IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice

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Supplementary material

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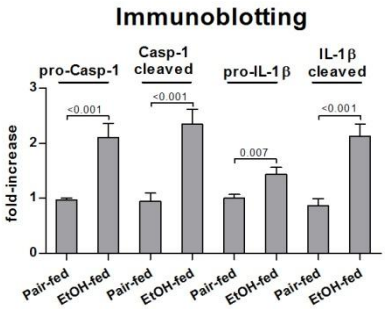
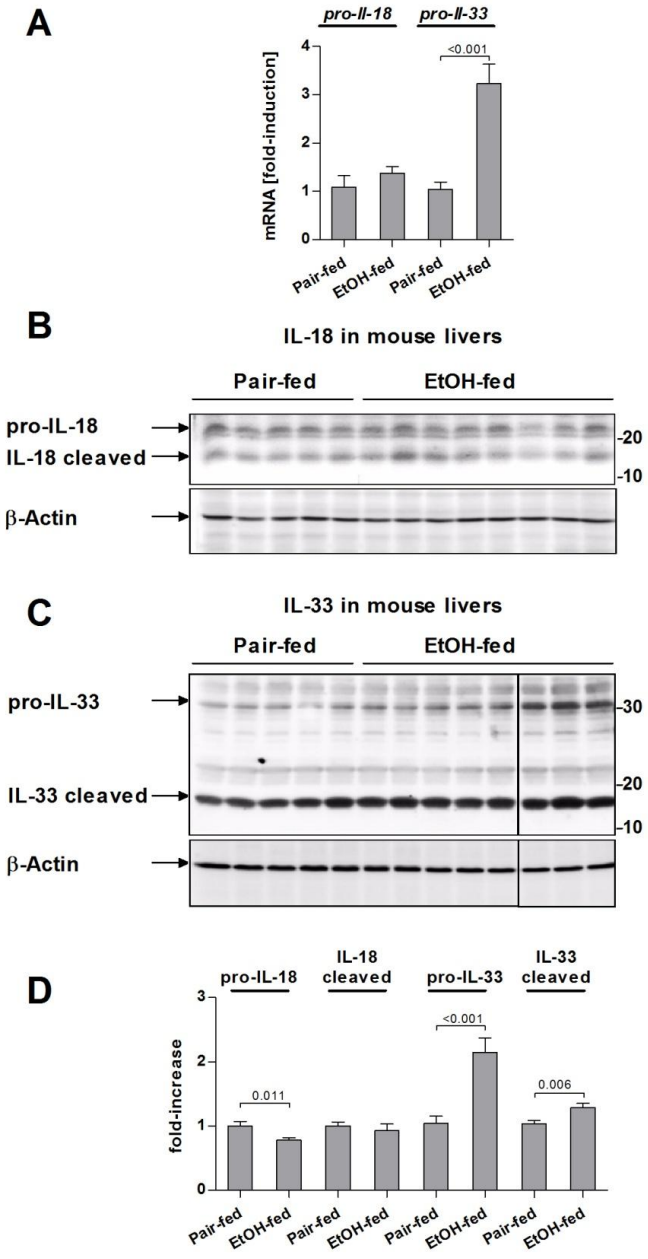
A. Supplementary figures



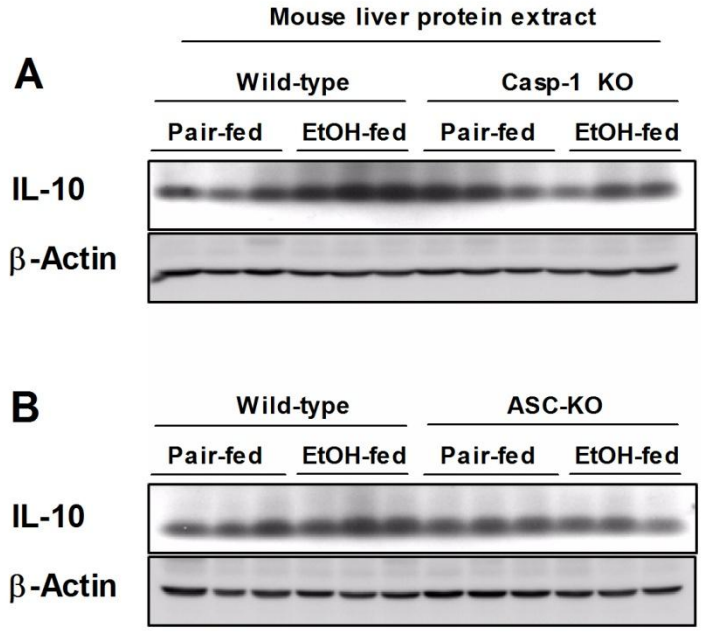
Supplementary Fig. 1 - *Activation of the inflammasome and IL-1β in alcohol-induced liver injury*

