Extracellular Vesicles From Mice With Alcoholic Liver Disease Carry a Distinct Protein Cargo and Induce Macrophage Activation Through Heat Shock Protein 90

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A salient feature of alcoholic liver disease (ALD) is Kupffer cell (KC) activation and recruitment of inflammatory monocytes and macrophages (MØs). These key cellular events of ALD pathogenesis may be mediated by extracellular vesicles (EVs). EVs transfer biomaterials, including proteins and microRNAs, and have recently emerged as important effectors of intercellular communication. We hypothesized that circulating EVs from mice with ALD have a protein cargo characteristic of the disease and mediate biological effects by activating immune cells. The total number of circulating EVs was increased in mice with ALD compared to pair-fed controls. Mass spectrometric analysis of circulating EVs revealed a distinct signature for proteins involved in inflammatory responses, cellular development, and cellular movement between ALD EVs and control EVs. We also identified uniquely important proteins in ALD EVs that were not present in control EVs. When ALD EVs were injected intravenously into alcohol-naïve mice, we found evidence of uptake of ALD EVs in recipient livers in hepatocytes and MØs. Hepatocytes isolated from mice after transfer of ALD EVs, but not control EVs, showed increased monocyte chemoattractant protein 1 mRNA and protein expression, suggesting a biological effect of ALD EVs. Compared to control EV recipient mice, ALD EV recipient mice had increased numbers of F4/80hi cluster of differentiation 11b (CD11b)h Mc and increased percentages of tumor necrosis factor alpha–positive/interleukin 12/23–positive (anti-inflammatory/M2) KCS and infiltrating monocytes (F4/80intCD11bhi), while the percentage of CD206+CD163+ (anti-inflammatory/M2) KCS was decreased. In vitro, ALD EVs increased tumor necrosis factor alpha and interleukin-1β production in MØs and reduced CD163 and CD206 expression. We identified heat shock protein 90 in ALD EVs as the mediator of ALD-EV-induced MØ activation. Conclusion: Our study indicates a specific protein signature of ALD EVs and demonstrates a functional role of circulating EVs containing heat shock protein 90 in mediating KC/MØ activation in the liver. (HEPATOLOGY 2018;67:1986-2000).

Excessive alcohol consumption leads to alcoholic liver disease (ALD), which can progress to alcoholic hepatitis (AH) and cirrhosis, often resulting in liver failure and death. Developing effective treatments for alcohol-related diseases requires a mechanistic understanding of the cellular processes involved. Recent studies have identified some characteristics of extracellular vesicles (EVs) in ALD, and we demonstrated that AH patients have increased numbers of circulating EVs/exosomes. EVs are heterogeneous,

Abbreviations: AH, alcoholic hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; CD, cluster of differentiation; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; EV, extracellular vesicle; Hsp, heat shock protein; iBAQ, intensity-based absolute quantification; IL, interleukin; IM, infiltrating monocyte; KC, Kupffer cell; LMNC, liver mononuclear cell; LPS, lipopolysaccharide; miR-122, microRNA 122; miRNA, microRNA; MØ, macrophage; MV, microvesicle; Prdx1, peroxiredoxin 1; SE, standard error; TNFα, tumor necrosis factor alpha.

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membranous, cell-derived vesicles that are classified as exosomes (40-150 nm) or microvesicles (MVs; 150-1,000 nm) based on their mode of biogenesis and size. Exosomes arise from the endosomal pathway and multivesicular bodies, while MVs are generated from the cell’s plasma membrane. Studies have shown differences in the number or composition of circulating EVs released by various cell types between healthy and diseased individuals. Thus, molecular and functional characterization of EVs should clarify their role in ALD pathogenesis.

Accumulating evidence suggests that EVs represent a novel mode of communication between hepatocytes and monocytes and macrophages (MØs); however, the mechanisms behind this potential role for EVs in ALD are only partially understood. Recent studies indicate that EVs may mediate intercellular communication by delivering their cargo to recipient cells. The cargo of circulating EVs contains mRNAs, microRNAs (miRNAs), proteins, and lipids and differs between healthy and diseased individuals, indicating their potential importance in disease pathogenesis. Studies on the RNA cargo of EVs show that internalization of EVs can result in changes in recipient cells. We demonstrated that EVs from AH patients have distinct miRNA signatures and that alcohol-treated hepatocytes release EVs that reprogram monocytes through transfer of miRNA 122 (miR-122). A model of experimental fatty liver disease showed that circulating EVs had specific proteome and liver miRNAs. In a gastric infusion model, ALD-specific miRNAs were identified in hepatocyte-derived EVs. Another group showed that alcohol-treated hepatocytes secrete EVs containing cluster of differentiation 40 (CD40) ligand, which stimulates MØ activation. Thus, by transporting bioactive molecules such as mRNAs, miRNAs, proteins, and lipids, EVs can serve as regulators of biological responses.

