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Supplementary Materials for

PDIA3 epitope-driven immune autoreactivity contributes to hepatic damage in type 2 diabetes

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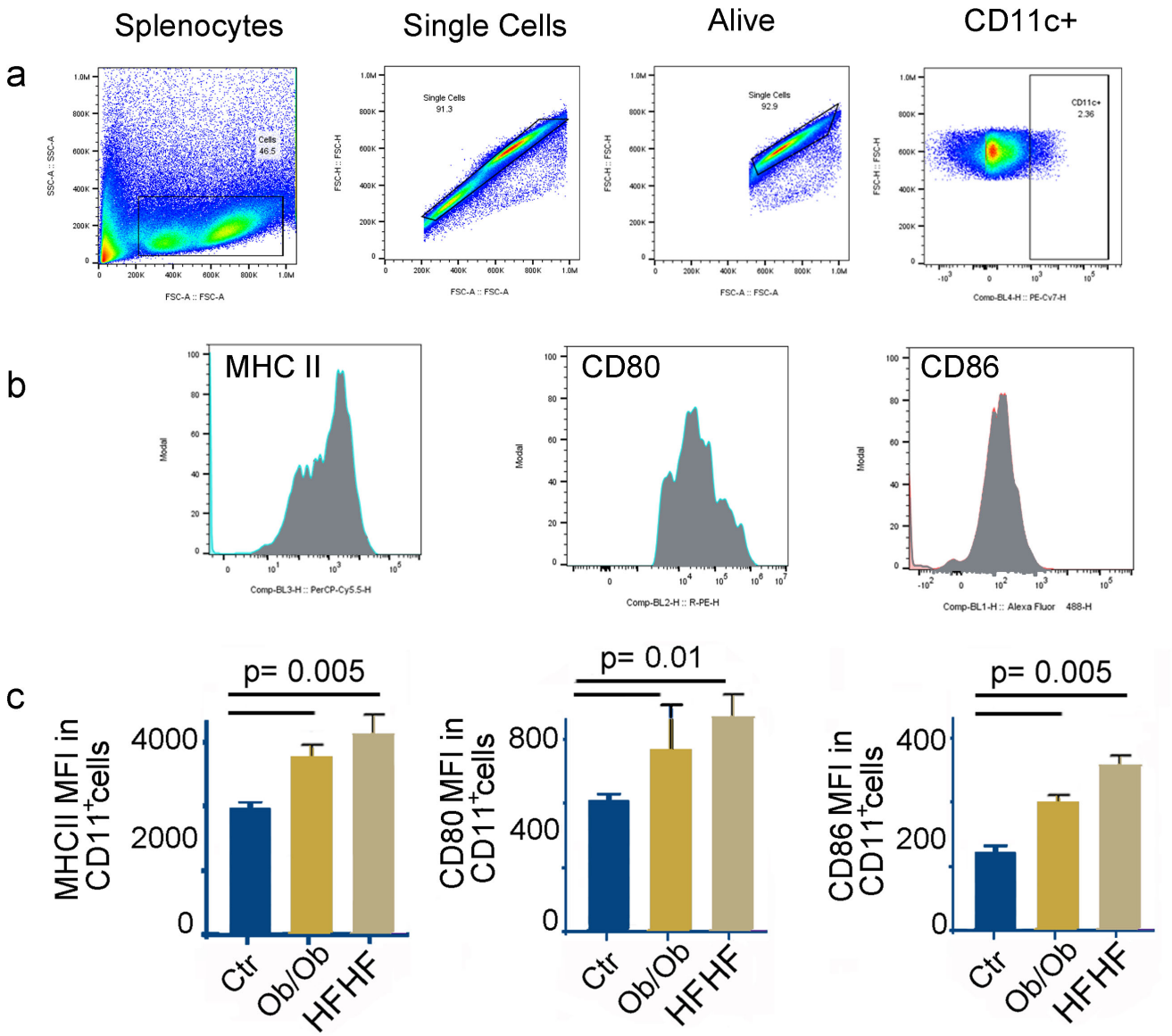
The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplemental Data

Figure S1 Dendritic cells gating strategy



a) Gating strategy for CD11c+ dendritic cells **b)** Representative plots of MHC II, CD80 and CD86 expression on CD11c gated cells. **c)** Bar graphs reporting surface amount of MHC II, CD80 and CD86 proteins on CD11c gated cells in B6, Ob/Ob and HFHF splenocytes. Values, from n=6 biologically independent replicates, are reported as mean relative expression ± SD and were statistically analyzed using a two-tailed paired-student's-t test.

Figure S2 PCA analysis (at 92% confidence) shows a differential clustering of the proteomes from control and HF mice, indicating differential protein expression profiles induced by the diet regime.

