Supporting Materials:

Materials and Methods:

Animals and Treatments. Female Balb/cJ mice (7-10 weeks old, 18-22 g) were purchased from Jackson Laboratory (Bar Harbor, ME) and before use were acclimated for at least one week to a 12-hour light/dark cycle in a humidity and temperature controlled, specific-pathogen-free environment. Mice were housed in microisolator autoclaved cages and given autoclaved food and water *ad libitum*. The mice were injected intraperitoneally with 30 mmol/kg of distilled halothane (Sigma, St. Louis, MO) dissolved in olive oil (Mild Olive Flavor Originale, Star Fine Foods, Fresno, CA) at a final concentration of 0.30 mmol/mL or vehicle only. All maintenance of animals conformed to the guidelines for humane treatment set by the Association for Assessment and Accreditation for Laboratory Animal Care International's *Guide for the Care and Use of Laboratory Animals* and approved by the National Institutes of Health (NIH).

Sera and Tissue Collection. Blood samples were collected at selected time points by retro-orbital sinus bleeding in microtainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). Serum was isolated and used for measurement of alanine aminotransferase (ALT) and other serum proteins. Sections of the left and right lateral liver lobes were fixed in 10% buffered formalin (Thermo-Fischer Scientific, Pittsburgh, PA) for 24 hours prior to being transferred into 70% ethanol solution. Fixed tissue was embedded in paraffin, processed by standard histological techniques, and stained with hematoxylin and eosin (H&E) (American Histolabs, Gaithersburg, MD).

Flow Cytometric Analysis of Hepatic Leukocyte Populations. All staining specified below was performed using 5% fetal bovine serum (FBS) in sterile phosphate-buffered saline (PBS) at 4°C in the dark. Hepatic leukocytes were stained with violet LIVE/DEAD Fixable Aqua Dead Cell stain kit (Invitrogen, Carlsbad, CA) for 30 minutes and blocked with 0.5 µg of anti-CD16/CD32 (2.4G2, BD Pharmingen, San Diego, CA) for 15 minutes. Cells were then stained for 45 minutes with combinations of 0.5 µg each of APC-Cy7-conjugated anti-CD11b (M1/70, BD Pharmingen), Alexa 488-conjugated anti-CD11c (N418, eBioscience, San Diego, CA), PacBlue-conjugated anti-Gr-1 (RB6-8C5, eBioscience), APC-conjugated Ly6C (clone AL21,BD Pharmingen), PE-conjugated Ly6G (clone 1A8, BD Pharmingen), PE-conjugated anti-sialic acid-binding immunoglobulin-like lectin-F (Siglec-F) (E50-2440, BD Pharmingen), PE-Cy5-conjugated anti-CD3ε (145-2C11, eBioscience), eFlour450-conjugated anti-CD4 (RM4-5, eBioscience), FITC-conjugated anti-CD8a (Ly-2, BD Pharmingen), FITC-conjugated anti-CD8b (eBioH35-17.2, eBioscience), PE-labeled PBS-57-loaded mouse CD1d (α-GalCer Tet) tetramer (The NIH Tetramer Facility at Emory University, Atlanta, GA), APC-conjugated anti-CD45/B220 (RA3-6B2, BD Pharmingen), PE-conjugated anti-CD19 (1D3, BD Pharmingen) or corresponding isotype controls. Cells were then fixed with BD Cytofix solution (BD Biosciences). Live cell events were measured on a LSRII flow cytometer (BD Biosciences) and data were analyzed with FlowJo Software (Ashland, OR). The absolute number of each cell type per liver was calculated by multiplying their

percentage (as determined by flow cytometric analysis) by the total number of viable hepatic leukocytes per liver. Cells gated as $CD3\varepsilon^{+}$ α-GalCer Tet^{+}, $CD3^{+}$ $CD4^{+}$ $CD25^{-}$, $CD3^{+}$ $CD8^{+}$ $CD25^{-}$, $CD11c^{-}$ $CD11b^{+}$ $Gr\text{-}1^{low}$ Siglec-F^{high}, $CD19^{+}$ $B220^{+}$, $CD11c^{+}$, and $CD11c^{-}$ $CD11b^{+}$ $Gr\text{-}1^{high}$ were determined to be NKT cells, $CD4^{+}$ T cells, $CD8^{+}$ T cells, eosinophils, B cells, dendritic cells and MDSCs respectively.