We hypothesized that circulating EVs from mice with ALD have a characteristic proteomic signature compared to EVs from pair-fed control mice. Our mass spectrometric analysis on the molecular composition of EVs revealed a distinct proteomic signature of circulating ALD EVs compared to control EVs. To test the functional significance of this observation, circulating ALD EVs were injected intravenously into naive mice, and liver mononuclear cells (LMNCs) and hepatocytes were isolated from the recipient mice for further analysis. We show that circulating ALD EVs have a functional effect in alcohol-naive mice and promote activation of MØs in the liver in vivo.

**Materials and Methods**

**ANIMAL STUDIES**

The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved the animal studies. Female C57BL/6 mice (8 weeks old; Jackson Laboratory, ME) were used because they have increased inflammatory responses to alcohol in their livers compared to male mice due to an estrogen effect and thus are useful as an ALD model. Female C57BL/6 mice (30) developed ALD after chronic ethanol feeding on the Lieber-DeCarli diet consisting of 5% (v/v) ethanol (36% ethanol-derived calories) for 5 weeks. Control animals (n = 20) received an isocaloric alcohol-free diet (Bio-Serv, NJ). We included at least five to eight mice per group in the experiments and used a two-tailed t test and parametric or nonparametric analysis of variance. To obtain sufficient EVs, 20-30 mice were used to develop the ALD model. Mice were killed at the end of the experiment and sera collected to measure alanine aminotransferase (ALT) activity, as described.
EV ISOLATION AND CHARACTERIZATION

EV isolation was carried out as described. Briefly, sera from mice were centrifuged at 1,500 g for 5 minutes to remove cells, followed by 10,000 g for 20 minutes to remove cellular debris. The serum was then filtered through a 0.8-μm syringe filter (Millipore, Billerica, MA), and EVs were precipitated with Exoquick-TC according to the manufacturer’s guidelines. After precipitation, the EV pellet was washed several times to remove any sera contaminant and resuspended in phosphate-buffered saline. The size and concentration of EVs were determined by nanoparticle tracking analysis, and EVs were visualized by electron microscopy as described.

MASS SPECTROMETRIC ANALYSIS

Liquid chromatography tandem mass spectrometry on isolated and enriched EVs from mice was performed on a NanoAcquity ultra-performance liquid chromatograph (Waters Corporation, Milford, MA) coupled to a Q Exactive (Thermo Scientific, Waltham, MA) mass spectrometer, described in the Supporting Information.

PROTEIN QUANTIFICATION AND HEATMAP GENERATION

For protein quantification, we used the label-free intensity-based absolute quantification (iBAQ) method applied in Scaffold (Proteome Software, Portland, OR). Briefly, precursor ion intensities of all peptides matching each protein were divided by the theoretical number of peptides derived from in silico tryptic digestion. Then we calculated the fold change of each protein by taking the logarithm of the ratio of individual protein iBAQ values normalized to the average of the corresponding iBAQ values in the control EVs (n = 6). For calculating the fold change, the 0 iBAQ values were replaced with 1. The heatmap was generated in R using the heatmap3 package and includes proteins that had an average fold change larger than 0.2 in ALD EVs versus control EVs. A volcano plot was generated using the same data.

INGENUITY PATHWAY ANALYSIS

Ingenuity Pathway Analysis, a web-based software application, was used to analyze, integrate, and categorize the data generated from proteomics experiments, based on the software manual guidelines. We generated heatmaps to compare the protein levels and expression in ALD EVs to control EVs.

IN VIVO ADMINISTRATION OF EVS TO NAIVE MICE AND ISOLATION OF HEPATOCYTES AND LMNCs

EVs from sera of alcohol-fed or pair-fed mice were resuspended in phosphate-buffered saline at 100 μg/100 μL of EV protein and stored at –80°C. Frozen EVs (100 μL) were thawed and vortexed gently, to form a homogenous suspension, before being injected into recipient mice intravenously. Mice were anesthetized 3 hours after EV administration and perfused through the portal vein with saline solution, followed by enzymatic digestion, as described. Hepatocytes were separated by centrifugation and LMNCs purified by centrifugation in Percoll gradient as described. Additional detail is in the Supporting Information.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error (SE). Based on the underlying distribution, a nonparametric Mann-Whitney test or a parametric t test was used. For statistical analysis GraphPad Prism v.6.03 (GraphPad Software Inc.) was used.