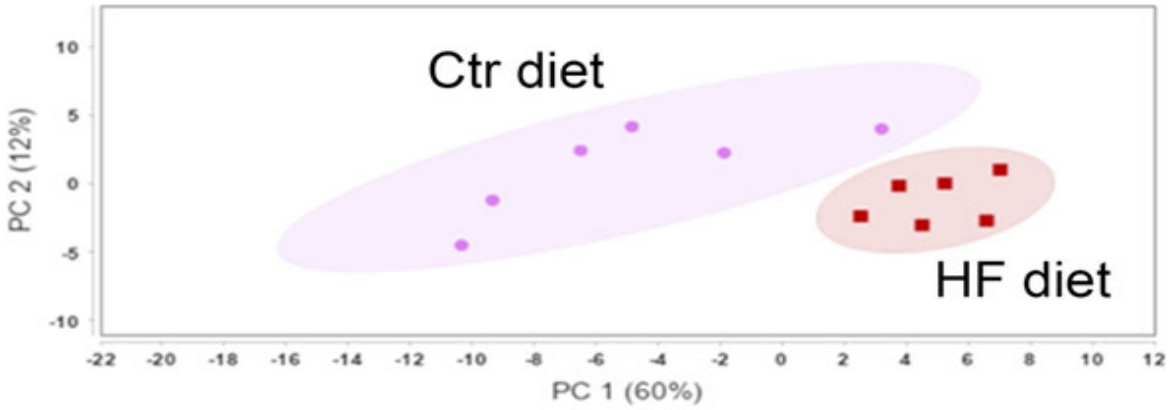
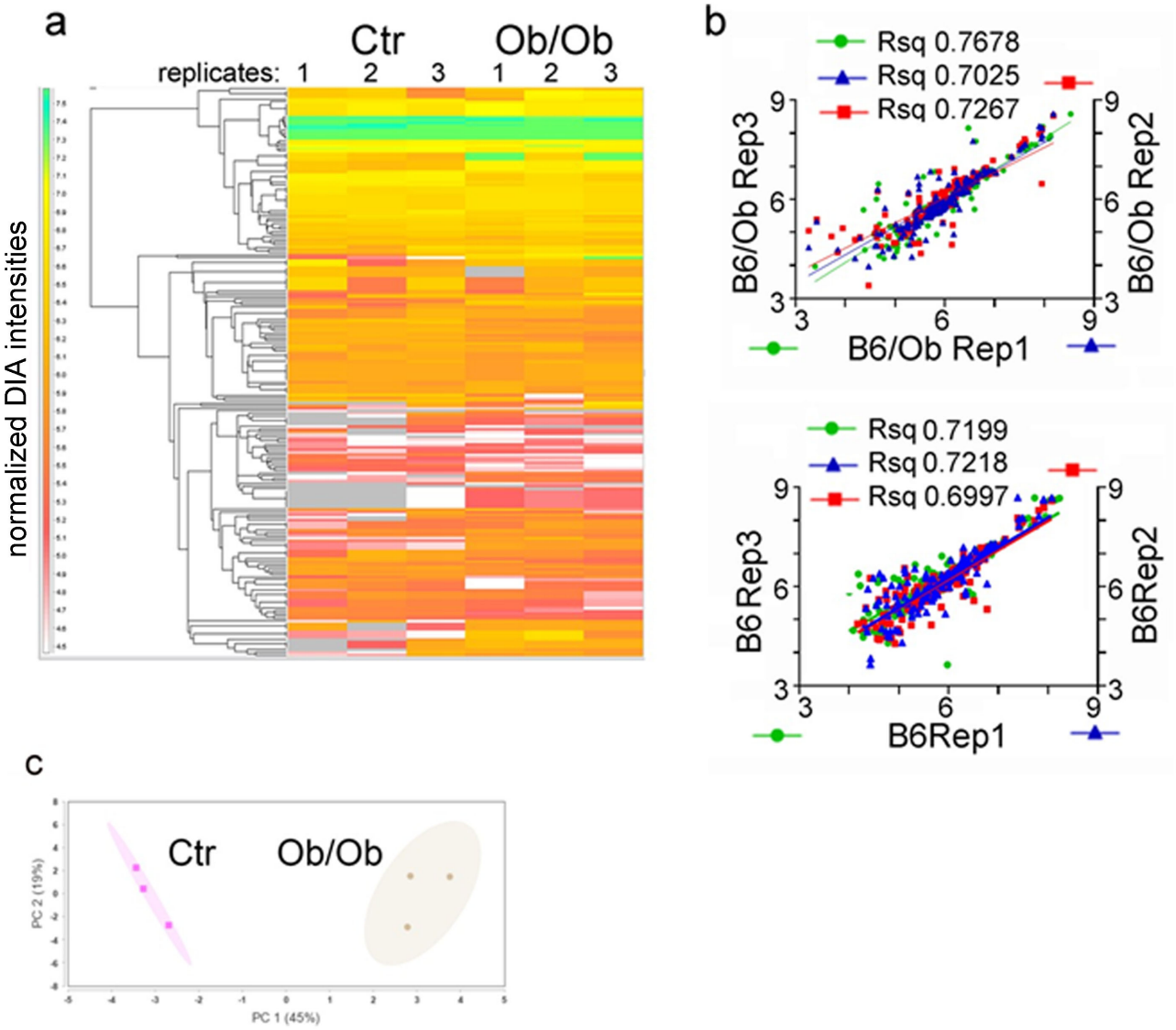


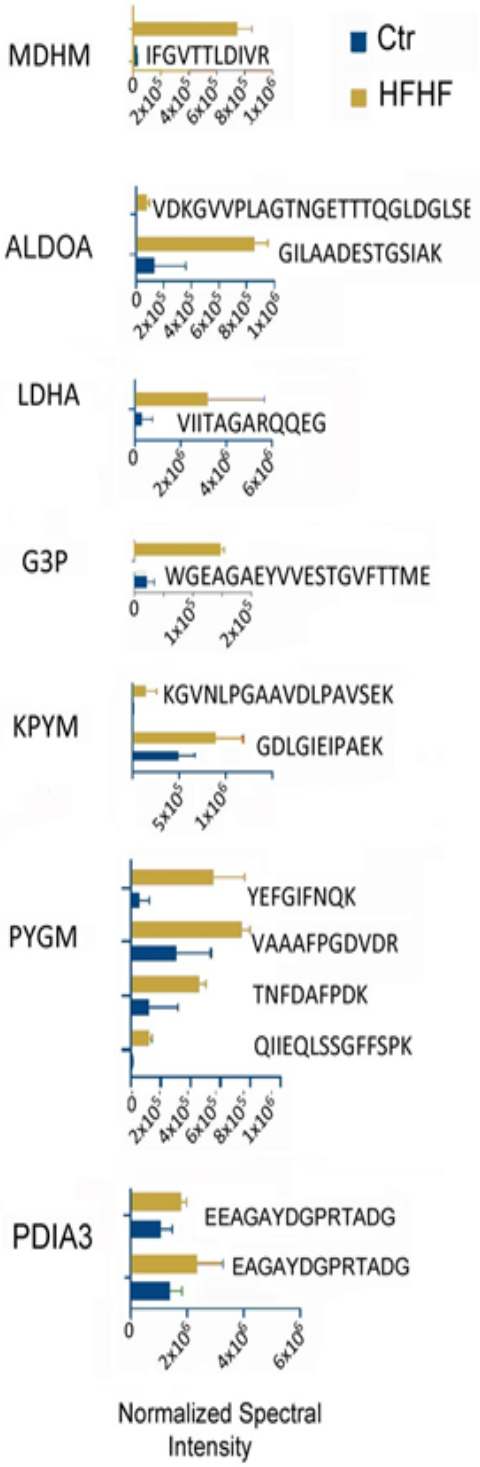
Figure S3: Qualitative and quantitative changes in the *I-Ab* eluted immunopeptidome from Ob/Ob as compared to control C57BL/6 mice.



a) Label-free quantitative analysis of changes in the expression profile of protein substrates derived from the DCs-I-Ab eluted immunopeptidomes from Ob/Ob vs control C57BL6 mice**.** The eluted immunopeptidome was analyzed by *nano*-LC DIA and the heat map generated in Scaffold DIA using the normalized DIA intensities (the dataset was reported in ***(49)***). **b)** Regression analysis of

normalized total DIA intensities, for the 148 target substrate proteins (identified with an attained 1.4 % FDR) contributing to the 544 endogenous peptides eluted from I-Ab molecule (0.8% attained FDR) in one representative experiment including n=3 biological replicates. The Pearson's correlation scores are displayed for each Ob/Ob and control biological triplicates. **c)** PCA analysis (with 95% confidence ellipses) shows a differential clustering for the protein substrates generating the I-Ab eluted immunopeptidomes from control (B6) as compared with Ob/Ob mice, thus acknowledging the quantitative changes in the protein/peptide abundances.

Figure S4: Analysis of I-Ab eluted peptides derived from glycolysis-related proteins (MDHM, ALDOA, LDHA, G3P, KPYM, PYGM) eluted from C57BL/6 and Ob/Ob mice.