Purification and Cytological Characterization of $CD11b^{+}$ $Gr\text{-}1^{high}$ Cells. Hepatic leukocytes were isolated and pooled from 5 female Balb/cJ mice sacrificed 24 hours after initial halothane treatment. $CD11b^{+}$ $Gr\text{-}1^{high}$ cells were stained for flow cytometry analysis as described and viable SSC^{high} $CD11c^{-}$ $CD11b^{+}$ $Gr\text{-}1^{high}$ Siglec $F^{low/neg}$ cells were sorted using an Aria II fluorescent-activated cell sorter (BD Biosciences). Sorted cells (50,000) in 100 µL of PBS containing 5% FBS were placed in a prewetted cytofunnel (Thermo Scientific, Rockford, IL) and centrifuged at 300*g* for 5 minutes at 4°C in a Cytospin 3 centrifuge (Thermo Scientific). Slides were stained with DiffQuick (Siemens, Newark, DE), dehydrated, and mounted using Shandon Consul-Mount Cytology permanent mounting media (Thermo Scientific). Cells were visualized by light microscopy.

T Cell Proliferation Assay. Proliferation of T cells in culture was measured by adding [^{3}H]-thymidine (1µCi/well) during the last 24 hours of incubation. Cell harvesting was performed using a Tomtec cell harvester (Wallac, Gaithersburg,

MD) and incorporated radioactivity was measured using a Wallac 1205 Betaplate liquid scintillation counter (Wallac).

Isolation of Hepatic CD11b^{+}Gr-1^{high} Cells and T Cell Suppression Assay.

Hepatic CD11b^{+}Gr-1^{high} cells isolated from mice 24 hours after halothane treatment were sorted using an Aria II fluorescent-activated cell sorter (BD Biosciences). To prepare target cells, CD4^{+}CD25^{-} or CD8^{+}CD25^{-} T cells were isolated from spleens of naïve female Balb/cJ mice using the above mentioned cell sorter. In addition, CD3^{-} MHC II^{+} cells were sorted from spleens of naïve female Balb/cJ mice and irradiated at 3000 rads for use as antigen presenting cells (APCs). CD4^{+}CD25^{-} or CD8^{+}CD25^{-} T cells ($5x10^{4}$ per well) were activated with plate bound anti-CD3ε antibody (clone 145-2C11, BD Biosciences) and co-cultured with hepatic CD11b^{+}Gr-1^{high} cells at different ratios (1:1 or 1:2) along with $1x10^{5}$ APCs. After 72 hours in culture, T cell proliferation was measured by [^{3}H]-thymidine incorporation during the last 24 hours of incubation (see Supporting Information for detailed methods). In specific wells, 0.5 mM of L-N^{6}-(1-Iminoethyl)lysine dihydrochloride (L-NIL, Sigma) was added at the beginning of the culture. Nitrite concentration in the culture supernatant was determined using Greiss Reagent kit (Molecular Probe, Eugene, OR) following the manufacturer's protocol.

The Detection of Trifluoroacetyl-Protein Adducts in Liver and Sera.

Detection of TFAPA in liver and serum samples was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

