DR. GINA EAGLE (Orcid ID : 0000-0002-7381-2355)

Article type : Original

TITLE PAGE

Exosomal transport of hepatocyte-derived drug-modified proteins to the immune system

Monday O. Ogese (ogese79@liv.ac.uk),[1,2] Rosalind E. Jenkins (rjenkins@liv.ac.uk),[2] Kareena Adair (Kareena.Adair2@liv.ac.uk),[2] Arun Tailor (hlatailo@liv.ac.uk),[2] Xiaoli Meng (xlmeng@liv.ac.uk),[2] Lee Faulkner L. (paxlf@liv.ac.uk),[2] Bright O. Enyindah (psbenyin@liv.ac.uk),[2] Amy Schofield (amyschof@liv.ac.uk),[2] Diaz-Nieto R (Rafael.diaz-nieto@aintree.nhs.uk),[4] Lorenzo Ressel (ressel@liv.ac.uk),[3] Gina L. Eagle (gleagle@liverpool.ac.uk),[2] Neil R. Kitteringham (neilk@liv.ac.uk),[2] Chris E. Goldring (chrissy@liv.ac.uk),[2] B. Kevin Park (bkpark@liv.ac.uk),[2] Dean J. Naisbitt (dnes@liv.ac.uk),[2] Catherine Betts (Catherine.Betts@astrazeneca.com).[1]

[1]Pathology Sciences, Drug Safety and Metabolism, IMED Biotech Unit, AstraZeneca, Cambridge, UK

[2]MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, University of Liverpool, Ashton Street, Liverpool L69 3GE, UK

[3]Department of Veterinary Pathology and Public Health, Institute of Veterinary Science, University of Liverpool, Leahurst Campus, Neston, CH647TE, UK

[4]North Western Hepatobiliary Unit, Aintree University Hospital NHS Foundation Trust, Liverpool L9 7AL, UK

Key words: Drug-induced liver injury, hepatocytes, immune system, human, antigen.

FOOTNOTE PAGE

Corresponding author: Professor Dean J. Naisbitt, MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, The University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, England

Telephone: 0044 151 7945346; Fax: 0044 151 7945540; e-mail: dnes@liv.ac.uk

List of abbreviations: drug-induced liver injury, DILI; peripheral blood mononuclear cells, PBMC; human leukocyte antigen, HLA; major histocompatibility complex, MHC.

Financial support: MOO received a post-doctoral Fellowship from AstraZeneca to support his studies. The project also received funding from the MRC Centre for Drug Safety Science (Grant number G0700654) and the BBSRC (Grant number BB/R008108/1).

ABSTRACT

Idiosyncratic drug-induced liver injury (DILI) is a rare, often difficult to predict adverse reaction with complex pathomechanisms. However, it is now evident that certain forms of DILI are immune-mediated and may involve the activation of drug-specific T-cells. Exosomes are cell-derived vesicles that carry RNA, lipids and protein cargo from their cell of origin to distant cells, and may play a role in immune activation. Herein, primary human hepatocytes were treated with drugs associated with a high incidence of DILI (flucloxacillin, amoxicillin, isoniazid and nitroso-sulfamethoxazole) to characterize the proteins packaged within exosomes that are subsequently transported to dendritic cells for processing. Exosomes measured between 50-100 nm and expressed enriched CD63. LC-MS/MS identified 2109 proteins, with 608 proteins being quantified across all exosome samples. Data are available via ProteomeXchange with identifier PXD010760. Analysis of gene ontologies revealed that exosomes mirrored whole human liver tissue in terms of the families of proteins present, regardless of drug treatment. However, exosomes from nitroso-sulfamethoxazole-treated hepatocytes selectively packaged a specific subset of proteins. LC-MS also revealed the presence of hepatocyte-derived exosomal proteins covalently modified with amoxicillin, flucloxacillin and nitroso-sulfamethoxazole. Uptake of exosomes by monocyte-derived dendritic cells occurred silently, mainly via phagocytosis, and was inhibited by latrunculin A. An, amoxicillin-modified 9-mer peptide derived from the exosomal transcription factor protein SOX30 activated naïve T-cells from HLA-A*02:01 positive human donors. *Conclusion.* This study shows that exosomes have the potential to transmit drug-specific hepatocyte-derived signals to the immune system and provides a pathway for the induction of drug hapten-specific T-cell responses.

INTRODUCTION

Drug-induced liver injury (DILI) is a complex, multistep and sometimes fatal adverse drug reaction (1). While type A reactions can be explained by the pharmacology of the drug, the molecular mechanisms of type B or idiosyncratic reactions remain the focus of intensive research. Idiosyncratic DILI is rare and difficult to predict; hence, it is reasonable to assume that these reactions are associated with specific patient risk factors. Genome-wide association studies have linked specific human leukocyte antigens (HLA) to DILI, and as HLA molecules present antigenic determinants to T-cells, the genetic studies implicate the adaptive immune system in the disease pathogenesis. Adverse reactions to amoxicillin clavulanate (2), flucloxacillin (3), lapatinib (4), lumiracoxib (5), minocycline (6), ticlopidine (7) and ximelagatran (8) are all associated with a specific risk allele. Although the exact role of drug-specific T-cells in DILI is not fully understood, recent studies have detected drug-specific T-cells in (i) the peripheral blood (9-11) and (ii) liver biopsies from patients with DILI (12, 13). Similarly, T-cells have also been shown to induce cytotoxicity of hepatocyte-like

cells transfected with the risk allele HLA-B*57:01 (13). It is often assumed that antigenic and stress-related signals from the liver are important for adaptive immune stimulation in patients with DILI; however, the origin and mechanism of transmission of these signals are difficult to define after systemic drug exposure (14). Since primary human hepatocytes are the principle target for DILI drugs, we hypothesize that they transmit drug-specific or at least drug-dependent signals to the immune system (15). Hence, the purpose of this study was to characterize the proteins encapsulated within exosomes derived from hepatocytes treated with DILI drugs and whether exosomes deliver drug-specific signals to dendritic cells that subsequently activate the adaptive immune system.

Exosomes, are membrane-bound nano vesicles that originate from the endosomal compartment. They are either degraded by lysosomes or secreted into the extracellular space upon fusion with the plasma membrane (16). The biogenesis, cargo sorting and ubiquitin-dependent degradation of exosomes are regulated by a combination of ESCRTs and multiprotein complexes (17, 18). These vesicles transport functional macromolecular components from their cells of origin to distant cells, and are thought to play an important role in intercellular communication (19). Although the biogenesis of exosomes is conserved in eukaryotes, Kruger et al demonstrated significant differences in proteomic and miRNA profiles of exosomes derived from MCF-7 and MDA-MB 231 cells (20). In addition the composition of exosomes can be regulated by factors like infection, stress and disease (21-22). Therefore, it is plausible that the sorting and packaging of hepatocyte-derived exosomes may be influenced by drug exposure, disease state and/or the unique phenotype of patients with drug-induced liver injury.

Interestingly, tumour-derived exosomes transport multiple membrane-bound and soluble factors that supress the function of human cytotoxic $CD4^+$ and $CD8^+$ T-cells (23). Administration of the immunotherapeutic agent, IRX-2 was shown to protect $CD8^+$ T-cells from tumour-derived exosome-induced apoptosis, resulting in enhanced T-cell-mediated anti-tumour activity (24). Paradoxically, the ability of exosomes to cross the cell membrane has also been explored for targeted drug delivery systems in cancer immunotherapy (25).

A dogma of drug immunogenicity research is that the binding of drugs to cellular proteins induces a tissue-specific signature to direct the immune response. In this respect, exosomes have the potential to transport drug-modified proteins from hepatocytes to dendritic cells for protein processing and ultimately the display of antigenic peptides. Thus, this study aimed to: (1) characterise protein profiles of hepatocyte-derived exosomes and identify specific drug modifications; and (2) explore the cellular uptake of hepatocyte-derived exosomes and the impact of this process on dendritic cell function and T-cell activation.

EXPERIMENTAL PROCEDURES

Isolation of hepatocytes from human liver tissue

Liver biopsies from consenting donors were used for primary human hepatocyte isolation. Biopsies were first perfused with HEPES buffer for 20-30 min. This was followed by tissue digest using collagenase type IV (Sigma Aldrich, UK). Hepatocytes were harvested using Williams E medium and washed twice by gradient centrifugation for 5 min, at 4^{o}C and 80 x g. Hepatocytes were cultured in Williams E supplemented with L-glutamine (2 mM), penicillin (100 µg/ml), streptomycin (100 U/ml), insulin-transferin-selenium (100x), and dexamethasone (1 µM/ml) using 6 well plates pre-coated

with collagen-I (Corning Flintshire, UK). Cells were then maintained in fresh culture media overnight before exposure to test drugs for 24 h.

Drug hepatocyte treatment and isolation of exosomes from culture supernatant

Culture supernatant from hepatocytes treated with sub-toxic concentrations of amoxicillin (0.05 mM), flucloxacillin (0.05 mM), isoniazid (0.03 mM) and nitroso-sulfamethoxazole (0.01 mM) (26) was collected for exosome isolation after 24 h and cells were lysed in RIPA buffer for further proteomic analysis. Amoxicillin, flucloxacillin and isoniazid were soluble in cell culture media while nitroso sulfamethoxazole was dissolved in 0.05% DMSO. The vehicle cell culture media contained 0.05% DMSO. Hepatocytes from 3 donors were used for the analysis. Supernatant was first centrifuged at 3000g for 15min to remove debris. Afterwards, supernatant was gently mixed with ExoQuick-TC solution (5:1; System Biosciences, Palo Alto, Canada). The mixture was incubated at 4 ^{o}C for 12 h. Samples were then centrifuged at 1500g for 30 min. Supernatant was discarded and the tubes were centrifuged for a further 5 min. Exosomes pellets were suspended in either RIPA buffer or PBS.