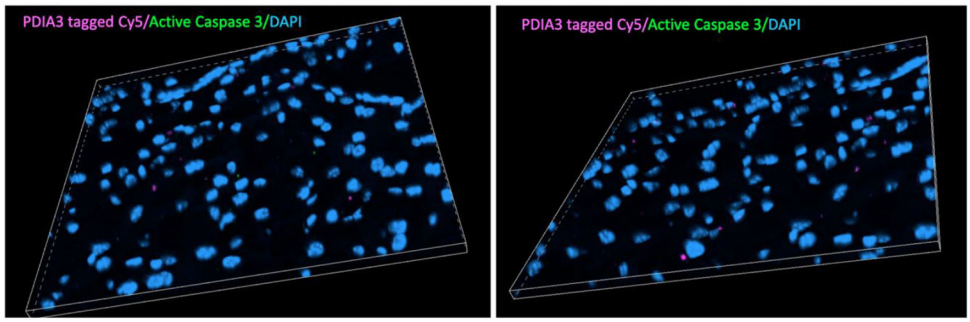


Comparative normalized DIA spectral counts of endogenous peptides derived from glycolysis-or ER-stress related proteins (MDHM, ALDOA, LDHA, G3P, KPYM, PYGM, PDIA3) eluted from C57BL/6 and Ob/Ob mice. The quantitative LFQ DIA analysis was performed on the I-Ab immunopeptidomes data set deposited at PRIDE database with the accession nr PXD023581 and PXD018779. The complete list of I-Ab eluted peptides is presented in **Supplemental Table S2.**

Figure S5. PDIA3-injected Abs bind to active caspase 3 positive hepatocytes in HF mice.

Representative 3D confocal images of infiltrating PDIA3-specific Ab (Cy5-tagged; magenta) in the liver of Ctr and HF mice binding to hepatocytes positive for active caspase 3 (green). Confocal images were collected in Z stack of 5 mm thickness.

Control diet



HFHF diet

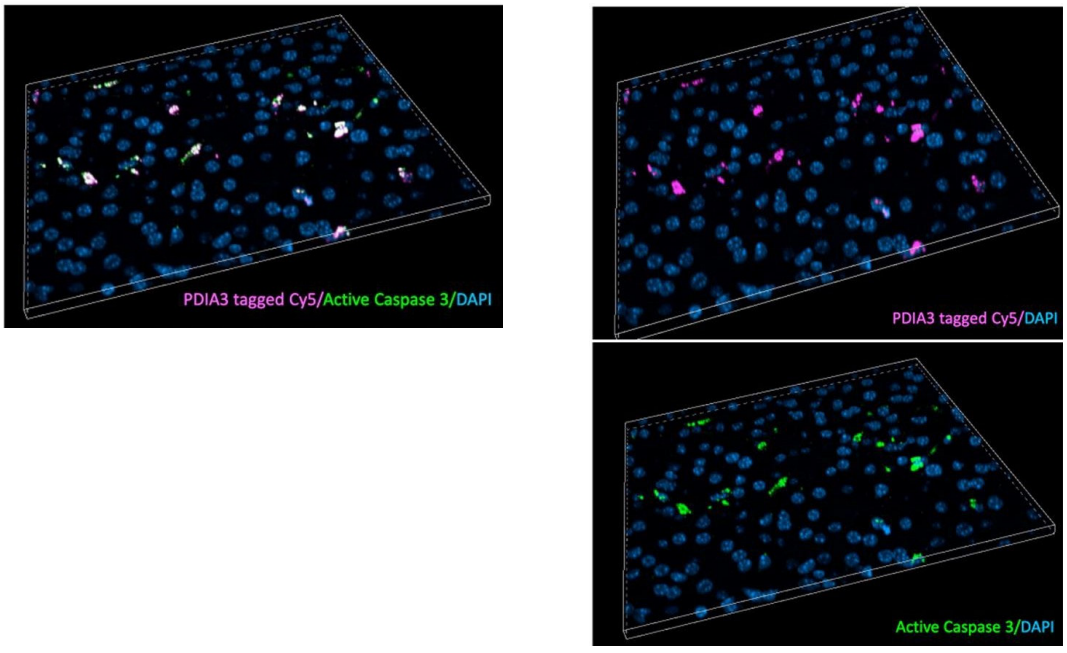
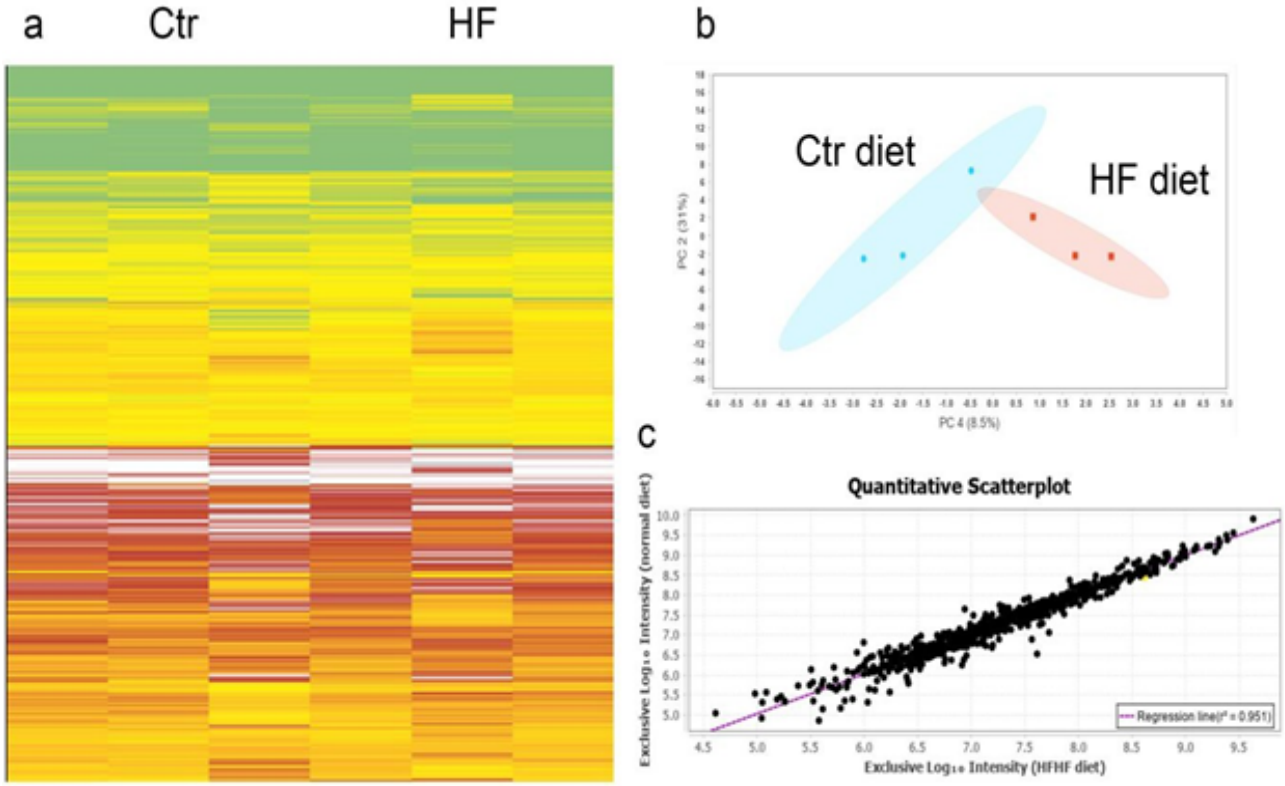