Results

MICE WITH ALD HAVE INCREASED NUMBERS OF CIRCULATING EVs COMPARED TO CONTROL MICE

We reported that mice fed a 5-week Lieber-DeCarli alcohol diet exhibit ALD features characterized by steatosis, inflammation, and moderate fibrosis in the liver. Consistent with this, serum ALT levels were significantly increased in these mice compared to pair-fed controls (Fig. 1A), and the total number of circulating EVs in the sera was also significantly increased (Fig. 1B). The majority of the EVs ranged in size from 40 to 150 nm, indicating an abundance of exosomes, while the number of MVs was ~3 logs lower (Fig. 1B; Supporting Fig. S1A,B). However, both the exosome and MV fraction in chronic alcohol-fed mice were
FIG. 1. Characterization and proteome profile of circulating (serum) EVs in alcohol-fed and pair-fed mice. (A) Serum ALT levels were measured in pair-fed (control) and alcohol-fed (ALD) mice. (B) Exosome, MV, and EV numbers were determined in the serum by Nanosight. Data are represented as mean ± SE (n = 30). (C,D) A total of 646 proteins were identified by mass spectrometry. Proteins are organized using annotation derived from the Gene Ontology database using Scaffold software based on biological process (C) and molecular function (D). (E) Heatmap showing log (10) fold change of proteins in EVs isolated from alcohol-fed mice (n = 6) versus pair-fed mice (n = 6, control), with depletion depicted in blue and enrichment in red. Abbreviations: EtFed, ethanol-fed; PF, pair-fed.
significantly increased compared to controls (Fig. 1B). EVs had a characteristic morphology, as shown by electron micrographs (Supporting Fig. S1C). EVs isolated from the sera of alcohol-fed and pair-fed mice had comparable CD63 expression (an exosomal marker) by western blot (Supporting Fig. S1D), while the cellular marker glucose-regulated protein 78 was only detected in primary hepatocytes and not EVs (Supporting Fig. S1D).

**EVs DERIVED FROM MICE WITH ALD HAVE A UNIQUE PROTEIN SIGNATURE COMPARED TO EVs FROM PAIR-FED MICE**

Next, we characterized the protein cargo of EVs by mass spectrometry using equal amounts of protein from alcohol-fed and control-fed mice. Proteomic analysis identified 646 proteins in the cargo of EVs isolated from alcohol-fed (ALD EVs) mice that were either differentially regulated or not detected in the cargo of EVs from control mice (Supporting Table S1). Table 1 lists the 10 highly up-regulated proteins in ALD EVs compared to control EVs. Proteins were categorized according to their biological processes and molecular clusters based on the Gene Ontology database using Scaffold software (Fig. 1C,D). The identified proteins included many well-known exosomal proteins: heat shock protein (Hsp) family A member 8, Hsp90kDa alpha member 1, elongation factor 1-alpha 1, pyruvate kinase isozymes M1, aldolase 1, protein kinase G1, Hsp90 alpha family class B member 1, and albumin.\(^{15}\)

We generated a volcano plot to identify changes between the protein expressions in ALD EVs and control EVs (Supporting Fig. S2) and observed a significant number of differentially regulated proteins in ALD EVs. A heatmap demonstrated quantitative changes, with many proteins up-regulated in ALD EVs compared to control EVs (Fig. 1E). We also identified proteins present exclusively in ALD EVs (Table 2). Interestingly, some of these proteins were involved in alcohol metabolism and redox processes.

**PROTEIN EXPRESSION PROFILING OF ALD EVs REVEALS DIFFERENCES IN PROTEINS ASSOCIATED WITH INFLAMMATORY RESPONSES, CELLULAR DEVELOPMENT, AND CELLULAR MOVEMENT**

Next, using the proteomic analysis software Ingenuity Pathway Analysis, we generated a heatmap to compare the functional characteristics associated with the proteome profile of the ALD EVs and control EVs. Compared to control EVs, ALD EVs had increased expression of proteins involved in inflammatory responses, immune responses, proliferation and differentiation of immune cells, and organ inflammation of the liver and pancreas, while levels of proteins involved in immune cell activation, fibroblast proliferation, and MØ and myeloid cell differentiation were down-regulated (Fig. 2A,B). Ingenuity Pathway Analysis revealed an increase in proteins associated with leukocyte movement, cellular infiltration, and migration of phagocytes and granulocytes in ALD EVs compared to control EVs (Fig. 2C), while proteins involved in chemotaxis of immune cells were down-regulated. Overall, we observed that alcohol feeding led to

<table>
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<tr>
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<tr>
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<td>P01186</td>
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<tr>
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*Fold changes were calculated for all of the proteins by converting the 0 iBAQ values to 1 for calculation purposes.

