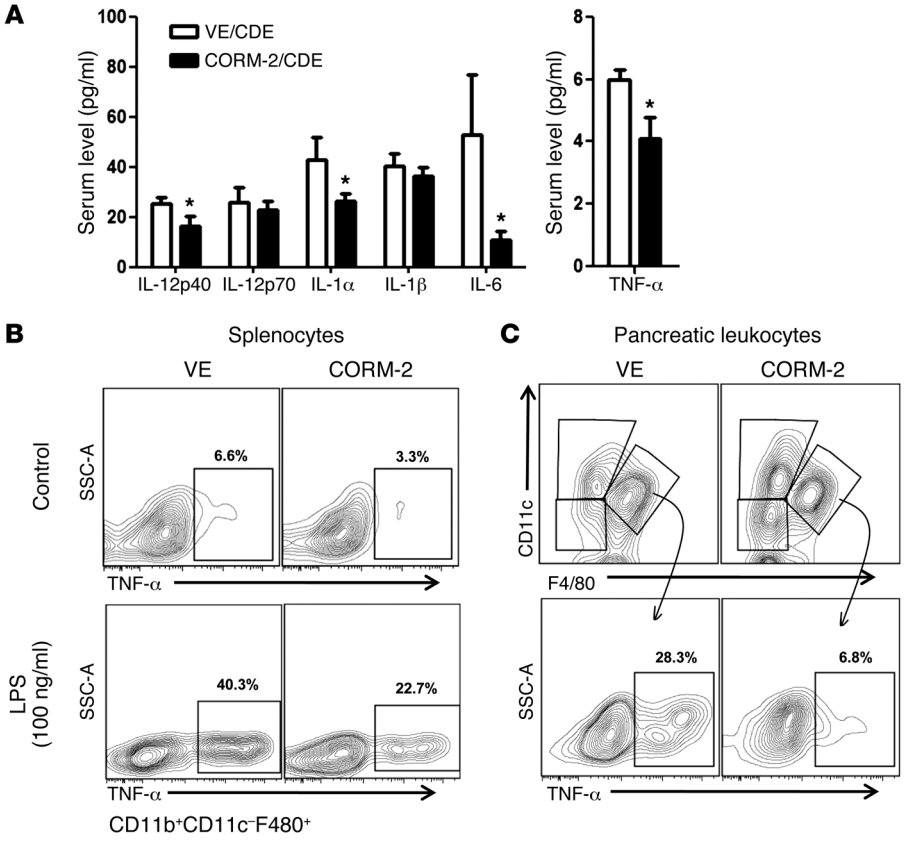

Figure 2
CORM-2 inhibits production of systemic proinflammatory cytokines and TNF-α by macrophages. (**A**) Balb/c mice were treated with 8 mg/kg CORM-2 or VE 24 hours after CDE feeding, then sacrificed at 72 hours. Serum cytokine and chemokine levels were determined by Luminex assay. Data are mean ± SEM. $^*P < 0.05$. (**B** and **C**) 24 hours after VE or CORM-2 treatment, splenocytes (**B**) and pancreatic leukocytes (**C**) were isolated and stimulated with control or LPS for 4 hours, then stained for surface markers as well as intracellular TNF-α. Macrophages were gated ($Lin^-CD11b^+CD11c^-F4/80^+$) and analyzed for TNF-α–positive cell frequency by flow cytometry. Representative FACS plots are shown from 3 independent experiments ($n \geq 3$ per group).

plex via surface plasmon resonance studies (14, 15). Similar to our findings with LPS, S100A8-mediated TNF-α production by mouse BM-derived macrophages (BMDMs) was significantly suppressed by CORM-2 treatment (Figure 3B). We confirmed TLR4's requirement for S100A8 using WT and TLR4 KO BMDMs with experiments similar to those in Figure 3A (data not shown). In order to address the translation potential of our study, we used human PBMCs and confirmed that CORM-2 suppressed LPS- and HMGB1-mediated TNF-α production by circulating human $CD14^+$ monocytes (Figure 3C).

CORM-2 reduces macrophage TLR4/MD2 receptor complex expression during AP. A robust TLR4 response to LPS requires additional components of the LPS recognition complex, which includes CD14 and MD2 (16). MD2 directly recognizes the lipid A domain of LPS and activates TLR4/MD2 complex formation, which is necessary for cellular response and downstream signaling. Previously, using an insulinoma cell line, Rocuts et al. showed that CO suppresses membrane activation of TLR4 by blocking TLR4 glycosylation and the physical interaction between TLR4 and MD2 (17). To understand the mechanism by which CORM-2 negatively regulates TLR4 activation in AP, we compared macrophage TLR4/MD2 receptor complex expression in mice treated with VE versus CORM-2. Using the mAb MTS510, which recognizes TLR4/MD2 receptor complex but not TLR4 alone, we found a significant decrease in pancreatic macrophage TLR4/MD2 receptor complex expression in CORM-2–treated mice at both 48 and 72 hours after CDE feeding (Figure 4, A and B). In addition, we used the murine macrophage cell line RAW264.7 to assess the effect of CORM-2 on membrane total TLR4 and TLR4/MD2 receptor complex expression using the specific mAbs UT51 and MTS510, respectively. CORM-2 suppressed surface TLR4/MD2 expression, but had no effect on surface total TLR4 expression (Figure 4C), which suggests that CORM-2 did not inhibit TLR4 expression, but rather blocked association of TLR4 and MD2.

CORM-2 decreases LPS binding to the TLR4/MD2 receptor complex. Since CORM-2 treatment suppressed TLR4/MD2 complex expression in vivo (Figure 4), we examined the effects of CORM-2 on LPS binding to the TLR4 receptor complex using FITC-conjugated LPS (referred to herein as FITC-LPS). RAW264.7 cells were pretreated with VE or CORM-2, then incubated with FITC-LPS for 30 minutes. FITC-LPS binding to CORM-2–treated RAW264.7 cells was significantly impaired compared with VE treatment, as determined by flow cytometry and confocal microscopy (Figure 5, A and B). CORM-2 did not alter TLR4 or MyD88 total protein expression (Figure 5A). We also observed suppressive effects of CORM-2 on surface TLR4/MD2 expression and FITC-LPS binding using freshly isolated mouse peritoneal macrophages (Figure 5C). iCORM-2 had no effect on TLR4/MD2 expression and was not as effective in decreasing FITC-LPS binding (Figure 5C), which suggests that the inhibitory function of CORM-2 was mediated through CO release. Taken together, our data indicate that CORM-2 inhibits TLR4/MD2 complex formation, a process that is required for functional TLR4 receptor activation.