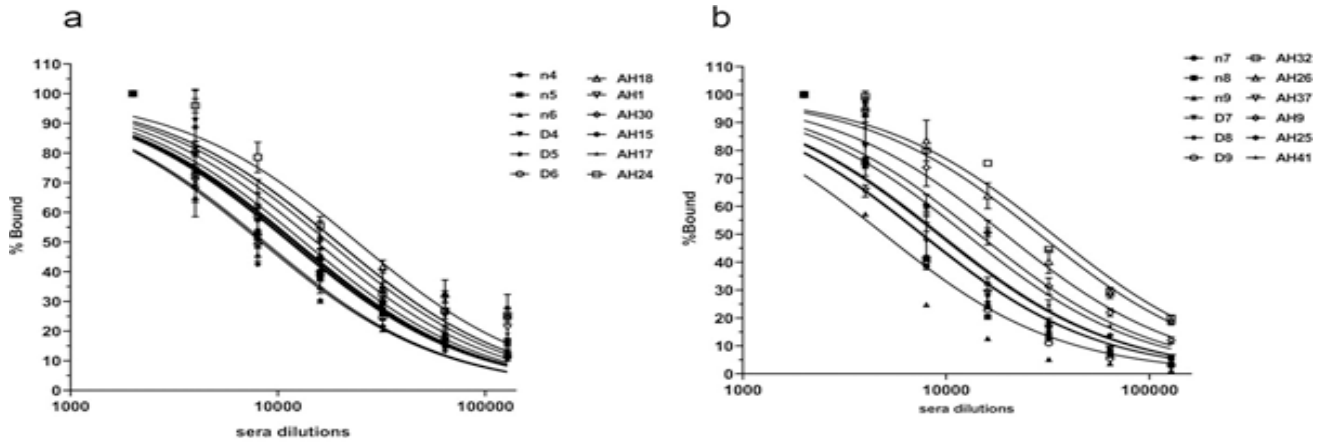
Figure S6 Qualitative and quantitative changes of the eluted immunopeptidomes from Ctr and HFHF liver immune infiltrates (n=3 replicates for each group).



a) Label-free quantitative analysis of changes in the expression profiles of protein substrates deriving from the eluted peptidome from liver immune infiltrates of Ctr and HF mice. The eluates were analyzed by *nano*-LC DIA and the heat map was generated in Scaffold DIA using the normalized DIA intensities for the corresponding protein substrates. A total of n=3 biological replicates retrieved 7392 endogenous peptides (FDR of 1.1%) derived from 803 protein substrates (FDR 5%) (**Supplement Table S5**). **b)** PCA analysis (with 95% confidence ellipses) shows two distinct clusters corresponding to the protein substrates generating the eluted peptidome from Ctr and HF mice (ANOVA/t-tests and $p<0.05$ for significance). c) Quantitative scatterplot and regression analysis shows the comparison of Log10(intensity) for the I-Ab peptidome eluted

from ctr and HF liver immune infiltrates (one of each replicate is shown). The Pearson's correlation score (0.951) indicates the high reproducibility for the peptidome elution and nano-LC/MS/MS DDA/DIA analysis of the samples.

Figure S7. Representative titration curves used to calculate the anti-PDIA3 antibodies titers



Supplemental Table S5: anti-PDIA3 peptide Ab titers calculated from the sera of Ctr and HF mice

Titers are shown as mean +/- SD (n=9 biological replicates and n=3 technical replicates for both Ctr and HF mice) for the PDIA3-3 peptide epitope [IFRDGEEAGAYDGPRTADGIVSHLK].

The Ab-titers (IC-50 sera dilutions) were calculated after fitting the experimental data using the equation "[Inhibitor] vs. normalized response" provided by GraphPad Prizm (version 9.0 for curve fitting). Supplemental Figure 6 shows representative binding curves from ELISA assays used to calculate all Ab titers reported in this table.

mice	Baseline	Ctr diet	HF diet
1	141.6 +/-7.08	146.8 +/- 7.34	177.8 +/- 10.668
2	127.1 +/-6.355	138.7 +/- 6.935	158.6 +/- 9.516
3	148 +/-7.4	138.5 +/- 9.695	155.9 +/- 9.354
4	143 +/-10.01	148.7 +/- 10.409	199.3 +/- 9.965
5	112.5 +/-7.875	126.8 +/- 8.876	181.5 +/- 9.075
6	120.9 +/-8.463	161.7 +/- 11.319	202 +/- 10.1
7	152 +/-10.64	172.9 +/- 8.645	185.8 +/- 9.29
8	124 +/-6.2	168.3 +/- 10.098	220.4 +/- 13.224
9	157.3 +/-7.865	151.1 +/- 9.066	205.6 +/- 12.336
Mean (SD)	**136.3** 15.56	**150.4** 15.03	**187.4** 21.58

Supplemental Table S7: anti-PDIA3 Ab titers calculated from the sera of healthy, T2D, autoimmune hepatitis, and autoimmune cholangitis patients.

The Ab-titers were calculated as described in Table S6a

	Ab-titer (IC-50 serum dilution) (Mean and Standard deviation)			
Donor	**Normal** (n=25)	**Diabetic** (n=48)	**Autoimmune Hepatitis** (n=28)	**PBC (Cholangitis)** (n=18)
1	7033 +/- 421.98	11210 +/- 560.5	11295 +/- 564.75	48402 +/- 2420.1
2	8696 +/- 521.76	15618 +/- 780.9	28944 +/- 1447.2	17268 +/- 863.4
3	7242 +/- 434.52	9615 +/- 480.75	13071 +/- 653.55	23725 +/- 1186.25
4	10068 +/- 302.04	15336 +/- 766.8	8929 +/- 446.45	7516 +/- 375.8
5	8511 +/- 255.33	8309 +/- 415.45	8648 +/- 432.4	12016 +/- 600.8
6	8636 +/- 259.08	11947 +/- 597.35	9534 +/- 476.7	6515 +/- 325.75
7	7667 +/- 460.02	9079 +/- 453.95	10832 +/- 541.6	25027 +/- 1251.35
8	7567 +/- 378.35	9372 +/- 468.6	6208 +/- 310.4	21448 +/- 1072.4
9	4942 +/- 247.1	7592 +/- 455.52	15387 +/- 769.35	30495 +/- 1524.75
10	5664 +/- 283.2	5199 +/- 311.94	28668 +/- 1146.72	11924 +/- 596.2
11	8194 +/- 409.7	9053 +/- 543.18	19451 +/- 778.04	33409 +/- 1670.45
12	5074 +/- 253.7	7822 +/- 469.32	12503 +/- 500.12	12303 +/- 615.15
13	7202 +/- 360.1	8022 +/- 481.32	14417 +/- 576.68	25400 +/- 1270
14	7877 +/- 315.08	4035 +/- 242.1	24041 +/- 961.64	25473 +/- 1273.65
15	4385 +/- 263.1	5917 +/- 355.02	17516 +/- 700.64	8741 +/- 437.05
16	5092 +/- 305.52	9405 +/- 564.3	11319 +/- 452.76	7436 +/- 371.8