Transmission electron microscopy

The exosome suspension was placed on formvar-coated copper grids for 20 minutes then washed several times with PBS. Exosomes were then fixed by placing grids on drops of 2% glutaraldehyde on parafilm for 5min. Exosomes were contrasted and embedded by transferring grids to 2% uranyl acetate for 5 min then adding a drop of 0.13% methyl cellulose and 0.4% uranyl acetate for 5 minutes. The grids were visualized using a Philips EM 208S transmission electron microscope at 80kv and images captured with a Gatan CCD camera.

Assessment of the expression of human exosomal markers and the presence of drug-modified exosomal proteins

20 µg of protein was separated using 12 %SDS-PAGE (170 V, 75 min) and transferred onto a nitrocellulose blotting paper (230 mA, 1 h). After blocking, blots were incubated in primary antibodies specific for CD9, CD63, CD81 and Hsp70 (System Biosciences, Palo Alto, Canada) overnight. Anti-rabbit and anti-goat HRP-conjugated secondary antibodies were then applied and the proteins were visualised using enhanced chemoluminescence substrate (Perkin Elmer, Netherlands).

Assessment of drug-modified exosomal proteins

Drug-modification within exosomes was visualized by immunoblotting using rabbit anti-flucloxacillin (custom antibody, Eurogentec, Belgium), rabbit anti-nitroso-sulfamethoxazole; 1:2000 (Panigen, USA) and mouse anti-penicillin (AbD Serotec, USA) antibodies.

Proteomic profiling of hepatocyte-derived exosomes

To investigate changes in exosomal protein expression and drug modification of exosomal proteins, protein lysates were separated using 12%SDS-PAGE (27). Each lane was rehydrated in 10 ng/µl trypsin solution and incubated at 37^{o}C overnight. The peptides were extracted and dried in a SpeedVac, followed by resuspension in 10 µl 0.1% formic acid for LC-MS/MS analysis. Samples were delivered into a Triple TOF 6600 mass spectrometer (Sciex) by automated in-line reversed phase liquid chromatography. A gradient of 2-50 % (v/v) ACN, 0.1 % (v/v) formic acid over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive

ion mode using information-dependent acquisition powered by Analyst TF 1.7 software (Sciex), using mass ranges of 400-1800 atomic mass units (amu) in MS and 100-1500 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle using a threshold of 300 counts per sec, with dynamic exclusion for 12 secs and rolling collision energy.

Proteomic data analysis

Proteins were identified by ProteinPilot software v5.0 (Sciex) using the Paragon^{TM} algorithm and the SwissProt database with biological modifications allowed. The mass tolerance for both precursor and fragment ions was 10 ppm. The data were also searched against the reversed database and only proteins that fell within a 1% global false discovery rate were included in further analyses.

Two approaches were used to assess potential differences in the exosomal protein profile as a result of each of the drug treatments. The decision to use two independent approaches for the data analysis was based on the observed heterogeneity of the hepatocyte donors and the passage of time between the acquisition of the first dataset and the last.

In approach 1, peptide intensities for each protein derived from ProteinPilot were summed and normalised to total ion count for each exosome sample. The data were sorted in RStudio (version 3.4.0) to produce a composite list of proteins present in every sample and log10 transformed. In approach 2, the data were analysed using label-free quantification in MaxQuant (28): all settings used were default apart from the minimal LFQ ratio count being set to 1 and the retention time window being increased to 5 minutes. The data were searched using human UniProt database. The MaxQuant output file was edited to remove contaminants and proteins matching the reverse database. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (29) partner repository with the dataset identifier PXD010760.

Data generated were then subjected to further analysis using the Partek Genomics Suite (version 7.18.0130). Hierarchical cluster analysis was performed using standardisation, whilst a correlative Principal Component Analysis was performed assuming all variables had an equal influence on the principal components. The data were then batch corrected for donor and the above analyses were repeated. Two-way ANOVA analysis was performed on the uncorrected data with drug treatment and donor as variables. Proteins significantly associated with drug treatment were classified using PANTHER.

Characterisation of the uptake of hepatocyte-derived exosomes by human monocyte-derived dendritic cells

Antibody-conjugated magnetic bead separation was used to isolate $CD14^{+}$ monocytes from healthy donor PBMC. Monocytes were then cultured with IL-4 (800 U/mL) and GM-CSF (800 U/mL) for 7 days to generate dendritic cells. To assess the uptake of hepatocyte-derived exosomes by dendritic cells, exosomes were first stained with PKH26 red fluorescent dye (Sigma-Aldrich, UK). Dendritic cells were then cultured with dye-stained exosomes on cover slips embedded in 12 well cell culture plates. In other experiments dendritic cells were cultured with exosomes isolated from hepatocytes exposed to either flucloxacillin or nitroso-sulfamethoxazole for 24 h followed by confocal microscopy to determine exosome uptake.

Dendritic cells were rinsed and fixed with 4% paraformaldehyde. After rinsing and permeabilisation, dendritic cells were incubated with primary antibodies; mouse anti-actin; 1:50 (Santa Cruz Biotechnology) and rabbit anti-flucloxacillin or rabbit anti-nitroso-sulfamethoxazole antibodies overnight at 4^{o}C. Actin was visualized using Alexa Fluor chicken anti-mouse 488 1:500 (molecular probes), while flucloxacillin and nitroso-sulfamethoxazole were visualized using Alexa Fluor chicken anti-rabbit 1:500 . Nuclei were stained with Dapi (Invitrogen, 5 µg/ml). Cells were visualized using a confocal microscope (Zeiss LSM 800, Germany) and dendritic cells uptake of hepatocyte-derived exosomes quantified using Fiji software. The amount of hepatocyte-derived exosomes taken up by dendritic cells was estimated by selecting exosomes within a representative field of view and the fluorescence intensity measured.

Flow cytometry assessment of activation and maturation markers on dendritic cells exposed to test drugs and drug-modified exosomes

To access the effect of hepatocyte-derived exosomes on dendritic cell activation and maturation, dendritic cells were cultured with exosomes derived from hepatocyte exposed to either cell culture media or the test compounds flucloxacillin and nitroso-sulfamethoxazole. In order to investigate the direct effect of soluble drug on dendritic cell surface markers, dendritic cells were also exposed to soluble flucloxacillin (0.05 mM) and nitroso-sulfamethoxazole (0.01 mM) for 24 h. Drug-induced changes in the expression of MHC class II, CD40, CD80, CD83 and HLA-DR expression were analysed by flow cytometry using FACS Canto II^{TM} system. A total of 10,000 events were acquired.

Peptide design and generation of amoxicillin-modified HLA-A*02:01 binding 9-mer peptides

Peptide binding prediction of MHC class I peptide epitopes for exosomal proteins was performed using the NetMHCpan Server 4.0 search criteria. The search parameters included restriction to 9mer peptides, the HLA-A*02:01 or the HLA-B*57:01 allele and the threshold for strong binders was set to 0.5%. Epitopes which ranked below 0.5% which contained a central modified residue were considered for immunogenicity studies. Finally, due to cysteine containing peptides being difficult to synthesise, these were excluded from the suitability criteria. The peptide SLLEPSVKI was chosen for the immunogenicity studies and was purchased with and without an Fmoc protection group on the n-terminus from Synpeptide Ltd. The n-terminus was protected to ensure site specific modification of the lysine at position 8 during synthesis.

Fmoc-SLLEPSVKI was incubated with amoxicillin at a 50:1 molar ratio in 70%ACN/ 30%H_2O for 48 hours at 37^{o}C. Analytes were acidified to 0.1% TFA and loaded onto a Phenomenex C_{18} Kinetex 5µm column coupled with an Agilent 1200 HPLC at λ214. The following HPLC gradient was applied over 30 minutes: (minutes, %B; 0, 2; 20, 75, 20.10, 98; 25, 98; 25.10, 2; 30, 2) with solvent A as 0.1% TFA and solvent B as ACN/ 0.1% TFA. The modified peptide peak was identified through a series of HPLC experiments to identify candidate peptide peaks. Fractions were collected and pooled from several HPLC runs, this was followed by the addition of piperidine at a 10:1 molar ratio in 70%ACN/ 30%H_2O for 24 hours to remove the Fmoc protecting group. The incubation was subjected to the same HPLC conditions to purify the deprotected drug-modified peptide. A full scheme of peptide purification and optimisation is shown in Supplementary Figure 3. Detection of the drug-modified peptide peak was analysed using LC-MS/MS. Samples were resuspended in 0.1% FA/ 2%ACN and loaded onto an AB Sciex TripleTof 5600 mass spectrometer for analysis. Peptides were manually sequenced using

PeakView 1.2 and annotated with the peptide sequence, modified amino acids and characteristic fragment ions of amoxicillin.

Determination of the activation of naïve human T-cells from healthy HLA-A*02:01+ donors with an amoxicillin-modified SLLEPSVKI peptide

PBMC from HLA*02:01+ healthy human donors were used to assess the T-cell immunogenicity of the amoxicillin-modified SLLEPSVKI peptide. Briefly, PBMC were isolated from venous blood of 3 donors using density centrifugation. This was followed by separation of CD14 positive monocytes and naïve T-cells using antibody-conjugated magnetic beads. Dendritic cells were generated from the CD14+ monocytes with a cocktail of IL-4 and GM-CSF over 7 days. On day 7, dendritic cells were loaded with unmodified (50 μM) or amoxicillin-modified (50 μM) SLLEPSVKI peptide for 6h followed by maturation with LPS and TNF-α overnight. Naïve T-cells ($2x10^5$/well; 24-32 wells per condition depending on the availability of naïve T-cells) were co-cultured with either with unmodified or amoxicillin-modified peptide pulsed dendritic cells ($8x10^3$) for 14 days. T-cells were then restimulated with a fresh batch of autologous dendritic cells loaded with the unmodified or amoxicillin-modified peptide. T-cell proliferative responses were evaluated through the addition of ^{3}[H]-thymidine incorporation for the final 16 h of the experiment.