Ablation of TLR4 in hematopoietic cells confers protection against AP. Sharif et al. showed significantly less severe AP in TLR4 KO compared with WT mice (8). TLR4 is also expressed in nonhematopoietic pancreatic cells, such as epithelium of the pancreatic duct

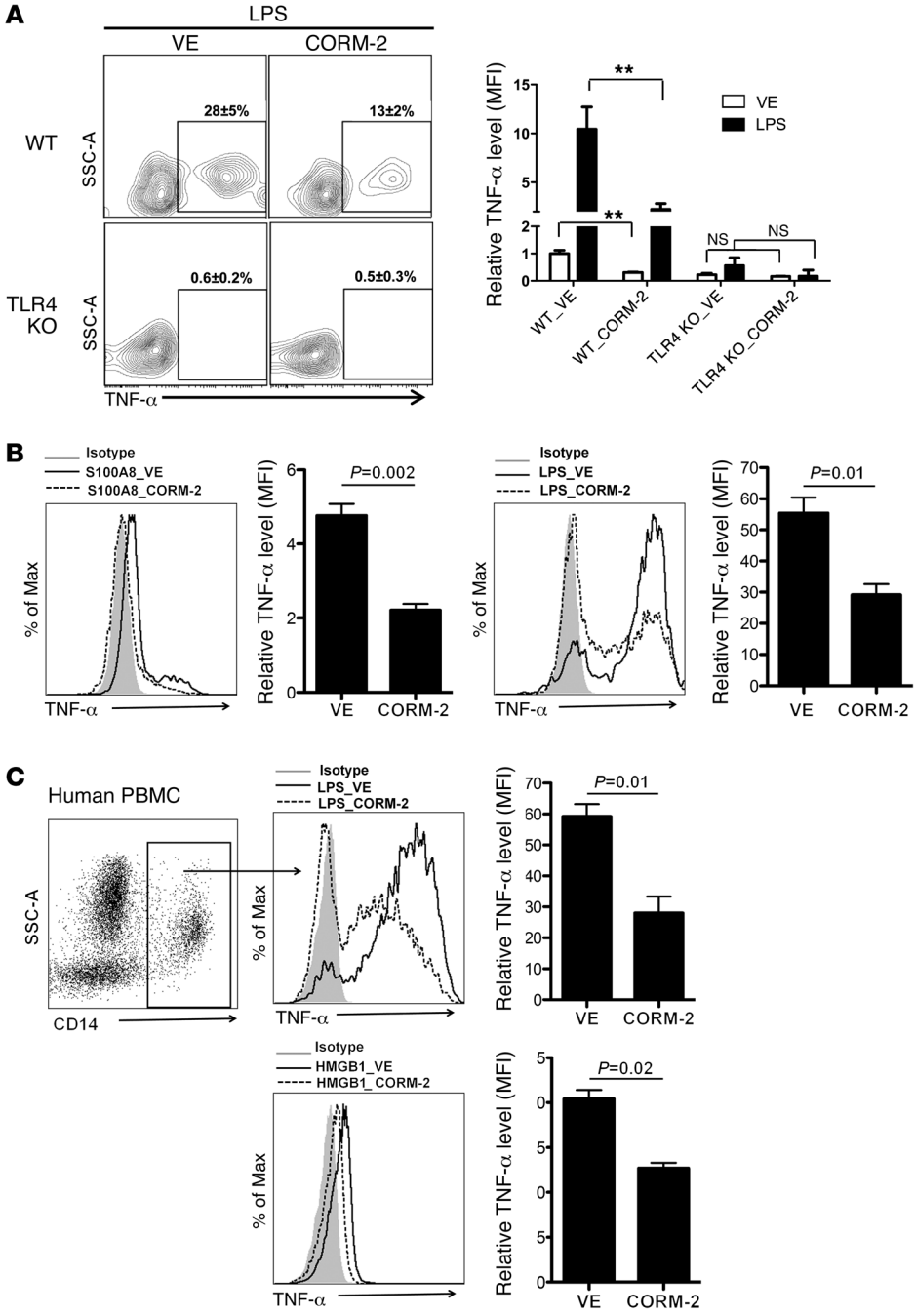

Figure 3
CORM-2 inhibits TLR4-mediated TNF-α production in mouse and human monocytes/macrophages. (**A**) Splenocytes from WT and TLR4 KO mice were isolated and pretreated with VE or CORM-2 for 1 hour, followed by stimulation with control or 100 ng/ml LPS for 4 hours in the presence of BFA. Frequency of TNF-α–positive macrophages ($Lin^-CD11b^+CD11c^-F4/80^+$) was determined by intracellular staining. Relative TNF-α expression was also quantified. Data are mean ± SEM. $^{**}P < 0.01$. (**B**) BMDMs were prepared and pretreated with VE or CORM-2 for 1 hour, then stimulated with 10 ng/ml LPS or 100 ng/ml S100A8 for 4 hours in the presence of BFA. Relative TNF-α expression was also quantified. (**C**) Monocytes from human PBMCs were enriched and pretreated with VE or CORM-2 for 1 hour, then stimulated with 10 ng/ml LPS or 100 ng/ml HMGB1 for 4 hours in the presence of BFA. Relative TNF-α frequency in $CD14^+$ circulating monocytes was determined by intracellular staining and quantified. Data are mean ± SEM of at least 3 independent experiments.