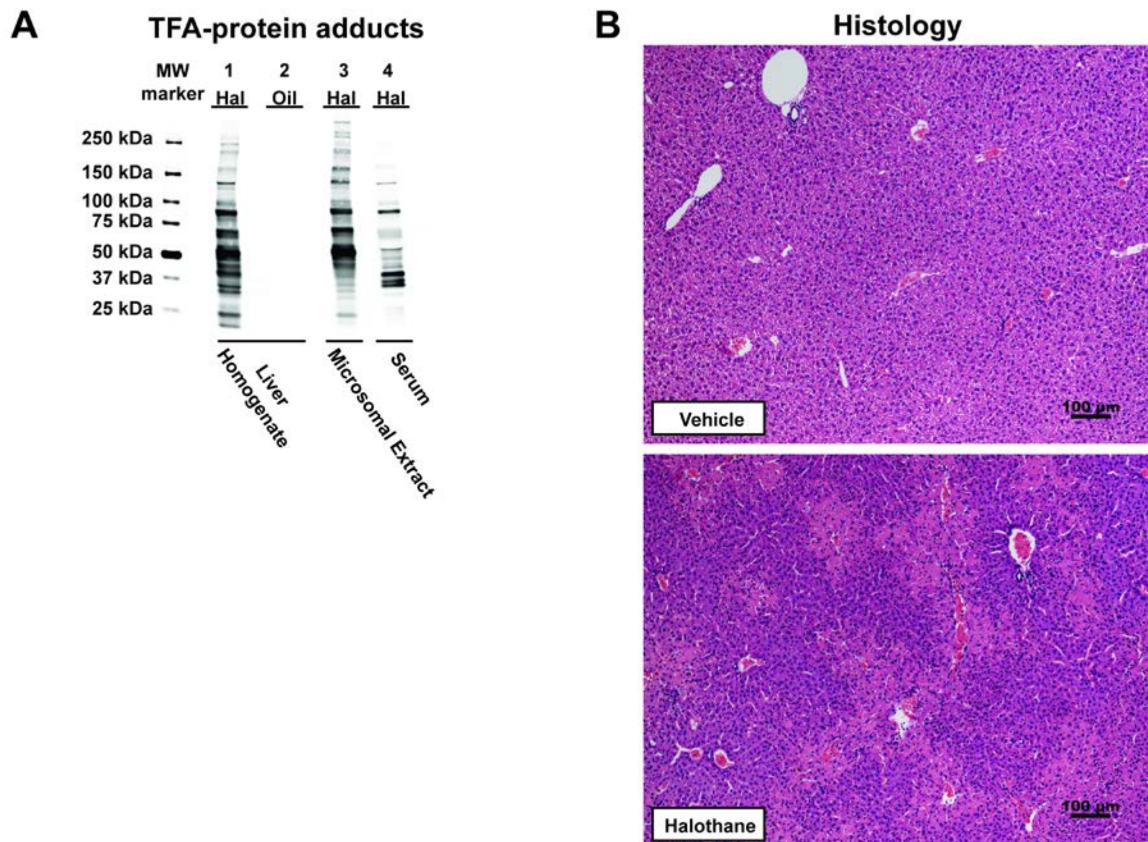
as previously described [1]. Briefly, female Balbc/J mice were treated with either 30 mmol/kg halothane or vehicle (oil) and liver homogenates were prepared from samples collected 10 hours post treatment. Additionally, DOC soluble hepatic microsomal protein extracts were prepared as described in Materials and Methods. Furthermore, mouse sera were collected 24 hours after halothane or vehicle treatment and albumin was removed using ProteaPrep Albumin Depletion Sample Prep kit (Sigma). 25 µg of liver homogenate protein, 5.5 µg of microsomal protein extract or 4 µL of serum (diluted 1:10 in PBS) were loaded onto a 4-20% polyacrylamide gel for SDS-PAGE. Protein loading was verified using MemCode Reversible Protein Stain Kit (Thermo Scientific) according to manufacture's protocol. Immunoblot analysis was performed by blocking the nitrocellulose membrane at room temperature with Odyssey blocking buffer (LI-COR biosciences, Lincoln NE) for 1 hour followed by a wash with 0.1% Tween 20 in PBS. The membrane was then stained for 2 hours with rabbit antiserum against TFA-protein adducts at 1:5000 dilution [2] and when possible protein loading was also verified by beta-tubulin staining using mouse monoclonal clone AA2 IgG1 (EMD Millipore, Billerica, MA). Following primary staining the membrane was washed and incubated for 1 hour at room temperature for secondary staining using IRDye 800CW goat anti-rabbit IgG (LI-COR biosciences) and IRDye 680LT goat anti-mouse IgG (LI-COR biosciences) at 1:15,000 dilutions (antiserum and antibodies were diluted with PBS containing 0.2% Tween 20). Following staining, membrane was washed and staining visualized using a LI-COR Odyssey infrared imager and Odyssey imaging software version 3.0 (LI-COR biosciences).