Statistical analysis

ANOVA or paired t test were used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology and proteomic characterisation of hepatocyte-derived exosomes

The hepatocyte-derived exosomes measured ≤ 100 nm in diameter (figure 1A) (19). Immunoblotting revealed the expression of CD63 but not CD81, CD9 and Hsp70 (figure 1B). Furthermore, CD63 was overexpressed in the exosomal fraction compared to the cell lysate.LC-MS analysis identified peptides consistent with CD63, CD81, CD9 and Hsp70 protein expression.

On average, approximately 1200 proteins were identified by LC-MS in untreated hepatocyte-derived exosomes across three donors (figure 1C). Comparison of the control hepatocyte exosome proteome with that of the whole liver (in-house database of 4739 proteins) revealed that they shared a similar protein profile based on molecular function, biological process, cellular component, protein class and pathway (figure 1D). Furthermore, exosomes expressed high-mobility group box 1 (HMGB1) and heat shock proteins (HSPs) that play important roles in inflammation (30).

Characterisation of global drug-induced changes in proteins packaged by hepatocyte-derived exosomes

When all data files were processed together using ProteinPilot, 2109 proteins were identified within an FDR of 1% (Supplementary Table 1 and in the PRIDE repository PXD010760). One third of the proteins identified have previously been published in the ExoCarta database (exocarta.org). There was a subset of 522 proteins for which there was intensity data in every sample (approach 1). MaxQuant identified 1571 proteins within an FDR of 1%, for which there was quantitative data in every sample for 608 proteins (approach 2). The data were subjected to hierarchical, PCA and ANOVA analysis using Partek Genomics Suite. Samples treated with nitroso-sulfamethoxazole

appeared to cluster away from the other samples when the data were processed using either approach 1 (Figures 2A and 2C) or approach 2 (Figure 2B and 2D). Two-way ANOVA analysis identified 148 proteins from approach 1 and 113 proteins from approach 2 that were significantly ($P<0.05$) associated with the nitroso-sulfamethoxazole treatment. Thirty five of these proteins were present in both datasets (Supplementary Figure 1) and are listed in Supplementary Table 2. Comparing each drug treatment individually to the control exosome sample revealed few significant changes (amoxicillin, 3 proteins; flucloxacillin, 10 proteins; isoniazid, 7 proteins) (Supplementary Tables 3A, B and C). However, the nitroso sulfamethoxazole sample compared to the control sample exhibited 35 differentially encapsulated proteins (Supplementary Table 3D). These overlapped (23/35) with the analysis of nitroso-sulfamethoxazole compared to all the other treatments combined. PANTHER analysis of the list of 35 proteins revealed that most of them are involved in catalytic activity (Figure 2E) and that this included a range of enzyme classes (Figure 2F).

Covalent modification of exosomal proteins by amoxicillin, flucloxacillin and nitroso-sulfamethoxazole

Immunoblotting with drug-specific antibodies detected nitroso-sulfamethoxazole and flucloxacillin adducts within hepatocyte-derived exosomes (Figure 3A). Furthermore, LC-MS/MS analysis of exosomes revealed amoxicillin modification of lysine residues on human transcription factor SOX30, albumin and apolipoprotein E (Figures 3B, 4A and 4B). Flucloxacillin-modified peptides from albumin were also observed (Figures 3C, 4A and C). In addition, sulphinamide and N-hydroxysulphinamide adducts on serotransferrin (Figures 3A, 4A and 4D) and transthyretin (figures 4A and 4E), respectively, were detected for nitroso- sulfamethoxazole-treated samples. Interestingly, both cysteine residues (C28 and C260) on human serotransferrin that were shown to have been modified by nitroso sulfamethoxazole would normally be present as disulphides with other cysteine residues. Figures 4A-E show a list of all the drug modified peptides identified and the structure of the proteins with sites of modification highlighted.

Human monocyte-derived dendritic cell uptake of exosomes by phagocytosis and endocytosis

Dendritic cells were found to engulf both unmodified and drug-modified hepatocyte-derived exosomes (figure 5A-C and supplementary figure 2A). Figure 5A shows exosomes stained with PKH26 red fluorescent dye, while figures 5B and 5C show red staining with anti-flucloxacillin and anti-nitroso- sulfamethoxazole antibodies, respectively. Pre-treatment of dendritic cells with either latrunculin-A (phagocytosis inhibitor), dynasore (endocytosis inhibitor) or both inhibitors for 2 h before addition of hepatocyte-derived exosomes for 24 h resulted in a significant reduction or inhibition of exosome uptake (figure 5D-G and supplementary figure 2B).

Effect of hepatocyte-derived exosomes on the activation of human monocyte-derived dendritic cells

Hepatocyte-derived exosomes did not significantly alter the expression of either dendritic cell maturation or activation markers (Figure 6). Furthermore, flucloxacillin and nitroso-sulfamethoxazole exposure did not interfere with the expression of most markers. However, as

described previously, nitroso-sulfamethoxazole treatment resulted in a significant increase in the expression of CD40 (31). Binding of CD40 on antigen presenting cells to CD40L on helper T-cells activates the antigen presenting cells leading to a pro-inflammatory response (32). Dendritic cell IFN-γ and IL-10 secretion were also assessed by ELIspot after drug or exosome treatment. No significant increase in secretion of either cytokine was observed.

Activation of naïve T-cells from HLA-A*02:01 human donors with the amoxicillin-modified 9-mer peptide SLLEPSVKI

Assessing the immunogenicity of whole proteins or exosomes *per se* was not possible. Therefore, peptides containing the drug-modified residues identified on the exosomal proteins, aligned to relevant HLA binding epitopes were synthesised to assess their immunogenicity. To do this, protein sequences were expanded to include 10 amino acids in both directions from the modified residue and these sequences were inputted into the NetMHCpan 4.0 server to search for potential MHC class I epitopes. Drug-induced liver injury to amoxicillin and flucloxacillin are associated with the class I alleles HLA-A*02:01 and HLA-B*57:01 respectively, while SMX has not shown any specific association with HLA alleles. Therefore, these alleles were selected to assess potential peptide epitopes for all four modified proteins. Peptide epitopes were selected for study if they scored less than 0.5 on the NetMHCpan 4.0 server and only if the modified residue fell within the anchor motifs. No peptide epitopes met this selection criteria for HLA-B*57:01, while, strong binding epitopes for HLA-A*02:01 were identified for the amoxicillin-modified SOX30 transcription factor protein (SLLEPSVKI – NetMHCpan 4.0 Score = 0.047) and the amoxicillin-modified apolipoprotein e protein (FLAGCQAKV – NetMHCpan 4.0 Score = 0.128). Both epitopes contained a modified lysine in position 8 falling within the P2 and PΩ anchors for HLA-A*02:01, however, the apolipoprotein peptide sequence also contained a central cysteine residue. Previous studies synthesising drug-modified peptides have shown that free cysteines cause difficulty due to peptide dimerization during synthesis resulting in low product yield and multiple oxidation states complicating assay variability. As such, the amoxicillin-modified peptide epitope for HLA-A*02:01, SLLEPSVK(Amox)I derived from SOX30 was the only epitope which met suitable criteria for immunogenicity studies (Figure 7A). The unmodified and amoxicillin-modified peptides yielded the expected peptide fragmentation pattern, with characteristic fragments for amoxicillin including a peak at 160 da indicative of the cleaved thiazolidine ring and a peak at 349 da indicative of the whole cleaved amoxicillin molecule. Several drug-modified fragment peaks were also identified (Figure 7 B-C; Supplementary figure 3).

Naïve T-cells isolated from three HLA-A*02:01 positive donors were co-cultured with autologous monocyte-derived dendritic cells that had been pulsed with either unmodified or amoxicillin-modified SLLEPSVKI peptide, derived from the transcription factor protein SOX30, for 2 weeks. The primed T-cells were then restimulated with a second batch of peptide-loaded dendritic cells prior to detection of proliferation using [^{3}H] thymidine. Naïve T-cells from the amoxicillin-modified peptide-primed cultures were stimulated to proliferate when restimulated with the amoxicillin-modified peptide (Figure 7D; $p<0.0001$). In contrast, no proliferative response was observed in similar experiments using dendritic cells pulsed with the unmodified peptide.