(18). In order to determine the contribution of and CORM-2's effect on hematopoietic TLR4 in AP, we generated chimeric mice by engrafting WT recipients with either WT or TLR4 KO BM cells (WT→WT or TLR4 KO→WT mice, respectively). At 8 weeks after engraftment, mice underwent caerulein-induced AP and were treated as above with either VE or CORM-2 (Figure 6A). VE-treated WT→WT mice had significantly more pancreatic injury than their CORM-2–treated counterparts (Figure 6, B–F). CORM-2 treatment protected WT→WT mice similar to the results seen in nonengrafted WT mice (Supplemental Figure 1). In contrast, the pancreatic injury of VE-treated TLR4 KO→WT mice was not as severe as that of VE-treated WT→WT mice. Furthermore, the protective function of CORM-2 was only observed in WT→WT mice, not in TLR4 KO→WT mice (Figure 6, B–F). These results indicate that the protective effect of CORM-2 in AP is mediated by inhibition of hematopoietic TLR4 activation. CORM-2 did not have a significant effect on isolated primary acinar cells, as assessed by caerulein-mediated NF-κB activation, amylase secretion, and calcium influx (Supplemental Figure 3).

CORM-2–primed cells ameliorate experimental AP. Given the potential toxicity of systemically administered CO-RMs or CO, we tested whether monocytes/macrophages primed via CORM-2 pretreatment can transfer protection and treat AP, using an adoptive cell transfer model to treat caerulein-induced pancreatitis (Figure

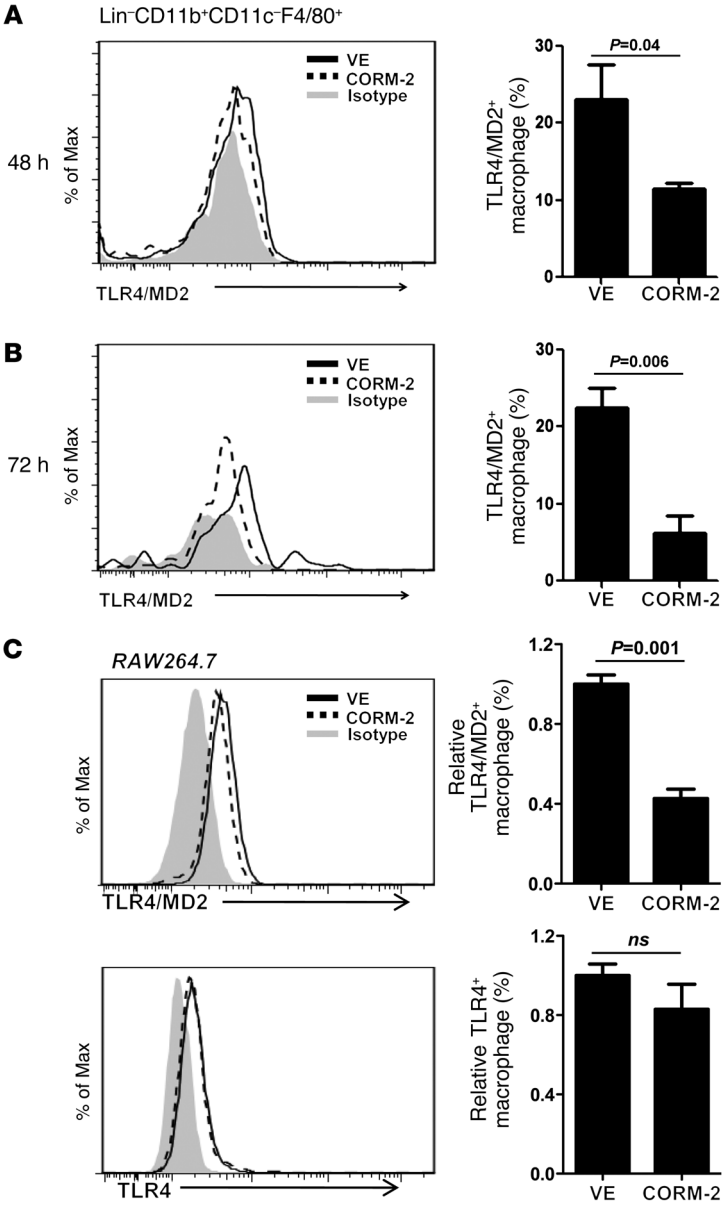

Figure 4
CORM-2 reduces macrophage TLR4/MD2 receptor expression during AP. (**A** and **B**) Balb/c mice were injected i.v. with CORM-2 or VE 24 hours after CDE feeding and euthanized at 48 (**A**) or 72 (**B**) hours. Pancreatic leukocytes were isolated for flow cytometry assay. Macrophages were gated ($Lin^-CD11b^+CD11c^-F4/80^+$) and analyzed for TLR4/MD2 expression. $TLR4/MD2^+$ cells are shown as a percentage of total macrophages. (**C**) RAW264.7 cells were treated with VE or 100 mM CORM-2 for 4 hours. Cells were stained with the mAbs MTS510 and UT51, which recognize surface TLR4/MD2 receptor complex and total TLR4, respectively, and then analyzed by flow cytometry. Data are mean ± SEM of at least 3 independent experiments.

Discussion

Despite significant morbidity and mortality, AP remains a clinical challenge, and no approved active therapies for this disease currently exist. We previously showed that HO-1 induction protects against experimental AP (19, 20). HO-1 is a rate-limiting enzyme that degrades heme into biliverdin, CO, and iron (2). The protective and antiinflammatory roles of HO-1 are thought to be mediated by the products biliverdin and CO (21). In agreement with this, we recently reported protective effects of biliverdin in AP that are mediated by downstream aryl hydrocarbon receptor activation and IL-22 induction (22). The unmet need for AP therapy and the limited understanding of the underlying immune responses in AP led us to explore the role of CO in AP and investigate potential mechanisms of action for CO.

To determine the therapeutic role of CO, we first established AP in mice using 24 hours of CDE feeding, as in our previous studies (19), and then treated the mice with either VE or CORM-2. CORM-2 was effective in treating local (pancreas), distant (lung), and systemic inflammatory effects of AP. Similar protective effects were also seen with the caerulein model of AP. Our results are consistent with the study by Chen et al. showing a protective effect of CORM-2 in a sodium taurocholate rat model of AP (23). Since the mechanisms for the beneficial and therapeutic effects of CORM-2 in AP are unknown, our next objective was to investigate potential mechanisms and identify CORM-2–mediated cellular targets.