T Cell Proliferation in Response to Hepatic TFAPA. Hepatic $CD4^+CD25^-$ T cells ($5x10^4$) were sorted using an Aria II fluorescent-activated cell sorter (BD Biosciences) and were cultured for 96 hours in the presence of microsomal protein extract (25 µg/mL concentration) from halothane- or vehicle-treated mice along with $5x10^5$ irradiated (3000 rad) splenocytes from naïve mice used as antigen presenting cells. After 72 hours in culture, T cell proliferation was measured by $[^3H]$-thymidine incorporation during the last 24 hours of incubation (see Supporting Information for detailed methods). The results were corrected by subtracting the values from wells incubated with microsomal extracts from vehicle-treated mice. Cell culture supernatant was also collected after 72 hours in culture and IFNγ was measured using ELISA Ready-Set-Go kit (ebiosciences).

Detection of Mouse Serum Antibodies Interacting with Hepatic TFAPA. To detect levels of TFAPA-specific Ig in serum, DOC soluble protein extracts of hepatic microsomes from halothane and vehicle-treated mice were coated in wells of a microtiter plate at 1 □g/100 □ PBS/well and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS and blocked with reagent diluent (#831380 R&D Systems, Minneapolis, MN) on a rotary shaker for 2 hours at room temperature. Mouse sera from experimental samples at 1:100 or 1:400 dilutions (all samples and antibodies were diluted with reagent diluent) were added to plates and incubated for 3 hours at room temperature. Plates were washed and incubated with alkaline phosphatase conjugated secondary antibodies including: goat anti-

mouse Ig(H+L) at 1:2000 dilution or rat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, or IgE (SouthernBiotech, Birmingham, AL) at 1:000,1:2000,1:2000,1:2000, 1:1000, 1:1000, 1:500, dilutions respectively for 1 hour. Plates were washed and developed using alkaline phosphatase substrate kit (BioRad, Hercules, CA) as per manufacture's protocol. The results were corrected by subtracting the OD (405 nm) values of wells coated with microsomal proteins from vehicle-treated mice.