DISCUSSION

Idiosyncratic drug reactions targeting the liver are a major challenge for patients, clinicians and the pharmaceutical industry as there is currently no way to accurately predict which individuals will develop tissue injury. This is partly because the adverse reactions are associated with individual susceptibility factors. The discovery of (1) strong associations between DILI and expression of specific HLA alleles and (2) the selective drug-specific activation of T-cells from patients with DILI indicates that the adaptive immune system is involved in the disease pathogenesis. However, the reason why a drug activates T-cells that target the liver has not been defined. One possibility is that drugs that induce liver injury form covalent adducts with critical hepatic proteins and that these adducts are taken up and processed within dendritic cells prior to presentation of the derived peptides to T-cells. Activation of the T-cells within the vicinity of the covalently-modified hepatic proteins would result in localized tissue damage. Given the importance of exosomes in the cross talk between tissue cells and the immune system and the accumulation of T-cells in liver of patients with early stage tissue injury (12, 13), we sought to understand the impact of drug exposure on the selective packaging and sorting of hepatocyte components into exosomes, and the interaction between hepatocyte-derived exosomes and immune cells. Of particular importance was the detailed assessment of exosomal proteins to identify sites of drug modification. Our study focused on three of the drugs commonly associated with idiosyncratic DILI in humans: amoxicillin, flucloxacillin and isoniazid (26). Although primary human hepatocytes express drug metabolising enzymes for up to 24 h (33), each drug selected has been shown to forms adducts with lysine residues on protein spontaneously, with no requirement for drug metabolism (34-36). Furthermore, we utilized nitroso-sulfamethoxazole, a synthetic metabolite of sulfamethoxazole that binds covalently to cysteine residues (37). Drug-responsive T-cells have been isolated from the peripheral blood of DILI patients exposed to each of these drugs, confirming the immune pathogenesis (9-11).

Global analysis of the hepatocyte exosome proteome identified 2109 proteins; of these, one third (681) of the proteins have previously been published in the ExoCarta database, which includes 5402 exosomal proteins derived from all tissues. Those listed in the database as having been derived from human liver (326 proteins) were predominantly identified in hepatocellular carcinoma cell lines. Of these, only 60 proteins were observed in the dataset presented here. This indicates that the exosome proteome derived from freshly isolated hepatocytes is markedly different to that of cancer cell lines. This study has provided the most complete proteome for hepatocyte-derived exosomes to date.

Comparison of the hepatocyte-derived exosome proteome with the whole liver proteome revealed that the distribution of proteins in terms of function, class and pathways was essentially the same between the two sources of hepatic material. This suggested that proteins were passively captured rather than selectively packaged into the vesicles. Whilst there is evidence for enrichment of RNA species and certain membrane-associated proteins in exosomes, the case for cytosolic proteins is less clear. When intraluminal vesicles are formed, they encapsulate cytosolic proteins in their lumen which remain in place as the vesicle matures into an exosome (38). Data presented here does indeed suggest that the hepatocyte exosome proteome provides a snapshot of the cytosolic proteome.

In order to determine whether drug treatment of hepatocytes resulted in a different subset of proteins being packaged and exported, a label-free, semi-quantitative analysis was performed.

Exosomes isolated from hepatocytes exposed to nitroso-sulfamethoxazole were most dissimilar. Thirty five proteins were revealed to be associated with the nitroso-sulfamethoxazole-treated sample Since the data presented here suggest that the exosomal proteome reflects the cellular proteome from which it is derived, we can extrapolate that the catalytic makeup of the hepatocyte is altered by treatment with nitroso-sulfamethoxazole. Several enzymes involved in xenobiotic metabolism, including epoxide hydrolase, UDP glucuronyl transferase and arylacetamide deacetylase, are present at lower levels in the exosomes from nitroso-sulfamethoxazole-treated hepatocytes. This suggests a possible dysfunction in pathways involved in response to chemical stress on treatment with the metabolite of sulfamethoxazole.

Drug protein adducts play an important role in immune activation. T-cells from patients with flucloxacillin- and amoxicillin clavulanate-induced DILI and sulfamethoxazole hypersensitivity can be activated via a pathway that is dependent on the processing of the protein adducts (9, 10). Recently, Sanchez-Gomez et al demonstrated that amoxicillin forms adducts with cellular proteins and that these adducts are detectable in exosomes found in B-cell culture supernatant (39). Hence, the exosome proteome derived from drug-treated hepatocytes was screened for drug modification. Adducts of amoxicillin, flucloxacillin and nitroso-sulfamethoxazole were detected on a total of five exosomal proteins. One of these, albumin, is targeted *in vitro* and *in vivo* by different β-lactam antibiotics and the modified protein stimulates patient T-cells *in vitro* (9, 34, 36). These data support the hypothesis originally proposed by Sanchez-Gomez et al that drug-modified proteins are encapsulated and transported within exosomes to the extracellular matrix. Importantly, however, the data also identifies hepatic protein targets modified by DILI drugs that might be critical for T-cell activation.

In order for haptenated proteins to trigger an immune response, they must be taken up by antigen presenting cells, processed and presented to T-cells in an HLA-restricted manner. Exosomes were shown to be internalized by both phagocytosis and endocytosis. Exosomes containing nitroso-sulfamethoxazole- and flucloxacillin-modified proteins were also internalized. Hence, our data show that exosomes transfer tissue-derived drug-modified proteins that have the potential to act as neoantigens.

To investigate whether drug-modified exosomal proteins activate T-cells, we utilized *in silico* peptide HLA binding software to identify the 9-mer peptide sequence containing an amoxicillin modification that was most likely to bind to HLA-A*02:01 (an HLA class I allele associated with amoxicillin-clavulanate-induced liver injury (2)). Restimulation of amoxicillin-modified peptide primed T-cells with peptide-loaded dendritic cells resulted in a significant proliferative response in all three donors. In contrast, the unmodified peptide subjected to the same extraction protocol did not activate the patient T-cells. These data demonstrate that the adducts generated in hepatocytes and transported to dendritic cells via exosomes can trigger antigen-specific T-cell responses and provide a foundation to explore in more detail the relationship between expression of specific HLA alleles and the development of drug-induced liver injury.

Danger signals stimulate Toll-like receptors and NLRP3 and this results in dendritic cell maturation, which can be visualized by upregulation of cell surface receptors and cytokine secretion (40). Haptenic chemicals and certain drugs have also been shown to stimulate dendritic cell maturation *in vitro* (32). Thus, the final objective of this project was to determine whether dendritic cell uptake of hepatocyte-derived drug-modified proteins encapsulated within exosomes occurs silently or with a concurrent stress response. The uptake of hepatic exosomes with or without drug treatment did not trigger increased dendritic cell surface receptor expression or cytokine release. These data show that drug-modified proteins can be transferred from tissue cells to the immune system via exosome transport to ultimately be displayed as peptide fragments on HLA molecules on the surface of dendritic cell without stimulating a stress response.

To conclude, we have demonstrated a significant heterogeneity in the proteome of exosomes isolated from hepatocytes obtained from different individuals. Exosomes derived from nitroso sulfamethoxazole treated hepatocytes contained a subset of proteins with altered levels of representation, suggesting perturbation of the stress response in hepatocytes exposed to nitroso-sulfamethoxazole. Furthermore, the drug-modified intracellular hepatic proteins transported by exosomes to dendritic cells serve as antigenic determinants for immune activation.

ACKNOWLEDGEMENTS

The authors would like to thank the patients and volunteers for their generous donations.

FIGURE LEGENDS

Figure 1. Morphology and proteomic characterization of hepatocyte-derived exosomes. (A) Hepatocyte-derived exosomes measured ≤ 100 nm in diameter, scale bar = 50 nm. **(B)** CD63 expression by hepatocyte-derived exosomes and corresponding whole cell lysates from 3 donors determined by immunoblotting using anti-CD63 specific antibody. **(C)** Proteins identified in hepatocyte-derived exosomes my mass spectrometry (n = 3). Dark bars represent total exosomal proteins identified across treatment groups and open bars represent human exosomal proteins published in the ExoCarta database. **(D)** Comparison of the whole liver tissue proteome with that of hepatocyte-derived exosomes based on molecular function, biological process, cellular component, protein class and pathway using PANTHER gene list analysis.

Figure 2. Characterization of global drug-induced changes in proteins expressed by hepatocyte-derived exosomes. (A) Hierarchical cluster analysis of data processed using approach 1 and batch corrected for hepatocyte donor. **(B)** Hierarchical cluster analysis of data processed using approach 2 and batch corrected for hepatocyte donor. Green and red represent low and high protein levels, respectively. **(C)** PCA of data processed using approach 1 and batch corrected for hepatocyte donor. **(D)** PCA of data processed using approach 2 and batch corrected for hepatocyte donor. **(E)** PANTHER molecular function classification of proteins differentially represented in exosomes from nitroso sulfamethoxazole-treated hepatocytes compared to those exposed to all other treatments. **(F)**

PANTHER protein class classification of proteins differentially represented in exosomes from nitroso sulfamethoxazole-treated hepatocytes compared to those exposed to all other treatments.

Figure 3. Detection of drug modification of exosomal proteins derived from drug-treated hepatocytes from a single donor. (A) Western blot characterisation of human exosomal proteins modified by either nitroso sulfamethoxazole or flucloxacillin using-specific anti-drug antibodies. **(B)** MS/MS spectrum of amoxicillin-modified peptide from SOX30. **(C)** MS/MS spectrum of flucloxacillin-modified peptide from HSA. **(D)** MS/MS spectrum of nitroso sulfamethoxazole-modified peptide from serotransferrin. Fragment ions derived from the drug are circled in red, the N-terminal y ion is circled in green (resulting from cleavage of the entire drug adduct prior to cleavage of the peptide backbone) and ions with full or partial drug adducts are marked with an asterisk.

Figure 4. Exosomal proteins modified with drugs. (A) List of the exosomal proteins modified by nitroso sulfamethoxazole, flucloxacillin and amoxicillin and the specific sites of modification. **(B-E)** Three-dimensional structures of exosomal proteins showing the sites of drug modification: **(B)** HSA (PDB-1O9X)-amoxicillin, **(C)** HSA (PDB-1O9X)-flucloxacillin, **(D)** serotransferrin (PDB-1DTG)-sulphinamide, **(E)** transthyretin (PDB-4N86)-N-hydroxysulphinamide.