7A). Compared with VE-primed $CD11b^+$ cells, CORM-2–primed cells were more effective in protecting against caerulein-induced AP (Figure 7, B–F). CORM-2–primed cells downregulated mRNA expression of the proinflammatory *Tnfa* and upregulated expression of the antiinflammatory *Il10* and *Il22* (Figure 7G). To address whether the CORM-2–primed cells ameliorate AP via cotransfer of CORM-2, we tested the transferred cells and the media in which they were cultured for the presence of ruthenium and the ability to release CO using inductively coupled plasma mass spectrometry (ICP-MS) and myoglobin assay, respectively. There was no active or inactive CORM-2 transferred with the cells, and the ruthenium remained in the culture media (Supplemental Figure 4). Taken together, these findings showed that CORM-2–primed $CD11b^+$ cells can transfer protection to mice undergoing AP and suggest that the therapeutic role of CORM-2 is mediated at least in part by monocytes/macrophages ($CD11b^+$ cells) that express higher levels of IL-10 and IL-22.

Monocytes/macrophages play a central role in the pathogenesis of AP, and the degree of macrophage activation is one of the important factors that may determine AP severity (24). We previously showed that HO-1–overexpressing macrophages could transfer protection in experimental AP (20, 25). Thus, we postulated that CO, a downstream antiinflammatory product of HO-1, is likely to modulate monocyte/macrophage activation in AP. In support of this hypothesis, we found that CORM-2 treatment during AP significantly inhibited macrophage TNF-α production in the pancreas as well as in distant tissue, such as the spleen.

High levels of TNF-α in mouse models and patients with AP are associated with severe disease (26, 27). TLR4 activation triggers generation of TNF-α as well as other proinflammatory cytokines and chemokines relevant to the pathogenesis of AP. Levels of endotoxins such as LPS and TLR4 ligand correlate with systemic complications, mortality, and disease severity in patients with AP (28). Patients with severe and complicated AP have elevated endotoxin levels in

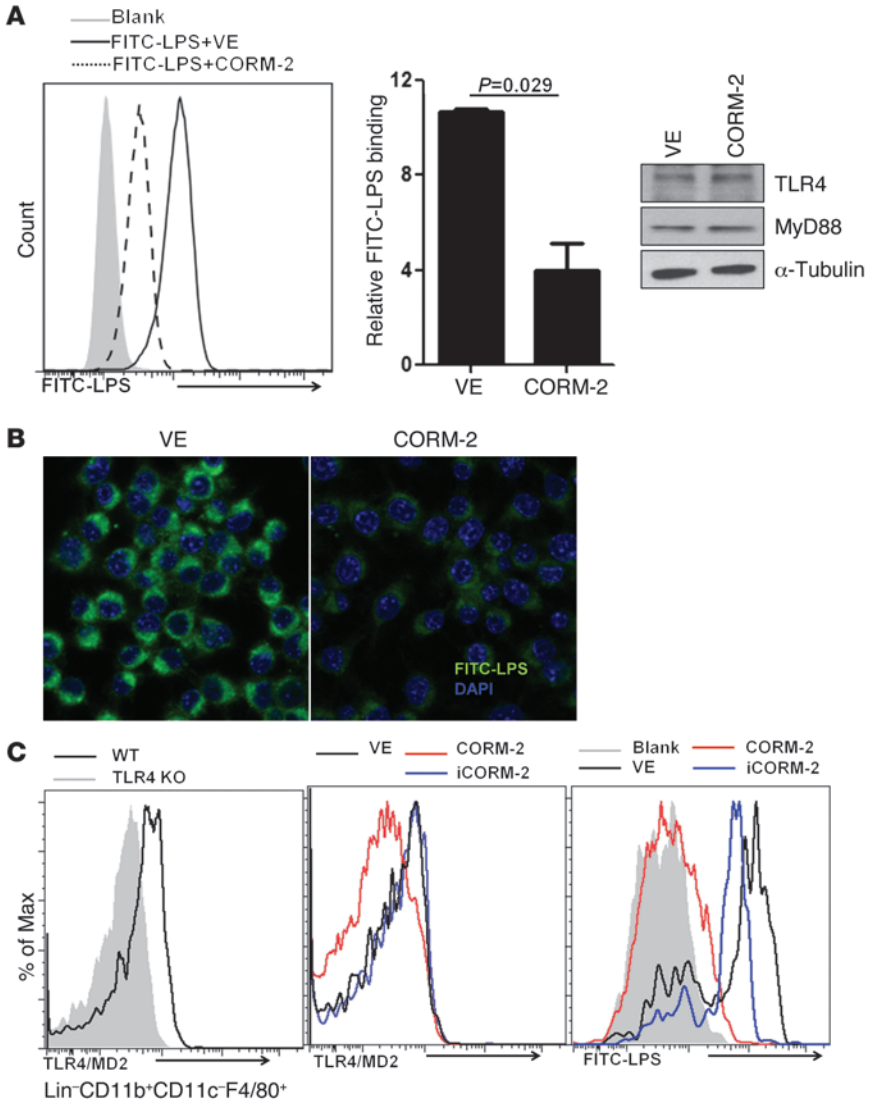

Figure 5
CORM-2 decreases LPS binding to TLR4/MD2 receptor complex. (**A**) Flow cytometry showing binding of FITC-LPS to RAW264.7 cells treated with VE or CORM-2. FITC-LPS–untreated cells (blank) are shown as a control. Relative FITC-LPS binding to RAW264.7 cells was quantified. Cell lysates were collected for TLR4 and MyD88 protein determination by Western blot. (**B**) RAW264.7 cells were pretreated with VE or CORM-2 for 30 minutes, then incubated with FITC-LPS. Cells were then washed and fixed for analysis by fluorescence microscopy. Green, FITC-LPS; blue, DAPI (nuclear stain). Original magnification, ×600. (**C**) Peritoneal macrophages from WT and TLR4 KO mice were isolated and treated with VE, CORM-2, or iCORM-2 for 4 hours, then analyzed by flow cytometry. Macrophages were gated ($Lin^-CD11b^+CD11c^-F4/80^+$) and analyzed for TLR4/MD2 expression and FITC-LPS binding. Shown is a representative FACS plot from at least 3 independent experiments. Data are mean ± SEM. $^*P < 0.05$.