17	5005 +/- 300.3	5509 +/- 330.54	19237 +/- 1154.22	10692 +/- 534.6
18	4245 +/- 254.7	14232 +/- 853.92	19371 +/- 1162.26	7667 +/- 383.35
19	4774 +/- 286.44	6248 +/- 374.88	55957 +/- 3357.42	
20	5435 +/- 326.1	6978 +/- 418.68	24971 +/- 1498.26	
21	5556 +/- 277.8	9476 +/- 568.56	10886 +/- 653.16	
22	4489 +/- 224.45	4832 +/- 289.92	5344 +/- 320.64	
23	3982 +/- 199.1	4394 +/- 263.64	7952 +/- 477.12	
24	5296 +/- 264.8	4208 +/- 252.48	9403 +/- 564.18	
25	5052 +/- 252.6	4407 +/- 264.42	7340 +/- 220.2	
26		4396 +/- 263.76	8043 +/- 241.29	
27		4482 +/- 179.28	8852 +/- 265.56	
28		4258 +/- 170.32	8683 +/- 260.49	
29		7298 +/- 291.92		
30		5374 +/- 214.96		
31		25282 +/- 1011.28		
32		13900 +/- 556		
33		17128 +/- 685.12		
34		11772 +/- 470.88		
35		7791 +/- 311.64		
36		24270 +/- 970.8		
37		10532 +/- 421.28		
38		20564 +/- 822.56		
39		7670 +/- 306.8		

40		22731 +/- 909.24		
41		16663 +/- 499.89		
42		8511 +/- 255.33		
43		4857 +/- 145.71		
44		5396 +/- 161.88		
45		3796 +/- 113.88		
46		4452 +/- 133.56		
47		5083 +/- 152.49		
48		5218 +/- 156.54		
Mean (SD)	**6307** (1728)	**9359** (5554)	**15243** (10394)	**18637** (11428)

Supplemental Table 8: List of Raw Data

Table 8: RAW data

7a FACS gating for dendritic cells in Figure 1e and f
7b Accession # for Proteomic Files for Figure 1g-m and Figure 2
7c PRM data for Figure 3a-g
7d Antibody quantification for Figure 3h-l
7e PDIA3 surface staining for Figure 4a,b
7f Liver Histology Figure 4d-k
7g Transaminases ALT and AST in control and HFHF sera, Figure 4l,m
7h anti-PDIA3 antibody sera titration for Figure 5a-c
7i anti-PDIA3 antibody isotyping for Figure 5g
7j Liver cytotoxicity assay for Figure 5l
7k Transaminases ALT and AST in control and HFHF sera following anti-PDIA3 antibody injection (Figure 5m,n)
7l PRM quantification of PDIA3 epitope eluted from liver infiltrates for Figure 6a,b
7m Proliferation of liver infiltrates to the PDIA3 epitope for Figure 6c
7n Gating strategy to analyze immune liver infiltrates for Figure 6d,e
7o Cytokines ELISA for liver immune infiltrates for Figure 6f,g
7p ELISPOT data for Figure 6h
7q Gating strategy to analyze anti-PDIA3 T cells infiltrating the liver for Figure 6i-k
7r Transaminases ALT and AST in control and HFHF sera following anti-PDIA3 T cell injection (Figure 6l)
7s Anti PDIA3 titer in subjects with T2D, Autoimmune Hepatitis and Primary Cholangitis in Figure 7

Supplemental materials and methods

Animal diets

Seven to eight week old C57BL/6 mice were used as experimental animals and were housed in metallic cages in an animal room with 12-h light and dark cycles. The animal room was maintained at a temperature range of 23 ± 1 °C. All mice were randomly divided into two groups including a control group in which the mice were fed the standard Laboratory Rodent Diet (Purina, St Louis, MO, USA) and HFHF (high fat and high fructose) group fed a high-fat diet (60kcal % fat) containing 245 grams of lard, 200 grams Casein, 3 grams cysteine, 125 grams lodex 10,72.80 grams Sucrose, 50 grams powdered cellulose (Solka Floc), 25 grams soybean oil, 50 grams mineral mix (S10026B), 2 grams Choline tartrate and 1 gram vitamin mix (V10001c) (cat# D12492, Research Diets Inc) and high fructose diet containing 64kcal% (cat # D10012G and D13061501, Research Diets Inc.,) in a 1:1 ratio for every day up to 3 months. Body weight was monitored in each animal every week; an average of 40% weight gain was observed after the 3 months HFHF diet.

Mouse DC preparation

Mice on control and HFHF diet were injected subcutaneously with $40x10^6$ B16-FLt3-L-producing melanoma cells to *in vivo* expand the number of DCs in secondary lymphatic organs (*50*). Twelve to 14 days after injection spleens and lymph nodes (cervical, axillary, inguinal and mesenteric) were harvested and DCs were purified by density-gradient centrifugation using 30% Bovine Albumin solution (Sigma-Aldrich) as described previously (*50, 51*). Briefly, 3 mL of sterile BSA 30% was mixed 1:1 (v/v) with RPMI1640 complete media on top of which an extra layer of 3 mL sterile BSA (30%) was added to finalize the gradient premixing solution; on top of the premix BSA gradient, 1-2 mL of cell suspension was added. The mixture was centrifuged in a polypropylene tube, fitting the Beckman *SW Ti41 rotor,* at 9200xg, for 1 hour at 4ºC using the Optima XPN-100k UltraCentrifuge (Bekman Coulter). The white ring of cells isolated after centrifugation which routinely contains 70 to 80% CD11c+ DCs (*50*) was

collected and washed three times with PBS before counting. Between 2–5 × 10^8 murine DCs cells were obtained from each spleen, to be further processed for proteomics analysis.

DC proteomics analysis

Pelleted DCs, purified by BSA gradient, were lysed on ice with cold MILLIPLEX MAP lysis buffer, (cat # S30043-MXA, Cell Signaling buffer) supplemented with 1mM DTT and complete cocktail of protease inhibitors (cat# 04693116001, Roche). The lysates were shaken on a rocker at 4ºC for 20 min followed by treatment with benzonase nuclease (5 units/µl lysate) on ice, for 20 min before being spun at 14,000 rpm in a microcentrifuge for 25 min, at 4ºC. The supernatant was further cleared with EMD Millipore filters (cat # UFC 0DV 25). Total protein quantitation was performed using the Micro BCA™ Protein Assay Kit, (cat # 23235 from Thermofisher Scientific). Fifty mg of whole DCs lysates from control and HF mice were reduced with 25 mM TCEP-HCl (Thermo Scientific) in 50 mM ammonium bicarbonate buffer, containing 8 M urea at pH 8.5 for 45 minutes at RT followed by alkylation with 100 mM iodoacetamide for 50 minutes in the dark at RT. The protein solutions were transferred on microcon-10kDa centrifugal filter units with Ultracel-10 membrane (catalog# MRCPRT010) from Millipore Sigma and washed with 50 mM ammonium bicarbonate buffer five times at 9000xg in a microcentrifuge, for 10 minutes each step. The reduced and alkylated samples were resuspended in 100 mL of 50 mM ABC buffer, pH 8.9 (urea <2M) and digestion was carried out at 37°C overnight (12 hours) using a combination of three enzymes: trypsin/LysC at 20:1 protein: enzyme ratio and GluC at 10:1 protein: enzyme ratio. The digestion was quenched with 0.5% acetonitrile and 1.5% formic acid. Processed peptides were then extracted through a 10-kDa MWCO (molecular weight cut-off) using 10kDa centrifugal filter units by spinning at 10,000xg for 15 minutes in a 20:1 microcentrifuge. The final peptide mixture, extracted from all enzymatic digestions, was desalted on C18 Prep clean columns (EMD Millipore) and reconstituted in 25 µl 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid for further nanoLC/MS/MS analysis.