Figure 5. The uptake of unmodified and drug-modified hepatocyte-derived exosomes by human monocyte-derived dendritic cells. Exosomes derived from drug-treated hepatocytes were cultured with human monocyte-derived dendritic cells for 24 h and exosome uptake was evaluated using confocal microscopy. Scale bar represents 5 micrometres. Green = F-actin, Blue = nucleus, red = unmodified or drug-modified exosomes. **(A)** Uptake of unmodified hepatocyte-derived exosomes by dendritic cells. Exosomes were first stained red with PKH26 dye before 24 h culture with dendritic cells and confocal microscopy. **(B, C)** Uptake of flucloxacillin- and nitroso-sulfamethoxazole-modified hepatocyte-derived exosomes by dendritic cells, respectively. Exosomes derived from drug-treated hepatocytes were cultured with dendritic cells for 24 h and drug-specific antibodies were used to tag drug-modified exosomal proteins followed by confocal microscopy to determine the uptake of drug-modified exosomes. **(D)** Exosome uptake by dendritic cells in the absence of inhibitor. **(E)** The effect of latrunculin A (phagocytosis inhibitor) on dendritic cell exosome uptake. **(F)** Effect of dynasore (endocytosis inhibitor) on dendritic cell exosome uptake. **(G)** Combined effect of latrunculin A and dynasore on dendritic cell exosome uptake.

Figure 6. Effect of hepatocyte-derived exosomes on dendritic cell surface marker expression. Dendritic cells were cultured with exosomes derived from either cell culture media-or drug-treated hepatocytes or soluble drugs for 24 h prior to analysis of **(A-C)** maturation markers (CD80, CD83, HLA-DR) and **(D-E)** activation markers (MHC class II, CD40) by flow cytometry. Dendritic cells were then stained with fluorochrome-conjugated antibodies and the expression of markers was evaluated using a FACS Canto II flow cytometer. A minimum of 10, 000 events were acquired.

Figure 7. LC-MS/MS characterization of an amoxicillin-modified HLA-A*02:01 binding peptide derived from transcription factor protein SOX30 and functional evaluation of T-cell immunogenicity. (A) SLLEPSVKI peptide design rationale. The tryptic peptide identified in exosomal proteins is highlighted in orange with the SOX30 amino acid sequence expanded to include 10 amino acids in either direction of the modified lysine. The HLA-A*02:01 binding epitope is highlighted in yellow labelled with the anchor residues in blue. The NetMHCpan score for this peptide was 0.047. **(B)** MS/MS Spectrum of the unmodified SLLEPSVKI peptide derived from SOX30. **(C)** MS/MS Spectrum of the amoxicillin modified SLLEPSVKI peptide derived from SOX30. Fragment ion peaks of the peptide sequence have been labelled with a red arrow and drug associated peaks labeled with a blue arrow. **(D)** Naïve T-cells from 3 HLA-A*02:01+ human donors were co-cultured with unmodified or amoxicillin-modified peptide-pulsed monocyte-derived dendritic cells at a 25:1 ratio in a 96 U-bottomed plate (24-32 wells per condition), in a final volume of 200 µl for 14 days. Cultures were re-stimulated with a fresh batch of autologous peptide loaded dendritic cells for 48 h. ^{3}[H]-thymidine added during the final 16 h of culture to measure the extent of T-cell proliferation. Wells were harvested and incorporated radioactivity analyzed using a beta counter. Each data point represents proliferative T-cell responses in a single well of a 96 well plate.

Supplementary Figure 1

Venn diagram showing the overlap between proteins that were differentially packaged (ANOVA) in exosomes from nitroso sulfamethoxazole-treated hepatocytes compared to all other treatments revealed using approach 1 (blue) and approach 2 (red).

Supplementary Figure 2

Quantification of the degree of uptake of hepatocyte-derived exosomes by human dendritic cells. Images of dendritic cell uptake of hepatocyte-derived dendritic cells were processed using ZEN imaging and Fiji software. The amount of exosome taken up was estimated by selecting exosomes, represented by the red elements in the confocal microscopy images, within a representative dendritic cell in a field of view and the fluorescence intensity measured. **(A)** Uptake of either unmodified of drug-modified exosomes. **(B)** Effect of latrunculin A and dynasore on dendritic cell uptake of hepatocyte-derived exosomes.

Supplementary Figure 3

Optimisation of amoxicillin-modified peptide purification using reversed phase HPLC. Purification of the SLLEPSVK(Amox)I from free drug and other contaminants was optimised by performing a series of HPLC experiments. Peptides were incubated with drug at a 50:1 molar ratio in 70%ACN/ 30%H_2O for 48 hours at 37^{o}C. Analytes were loaded onto a Phenomenex C_{18} Kinetex 5µm column and analysed using an Agilent 1200 HPLC at λ214. **(A)** Unmodified Fmoc-SLLEPSVKI peptide alone at 5mM concentration prior to incubation. **(B)** Amoxicillin alone at 250mM concentration prior to incubation. Penicillins are prone to degradation in water, thus several degradation peaks are observed. **(C)** Fmoc-SLLEPSVKI incubated with amoxicillin after 5 minutes labelled with amoxicillin peaks and the

unmodified Fmoc-protected peptide peaks. **(D)** Fmoc-SLLEPSVKI incubated with amoxicillin after 4 hours, labelled with the amoxicillin-modified fmoc-peptide peak. **(E)** Fmoc-SLLEPSVKI incubated with amoxicillin after 24 hours, labelled with the amoxicillin-modified fmoc-peptide peak. **(F)** Fmoc-SLLEPSVKI incubated with amoxicillin after 48 hours, labelled with the amoxicillin-modified fmoc-peptide peak. **(G)** Fmoc-SLLEPSVK[AX]I alone after purification and collection. Some amoxicillin dimer modified peptide is observed. **(H)** Amoxicillin-modified peptide following piperidine incubation. **(I)** Final product of deprotected SLLEPSVK[AX]I used for cell culture studies.

Supplementary Table 1

List of proteins identified in hepatocyte exosomes. LC-MS/MS data were acquired on a Triple TOF 6600 and processed using ProteinPilot 5. All proteins lie within a 1% FDR. The proteins are listed in order of their confidence.

Supplementary Table 2

List of exosomal proteins associated with treatment of hepatocytes with nitroso sulfamethoxazole. ANOVA analysis was performed to compare the protein profile of the nitroso sulfamethoxazole-treated samples to all the others. This identified 148 proteins from approach 1 and 113 proteins from approach 2 that were significantly ($P<0.05$) associated with the nitroso-sulfamethoxazole treatment. Thirty five of these proteins were present in both datasets and are listed here.

Supplementary Table 3

List of exosomal proteins associated with treatment of hepatocytes with individual drugs. ANOVA analysis was performed to compare the protein profile of each of the drug-treated samples to the control sample. **(A)** Amoxicillin. **(B)** Flucloxacillin. **(C)** Isoniazid. **(D)** Nitroso-sulfamethoxazole. The shaded proteins in D correspond to proteins also highlighted in the comparison between nitroso-sulfamethoxazole sample and all other treatments combined listed in Supplementary Table 2.

REFERENCES

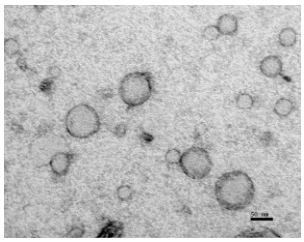
1. Laverty H, Benson C, Cartwright E, Cross M, Garland C, Hammond T, et al. How can we improve our understanding of cardiovascular safety liabilities to develop safer medicines? Br J Pharmacol. 2011;163:675-93.
2. Lucena MI, Molokhia M, Shen Y, Urban TJ, Aithal GP, Andrade RJ, et al. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. Gastroenterology. 2011;141:338-47.
3. Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet. 2009;41:816-9.
4. Spraggs CF, Budde LR, Briley LP, Bing N, Cox CJ, King KS, et al. HLA-DQA1*02:01 is a major risk factor for lapatinib-induced hepatotoxicity in women with advanced breast cancer. J Clin Oncol. 2011;29:667-73.

5. Singer JB, Lewitzky S, Leroy E, Yang F, Zhao X, Klickstein L, et al. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. Nature genetics. 2010;42:711-4.
6. Urban TJ, Nicoletti P, Chalasani N, Serrano J, Stolz A, Daly AK, et al. Minocycline hepatotoxicity: Clinical characterization and identification of HLA-B *35:02 as a risk factor. J Hepatol. 2017;67:137-44.
7. Hirata K, Takagi H, Yamamoto M, Matsumoto T, Nishiya T, Mori K, et al. Ticlopidine-induced hepatotoxicity is associated with specific human leukocyte antigen genomic subtypes in Japanese patients: a preliminary case-control study. Pharmacogenomics J. 2008;8:29-33.
8. Kindmark A, Jawaid A, Harbron CG, Barratt BJ, Bengtsson OF, Andersson TB, et al. Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. Pharmacogenomics J. 2008;8:186-95.
9. Monshi MM, Faulkner L, Gibson A, Jenkins RE, Farrell J, Earnshaw CJ, et al. Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. Hepatology. 2013;57:727-39.
10. Kim SH, Saide K, Farrell J, Faulkner L, Tailor A, Ogese M, et al. Characterization of amoxicillin- and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate-induced liver injury. Hepatology. 2015;62:887-99.
11. Usui T, Meng X, Saide K, Farrell J, Thomson P, Whitaker P, et al. From the Cover: Characterization of Isoniazid-Specific T-Cell Clones in Patients with anti-Tuberculosis Drug-Related Liver and Skin Injury. Toxicol Sci. 2017;155:420-31.
12. Mennicke M, Zawodniak A, Keller M, Wilkens L, Yawalkar N, Stickel F, et al. Fulminant liver failure after vancomycin in a sulfasalazine-induced DRESS syndrome: fatal recurrence after liver transplantation. Am J transplant. 2009;9:2197-202.
13. Wuillemin N, Terracciano L, Beltraminelli H, Schlapbach C, Fontana S, Krahenbuhl S, et al. T cells infiltrate the liver and kill hepatocytes in HLA-B(*)57:01-associated floxacillin-induced liver injury. Amer J Pathol. 2014;184:1677-82.
14. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. Nat Immunol. 2015;16:343-53.
15. Gomez-Lechon MJ, Donato MT, Castell JV, Jover R. Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. Curr Drug Metab. 2004;5:443-62.
16. Bobrie A, Colombo M, Raposo G, Thery C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic. 2011;12:1659-68.
17. Babst M. A protein's final ESCRT. Traffic. 2005;6:2-9.
18. Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. J Proteome Res. 2008;7:5157-66.
19. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Ann Rev Cell Dev Biol. 2014;30:255-89.
20. Kruger S, Abd Elmageed ZY, Hawke DH, Worner PM, Jansen DA, Abdel-Mageed AB, et al. Molecular characterization of exosome-like vesicles from breast cancer cells. BMC cancer. 2014;14:44.
21. Bhatnagar S, Schorey JS. Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. J Biol Chem. 2007;282:25779-89.
22. Clayton A, Turkes A, Navabi H, Mason MD, Tabi Z. Induction of heat shock proteins in B-cell exosomes. J cell Sci. 2005;118:3631-8.
23. Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL. Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. Sci Rep. 2016;6:20254.

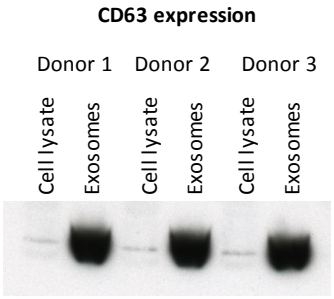
24. Czystowska M, Han J, Szczepanski MJ, Szajnik M, Quadrini K, Brandwein H, et al. IRX-2, a novel immunotherapeutic, protects human T cells from tumor-induced cell death. Cell Death Differ. 2009;16:708-18.
25. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998;4:594-600.
26. Ogese MO, Faulkner L, Jenkins RE, French NS, Copple IM, Antoine DJ, et al. Characterization of Drug-Specific Signaling Between Primary Human Hepatocytes and Immune Cells. Toxicol Sci. 2017;158:76-89.
27. Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K. Gel-based and gel-free quantitative proteomics approaches at a glance. Int J Plant Genomics. 2012;2012:494572.
28. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotech. 2008;26:1367-72.
29. Vizcaino JA, Csordas A, del-Toro N, Dianes JA, Griss J, Lavidas I, et al. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 2016;44:D447-56.
30. Pockley AG. Heat shock proteins as regulators of the immune response. Lancet. 2003;362(9382):469-76.
31. Sanderson JP, Naisbitt DJ, Farrell J, Ashby CA, Tucker MJ, Rieder MJ, et al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. J Immunol. 2007;178:5533-42.
32. Karnell JL, Rieder SA, Ettinger R, Kolbeck R. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. Adv Drug Deliv Rev. 2018 pii: S0169-409X(18)30308-9. doi: 10.1016/j.addr.2018.12.005. [Epub ahead of print]
33. Heslop JA, Rowe C, Walsh J, Sison-Young R, Jenkins R, Kamalian L, et al. Mechanistic evaluation of primary human hepatocyte culture using global proteomic analysis reveals a selective dedifferentiation profile. Arch Toxicol. 2017;91:439-452
34. Meng X, Earnshaw CJ, Tailor A, Jenkins RE, Waddington JC, Whitaker P, et al. Amoxicillin and Clavulanate Form Chemically and Immunologically Distinct Multiple Haptenic Structures in Patients. Chem Res Toxicol. 2016;29:1762-72.
35. Meng X, Maggs JL, Usui T, Whitaker P, French NS, Naisbitt DJ, et al. Auto-oxidation of Isoniazid Leads to Isonicotinic-Lysine Adducts on Human Serum Albumin. Chem Res Toxicol. 2015;28:51-8.
36. Jenkins RE, Meng X, Elliott VL, Kitteringham NR, Pirmohamed M, Park BK. Characterisation of flucloxacillin and 5-hydroxymethyl flucloxacillin haptenated HSA in vitro and in vivo. Proteomics Clin Appl. 2009;3:720-9.
37. Callan HE, Jenkins RE, Maggs JL, Lavergne SN, Clarke SE, Naisbitt DJ, et al. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. Chem Res Toxicol. 2009;22:937-48.
38. Edgar JR. Q&A: What are exosomes, exactly? BMC biology. 2016;14:46.
39. Sanchez-Gomez FJ, Gonzalez-Morena JM, Vida Y, Perez-Inestrosa E, Blanca M, Torres MJ, et al. Amoxicillin haptenates intracellular proteins that can be transported in exosomes to target cells. Allergy. 2017;72:385-96.
40. Martin SF, Rustemeyer T, Thyssen JP. Recent advances in understanding and managing contact dermatitis. F1000Research. 2018;7.

Figure 1

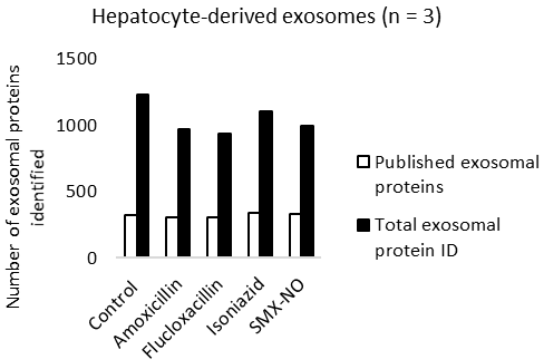
A.



B.



C.



D.