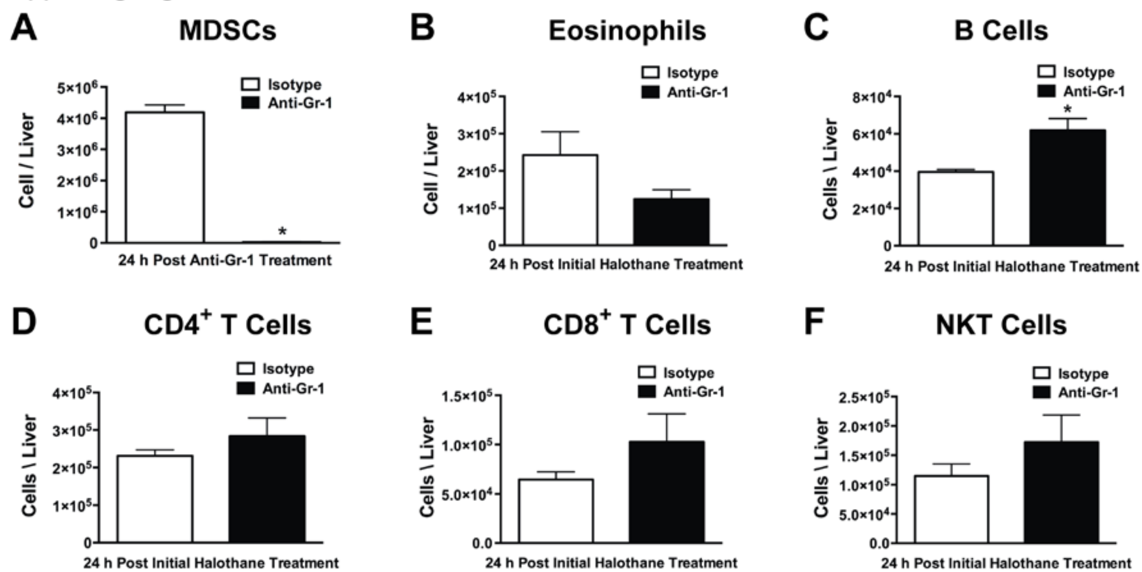
Supporting Figure 1



Supporting Figure 1. Detection of TFAPA in mouse liver and serum after halothane induced liver injury. (A) Liver homogenates and mouse sera collected at 10 hours and 24 hours respectively after halothane treatment were

immunoblotted with rabbit antiserum (1:5000 dilution) that recognizes the TFA group covalently bound to lysine residues of proteins. Lane 1 - Liver homogenates from halothane-treated mice, Lane 2 - Liver homogenate from vehicle-treated mice, Lane 3 - Extract of liver microsomal fraction from halothane-treated mice and Lane 4 - Mouse sera from halothane-treated mice. Molecular markers (kDa) are indicated on the left. **(B)** Representative photomicrographs of H&E stained liver sections from mice 24 hours post treatment. Sections from mice treated with halothane showed perivenous necrosis, while those treated with vehicle lacked liver injury.

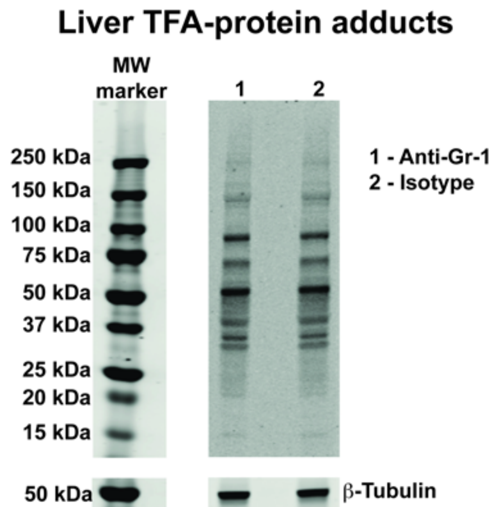
Supporting Figure S2



Supporting Figure 2. Effect of anti-Gr-1 treatment on hepatic leukocyte populations. Total number of hepatic **(A)** MDSCs, **(B)** eosinophils, **(C)** B cells, **(D)** CD4+ T cells, **(E)** CD8+ T cells and **(F)** NKT cells were determined in isotype control and anti-Gr-1 treated mice 24 hours post initial halothane treatment. All

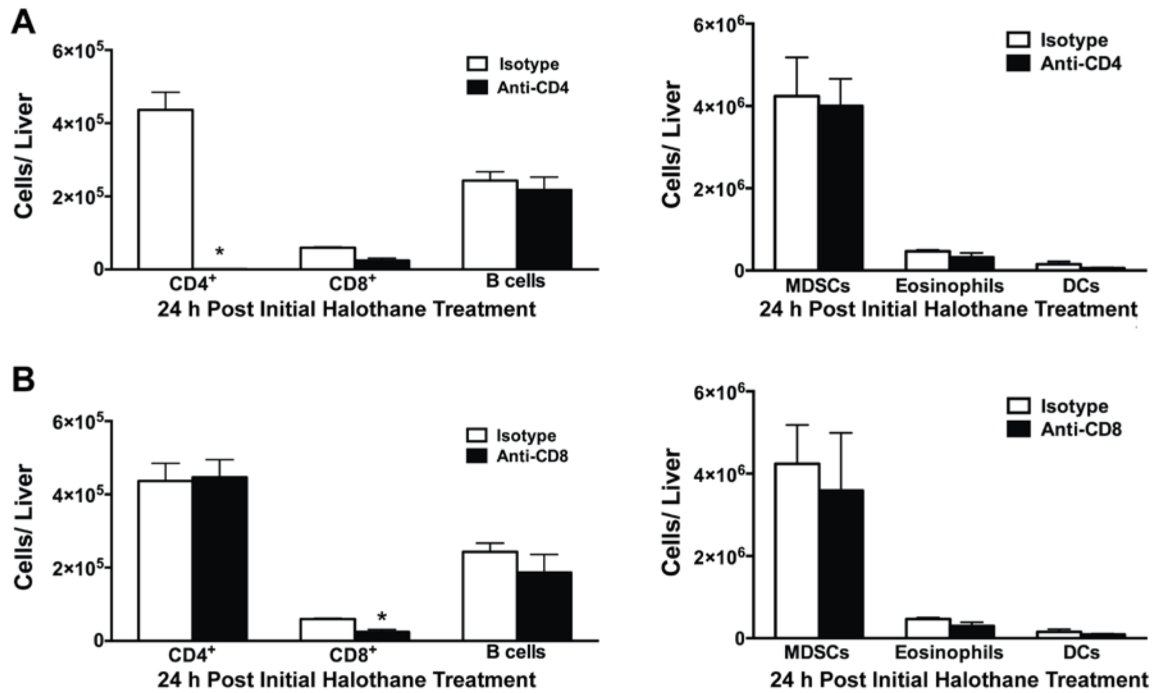
data reported as mean ± SEM with N=6 per group. * $P<0.05$ versus the isotype-treated group.