Wild-type mice were fed with control (pair-fed) or alcohol (EtOH-fed) diet and sacrificed 4 weeks later. Cleaved forms of caspase-1 and IL-1β in the livers were analyzed using antibodies that identify both full-length pro-form and cleaved forms, normalized to beta-actin and subjected to densitometry. Total number of mice used for statistical analysis were n=14 (pair-fed); n=19 (EtOH-fed). Means and SEM values are shown. Numbers in the graph denote *P* values. Representative Western blots are shown in Fig. 1 G, H.



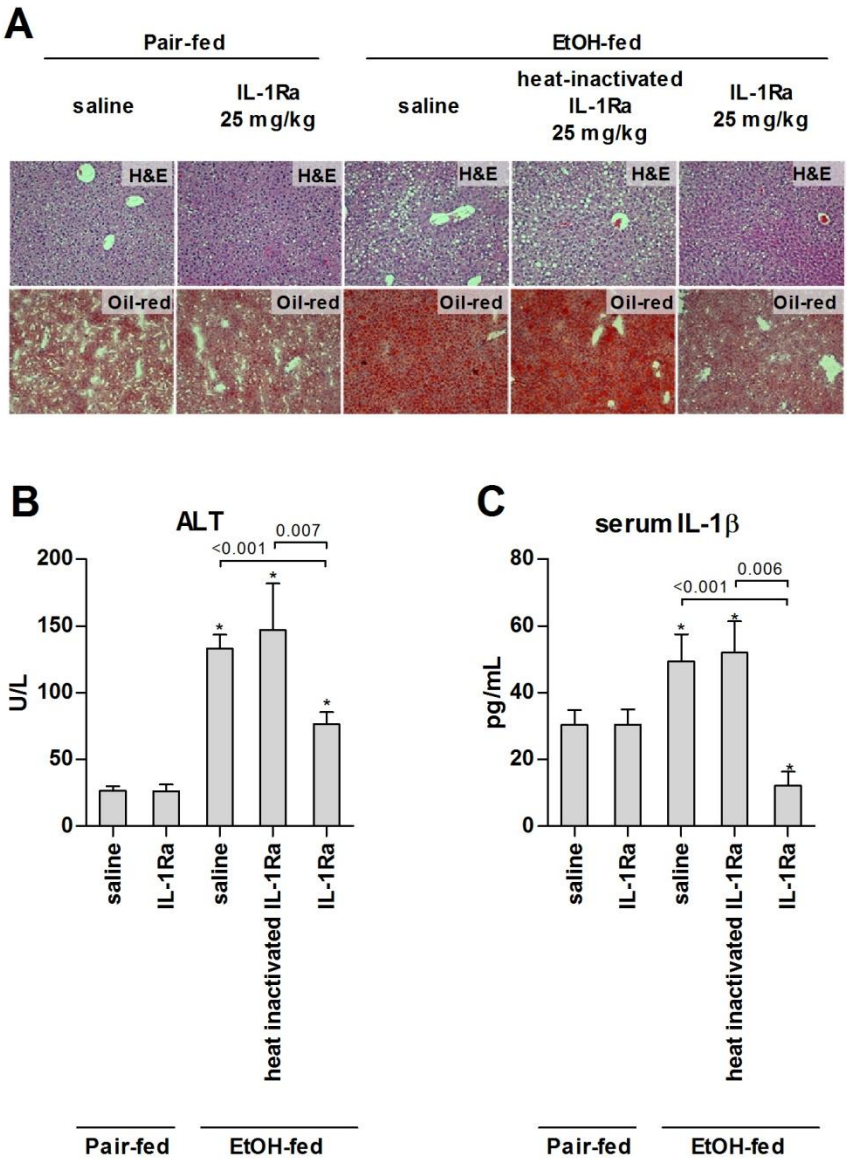
Supplementary Fig. 2- *Alcoholic liver disease is associated with increased IL-33, but not IL-18, in the liver*

Wild-type mice were fed with control (pair-fed) or alcohol (EtOH-fed) diet and sacrificed 4 weeks later. IL-18 and IL-33 (encoded by *pro-Il-18* and *pro-Il-33*, respectively) in the livers were analyzed by qPCR (A). IL-18 and IL-33 proteins in the liver were analysed in the livers using antibodies that identify both full-length and cleaved forms (B,C), normalized to beta-actin, and measured using densitometric analysis (D). Total number of mice used were n=5 (pair-fed); n=8 (EtOH-fed). Vertical line in (C) is used to divide two non-contiguous parts of the same blot. Numbers in graphs denote *P* values.



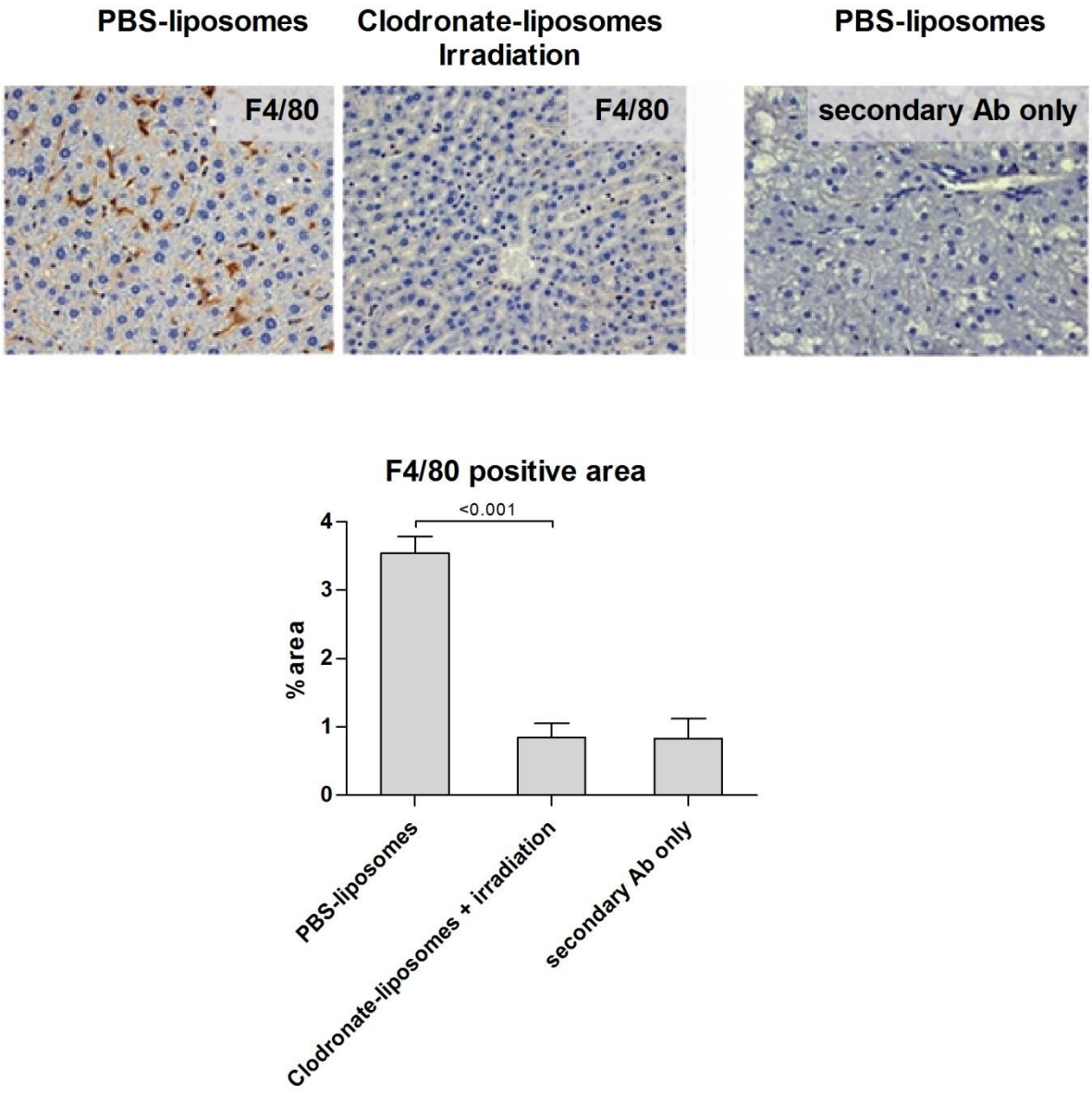
Supplementary Fig. 3 - *Deficiency of caspase-1 or ASC prevents upregulation of IL-10 in the livers of alcohol-fed mice*

Wild-type, caspase-1- or ASC-deficient mice were fed with control (pair-fed) or alcohol (EtOH-fed) diet and sacrificed 4 weeks later. IL-10 in the livers was analyzed using immunoblotting, and normalized to beta-actin. Densitometric analysis of these blots is presented in Fig. 2 I (for A) or Fig. 4 G (for B).



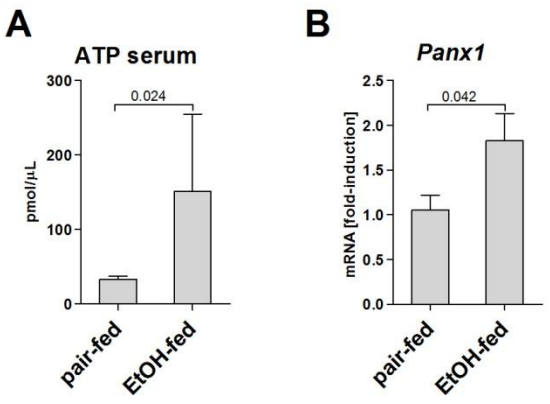
Supplementary Fig. 4- *Active form of human recombinant IL-1Ra is required for protection from alcoholic liver disease*

Wild-type mice were fed with control (pair-fed, n=5) or alcohol diet (EtOH-fed, n=10 per treatment group), treated daily with saline, recombinant human IL-1Ra (Anakinra) or heat-inactivated IL-1Ra i.p., as described in Methods. Mice were sacrificed 4 weeks later. Liver injury and steatosis was quantified by H&E and Oil-red-O staining (A) and by serum ALT (B). Serum levels of the inflammatory cytokine IL-1β (C) were measured using specific ELISA. Total number of mice used was n=5 (pair-fed), n=10 (EtOH-fed, per treatment). Means and SEM values are shown. Numbers in graphs denote *P* values. Original magnification 200x. **P* < 0.05 vs. pair-fed, saline-treated mice.



Supplementary Fig. 5 - *Depletion of Kupffer cells in the liver following treatment with clodronate and whole-body irradiation*

Wild-type mice were injected with clodronate-liposomes or control PBS-liposomes i.v. (n=3 per group) and subjected to whole body irradiation, as described in Methods. Depletion of Kupffer cells was assessed 48 hours after clodronate or PBS-liposome injection using the F4/80 staining. Means and SEM values are shown. Number in the graph denotes *P* value. Original magnification 200x.



Supplementary Fig. 6 - *Alcoholic liver disease is associated with increased levels of ATP in the serum and upregulation of pannexin-1 in the liver*

Wild-type mice were fed with control (pair-fed, n=5) or alcohol (EtOH-fed, n=10) diet and sacrificed 4 weeks later. Levels of ATP were measured in the serum (A) and expression of pannexin-1, encoded by *Panx1* (B) was measured in the liver by qPCR, as described in Methods. Means and SEM values are shown. Numbers in graphs denote *P* values.

B. Supplementary table

qPCR primers

Target gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
Asc	gaa gct gct gac agt gca ac	gcc aca gct cca gac tct tc
Nlrp3	agc ctt cca gga tcc tct tc	ctt ggg cag cag ttt ctt tc
Panx1	tgt ggc tgc aca agt tct tc	aca gac tct gcc cca cat tc
pro-Casp-1	aga tgg cac att tcc agg ac	gat cct cca gca gca act tc
pro-Col1a1	gct cct ctt agg ggc cac t	cca cgt ctc acc att ggg g
pro-Il-1a	ggc acg ggg act gcc ctc tat	tgt cgg ggt ggc tcc act
pro-Il-1b	tct ttg aag ttg acg gac cc	tga gtg ata ctg cct gcc tg
pro-Il-18	cag gcc tga cat ctt ctg caa	tct gac atg gca gcc att gt
pro-Il-33	agc tct cca ccg ggg ctc ac	gcc tgc ggt gct gct gaa ct
Tgfb1	att cct ggc gtt acc ttg	ctg tat tcc gtc tcc ttg gtt

Quantitative polymerase chain reaction (qPCR) was performed using the iCycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions were: 95°C for 15 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 30 seconds. The reaction mixture for the SYBR Green assay contained 12.5 μL SYBR Green PCR Master Mix (Bio-Rad laboratories, Hercules, CA, USA), 0.5 μM of forward and reverse primer and 1 μL of complementary DNA (corresponding to 100 ng RNA) for a total volume of 25 μL. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by double-stranded DNA-binding SYBR Green. After PCR, a dissociation melting curve was constructed in the range of 55°C to 95°C. All data were analyzed using Bio-Rad iCycler software. The 18S was used for normalization of all experiments. Data was analyzed using the comparative Ct method (ΔΔCt method) using the following formula: ΔCt = Ct (target) - Ct (normalizer). The comparative ΔΔCt calculation involved finding the difference between the sample ΔCt and the baseline ΔCt. Fold increase in the expression of specific mRNA compared with 18S was calculated as $2^{-(\Delta\Delta Ct)}$.

C. Supplementary references

Adachi, M., and Brenner, D.A. 2005. Clinical syndromes of alcoholic liver disease. Dig. Dis. 23:255-263.

Adachi, Y., Moore, L.E., Bradford, B.U., Gao, W., and Thurman, R.G. 1995. Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology 108:218-224.

Alcoholic liver disease by country, World Health Organisation Statistical Information System. Retrieved from http://www.NationMaster.com/graph/mor_alc_liv_dis-mortality-alcoholic-liver-disease.

Baeck, C., Wehr, A., Karlmark, K.R., Heymann, F., Vucur, M., Gassler, N., Huss, S., Klussmann, S., Eulberg, D., Luedde, T. et al. 2012. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. Gut 61:416-426.

Ceccanti, M., Attili, A., Balducci, G., Attilia, F., Giacomelli, S., Rotondo, C., Sasso, G.F., Xirouchakis, E., and Attilia, M.L. 2006. Acute alcoholic hepatitis. J. Clin. Gastroenterol. 40:833-841.

Clement, S., Juge-Aubry, C., Sgroi, A., Conzelmann, S., Pazienza, V., Pittet-Cuenod, B., Meier, C.A., and Negro, F. 2008. Monocyte chemoattractant protein-1 secreted by adipose tissue induces direct lipid accumulation in hepatocytes. Hepatology 48:799-807.

Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27:519-550.

Granowitz, E.V., Porat, R., Mier, J.W., Pribble, J.P., Stiles, D.M., Bloedow, D.C., Catalano, M.A., Wolff, S.M., and Dinarello, C.A. 1992. Pharmacokinetics, safety and immunomodulatory effects of human recombinant interleukin-1 receptor antagonist in healthy humans. Cytokine 4:353-360.

Hritz, I., Velayudham, A., Dolganiuc, A., Kodys, K., Mandrekar, P., Kurt-Jones, E., and Szabo, G. 2008. Bone marrow-derived immune cells mediate sensitization to liver injury in a myeloid differentiation factor 88-dependent fashion. Hepatology 48:1342-1347.

Huang, Y.S., Chan, C.Y., Wu, J.C., Pai, C.H., Chao, Y., and Lee, S.D. 1996. Serum levels of interleukin-8 in alcoholic liver disease: relationship with disease stage, biochemical parameters and survival. J. Hepatol. 24:377-384.

Iimuro, Y., Gallucci, R.M., Luster, M.I., Kono, H., and Thurman, R.G. 1997. Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. Hepatology 26:1530-1537.

Lemmers, A., Moreno, C., Gustot, T., Marechal, R., Degre, D., Demetter, P., de Nadai, P., Geerts, A., Quertinmont, E., Vercruysse, V. et al. 2009. The interleukin-17 pathway is involved in human alcoholic liver disease. Hepatology 49:646-657.

Listing, J., Strangfeld, A., Kary, S., Rau, R., von Hinueber, U., Stoyanova-Scholz, M., Gromnica-Ihle, E., Antoni, C., Herzer, P., Kekow, J. et al. 2005. Infections in patients with rheumatoid arthritis treated with biologic agents. Arthritis Rheum. 52:3403-3412.

Mandal, P., Pritchard, M.T., and Nagy, L.E. 2010. Anti-inflammatory pathways and alcoholic liver disease: role of an adiponectin/interleukin-10/heme oxygenase-1 pathway. World J. Gastroenterol. 16:1330-1336.

McClain, C.J., Barve, S., Barve, S., Deaciuc, I., and Hill, D.B. 1998. Tumor necrosis factor and alcoholic liver disease. Alcohol. Clin. Exp. Res. 22:248S-252S.

Menon, K.V., Stadheim, L., Kamath, P.S., Wiesner, R.H., Gores, G.J., Peine, C.J., and Shah, V. 2004. A pilot study of the safety and tolerability of etanercept in patients with alcoholic hepatitis. Am. J. Gastroenterol. 99:255-260.

O'Shea, R.S., Dasarathy, S., and McCullough, A.J. 2010. Alcoholic liver disease. Am. J. