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Extracellular vesicles from mice with alcoholic liver disease carry a distinct protein cargo and induce macrophage activation via Hsp90

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Abstract

A salient feature of alcoholic liver disease (ALD) is Kupffer cell (KC) activation and recruitment of inflammatory monocytes/macrophages (Mo/MØ). These key cellular events of ALD pathogenesis may be mediated by extracellular vesicles (EVs). EVs transfer biomaterials, including proteins and miRNAs, 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 (ALD-EV) compared to pair-fed controls (control-EV). Mass spectrometry 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 MCP-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 F4/80^{hi}CD11b^{lo} KCs and increased percentages of TNFα⁺IL-12/23⁺ (inflammatory/M1) KCs and infiltrating monocytes (F4/80^{int}CD11b^{hi}) while the percentage of CD206⁺CD163⁺ (anti-inflammatory/M2) KCs was decreased. *In vitro*, ALD-EVs increased TNFα and IL-1β production in MØ and reduced

CD163 and CD206 expression. We identified Heat shock protein 90 (Hsp90) 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 Hsp90 in mediating KC/MØ activation in the liver.

Keywords

inflammatory; alcoholic hepatitis; proteomics; cell communication; exosome

Introduction

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 (1). Developing effective treatments for alcohol-related diseases requires a mechanistic understanding of the cellular processes involved. Recent studies identified some characteristics of extracellular vesicles (EVs) in ALD and we demonstrated that AH patients have increased numbers of circulating EVs/exosomes (2). EVs are heterogeneous, membranous, cell-derived vesicles that are classified as exosomes (40–150 nm) or microvesicles (MVs) (150–1000 nm) based on their mode of biogenesis and size (3). Exosomes arise from the endosomal pathway and multivesicular bodies while MVs are generated from the cell's plasma membrane (4). 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/macrophages (Mo/MØ); 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 (5). The cargo of circulating EVs contain mRNAs, microRNAs (miRNAs), proteins and lipids, and differs between healthy and diseased individuals, indicating their potential importance in disease pathogenesis (5). Previous studies on the RNA cargo of EVs show that internalization of EVs can result in changes in recipient cells (6). We demonstrated that EVs from AH patients have distinct miRNA signatures (2) and that alcohol-treated hepatocytes release EVs that reprogram monocytes via transfer of miR-122 (7). A model of experimental fatty liver disease showed that circulating EVs had specific proteome and liver miRNAs (8). In a gastric infusion model, ALD-specific miRNAs were identified in hepatocyte-derived EVs (9). Another group showed that alcohol-treated hepatocytes secrete EVs containing CD40 ligand that stimulate MØ activation (10). 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 (ALD-EVs) have a characteristic proteomic signature compared to EVs from pair-fed mice (control-EVs). Our mass spectrometry 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 naïve 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-naïve mice and promote activation of MØs in the liver *in vivo*.

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, from Jackson Laboratory, ME, USA) were used because they have increased inflammatory responses to alcohol in their livers compared to males due to an estrogen effect and thus are useful ALD model (11, 12). Mice (n = 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. The control animals (n=20) received an isocaloric alcohol-free diet (Bio-Serv, New Jersey, USA) (13). We included at least 5–8 mice per group in the experiments and used two-tailed t test, parametric or non-parametric ANOVA for analysis. To obtain sufficient EVs, 20–30 mice were used to develop the ALD model. Mice were euthanized at the end of the experiment and sera collected to measure alanine aminotransferase (ALT) activity as described (13).

EV isolation and characterization

EV isolation was carried out as described (2). Briefly, sera from mice were centrifuged at 1,500g for 5 min to remove cells followed by 10,000g for 20 min to remove cellular debris. The serum was then filtered through a 0.8µm syringe filter (Millipore, Billerica, MA, USA), and EVs 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 PBS. The size and concentration of EVs was determined by Nanoparticle Tracking Analysis and EVs were visualized by electron microscopy as described (2).