Supporting Figure S3



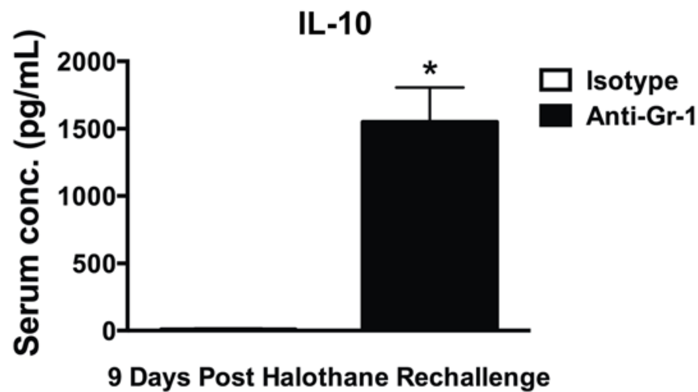
Supporting Figure 3. Effect of anti-Gr-1 treatment on halothane-induced generation of hepatic TFAPA. Hepatic TFA-protein adducts were detected in isotype and anti-Gr-1 treated mice, 9 days post halothane rechallenge, by SDS-PAGE and immunoblotting with anti-TFA rabbit serum.

Supporting Figure S4



Supporting Figure 4. Effects of anti-CD4 or anti-CD8 treatment on hepatic leukocytes. To deplete hepatic CD4+ or CD8+ T cells, Balb/cJ mice were administered either 60 µg/mouse CD4 mAb **(A)**, 40 µg CD8 mAb **(B)** or respective isotype control antibodies 24 h prior to initial halothane treatment. Total number of hepatic CD4+ T cells, CD8+ T cells, B cells, MDSCs, eosinophils and DCs were determined in mice at 24 h post initial halothane treatment. All data reported as mean ± SEM with N=3-4 mice per group. * *P*<0.05 versus the respective isotype control-treated group.

Supporting Figure S5



Supporting Figure 5. Serum cytokine levels following rechallenge with halothane. Serum protein levels of IL-10 **(A)** in isotype and anti-Gr-1 treated mice 9 days post rechallenge with halothane. All data reported as mean ± SEM with N=5-15 per group from 3 independent experiments. * $P<0.05$ versus the isotype-treated group.

1. Proctor WR, Chakraborty M, Chea LS, Morrison JC, Berkson JD, Semple K*, et al.* Eosinophils mediate the pathogenesis of halothane-induced liver injury in mice. *Hepatology* 2013,**57**:2026-2036.
2. Satoh H, Fukuda Y, Anderson DK, Ferrans VJ, Gillette JR, Pohl LR. Immunological studies on the mechanism of halothane-induced hepatotoxicity: immunohistochemical evidence of trifluoroacetylated hepatocytes. *J Pharmacol Exp Ther* 1985,**233**:857-862.