blood and peritoneal fluid (29). However, the initial injury and early complications associated with AP are likely related to sterile inflammatory process or endogenous ligand-mediated TLR activation, not to endotoxins (30). More recently, substances released by stressed or damaged cells, such as HMGB1, S100A8/9, and HSPs, have been shown to trigger TLR4 signaling, generate proinflammatory cytokines, and mediate necrosis-induced sterile inflammation (13, 31). Pancreatic acinar cell necrosis is an early event in AP, and serum HMGB1 levels are significantly elevated in patients with AP. Furthermore, high HMGB1 serum levels correlate with worsening clinical course and mortality (32). Similar results have been observed in preclinical animal models of AP (33). Using both human and mouse monocytes/macrophages, we showed here that CORM-2 inhibited both endogenous (HMGB1 and S100A8) and exogenous (LPS) ligand-mediated TLR4 activation, supporting a potential therapeutic role for CORM-2 in both early and late stages of human AP.

TLR4 and MD2 form a heterodimer to recognize LPS, and the physical interaction between TLR4 and MD2 is essential for TLR4 maturation and binding efficiency to its ligands (16). Nakahira et al. showed that CO inhibits TLR signaling by regulating reactive oxygen species–induced trafficking of TLRs to lipid rafts (34). Moreover, Rocuts et al. used the βTC3 cell line to show that CO inhibits the physical interaction between TLR4 and MD2 and also blocks TLR4 glycosylation, a necessary step for TLR4 trafficking to the cell membrane (17). Based on these studies, we hypothesized that CORM-2 treats AP by inhibiting TLR4 receptor complex formation, thereby interfering with TLR4 activation. Using in vivo and in vitro studies and mAbs that recognize the TLR4/MD2 complex versus total TLR4, we found that CORM-2 suppressed TLR4/MD2 complex expression. This also translated to lower TLR4 ligand binding, as shown by our FITC-LPS studies using a mouse macrophage cell line and freshly isolated primary/peritoneal macrophages. High-resolution crystal structure of murine TLR4/MD2 complex revealed several molecular recognition sites on the TLR4/MD2 interface that could serve as potential targets for blocking the TLR4 signaling pathway (35). Future studies will need to address the exact mechanism by which CO disrupts the association of TLR4 and MD2.

TLR4 KO mice are less susceptible to AP than their WT counterparts (8). Because TLR4 is expressed by a broad range of cells, we sought to determine the contribution of immune cell TLR4 to

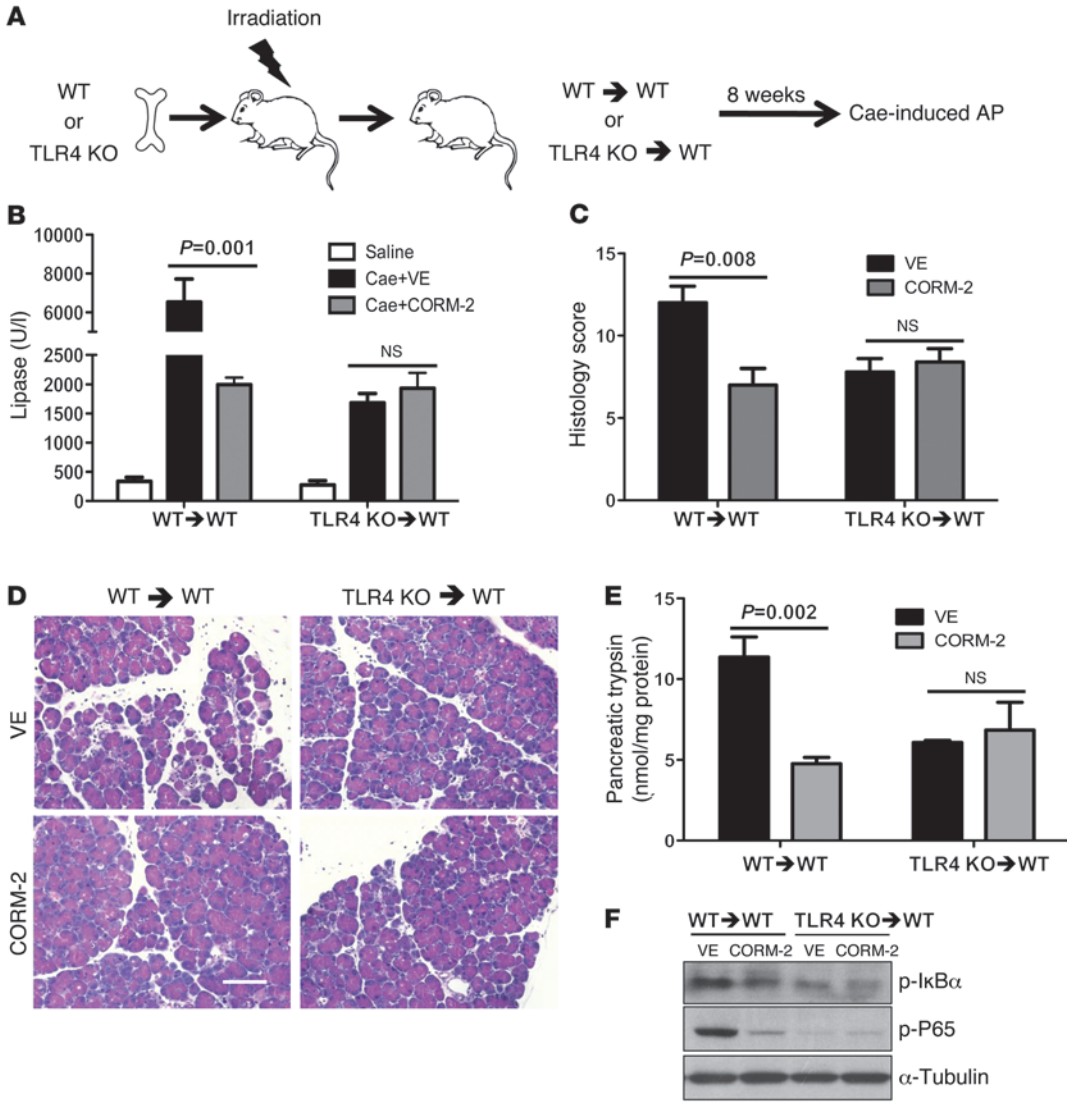

Figure 6

Ablation of TLR4 in hematopoietic cells confers protection against AP. (**A**) BM chimeric mice were generated by transferring donor WT or TLR4 KO BM cells (5×10^6 cells/mouse) into recipient WT mice that had been lethally irradiated with a signal dose of 9.5 Gy. The resulting WT→WT and TLR4 KO→WT chimeric mice were allowed to recover for at least 8 weeks to ensure stable engraftment. Mice were then injected hourly with caerulein (Cae) a total of 7 times to induce AP. They were treated with CORM-2 or VE 30 minutes after the first caerulein injection and euthanized 12 hours after the first caerulein injection. (**B**) Sera were collected for lipase measurements. (**C** and **D**) Pancreata were collected for histology scoring (**C**; see Methods) and H&E staining (**D**). Scale bar: 100 μm. (**E**) Pancreatic trypsin activity. (**F**) Pancreatic p-IκBα, p-P65, and α-tubulin protein expression, determined by Western blot. Data are mean ± SEM of 3 independent experiments ($n = 5$ per group).

the pathogenesis of AP. Chimeric TLR4 KO→WT mice had less severe AP; moreover, in the absence of leukocyte TLR4 expression, CORM-2 did not offer additional protection. These results, together with the observed inhibitory effects of CORM-2 on monocyte/macrophage TNF-α expression, suggest that the therapeutic effects of CORM-2 in AP are likely mediated by disruption of monocyte/macrophage TLR4 activation.

In addition to CO release, CORM-2 has other effects, including inhibition of potassium channel (Kv2.1), activation of nonselective cation current, and production of reactivate oxygen species (36–38). Although it is hard to rule out these effects in our model, we did not see improvement or worsening of AP using iCORM-2 and $RuCl_3$, which are depleted of and incapable of releasing CO, respectively. Even though we did not test the effect of inactive CORM-2 forms on freshly isolated acinar cells, with the exception of slightly higher calcium influx, we did not see significant acute effects of CORM-2 based on amylase secretion or NF-κB activation. It is possible that prolonged and/or repeated exposure may have an effect; alternatively, the CO may have circumvented the effects of the ruthenium complex.

Despite the beneficial and antiinflammatory effects of CO in various diseases and injury models, there has been little progress with respect to its translation and use in clinical settings. Low-dose inhalational CO has been shown to have antiinflammatory

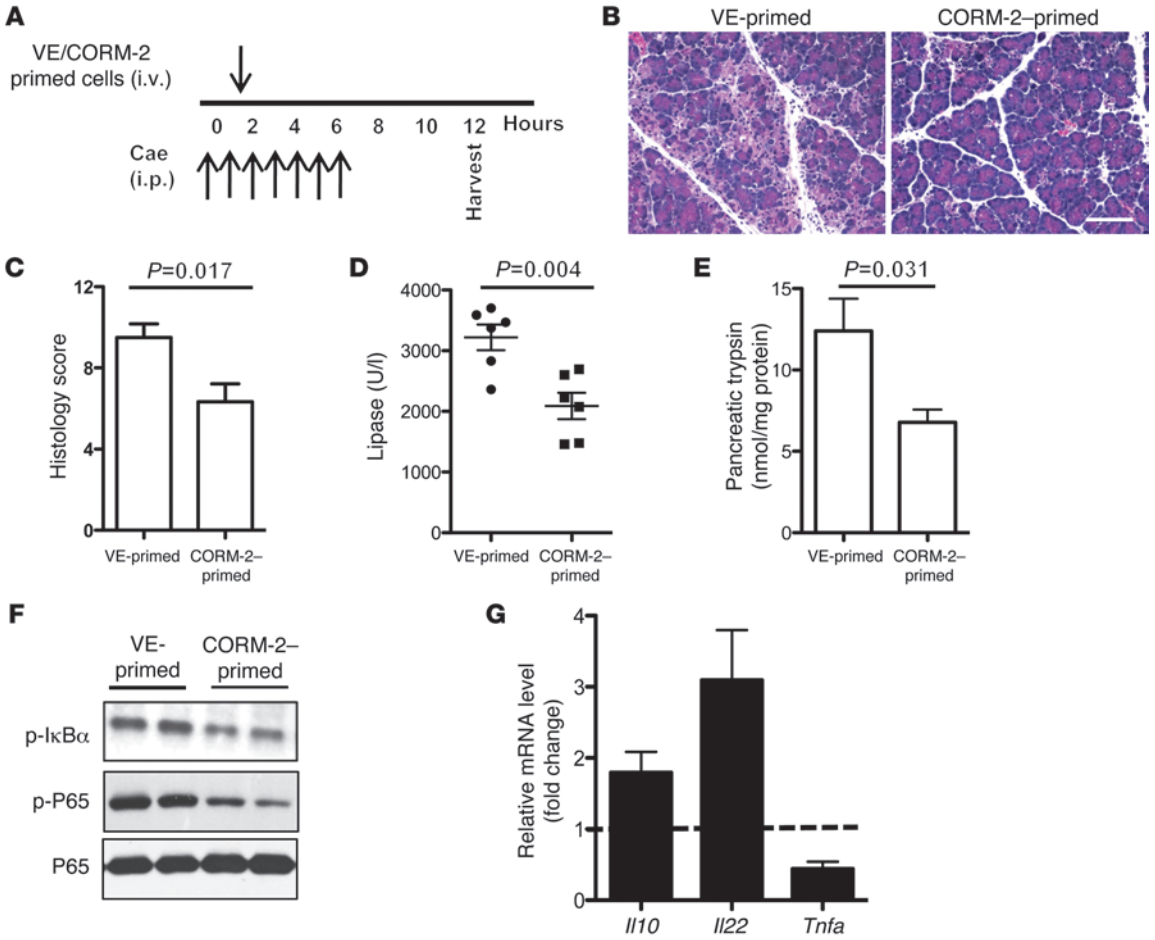

Figure 7

CORM-2–primed cells ameliorate experimental AP. (**A**) Mice were injected hourly with caerulein a total of 7 times, treated with CORM-2– or VE-primed CD11b$^+$ monocytes/macrophages 90 minutes after the first caerulein injection, and euthanized 12 hours after the first caerulein injection. (**B** and **C**) Pancreatic tissues were collected for H&E staining (**B**) and histology scoring (**C**). Scale bar: 100 μm. (**D**) Serum lipase measurements. (**E**) Pancreatic trypsin activity. (**F**) Pancreatic p-IκBα, p-P65, and P65 protein expression, determined by Western blot. (**G**) RNA from VE- and CORM-2–primed monocytes/macrophages was isolated for quantitative PCR analysis. Results are shown as fold change relative to VE (assigned as 1; dashed line). Data are mean ± SEM of at least 3 independent experiments.

effects against ventilatory-induced lung injury, a common hurdle in intensive care units, and was associated with decreased TNF-α (39). The toxicity of CO has long been known, and experts in CO-related toxicity are concerned about even modest doses of therapeutic CO (40). Active or inactive CO-RMs, at the concentrations used in our present studies, have previously been shown not to be toxic to mammalian cells in vitro and to rats in vivo (7, 41). However, Winburn et al. reported in vitro cytotoxic effects associated with ruthenium-based CO-RM byproducts, but not with CO, and suggested that accumulation of ruthenium in vivo might limit clinical application of these compounds (42). With these concerns in mind, we tested whether CORM-2–activated cells have the ability to transfer protection and treat experimental AP. Our data showed that CORM-2–activated monocytes/macrophages have a beneficial role in experimental AP. CORM-2–activated monocytes/macrophages upregulated the genes encoding the antiinflammatory cytokines IL-10 and IL-22, which were previously shown to be protective in experimental AP (22, 43). These cytokines may contribute to a mechanism whereby CORM-2–pretreated cells are able to ameliorate AP once adoptively transferred. Moreover, the pretreated cells did not transfer any inactive or active CORM-2. This finding is highly notable, and the therapeutic approach is likely to have utility in other diseases in which CO or active CO-RMs have been shown to be effective. Our findings support the potential use of ex vivo CORM-2–primed monocytes (which can be obtained easily from patients' blood) in a clinical setting, thereby avoiding the feared systemic toxicities associated with CO therapy.

In summary, we demonstrated here that CORM-2 inhibits monocyte/macrophage TLR4/MD2 receptor complex activation and has a therapeutic role in treating mouse models of AP. Using mouse and human monocytes/macrophages, we showed that CORM-2 inhibited both endogenous and exogenous ligand-dependent TLR4 activation and TNF-α generation. Thus, CORM-2 is likely to be effective in the early stages of AP, in which sterile inflammation and necrosis are key, as well as in the later and severe stages of AP, in which bacterial translocation and endotoxin-mediated inflammatory events play a critical role.

Furthermore, our data demonstrated that ex vivo CORM-2–primed monocytes/macrophages have the ability to transfer protection and treat experimental AP. Such a cellular therapeutic approach not only offers an alternative treatment route, but also avoids the toxicities associated with systemic administration of ruthenium and CO-based therapies.

Methods

Mice. Mice (including Balb/c, C57B6/J, and TLR4 KO mice) were purchased from Jackson Laboratory and housed under pathogen-free conditions.

AP models. For caerulein-induced pancreatitis, age- and sex-matched mice were fasted for 12–16 hours with free access to water. Mice then received 7 hourly i.p. injections of saline as control or 50 μg/kg caerulein (Sigma-Aldrich) and were followed up to 12 hours. For the CDE model of pancreatitis, young female mice (16–20 g) were fasted, then fed with choline-deficient diet (Harlan Teklad) supplemented with 0.5% DL-ethionine (Sigma-Aldrich) or normal chow as control (22).

CORM-2 treatment. CORM-2 dosing was based on past dose titration studies and reported effectiveness of a single systemic 8-mg/kg dose in rodents (44–46). CORM-2 (8 mg/kg i.v.; Sigma-Aldrich) was administered to mice at the indicated times. iCORM-2 was generated as described previously by addition to cell culture medium overnight (18 hours) at 37°C and bubbling with air (N_2) to remove residual CO (47). $RuCl_3$ (Sigma-Aldrich) was also used, as previously reported by others (7).

Histology. Mice were euthanized by CO_2 inhalation. Pancreata and other tissues were rapidly fixed in 10% formalin and embedded in paraffin. Fixed tissues were sectioned and then stained with H&E (performed by Histo-Tec Laboratory). Pancreatitis severity was scored in a blinded fashion as described previously (48).

Biochemical analysis. Blood was collected from mice via intracardiac puncture, and serum was isolated from these samples for subsequent lipase and amylase level determination. Lipase and amylase levels were determined by diagnostic laboratory at Stanford University. Lung and pancreas were collected for detection of MPO and trypsin activity using commercial kits (Biovision), following the manufacturer's guidelines.

Luminex assay. Luminex assay was performed as recommended by the manufacturer (Panomics/Affymetrix). Assays were performed in duplicate using the Luminex 200 IS System (Luminex Corp.). Individual cytokines and chemokines were identified and classified by the red laser, and levels were quantified using the green laser. Digital images of the bead array were captured after laser excitation and processed on a computer workstation. Standard curves and reports of unknown samples were prepared using BeadView and MiraiBio software.

Cell preparation and isolation. Pancreatic acinar cells and leukocytes were prepared as previously described (22). Peritoneal cavity cells were isolated according to a previously described method (49). For BMDM preparation, in brief, both ends of femur and tibia were cut and flushed with a syringe filled with complete RPMI 1640 containing 10% fetal bovine serum (cRPMI) to extrude BM cells into a sterile petri dish. After gentle resuspension and centrifugation, BM cells were cultured using 20% L929 cell conditioned medium (as a source of granulocyte/macrophage colony-stimulating factor) in cRPMI. On day 4, unattached cells were discarded, and medium was replaced with a fresh batch containing the L929 cell conditioned medium as above. Cells were ready for use on day 6 (50). Human PBMCs were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation, and monocytes were further enriched by Percoll gradient centrifugation.

Flow cytometry. For surface staining, cells were stained with the following antibodies: APC-conjugated CD45.2, PE/Cy7-conjugated CD4, PE/Cy7-conjugated CD19, PE/Cy7-conjugated NK1.1, Percp/Cy5.5-conjugated CD11b, FITC-conjugated F4/80, APC/Cy7-conjugated CD11c, PE-conjugated TLR4/MD2 (clone MTS510; Biolegend), and Alexa Fluor 488–conjugated TLR4 (clone UT51; eBioscience). For intracellular TNF-α staining, immediately after isolation, cells were cultured in cRPMI and stimulated with VE, 10–100 ng/ml LPS (Invivogen), 100 ng/ml mouse S100A8 (ProSpec), or 100 ng/ml human HMGB1 (ProSpec) for 4 hours in the presence of 10 μg/ml brefeldin A (BFA; eBioscience). Cells were washed, stained with surface markers, and then fixed/permeabilized using a kit (eBioscience) according to the manufacturer's guidelines. Mouse PE-conjugated TNF-α or isotype control PE-conjugated IgG1 (BD Biosciences) was used for intracellular staining. Dead cells were excluded from analysis using violet viability stain (Invitrogen). Cells were acquired on a LSRII or Fortessa (BD Biosciences) and analyzed with FlowJo software (Treestar Inc).

LPS-FITC binding assay. RAW264.7 cells were treated with VE or CORM-2 for 4 hours followed by incubation with 1 μg/ml FITC-LPS (Invitrogen) for 30 minutes at 37°C in cRPMI. RAW264.7 cells were washed with PBS 3 times and fixed with 4% PFA, and then binding of FITC-LPS to RAW264.7 cells was analyzed by flow cytometry and fluorescence confocal microscopy.

Generation of BM chimeric mice. BM cells were collected from WT or TLR4 KO mice by flushing femur and tibia as above with HBSS containing 2% bovine calf serum (BCS). Chimeric mice were generated by transferring donor WT or TLR4 KO BM cells into irradiated WT recipient mice (n = 10 per group). Recipient mice were lethally irradiated with a signal dose of 9.5 Gy, after which 5×10^6 BM cells/mouse were transferred via retro-orbital injection. The resulting WT→WT and TLR4 KO→WT mice were allowed to recover for at least 8 weeks to ensure stable engraftment before being subjected to AP induction.

CORM-2–primed cell transfer. BM cells were isolated from Balb/c mice as above, and $CD11b^+$ cells were enriched with CD11b microbeads (Miltenyi Biotec). The CD11b-enriched cells were treated with either VE or 100 μM CORM-2 overnight. After washing with PBS, 5×10^6 cells were transferred i.v. into mice undergoing caerulein-induced pancreatitis, 90 minutes after the first caerulein injection.

Western blot and quantitative PCR assay. Mouse pancreata or cells were homogenized in RIPA buffer containing protease inhibitors, and IκBα, phospho-IκBα, P65, and phospho-P65 antibodies (Cell Signaling) were used for Western blot. CORM-2–primed cells were lysed with TRIzol for RNA extraction. cDNAs were generated using GoScript reverse transcription system (Promega). Quantitative PCR was performed with ABI-7900 sequence detection system (Applied Biosystems) using the following specific TaqMan probes and primers: *Il22* forward, 5′-GACAGGTTCCAGCCCTACAT-3′; *Il22* reverse, 5′-CTGGATGTTCTGGTCGTCAC-3′; *Il22* probe, 5′-CAGGAAAGGCACCACCTCCTGC-3′; *Il10* forward, 5′-CCCAGAAATCAAGGAGCATT-3′; *Il22* reverse, 5′-TCACTCTTCACCTGCTCCAC-3′; *Il22* probe, 5′-TCGATGACAGCGCCTCAGCC-3′; *Tnfa* forward, 5′-CCAAAGGGATGAGAAGTTCC-3′; *Tnfa* reverse, 5′-CTCCACTTGGTGGTTTGCTA-3′; *Tnfa* probe, 5′-TGGCCCAGACCCTCACACTCA-3′. Samples were normalized to *Gapdh* and displayed as fold change relative to control.

Ca^{2+} influx assay. Fresh isolated pancreatic acinar cells were treated with VE or CORM-2 for 1 hour, loaded with 2 μM Fura 2-AM (MP Biomedicals) for 30 minutes at 37°C, and then washed extensively. Prior to Ca^{2+} influx measurements, 100 nM caerulein was added. Ca^{2+} influx was quantified using a dual-excitation spectrofluorometer as described previously (51, 52). Excitation at 340 and 380 nm and emission at 510 nm were measured, and results are expressed as fluorescence ratios.

CO and ruthenium determination. Release of CO from medium or cells was assessed by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy-myoglobin (MbCO). The amount of MbCO formed was quantified by measuring absorbance at 540 nm as described

previously (7). In brief, myoglobin solution (final concentration, 66 μmol/l; Sigma-Aldrich) was prepared fresh by dissolving the protein in 0.04M-phosphate buffer (pH 6.8). Sodium dithionite (0.1%) was added to convert myoglobin to deoxy-Mb prior to each reading. For standard curves, different concentrations of CORM-2 were directly added to the myoglobin solution. The presence of CORM-2 was determined by ICP-MS at the School of Earth Sciences Environmental Measurement Center of Stanford University.

Statistics. Unpaired Student's *t* test was used to determine statistical significance. A *P* value less than 0.05 was considered significant. Values are expressed as mean ± SEM (Prism 4; GraphPad Software). Unless otherwise indicated, results are from at least 3 independent experiments.

Study approval. All animal experiments were approved by Stanford University institutional animal care and use committees.

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