Mass spectrometry analysis

Liquid chromatography tandem mass spectrometry on isolated and enriched EVs from mice was performed on a NanoAcquity UPLC (Waters Corporation, Milford, MA) coupled to a Q Exactive (Thermo Scientific, Waltham, MA) mass spectrometer, described in the Supplementary Methods.

Protein quantification and Heatmap generation

For protein quantification, we used the label-free iBAQ (intensity-based absolute quantification) method applied in Scaffold (Proteome Software, Portland, OR) (14). 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 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 (IPA)

IPA, 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 naïve mice and isolation of hepatocytes and LMNCs**

EVs from sera of alcohol- or pair-fed mice were resuspended in PBS at 100µg/100µl of EV protein and stored at -80°C. Frozen EVs (100µl) were thawed, vortexed gently, to form a homogenous suspension before injecting into recipient mice intravenously. Mice were anesthetized 3 hrs after EV administration and perfused via portal vein with saline solution, followed by enzymatic digestion, as described (13). Hepatocytes were separated by centrifugation, and LMNCs purified by centrifugation in percoll gradient as described (13).

Additional information is in the supplementary section.

Statistical analysis

Data are expressed as mean±standard error (SE). Based on the underlying distribution, non-parametric Mann-Whitney test or 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 5-week Lieber-DeCarli alcohol diet exhibit ALD features characterized by steatosis, inflammation and moderate fibrosis in the liver (13). 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 40–150 nm, indicating an abundance of exosomes; while the number of MVs was ~3 logs lower (Fig 1B, S1A & B). However, both the exosome and MV fraction in chronic alcohol-fed mice were significantly increased compared to controls (Fig 1B). EVs had a characteristic morphology, as shown by electron micrographs (Fig S1C). EVs isolated from the sera of alcohol-fed and pair-fed mice had comparable CD63 expression (an exosomal marker) by western blot (Fig S1D), while the cellular marker, GRP-78, was only detected in primary hepatocytes and not EVs (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-EV) mice that were either differentially-

regulated or not detected in the cargo of EVs from control mice (control-EVs) (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 (GO) 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; Aldolase1; 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 (Fig S2) and observed a significant number of differentially-regulated proteins in ALD-EVs. A heatmap demonstrated quantitative changes with many proteins upregulated 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, IPA, 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 downregulated (Fig 2A,B). IPA 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 downregulated. Overall, we observed that alcohol feeding led to 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-naïve 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 hrs 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 (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 (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 (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 (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 (Fig S4F).

Additional evidence for the biological effects of transferred ALD-EVs was indicated by significantly increased MCP-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 hrs after EV transfer (Fig 3C).

Finally, to demonstrate the uptake of EVs by hepatocytes and KCs *in vivo*, we intravenously administered fluorescently labeled-EVs derived from control or alcohol-fed mice into naïve 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, SV1–4).

***In vivo* transfer of ALD-EVs increases inflammatory MØs in the livers of alcohol-naïve 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^{int}CD11b^{hi}) and for KCs (F4/80^{hi}CD11b^{low}) (Fig 4A). We found an increase in the CD45⁺ 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 percentage of F4/80^{int}CD11b^{hi} IMs were unchanged, we found a significant increase in the percentage of F4/80^{hi}CD11b^{low} KCs in mice administered ALD-EVs compared to control-EVs (Fig 4B, Fig S5). We also evaluated other immune cell populations in the liver including dendritic cells, natural killer (NK) cells, neutrophils, NKT 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 (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 TNFα and IL-12/23 is characteristic of an activated inflammatory MØ phenotype (17). We found a significant increase in the frequency of TNFα and IL-12/23 expression from ALD-EVs in F4/80^{int}CD11b^{hi} IMs (Fig 4C) and in the F4/80^{hi}CD11b^{low} KCs (Fig 4D). The TNFα and IL-12/23 increase was evident not only in the percent of cells expressing these cytokines but also in the mean fluorescence intensity (MFI) of cytokine expression (Fig S7A&B). Expression of anti-inflammatory M2 markers, CD206⁺CD163⁺ was increased in IMs in mice that received ALD-EVs compared to control-EVs (Fig 4C) and