Desalted peptides were injected onto the EASY-Spray PepMap RSLC C18 50 cm x 75 μm column (Thermo Scientific), coupled to the Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides were eluted with a non-linear 130 min gradient of 5-30% buffer B (0.1% (v/v) formic acid, 100% acetonitrile) at a flow rate of 250 nl/min. The column temperature was maintained at a constant 50 ˚C during all experiments.

DIA method for peptide MS/MS analysis was employed by performing survey scans of peptide precursors from 350-1200 *m/z* at 120K FWHM resolution (at 200 *m/z*) with a 1 x 10^6 ion count target and a maximum injection time of 60 ms. The instrument was set to run in top speed mode with 3 s cycles for the survey and the MS/MS scans. After a survey scan, 26 m/z DIA segments (windows) were acquired at from 200-2000 *m/z* at 60K FWHM resolution (at 200 *m/z*) with a 1 x 10^6 ion count target and a maximum injection time of 118 ms. HCD fragmentation was applied with 27% collision energy and resulting fragments were detected using the rapid scan rate in the Orbitrap. The spectra were recorded in profile mode. DIA-MS samples were analyzed using Scaffold DIA (2.2.1) after DIA-MS data files were converted to mzML format using ProteoWizard (3.0.11748). *Spectral Library Search* The analytic samples were aligned based on retention times and individually searched against an *in-house* spectral library derived from the proteomics analysis of DDA-MS/MS files (PRIDE accession number **PXD024239**), using a peptide mass tolerance of 15.0 ppm and a fragment mass tolerance of 20.0 ppm. Fixed modifications were: Carbamidomethylation (Cys). Variable modifications were: Carboxymethylation (CML, CMA) (+58.005; K, R); Carboxyethylation (CEL) (+72.021; K, R); Fructosylation (N-hexosyl lysine) (+162.053; K) Pyrraline (+108.021; K); GLAP (+109.029; K); Glycerinylation (+88.016; K); Acetylation (+42.011; K); Formylation (+27.995; K); Glarg (+39.995; R); MG-H (+54.011; R); G-H1 (+39.990; R); Argpyrimidine (+79.966; R) Tetrahydropyrimidine (3-deoxyglucosone) (+144.042; R); Dihydroxyimidazolidine (CEAMGDHI) (+72.020; R); N-Pentosyl Lysine (+132.0575; K); N-tetrosyl Lysine (+102.0317; K) (as reported in other proteomic AGE analyses. In addition, the following posttranslational modifications were specified in PEAKS X/X+ as variable

modifications for carbonylation: Lys->Allysine (-1.03; K); Lys->AminoadipicAcid (+14.96; K); Amino (Y) (+15.01, Y); Trp->Oxolactone (W) (+13.98, W); Pro->Pyrrolidone (P) (-27.99; P); Pro->Pyrrolidinone (P) (-30.01; P); Arg->GluSA (-43.05; R); Dehydrated 4-hydroxynonenal (HNE-Delta:H(2)O(H)) (+138.21, C,H,K); reduced 4-Hydroxynonenal (HNE+Delta:H(2) (H)) (+158.13, C,H,K); HNE (+156.12, C,H,K); 4-Oxononenal (ONE) (4-ONE) (+154.10, C,H,K); dehydrated 4-Oxononenal Michael adduct (4-ONE+Delta:H(-2)O(-1)) (+136.09; C,H,K). The digestion enzyme was assumed to be Trypsin with a maximum of 3 missed cleavage site(s) allowed. Only peptides with charges in the range of 2+ to 4+ and length in the range of 5 to 25 aa were included for further quantitative analysis. Peptides identified in each sample were filtered by Percolator (3.01. nightly-13-655e4c7-dirty) to an FDR of 0.05. For each peptide, the 5 highest quality fragment ions were selected for quantitation, performed by Encyclopedia (0.9.6). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony.

Peptidome extraction from the immune infiltrates in the livers of Ctr and HF mice

The immune cells infiltrate in the liver of mice kept on normal or HF diet were obtained as described above. Pelleted cells were subjected to mild acid extraction method as previously reported (*51*). The extracted peptides from the filtrates (MW<10kDa) were lyophilized and further subjected to the nano-LC/MS/MS analysis using a combination of DDA and DIA methods described above for the I-Ab eluted immunopeptidomes. Peptide epitopes derived from MDH, ALDOA, PKM and PDIA3 were selected for labeling with heavy isotopes and standards were spiked into the total cellular peptidomes of liver's immune infiltrates (see PRM methods section for further details).

Absolute peptide quantification using stable isotope-labeled peptides and parallel reaction monitoring (PRM)

A peptide spanning the region 105-129 of the PDIA3 protein (DGEEAGAYDGPRTADG) was selected for synthesis and absolute quantification in the total I-Ab eluted immunopeptidomes from mice kept on

different diet regime. The heavy labeled peptide PDIA3 was synthesized with 13C and 15N labeled amino acids (e.g. R+10Da: 13C(6)15N (4) in DGEEAGAYDGP**R[+10]**TADG). The PRM analysis was also employed to quantify additional endogenous epitopes identified in the I-Ab eluted immunopeptidome including: 1) MDHM (epitope IFGVTT**[L]**DIVR); 2) ALDOA (epitope GI**[L]**AADESTGSIAK); 3) KPYM (epitope GD**[L]**GIEIPAEK); 4) LDHA (epitope VIITAGA**[R]**QQEG) and 5) MDHC (epitope FVEG**[L]**PINDFSR), where in bolded parentheses are shown the heavy labeled amino acids: Leucine (L), (+7Da) and Arginine (R), (+10Da). All heavy labeled peptides were synthesized by Pierce Custom Peptides (Thermo Fisher Scientific, Rockford, IL, USA). The heavy labeled standard peptides were spiked at 1 or 2 fmol/peptide/injection into the immunopeptidomes to quantify the chemically identical, but unlabeled (light) endogenous peptide present in the eluted peptidomes. Peptides were resuspended in 10 uL of 3% ACN/0.1% formic acid and injected as a mixture with the 1-2 fmol of spiked heavy peptide in the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer using the PRM method for MS1 and MS/MS analysis. The UltiMate 3000 UHPLC system (Thermo Scientific) and EASY-Spray PepMap RSLC C18 50 cm x 75 μm ID column (Thermo Fisher Scientific) coupled with Orbitrap Fusion (Thermo) were used to separate fractioned peptides with a 5-30% acetonitrile gradient in 0.1% formic acid over 70 min at a flow rate of 250 nl/min. After each gradient, the column was washed with 90% buffer B for 5 min and re-equilibrated with 98% buffer A (0.1% formic acid, 100% HPLC-grade water) for 20 min. MS data were acquired by combining to scan events corresponding to full scan and a Parallel Reaction Monitoring (PRM) method targeting the ten I-Ab and liver leukocytes whole cell eluted peptides. A target value for the full scan MS spectra was 1 × 106 ions in the 300-1500 m/z range with a maximum injection time of 60 ms and resolution of 120,000 at 200 m/z with data collected in profile mode. The PRM method was utilized at a resolution of 30,000 at 200 m/z, a target AGC value 1×10^6, and maximum fill times of 110 ms. The precursors ions of each targeted peptide were isolated using a 1.7 m/z unit window and fragmented by higher-energy C-trap dissociation with a normalized collision energy of 27 eV. Data analysis was performed using Skyline (version: 20.2.0.286) which extracted all valid transitions for MS1 and MS2

(presented in the **Supplement Table S4**) and provided the area under the MS1 and MS2 peaks which were further used to determine the quantity of the respective endogenous vs heavy reference peptide.

Isolation of I-Ab bound peptides from mouse DCs

I-Ab peptide complexes from DCs of control mice, Ob/Ob mice and HF mice were isolated using immunoaffinity chromatography. Three independent samples of C57Bl6 controls, HF and Ob/Ob were isolated in parallel. Cell pellets were resuspended in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, containing protease inhibitors and 5% b-octylglucoside, freeze-thawed for 4 times, homogenized, and the solubilized whole cell fraction was recovered by centrifugation at 100,000 χ g for 1 h at 4°C. The supernatant was used for the isolation of the MHC-class-II peptide complexes using an immunoaffinity column of M5/114 monoclonal antibody immobilized onto CNBr activated Sepharose. The column was equilibrated with buffer (50 mM Tris-HCl, 150 mM NaCl pH 8.0, containing protease inhibitors) for 2 h. The lysates were pre-cleared with the beads only followed by isotype control antibody slowly for 1 h each at 4°C to prevent nonspecific binding of the proteins to the beads. After pre-clearing the lysate was incubated with M5/114 conjugated beads and allowed to mix slowly for 1 h at 4°C. The column was washed with several buffers in succession as follows: (1) 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, containing protease inhibitors and 5% b-octylglucoside (5 times the bead volume); (2) 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, containing protease inhibitors and 1% b-octylglucoside (10 times the bead volume); (3) 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, containing protease inhibitors (30 times the bead volume); (4) 50 mM Tris-HCl, 300 mM NaCl, pH 8.0, containing protease inhibitors (10 times the bead volume); (5) 1X PBS (30 times the bead volume); and (6) HPLC water (100 times the bead volume). The total amounts of I-Ab were measured by ELISA from each preparation. The lysates containing similar amounts of I-Ab was used for the isolation of peptides. Peptides were further separated using a Vydac C4 macrospin column (The Nest Group, USA). I-Ab peptides were eluted using 30% acetonitrile in 0.1% TFA. Eluted peptides were lyophilized using a Speed-Vac.

Analysis of the I-Ab-eluted immunopeptidome

Survey scans of peptide precursors were performed from 400 -1500 *m/z* at 120K FWHM resolution (at 200 *m/z*) with a 4 x 10^5 ion count target and a maximum injection time of 50 ms. The instrument was set to run in top speed mode with 3 s cycles for the survey and the MS/MS scans. After a survey scan, tandem MS was performed on the most abundant precursors exhibiting a charge state from 2 to 6 of greater than 5 x 10^3 intensity by isolating them in the quadrupole at 1.6 Th. HCD fragmentation was applied with 35% collision energy and resulting fragments were detected using the rapid scan rate in the ion trap. The AGC target for MS/MS was set to 1 x 10^4 and the maximum injection time limited to 35 ms. The dynamic exclusion was set to 60 s with a 10-ppm mass tolerance around the precursor and its isotopes. Monoisotopic precursor selection was enabled.

DDA raw files were filtered, *de novo* sequenced and assigned with protein ID using Peaks X/X+/XPro software (Bioinformatics Solutions, Waterloo, Canada), by searching against the Swiss-Prot FASTA database (https://www.uniprot.org), using *Mus musculus* as specie corresponding to the processed antigens (17,073 entries, reviewed Swiss-Prot, January 2021). The following settings were applied for peptide search: "*no enzyme*", parent mass tolerance of 12 ppm using monoisotopic mass, and fragment ion mass tolerance of 0.02 Da. Methionine, lysine, proline, arginine, cysteine and asparagine oxidations (+15.99 on CKMNPR), deamidation of asparagine and glutamine (NQ-0.98) and pyro-Glu from glutamine (Q-18.01 N-term) were set as variable modifications. The estimation of false discovery rate (FDR) using decoy-fusion algorithm was enabled during the PEAKS DB search in PEAKS. Data were validated using the false discovery rate (FDR) method built in PEAKS X/X+/XPro and protein identifications were further accepted if they could be identified with a confidence score (-10lgP)>20 for peptides and (-10lgP)>20 for proteins (corresponding to $p<0.01$); a minimum of 1 peptide per protein after data were filtered for less than 1.0-2.0% FDR for peptides and less than 1.5 % FDR for proteins identifications. In addition, selected peptides epitopes with 10< (-10lgP) <20 were included in the data set after manual inspection of their MS/MS spectra.

The DIA (data independent analysis) of the I-Ab immunopeptidome of B6 and Ob/OB mice was previously published (*49, 50*) and deposited at the PRIDE database with the **ProteomeXchange accession numbers:** PXD023581 and PXD018779.

Gene ontology (GO) annotations and functional analysis of networks and cellular pathways

GO annotations and functional networks analysis leading to the identification of major biochemical and cellular pathways characterizing the relative changes in the protein expression profiles or RNAseq quantitative readout from murine DCs, were generated in the ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA, USA). For network analysis, datasets containing gene identifiers (gene symbols) were uploaded into the IPA application together with their rescaled log2 transformation of protein's area ratios extracted from the normalized DIA intensities and label free quantitative (LFQ) analysis provided by the Scaffold DIA software. A similar protocol was used for the corresponding RNA sequences. The probability of having a relationship between each IPA indexed biological function and the experimentally determined genes were calculated by the right-tailed built-in Fisher's exact test using the Benjamini-Hochberg correction. The level of significance was set to a p-value of <0.05. For the relative quantitative analysis of the overrepresented pathways, IPA assigned the "z-score" function to all eligible canonical and cellular pathways: where a "$z<-2$ represent significant down-regulation while a $z>2.0$ represent a significant up-regulation of the selected pathway.

Histology

After perfusion the liver tissue were placed in tissue cassette and fixed in 10% formalin for 24 hrs. Liver tissue was sent to Applied Pathology Systems (MA) for tissue processing with a standard cycle, paraffin embedding, cutting, and staining. All tissue sections were cut at 5µm for histology. Hematoxylin and eosin stain (H&E) was performed with an automated stainer (Leica Autostainer XL) following standard procedure. Histomorphology of each H&E slides was evaluated by a board-certified pathologist, and the images were captured with Olympus cellSens Entry software at indicated magnifications. Steatosis was

scored twice at one week distance to minimize intraobserver variability on H&E stained sections from 0 to 3 according to the percentage area of parenchyma occupied by fat micro and macro vacuoles (0<5%; 1 (mild)= 5 to <20%; 2 (moderate)= 20 to 50%; 3 (severe) = >50%).. Lobular inflammation (expressed as presence of foci of monuclear cell infiltrate) was scored from 0 to 3 at 200x (0 = no foci; 1= 1-2 foci; 2= 3-4 foci; 3= > 4 foci). The presence of steatosis was confirmed by oil red o staining performed by standard procedure on frozen sections of liver tissue slices adjacent to those used for paraffin embedding.. Hepatocellular ballooning (defined as enlarged hepatocytes with flocculent cytoplasm, hyperchromatic nucleus, location near terminal hepatic venule) was scored from 0 to 2 (0= none; 1= occasional balloon cells; 2= multifocal balloon cells).

Confocal Analysis

Liver harvested from Ctr or HF mice, injected with anti-PDIA3 Ab-Cy5 conjugated 24 hours prior, were OCT-embedded and sectioned at 5-7 μm thickness. Frozen sections were fixed in cold 2% paraformaldehyde for 15 minutes. Slides were incubated with 5 μg/mL Wheat Germ Agglutinin, Alexa Fluor™ 594 Conjugate (Catalog number: W11262, Molecular Probes TM, Thermo Fisher Scientific Inc, Eugene, Oregon) for 30 min at room temperature; followed by 3 times 10 min washes in PBS. For active caspase staining antigen retrieval was performed in a microwave oven in Tris buffer (pH 9.0). Slides were incubated with 4μg/mL active caspase 3 rabbit polyclonal antibody (Catalog number: AF835, R&D Systems Bio-tech, Minneapolis, MN) at 4°C overnight; followed by incubation with goat anti-rabbit Alexa flour 488 conjugated secondary antibody for 1 hour room temperature, followed by washing with PBS/0.1 % Tween-20. Fifty μL of Prolong Gold Antifade Mounting media with DAPI (Molecular Probes TM, Thermo Fisher Scientific Inc.) were added to the slide sections before closing with a coverslip. Confocal images were collected with Nikon A1R-Si Confocal Microscope System equipped with solid state lasers (405nm, 561nm and 640 nm).

Cytokines quantification

Cytokines in liver infiltrates were analyzed with the mouse cytokine array kit (Quantibody® Mouse Th17 Array 1, QAM-TH17-1 from RayBiotech Inc., Norcross, GA), which detects 18 mouse Th1, Th2, and Th17 cytokines. Briefly, between 100-200 μg of total liver extracts were loaded on each well of the protein array slide and processed according to the manufacturer instructions. An Axon scanner 4000B with GenePix software was used to collect fluorescence intensities. The QAM-TH17-1 Q-Analyzer v8.18.4 excel software was further employed to quantify the median fluorescence intensities corrected for the background, as recommended by the manufacturer. Individual calibration curves for each cytokine (fitted to linear or log-log regression functions) were generated to obtain the best fit (R^2>0.98). The final cytokine concentrations (pg/mL) were interpolated from the calibration curves and plotted as average ± standard deviation. The statistical significance was determined using the Holm-Sidak method, with alpha = 0.05 by applying multiple t-tests analysis where each row (corresponding to individual cytokine data) was analyzed individually, without assuming a consistent SD in GraphPad Prism 8.1.1. An independent analysis for statistics significance was performed using one-way or two-way ANOVA and followed by Tukey's multiple comparison test when appropriate.

Granzyme B quantification

Granzyme B was quantified using a sandwich ELISA assay (Duo-Set-ELISA cat# DY1865-05, R&D systems). Briefly the supplied goat anti-mouse Granzyme B capture antibody was diluted in PBS and then in bicarbonate/carbonate buffer pH 9.0 (at 300 ng/well) and incubated in 96 well plates (Immulon 4 HBXND541225) at 4°C, O/N. The plates were then washed four times with the provided 1xPBST washing buffer (PBS and 0.05% Tween20) followed by blocking with 2% BSA in 1X PBS for 2hr at RT. Following 2 washes in PBS, 100 μg of total liver protein lysates was loaded in the wells in technical triplicates and incubation for 2 hours at RT with gentle shaking. A calibration curve containing recombinant mouse Granzyme B (spanning 62.5-4200 pg/mL concentration) was also generated in technical duplicates. Following incubation, samples were decanted, and the plate was further washed three times in 1xPBST before being loaded with 100 μL of the biotinylated goat anti-mouse granzyme B

detection antibody, followed by 2 hours incubation at RT with gentle shaking. The samples were then decanted, and after three washes incubated with 100 μL/well of Streptavidin-HRP (1/1000 in PBST+ 0.2%BSA) for 20 minutes at RT. The streptavidin-HRP solution was then removed and after three washes the plate was developed with 100 μL/well of TMB substrate solution (1:1 mixture of color reagent A (H2O2) and color reagent B (Tetramethylbenzidine) (R&D Systems®, Catalog # DY999) for 10 minutes at RT in the dark. The reaction was stopped with 50 μL/well of stop solution (2N H2SO4) for each well. The optical density was quantified at 450 nm using a microplate reader (FlexStation 3, from Molecular Devices).

Purification and proliferation of liver immune infiltrates

Livers were harvested from control and HFHF mice following perfusion with PBS 1x (Corning #21-040-CV). To obtain single cell suspensions, livers were processed using the liver dissociation kit (Miltenyi Biotec #130105807) following provider instructions for gentleMACS Octo Dissociator (Miltenyi Biotec # 130-095-937) with heaters (Miltenyi Biotec # 130-096-427). Cell suspensions were filtered through 100 μm cell strainers and resuspended in 2 mL of RPMI media. Cells were then purified over a Ficoll gradient and seeded ($5x10^5$ cells/100 mL/well) in MICROTEST U-Bottom 96-well polystyrene sterile plates (cat. #353077 Fisher Scientific) with titrated amounts (0-2-20 mg/mL) of the PDIA3 peptide (IFRDGEEAGAYDGPRTADG) in quadruple replicates for each peptide concentration. After 3 days incubation cell proliferation was measured on the fourth day using Click-iT EdU Microplate Assay (cat # C10214, Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. Data were recorded in a microplate reader (excitation/emission 565/585 nm). Independent proliferation assays, using the same experimental conditions, were also performed using the BrDU labeling, and the corresponding ELISA based method as described in the manufacturer instructions (cat#11647229001; Roche). In both assays the final processed data (after background subtraction) were expressed as mean ± SD of biological replicates. Statistical significance was determined using the Holm-Sidak method, with

alpha = 0.05 or a 2-tailed unpaired 1-way ANOVA with followed by Tuckey multiple comparison test. A *p* value less than 0.05 was considered significant.

Generation of anti-PDIA3 T cell line

Control and HFHF mice were immunized with 100 μg of the PDIA-3 peptides as mentioned above. After 14 days, inguinal and axillary lymph nodes were collected in sterile PBS solution and a single- cell suspension was prepared through a 100 μm cell strainer (Falcon, 352360). Cells were cultured in complete DMEM (Corning, 15-017-CV) medium containing 4.5g/L glucose, L-glutamine, sodium pyruvate, HEPES buffer, 10% Fetal bovine serum, antibiotic (streptomycin and penicillin) and stimulated with 20 μg/ml of PDIA3 peptide. After 48 hours of peptide stimulation, cell cultures were supplemented with 8 ng/ml of mouse IL-2 (R&D, 402-ML-020). The same IL2 concentration was added every other day until day 14 when cells were re-stimulated with feeder cells (syngeneic irradiated spleen cells) and with 20 μg /ml of PDIA3 peptide. BrDU incorporation (BrDU labeling as described in the manufacturer instructions; cat#11647229001; Roche) was measured at the time of each stimulation and the proliferation index calculated (ratio of BrDU incorporation in presence vs absence of PDIA3 peptide).