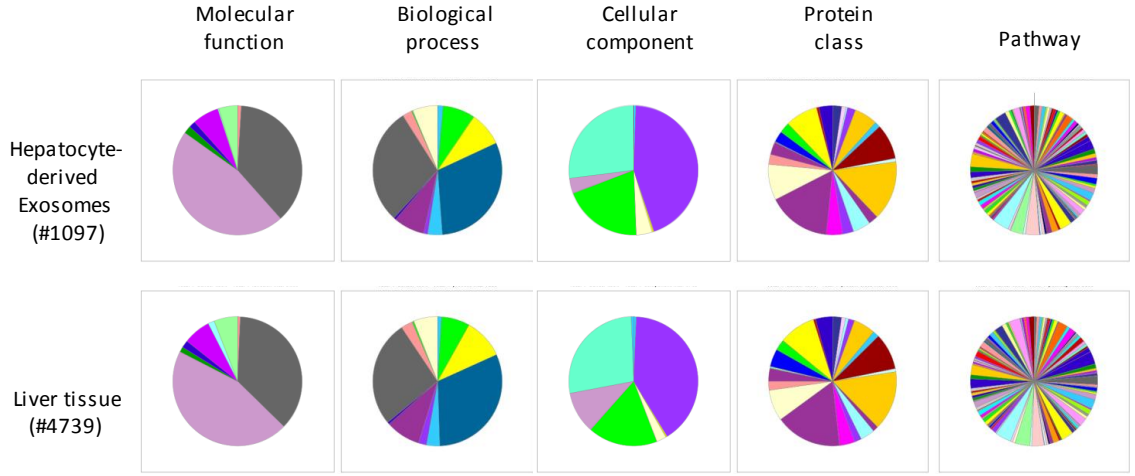


Figure 2

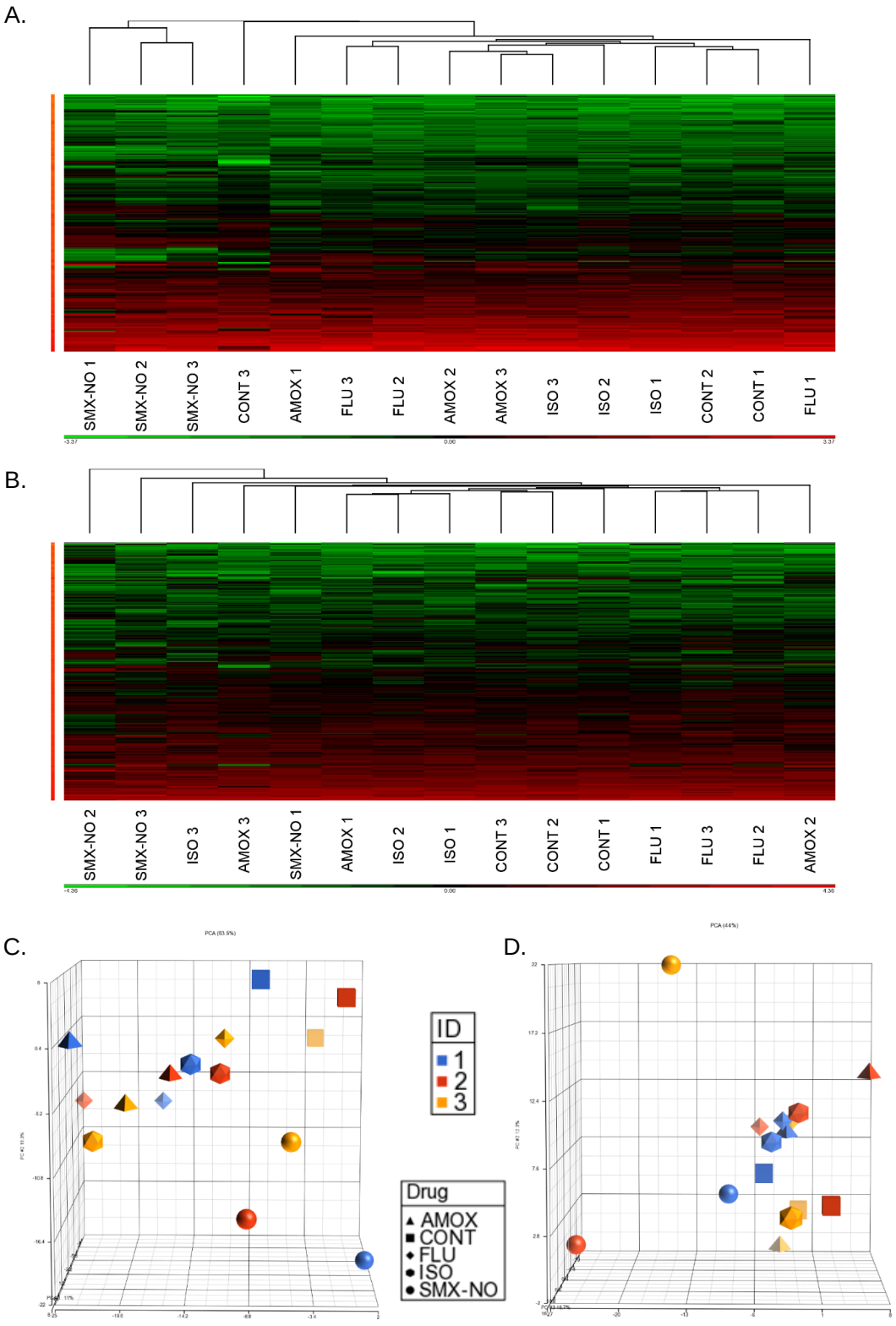
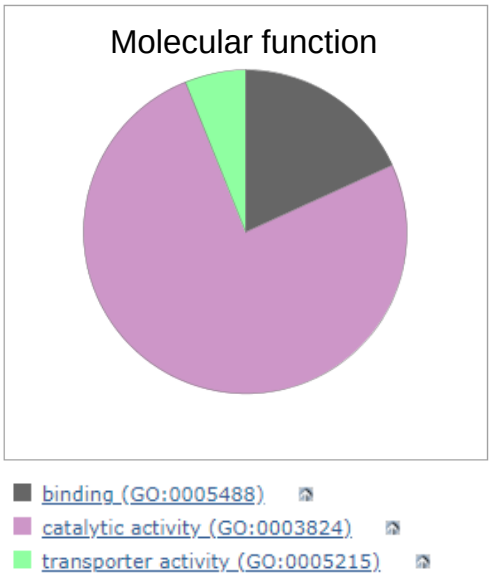


Figure 2

E.



F.

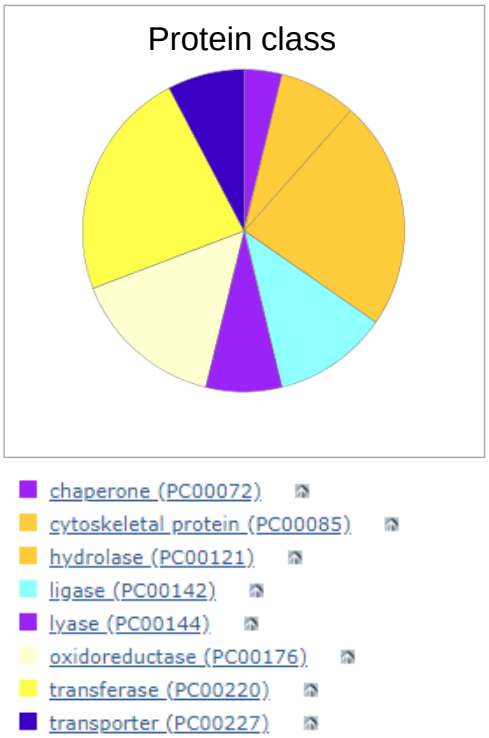


Figure 3

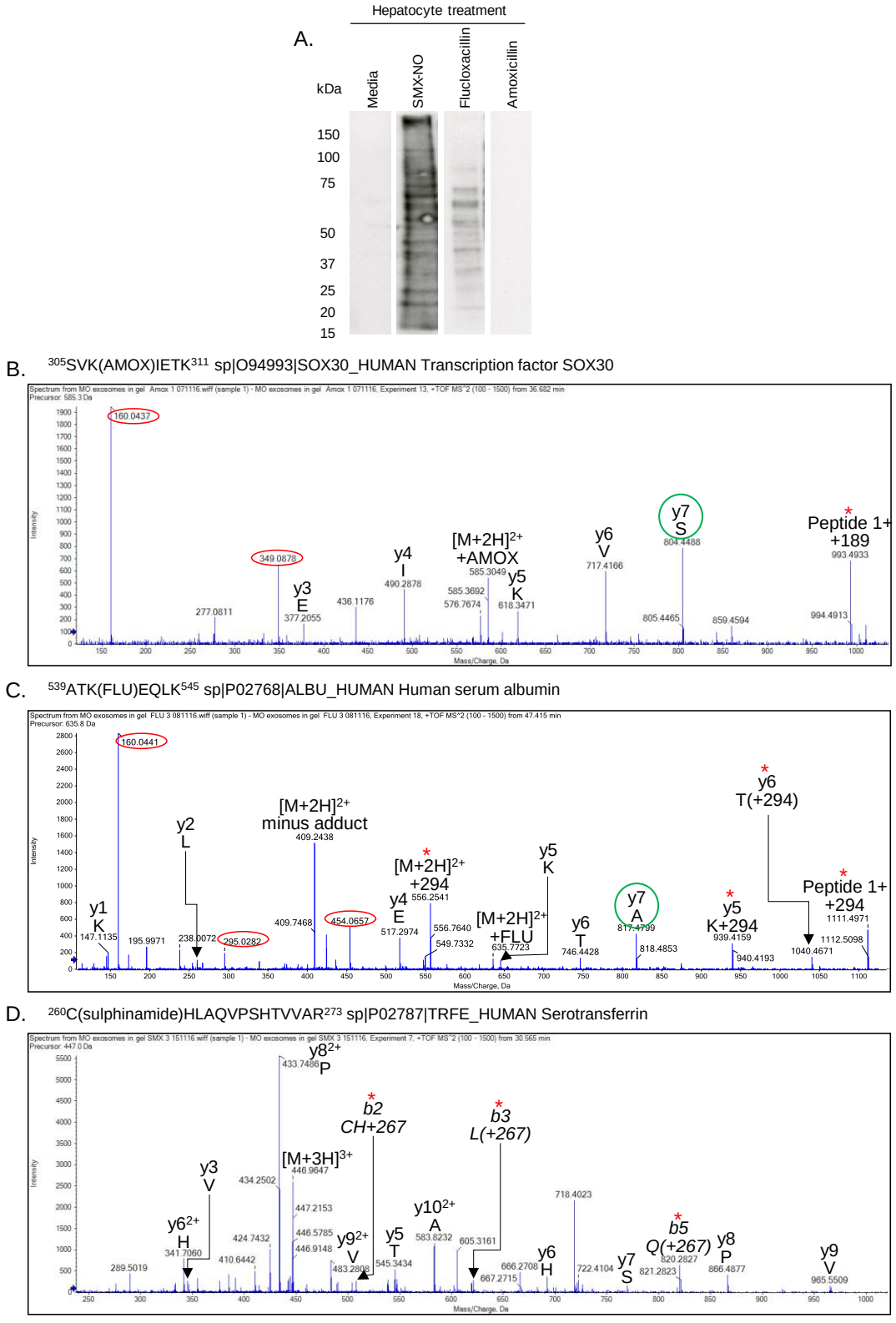
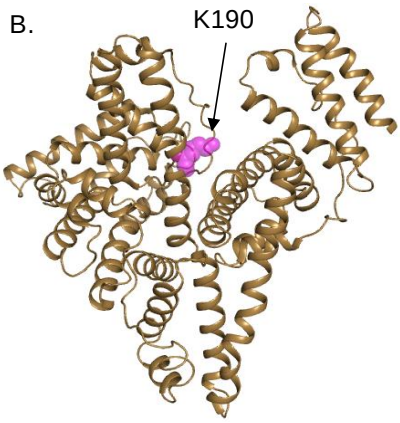


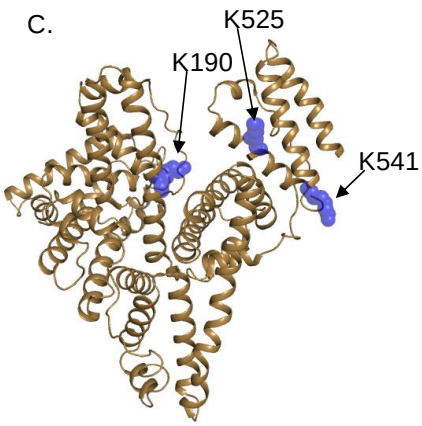
Figure 4

A.