Abbreviation: BPI, bactericidal/permeability increasing.
significant changes in the protein cargo of circulating EVs, and many of these were related to MØ functions.

**INCREASED miR-192 EXPRESSION IN HEPATOCYTES OF RECIPIENT MICE AFTER ALD EV TRANSFER INDICATES EV UPTAKE**

Next, to test the *in vivo* biological effect of circulating ALD EVs, we intravenously injected ALD EVs or control EVs into alcohol-naive recipient mice. We previously demonstrated a rapid biodistribution of exosome-rich plasma and its miRNA cargo to the liver after intravenous administration of EVs (16); thus, we harvested and analyzed recipient livers 3 hours after EV transfer and collected hepatocytes and LMNCs for further analysis (Fig. 3A).

We assessed indicators of EV uptake. First, to show hepatocyte uptake of EVs *in vitro*, we treated isolated primary hepatocytes with fluorescently labeled ALD or control EVs and observed that they were equally taken up by hepatocytes (Supporting Fig. S3). Second, we evaluated evidence of EV uptake *in vivo* and found a significant increase in miR-192 and miR-30a levels in ALD EVs compared to control EVs (Supporting Fig. S4A,B), consistent with our previous study of potential ALD biomarkers (2). After *in vivo* transfer of EVs, we observed a significant increase in miR-192 and miR-30a expression in hepatocytes isolated from recipient mice that received ALD EVs but not control EVs (Supporting Fig. S4C,D), most likely due to transfer of these miRNAs from ALD EVs. To further establish that the increased miR-192 and miR-30a levels in hepatocytes after ALD-EV transfer represented EV uptake and not *de novo* miRNA synthesis, we tested pre-miRNA levels. We found that pre-miR-192 expression was unchanged, supporting transfer of miR-192 from ALD EVs rather than synthesis in the recipient hepatocytes (Supporting Fig. S4E). Interestingly, both mature and pre-miR-30a expressions were increased in hepatocytes after ALD EV transfer compared to pair-fed EVs, suggesting a biological role for the transferred EVs (Supporting Fig. S4F).

Additional evidence for the biological effects of transferred ALD EVs was indicated by significantly increased monocyte chemoattractant protein 1 RNA and protein levels in hepatocytes of ALD EV recipient mice compared to mice receiving control EVs (Fig. 3B). These data suggested that hepatocytes from mice receiving ALD EVs show increased expression of inflammatory markers in the liver. In contrast, ALT levels in mice receiving ALD EVs or control EVs were unchanged 3 hours after EV transfer (Fig. 3C).

Finally, to demonstrate the uptake of EVs by hepatocytes and Kupffer cells (KCs) *in vivo*, we intravenously administered fluorescently labeled EVs derived from control or alcohol-fed mice into naive recipient mice. We observed *in vivo* uptake of both control EVs and ALD EVs in hepatocytes and KCs of the recipient mice (Fig. 3D,E; Supporting Videos S1-S4).

**IN VIVO TRANSFER OF ALD EVs INCREASES INFLAMMATORY MØs IN THE LIVERS OF ALCOHOL-NAIVE RECIPIENT MICE**

Because of the important role of MØs in ALD, we assessed the effect of ALD EVs on LMNCs. LMNC isolation resulted in ~97% live cells, and ~91% of these cells were CD45+ immune cells that were then
gated for infiltrating monocytes (IMs; F4/80<sup>int</sup>CD11b<sup>hi</sup>) and for KCs (F4/80<sup>hi</sup>CD11b<sup>low</sup>) (Fig. 4A). We found an increase in the CD45<sup>+</sup> immune cell population in the livers of mice that received ALD EVs compared to control EVs, indicating recruitment of immune cells to the liver (Fig. 4B). While the

FIG. 2. Heatmaps of the immune proteome profile of circulating EVs modulated in alcohol-fed compared to pair-fed mice. Heatmap showing the proteins that were modulated in EVs isolated from alcohol-fed mice (n = 6) compared to pair-fed mice (n = 6), which represent (A) inflammatory response, (B) cellular development, and (C) cellular movement. The up-regulated proteins in ALD EVs compared to control EVs are represented as orange boxes and the down-regulated proteins as blue boxes.
percentage of F4/80\textsuperscript{int}CD11b\textsuperscript{hi} IMs was unchanged, we found a significant increase in the percentage of F4/80\textsuperscript{hi}CD11b\textsuperscript{low} KCs in mice administered ALD EVs compared to control EVs (Fig. 4B; Supporting Fig. S5). We also evaluated other immune cell populations in the liver, including dendritic cells, natural killer cells, neutrophils, natural killer T cells, and B cells, and observed no significant change in their frequency; notably, T cells were increased in mice that received ALD EVs compared to control EVs (Supporting Fig. S6).

Because MØ/KCs play a pivotal role in ALD pathogenesis, we investigated the phenotype and activation of the monocyte/MØ populations in the EV recipient mice. Intracellular staining for tumor necrosis factor alpha (TNF\textalpha) and interleukin 12/23 (IL-12/23) is characteristic of an activated inflammatory MØ phenotype.\textsuperscript{(17)} We found a significant increase in the frequency of TNF\textalpha and IL-12/23 expression from ALD EVs in F4/80\textsuperscript{int}CD11b\textsuperscript{hi} IMs (Fig. 4C) and in F4/80\textsuperscript{hi}CD11b\textsuperscript{low} KCs (Fig. 4D). The TNF\textalpha and IL-12/23 increase was evident not only in the percent of cells...
expressing these cytokines but also in the mean fluorescence intensity of cytokine expression (Supporting Fig. S7A,B). Expression of the anti-inflammatory M2 markers CD206+CD163+ was increased in IMs in mice that received ALD EVs compared to control EVs (Fig. 4C), and CD163 mean fluorescence intensity was also increased (Supporting Fig. S7B). Furthermore, the percentage of CD206/CD163+ expression was decreased in F4/80hiCD11blo KCs (Fig. 4D) with no change in the mean fluorescence intensity of CD206 or CD163 (Supporting Fig. S7A) after ALD EV transfer. These data demonstrated inflammatory activation of liver MØ/KCs and the presence of IMs with both inflammatory and anti-inflammatory markers in ALD EV recipient mice. These results indicated that ALD EVs have a biological effect on the MØ/KC populations in the livers of recipient mice.

**ALD EVs ACTIVATE MØs IN VITRO**

Next, we tested whether MØs/KCs can take up circulating EVs using fluorescently labeled ALD EVs or control EVs. There was no significant difference in the KC uptake of ALD EVs or control EVs (Supporting Fig. S8). We also found that, after in vivo transfer of...
ALD EVs, there was a significant increase in the expression of miR-192 and miR-30a in LMNCs isolated from ALD EV recipient mice compared to LMNCs isolated from control EV recipient mice (Supporting Fig. S9A,B). These results suggested uptake of ALD EVs by MØ/KCs and indicated mediation of a biological effect on MØ in recipient mice.

To further investigate the biological effects of ALD EVs on MØ in vitro, RAW MØs were exposed to ALD EVs or control EVs. MØ treatment with ALD EVs, but not control EVs, resulted in increased expression of the MØ marker CD68 and activation marker TNF-α (Fig. 5A). Lipopolysaccharide (LPS) served as positive control for MØ activation and induced a significant increase in TNF-α levels (Fig. 5A). Gene expression analysis revealed a reduction in levels of the M2 MØ marker CD163 and no significant change in CD206 expression in RAW MØs after treatment with ALD EVs compared to control EVs (Fig. 5A). The secretion of TNF-α and IL-10 cytokines was significantly increased in MØs treated with ALD EVs compared to control EVs (Fig. 5B). Overall, these results suggested that ALD EVs can be taken up by normal MØs, leading to their activation and polarization.

**Hsp90 IN THE CARGO OF ALD EVs INDUCES MØ ACTIVATION**

Mass spectrometry identified Hsp90 as a highly enriched protein in ALD EVs compared to control EVs (Table 1), which we confirmed by western blot (Fig. 6A). To identify the origin of Hsp90 in the sera EV of alcohol-fed mice, we performed western blot analysis of the liver and hepatocytes isolated from

![FIG. 5. Hepatic immune cell profiling of EV recipient mice showed changes in the immune population. (A) RAW MØs were treated with LPS (100 ng/mL) or EVs isolated from pair-fed (Control-EVs) or ethanol-fed (ALD-EVs) or untreated (Control) for 24 hours. Cells were harvested, and cell culture supernatant was collected. RNA was isolated from cells, complementary DNA was transcribed, and PCR was performed for CD68, TNF-α, CD206, and CD163. (B) An enzyme-linked immunosorbent assay was performed for TNF-α and IL-10 from the cell culture supernatant. Data are represented as mean ± SE from three independent experiments.](image)
FIG. 6. Role of EV-bound Hsp90 in MØ activation. (A) Hsp90 and CD63 levels were assessed by western blot analysis in control EVs and ALD EVs. (B) RAW cells were treated with 0, 0.1, and 1 μg of recombinant Hsp90 proteins for 24 hours. Cells were harvested, RNA was isolated, complementary DNA was transcribed, and PCR was performed for TNFα, IL-1β, CD206, and CD163. (C) An enzyme-linked immunosorbent assay was performed from the cell culture for TNFα and IL-10. (D) RAW cells were pre-incubated with 0.5 μM of 17-DMAG and then treated with either 1 μg of Hsp90 or control EVs or ALD EVs for 24 hours. Cells were harvested, RNA was isolated, complementary DNA was transcribed, and PCR was performed for TNFα and CD163. (E) An enzyme-linked immunosorbent assay was performed from the cell culture for TNFα and IL-10. Data are represented as mean ± SE from three independent experiments. Multiple groups were compared using one-way analysis of variance.
chronic alcohol-fed mice, the same mice from which circulating EVs were isolated for the in vivo EV transfer experiment (Fig. 3). There was a significant increase in levels of Hsp90 in both the liver and isolated hepatocytes of ALD mice compared to pair-fed controls (Supporting Fig. S10A,B), indicating that the increased Hsp90 levels in the hepatocytes of the ALD mice are likely sequestered in the EVs secreted from these hepatocytes into the circulation.

We also studied two other highly expressed proteins in the ALD EVs, alcohol dehydrogenase 1 and fatty acid synthase, both of which are important in ALD pathogenesis. Based on our work and studies by others, hepatocytes are a major source of circulating EVs during ALD. Western blot analysis of hepatocytes showed increased expression of alcohol dehydrogenase 1 and fatty acid synthase in hepatocytes from alcohol-fed mice (Supporting Fig. S11), suggesting hepatocytes as the likely source of the Hsp90, fatty acid synthase, and ADH in circulating EVs.

Because Hsp90 has recently been implicated in MØ activation in ALD, we tested the hypothesis that Hsp90 mediates the effects of ALD EVs in inducing MØ activation. We demonstrated that increasing doses of recombinant Hsp90 on RAW MØs resulted in increased TNFα and IL-1β expression, while CD163 and CD206 expression decreased (Fig. 6B). The protein level of TNFα increased, whereas IL-10 decreased in cells treated with recombinant Hsp90 (Fig. 6C). These results demonstrated a MØ activating effect of Hsp90 similar to the effects we observed after ALD EV treatment of MØs (Fig. 5). Furthermore, MØs pretreated with 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; a competitive inhibitor of Hsp90) before exposure to ALD EVs or control EVs inhibited TNFα induction and prevented reduction of CD163 by ALD EV treatment or by the positive control, Hsp90 (Fig. 6D). TNFα levels were decreased in cells treated with 17-DMAG plus ALD EV compared to ALD EV alone (Fig. 6D). Together, these results indicate a role for Hsp90 in the cargo of ALD EVs as a mediator of MØ activation and function.

Discussion

EVs are involved in many biological processes including, but not limited to, inflammation, cell damage, regeneration, and cancer. EVs are derived from different cell types and are involved in physiological functions as well as disease pathogenesis. Studies from our group and others have reported changes in the numbers and pathologic roles of EVs in various types of liver diseases. We found a significant increase in the numbers of circulating EVs in the sera of chronic alcohol-fed mice compared to pair-fed mice with vesicles mostly in the size range of exosomes (the smaller subpopulation), similar to what has been reported in ALD patients. Depending on the state and type of the cells of origin, EVs carry unique and specific cargo that has been exploited in biomarker discovery and implicated in intercellular or interorgan communication.

Mass spectrometric analysis of the protein content of EVs from hepatocytes and biological fluids (e.g., urine) showed unique protein signatures in diseases. Proteome signatures have been identified in circulating EVs from a mouse model of fatty liver disease. We previously identified increases in specific miRNA cargos in circulating EVs of AH patients which are potential novel biomarkers. In this study, proteomic analysis of ALD EVs revealed enrichment of proteins involved in various immune responses, including inflammatory responses, cellular development, and cellular movement compared to control EVs. We identified at least 10 proteins uniquely expressed in the cargo of ALD EVs and not in control EVs, indicating a unique protein signature in ALD EVs. Interestingly, these proteins were induced in metabolism, alcohol metabolism, and redox regulation and may define EVs as a potential conductor of ALD or alcohol use. ALD EVs were also enriched for several proteins and pathways characteristic of ALD and AH including proteins involved in the migration of granulocytes, phagocytosis, and cellular infiltration by leukocytes. For example, increased granulocyte accumulation in the liver is a hallmark of AH, and we found increased neutrophil leukocyte numbers in our murine ALD model. We also found activation of protein clusters involved in organ inflammation as opposed to proteins involved in the initiation of inflammation, likely due to the fact that EVs reflect the present status of liver pathology and EV samples were analyzed in an already established phase of ALD characterized by established inflammation. Given the dynamic nature of EV cargo in other disease models, we cannot rule out that acute binge alcohol drinking or earlier time points of chronic alcohol feeding could show a different signature profile of EVs.

While some functional protein clusters were increased in ALD EVs, others were decreased compared to control
EVs. For example, a protein cluster associated with MØ differentiation was reduced. This could be consistent with previous observations of recruitment and activation of MØs in ALD and the particular enrichment of M1-type proinflammatory MØs (Prdx1) was up-regulated, similar to ALD EVs. Identifying proteins involved in cytoskeletal structures; carbohydrate, lipid, and xenobiotic metabolism; and redox homeostasis. Arginase 1, identified in ALD EVs, was up-regulated in galactosamine-treated, but not LPS-treated, hepatocytes. Circulating EVs from acetaminophen-treated mice and ALD patients showed that the inflammation-related protein peroxiredoxin 1 (Peroxiredoxin 1) was up-regulated, similar to ALD EVs. Interestingly, Prdx1, Prdx2, and Prdx4 were up-regulated in the circulating EVs of mice with fatty liver disease. Hsp90b, but not Hsp90a, was up-regulated in acetaminophen EVs by mass spectrometry. Thus, we conclude that there are similarities in some of the up-regulated proteins in the various models of liver diseases, but there are also unique proteins that can serve as disease biomarkers.

EVs can actively transfer biomacromolecules. We found a selective enrichment of miR-192 and miR-30a in ALD EVs, consistent with our previous report of miRNA profiling of EVs in chronic alcohol feeding. Here, we demonstrate that increased miR-192 levels in hepatocytes from ALD EV recipient mice were due to the horizontal transfer of miRNAs by EVs and not to de novo pre-miR-192 production in hepatocytes of recipient mice. Hepatocyte uptake of EVs was confirmed using fluorescein isothiocyanate–labeled EVs and in vitro experiments. We also found increased expression of miR-30a in mice injected with ALD EVs. Interestingly, the expression level of pre-miR-30a was increased in the hepatocytes of ALD EV recipient mice, indicating activation of upstream pathways that induce transcription of miR-30a. Notably, miR-30a target mRNAs were enriched in the ALD EVs, consistent with the proteomics analysis of EVs that showed specific pathway enrichment in ALD EVs. For example, miR-30a overexpression was associated with decreases in cellular migration and tumor invasiveness in breast cancer patients. Consistent with these effects, our functional enrichment pathway analysis showed that ALD EVs isolated from mice showed lower enrichment in proteins inducing cellular movement and migration compared to control EVs.

Previous studies indicated that alcohol can affect MØ polarization both in vitro and in vivo, and notably we found a reduction in the expression of CD206 and CD163 in livers of recipients of ALD EV transfer in vivo. Additionally, after in vivo transfer of EVs, we observed increased activation and frequency of the MØ/KC–secreting inflammatory cytokines TNFα and IL-12 in the liver of the ALD EV recipient mice compared to control EV recipient mice, suggesting an effect of EVs in liver inflammation. In vivo experiments performed with fluorescently labeled EVs also demonstrated increased uptake of ALD EVs in KCs in naive recipient mice. Although we did not observe a significant change in the total numbers of IMs infiltrating the liver of ALD EV recipient mice, we did observe increased activation of MØ/KCs with a mixed M1/M2 phenotype. This is reminiscent of the monocyte/MØ population observed in chronic alcohol–treated mice. Some evidence in the literature demonstrates selective uptake of EVs by immune cells in the liver, especially KCs, that leads to increased activation and proinflammatory signaling. Additionally, uptake of exosomes can lead to changes in the phenotype of resident macrophages (KC). Also, studies suggest that, along with monocyte recruitment to the liver, resident macrophages can proliferate locally within the liver. Thus, we interpret our results as indicating that the KC increase in the liver might be due to local proliferation and the effect of ALD EV uptake. Also, the increased proinflammatory and anti-inflammatory phenotype of the infiltrating monocytes could result from the uptake of ALD EVs as supported by the in vitro stimulation data. However, further studies may help to better understand this effect of EVs on liver immune cells.

Immunomodulatory activities including both immune suppression and immune activation have been reported for EVs in other pathological conditions. Di Trapani et al. demonstrated the immunomodulatory effects of EVs derived from mesenchymal stromal cells on immune cell functions. Exosomes isolated from human neural stem cell–derived exosomes suppress the activation and proliferation of T cells by inducing cell cycle arrest and apoptosis. Alcohol-treated hepatocytes secrete CD40 ligand–containing EVs that activate MØs and induce inflammatory cytokine secretion. Here, we show that in vivo activation of MØs can occur through uptake of EVs from mice with ALD. After ALD exosome transfer, we found an increased percentage of the F4/80hiCD11b hi cell population that is typical of KCs. While several studies have shown that elimination of KCs/MØs with clodronate or other methods prevents alcohol-induced...
liver inflammation, the plasticity and phenotype of KCs in ALD remain to be elucidated. (42) We provide evidence suggesting that EVs can modulate the KC phenotype and result in their inflammatory activation.

Finally, our study demonstrates that Hsp90 is increased in circulating ALD EVs and identifies the role of EV-bound Hsp90 in mediating MØ activation. The Hsps are key proteins in peptide folding and protein transportation. They also prevent protein aggregation during cellular stress conditions. (43) Hsp40, Hsp70, and Hsp90 are secreted from cells through exosomes and mediate communication between cells and tissues during the stress response. (44) Hsp70 also has an immunological function to stimulate cytokine production in monocytes. (44) We found increased expression of Hsp90 in ALD EVs compared to control EVs. Hsp90 is induced in the liver during the alcohol-induced inflammatory ALD EVs compared to control EVs. Hsp90 is induced in the liver during the alcohol-induced inflammatory response and modulates IkB kinase activation and the Toll-like receptor 4 pathway, leading to activation of nuclear factor kappa B. (46) Hsp90 is crucial in alcohol-induced steatosis and TNFα production in MØs, leading to alcohol-induced inflammation. (45) Hsp90 levels were diminished during short-term alcohol administration but increased after chronic alcohol exposure and induced a proinflammatory phenotype. (45) Our findings and a new level of understanding on increased Hsp90 by alcohol in liver parental cells demonstrate that Hsp90 is increased in ALD EVs. Furthermore, we show that ALD EVs can activate the proinflammatory effects of ethanol treatment through their Hsp90 cargo. The functional role of Hsp90 in mediating this effect was supported by our experiments with 17-DMAG, a selective Hsp90 inhibitor. (47) Consistent with our findings with EVs, in vivo inhibition of Hsp90 attenuated ALD in mice; however, we did observe that addition of exogenous ALD EVs elevated IL-10 levels, whereas Hsp90 addition led to a decrease in IL-10 levels, suggesting an Hsp90-independent effect of ALD EVs. Thus, it is likely that Hsp90 may not be the only effector in ALD EVs and that other proteins in the ALD EVs may also contribute to its immunomodulatory functions.

In conclusion, our results indicate that chronic alcohol administration results in a characteristic signature in the protein cargo of circulating EVs. Furthermore, we demonstrated that ALD EVs can induce functional changes in hepatocytes and induce a proinflammatory MØ phenotype in naive recipient mice after injection. Our proteomic analysis revealed EV enrichment of proteins associated with pathways involved in alcohol-induced liver injury. We also discovered a role for ALD EV–bound Hsp90 in mediating MØ activation in the liver in vivo. These data indicate that circulating ALD EVs have a unique proteomic signature and a functional role in the regulation of liver inflammation and MØ activation in ALD.

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