CD163 MFI was also increased (Fig S7B). Furthermore, the percentage of CD206/CD163⁺ expression was decreased in the F4/80^{hi}CD11b^{low} KCs (Fig 4D) with no change in the MFI of CD206 or CD163 (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 MØ/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 (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 (Fig S9A and 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). 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 the M2 MØ marker CD163 levels and no significant change in CD206 expression in RAW MØ after treatment with ALD-EVs compared to control-EVs (Fig 5A). The secretion of TNF α and IL-10 cytokines was significantly increased in MØ treated with ALD-EVs compared to control-EVs (Fig 5B). Overall, these results suggested that ALD-EVs can be taken up by normal MØ and lead to their activation and polarization.

Hsp90 in the cargo of ALD-EVs induces MØ activation

Mass spectrometry analysis 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 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 the levels of Hsp90 in both the liver and isolated hepatocytes of ALD mice compared to pair-fed controls (Fig S10A & B); thus, indicating that the increased Hsp90 levels in the hepatocytes of the ALD mice is 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 (ADH1) and Fatty acid synthase (FAS), both of which are important in ALD pathogenesis (18–20). Based on our work and studies by others, hepatocytes are a major source of circulating EVs during ALD (7, 21). Western blot analysis of hepatocytes showed increased expression of ADH1 and FAS in hepatocytes from alcohol-fed mice (Fig S11), suggesting hepatocytes as the likely source of the Hsp90, FAS, and ADH in circulating EVs.

Because Hsp90 has recently been implicated in MØ activation in ALD (22), 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 TNF α and IL-1 β expression, while CD163 and CD206 expression decreased (Fig 6B). The protein level of TNF α 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 MØ (Fig 5). Furthermore, MØs pretreated with 17-DMAG (a competitive inhibitor of Hsp90) before exposure to ALD-EVs or control-EVs inhibited TNF α induction and prevented reduction of CD163 by ALD-EV treatment or by the positive control, Hsp90 (Fig 6D). The TNF α 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 (5). EVs are derived from different cell types and are involved in physiological functions as well as in disease pathogenesis (5). Studies from our group and others reported changes in the numbers and pathologic roles of EVs in various types of liver diseases (2, 3, 7, 8, 10, 23, 24). 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 (2, 25). 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 inter-organ communication (7, 25, 26).

Mass spectrometry analysis of the protein content of EVs from hepatocytes and biological fluids (e.g., urine) showed unique protein signatures in diseases (27–29). Proteome signatures have been identified in circulating EVs from a mouse model of fatty liver disease (8). We previously identified increases in specific miRNA cargos in circulating EVs of AH patients which are potential novel biomarkers (2). 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 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 (30). 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 (30). Given the dynamic nature of EV cargo in

other disease models (5), 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 MØ in ALD and the particular enrichment of M1-type pro-inflammatory MØs (31) and requires follow-up. Other studies investigated the content of EVs in drug-, alcohol-induced liver injury and nonalcoholic steatohepatitis (8, 21, 24). A proteomic analysis of the hepatocyte-secreted EVs after galactosamine (GalN) and lipopolysaccharide (LPS) treatment, that cause liver inflammation and damage (24), identified proteins involved in cytoskeletal structures, carbohydrate, lipid and xenobiotic metabolism, and redox homeostasis. Arginase 1 (Arg1), identified in ALD-EVs, was upregulated in GalN but not LPS-treated hepatocytes (24). Circulating EVs from APAP-treated mice and ALD patients showed inflammation-related protein, peroxiredoxin 1 (Prdx1), upregulated, similar to ALD-EVs (21). Interestingly, Prdx1, Prdx2 and Prdx4 were upregulated in the circulating EVs of mice with fatty liver disease (8). Hsp90b, but not Hsp90a, was upregulated in APAP-EVs by mass spectrometry analysis (21). Thus, we conclude that there are similarities in some of the upregulated 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 (7, 32, 33). We found a selective enrichment of miRNA-192 and miRNA-30a in ALD-EVs, consistent with our previous report of miRNA profiling of EVs in chronic-alcohol feeding (2). Here, we demonstrated that increased miRNA-192 levels in hepatocytes from ALD-EV recipient mice were due to the horizontal transfer of miRNAs by EVs, and not due to *de novo* pre-miRNA-192 production in hepatocytes of recipient mice. Hepatocyte uptake of EVs was confirmed using FITC-labeled EVs and *in vivo* experiments. We also found increased expression of miRNA-30a in mice injected with ALD-EVs. Interestingly, the expression level of pre-miRNA-30a was increased in the hepatocytes of ALD-EV recipient mice indicating activation of upstream pathways that induce transcription of miRNA-30a. Notably, miRNA-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, miRNA-30a overexpression was associated with decreases in cellular migration and tumor invasiveness in breast cancer patients (34). 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* (31, 35, 36). Additionally, after *in vivo* transfer of EVs, we observed increased activation and frequency of MØ/KCs secreting inflammatory cytokines, TNF α 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 naïve 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 (31, 37). Some evidence in the literature demonstrate selective uptake of EVs by immune cells in the liver, especially KCs, that leads to increased activation and pro-inflammatory signaling (38, 39). Additionally, uptake of exosomes can lead to changes in the phenotype of resident macrophages (KCs) (38). Also, studies suggest that, along with monocyte recruitment to the liver, resident macrophages can proliferate locally within the liver (40, 41). Thus, based on our results we interpret that the KC increase in the liver might be due to local proliferation and the effect of ALD-EV uptake. Also, the increased pro-inflammatory 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. Trapani et al. demonstrated the immunomodulatory effects of EVs derived from mesenchymal stromal cells on immune cell functions (42). Exosomes isolated from human neural stem cell-derived exosomes suppress the activation and proliferation of T cells by inducing cell cycle arrest and apoptosis (26). Alcohol-treated hepatocytes secrete CD40L-containing EVs that activate MØ and induce inflammatory cytokine secretion (10). Here, we show that *in vivo* activation of MØ can occur through uptake of EVs from mice with ALD. After ALD-exosome transfer, we found an increased percentage of the F4/80^{hi}CD11b^{lo} cell population that is typical of KCs (31). While several studies showed that elimination of KCs/MØ with clodronate or other methods prevents alcohol-induced liver inflammation, the plasticity and phenotype of KCs in ALD remains to be elucidated (43). We provide new evidence suggesting that EVs can modulate the KC phenotype and result in their inflammatory activation.

Finally, our study demonstrated that Hsp90 is increased in circulating ALD-EVs and identified 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 (44). Hsp40, Hsp70 and Hsp90 are secreted from cells via exosomes and mediate communication between cells and tissues during the stress response (45). Hsp70 also has an immunological function to stimulate cytokine production in monocytes (45). We found increased expression of Hsp90 in ALD-EVs compared to control-EVs. Hsp90 is induced in the liver during the alcohol-induced inflammatory response (22, 46) and modulates IκB kinase activation and the TLR4 pathway leading to activation of NF-κB (47). Hsp90 is crucial in alcohol-induced steatosis and TNFα production in MØ leading to alcohol-induced inflammation (46). Hsp90 levels were diminished during short-term alcohol administration but increased after chronic alcohol exposure and induced a proinflammatory phenotype (46). Our findings and a new level of understanding on increased Hsp90 by alcohol in liver parental cells demonstrate for the first time that Hsp90 is increased in ALD-EVs. Furthermore, we showed that ALD-EVs can mediate the proinflammatory effects of ethanol treatment via their Hsp90 cargo. The functional role of Hsp90 in mediating this effect was supported by our experiments with 17-DMAG, a selective Hsp90 inhibitor (48). Consistent with our findings with EVs, *in vivo* inhibition of

Hsp90 attenuated ALD in mice; however, we did observe that addition of exogenous ALD-EV 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 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 pro-inflammatory MØ phenotype in naïve 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 new 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 regulation of liver inflammation and MØ activation in ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AH	Alcoholic Hepatitis
ALD	Alcoholic Liver Disease
ADH1	Alcohol Dehydrogenase 1
ALT	Alanine aminotransferase
EVs	Extracellular Vesicles
FAS	Fatty acid synthase
Hsp	Heat shock protein
IMs	Infiltrating monocytes
iBAQ	intensity-based absolute quantification
IPA	Ingenuity Pathway Analysis
KCs	Kupffer Cells
LMNCs	Liver Mononuclear Cells

MØ	Macrophages
MFI	Mean Fluorescence Intensity
MVs	Microvesicles
miRNA	microRNA
NK cells	natural killer cells

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Figure 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 (PF, control) and alcohol-fed (EtFed, ALD) mice. (B) Exosome, Microvesicle and EV numbers were measured in the serum by Nanosight. The data is represented as Mean $\pm$ SE (n=30). (C, D) A total of 646 proteins were identified by mass spectrometry analysis. Proteins are organized using annotation derived from the Gene Ontology (GO) database using Scaffold software based on biological process (C) and molecular function (D). (E) Heatmap showing log₁₀ fold change of proteins in EVs isolated from alcohol-fed mice (n=6, alcohol) versus pair-fed mice (n=6, control) with depletion depicted in blue and enrichment in red.

Figure 2. Heatmaps of the immune proteome profile of circulating EVs modulated in alcohol-fed as 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 upregulated proteins in ALD-EVs compared to control-EVs are represented as orange boxes and the downregulated proteins as blue boxes.

Figure 3. *In vivo* delivery of EVs from alcohol-fed and pair-fed mice to naïve mice led to hepatocyte activation

(A) Schematic showing the experimental setup for the *in vivo* administration of EVs in naïve mice. (B) MCP-1 RNA, (B) MCP-1 protein levels, and (C) ALT levels in the hepatocytes from naïve mice which received ALD-EVs or control-EVs. The data is represented as Mean $\pm$ SE ($n=6$). (D, E) Fluorescently-labeled control-EVs and ALD-EVs (100µg) were administered into recipient mice intravenously and hepatocytes and KCs were isolated 3 hrs after administration from the mice and studied by confocal microscopy. The nucleus was stained by DAPI and the EVs were labeled with PKH67 (green). The bar graphs show the quantification of the fluorescence for each of the conditions.

Figure 4. Increased inflammatory markers in macrophages of mice receiving ALD-EVs as compared to control-EVs

(A) LMNCs were immunostained with Live dead stain, CD45 APC-Cy7, CD11b PE and F4/80 APC. Live cells were gated based on Indo-1 violet live cell staining. CD45⁺ cells were gated and the percentage of F4/80^{hi}CD11b^{lo} KCs and F4/80^{int}CD11b^{hi} infiltrating monocytes were determined. A representative gating strategy is shown. (B) In mice receiving ALD-EVs or control-EVs the frequency of total immune cell population (CD45⁺), F4/80^{hi}CD11b^{lo} KCs and F4/80^{int}CD11b^{hi} infiltrating monocytes were determined. (C, D) The M1 (TNFα⁺IL-12/23⁺) and M2 (CD206⁺CD163⁺) KCs and infiltrating monocyte population are shown. The data is representative of n=5–6 mice per group and shown as Mean±SE.

Figure 5. Hepatic immune cell profiling of EV recipient mice showed changes in the immune population

(A) RAW macrophages were treated with LPS (100ng/ml) or EVs isolated from pair-fed (PF, Control-EVs) or ethanol fed (ALD-EVs) or were untreated (Control) for 24 hrs. The cells were harvested and cell culture supernatant was collected. RNA was isolated from cells, cDNA transcribed and PCR was performed for CD68, TNF α , CD206 and CD163. (B) ELISA was performed for TNF α and IL-10 from the cell culture supernatant. The data is represented as Mean $\pm$ SE from 3 independent experiments.

Figure 6. Role of EV bound Hsp90 in macrophage 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 μ g of recombinant Hsp90 proteins for 24 hr. The cells were harvested, RNA isolated, cDNA transcribed and PCR performed for TNF α , IL-1 β , CD206, and CD163. (C) ELISA was performed from the cell culture for TNF- α and IL-10. (D) RAW cells were pre-incubated with 0.5 μ M of 17-DMAG and then treated with either 1 μ g of Hsp90 or Control-EVs or ALD-EVs for 24 hrs. The cells were harvested, RNA isolated, cDNA transcribed and PCR performed for TNF α and CD163. (E) ELISA was performed from the cell culture for TNF α and IL-10. The data is represented as

Mean $\pm$ SE from 3 independent experiments. Multiple groups were compared using one-way ANOVA.

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Table 1

A. List of 10 upregulated proteins based on fold change in EVs from ALD mice as compared to control mice (Total 646 identified proteins)

	Identified proteins	Accession number	Molecular Weight	Fold changes*
1	Cluster of Heat shock protein HSP 90-alpha	sp P07901 HS90A_MOUSE	85 kDa	5.87E+05
2	Cluster of Cathepsin D	sp P18242 CATD_MOUSE	45 kDa	2.76E+06
3	Cluster of Ig kappa chain V-II region 2S1.3	sp P01629 KV2A4_MOUSE	12 kDa	2.75E+06
4	Cluster of Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	sp Q9R0E2 PLOD1_MOUSE	84 kDa	2.99E+05
5	Fructose-bisphosphate aldolase B	sp Q91Y97 ALDOB_MOUSE	40 kDa	486
6	BPI fold-containing family A member 2	sp P07743 BPIA2_MOUSE	25 kDa	347
7	Complement C1q subcomponent subunit	sp Q02105 C1QC_MOUSE	26 kDa	172
8	Homogentisate 1,2-dioxygenase	sp O09173 HGD_MOUSE	50 kDa	7.84E+01
9	Fatty acid synthase	sp P19096 FAS_MOUSE	272 kDa	3.58E+01
10	Isoform 2 of Reelin	sp Q60841-2 RELN_MOUSE	387 kDa	3.31E+01

* Fold changes were calculated for all the proteins by converting the 0 Ibaq values to 1 for calculation purposes.

Table 2

List of upregulated proteins uniquely present in EVs from ALD mice and not in EVs of control mice (Total 646 identified proteins)

	Identified proteins	Accession number	Molecular Weight
1	Cluster of Betaine--homocysteine S-methyltransferase 1	sp O35490 BHMT1_MOUSE	45 kDa
2	Argininosuccinate lyase	sp Q91YI0 ARLY_MOUSE	52 kDa
3	Arginase-1	sp Q61176 ARGI1_MOUSE	35 kDa
4	Glycine N-methyltransferase	sp Q9QXF8 GNMT_MOUSE	33 kDa
5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein	sp A2AVA0 SVEP1_MOUSE	387 kDa
6	Glutamate dehydrogenase 1	sp P26443 DHE3_MOUSE	61 kDa
7	Granulins	sp P28798 GRN_MOUSE (+1)	63 kDa
8	Complement C1q subcomponent subunit B	sp P14106 C1QB_MOUSE	27 kDa
9	Peroxiredoxin-1	sp P35700 PRDX1_MOUSE	22 kDa
10	Alcohol dehydrogenase 1	sp P00329 ADH1_MOUSE	40 kDa
11	Chymotrypsin-like elastase family member 1	sp Q91X79 CELA1_MOUSE	29 kDa
12	Platelet glycoprotein 4	sp Q08857 CD36_MOUSE	53 kDa