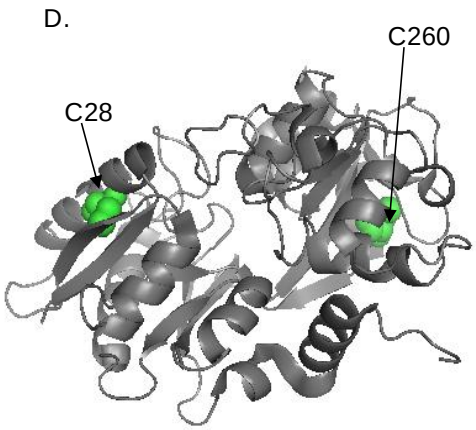
S/No.	Drug	Exosomal proteins modified	Peptides modified
1.	Amoxicillin	Human transcription factor, SOX30 Human serum albumin Human apolipoprotein E	^{305}SVK(AMOX)IETK^{311} ^{182}LDELRDEGK(AMOX)ASSAK^{195} ^{19}K(AMOX)VEQAVETEPEPELR^{33}
2.	Flucloxacillin	Human serum albumin	^{539}ATK(FLU)EQLK^{545} ^{182}LDELRDEGK(FLU)ASSAK^{195} ^{525}KQ(FLU)TALVELVK^{534}
3.	SMX-NO	Human serotransferrin Human transthyretin	^{260}C(sulphinamide)HLAQVPSHTVVAR^{273} ^{24}T(Me)VRWC(sulphinamide)AVSEHEATK^{37} ^{1}GPTGTGESKC(N-hydroxysulphinamide)PLM(Ox)VK^{25}
4.	Isoniazid	No protein adducts identified	-



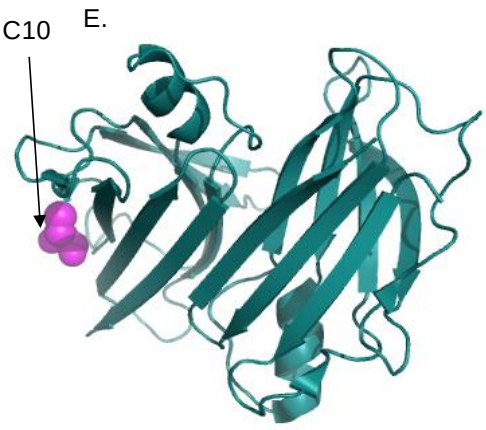
HSA-Amoxicillin adduct



HSA-Flucloxacillin adduct



Serotransferrin-SMX-NO adduct



Transthyretin-SMX-NO adduct

Figure 5

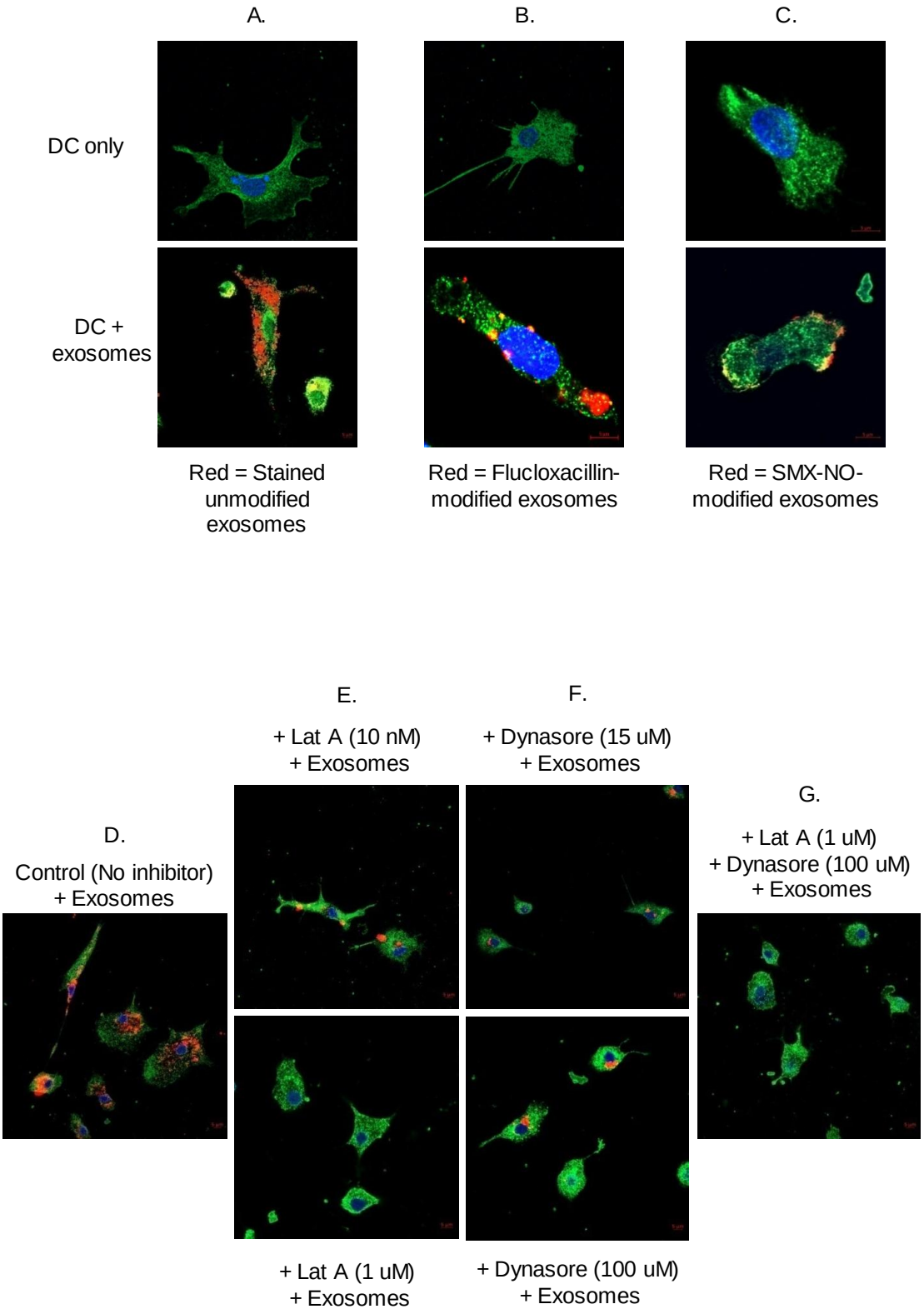


Figure 6

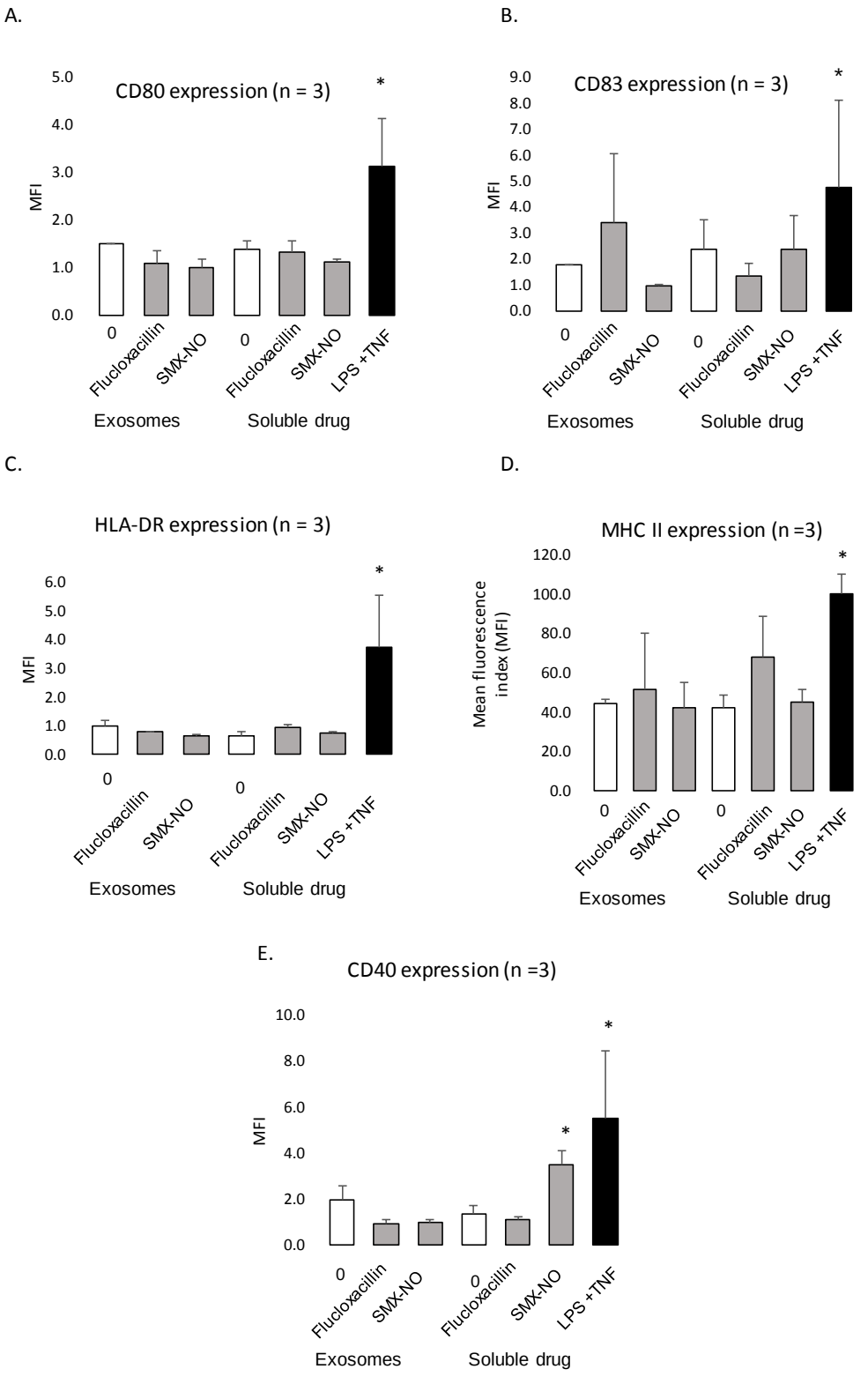


Figure 7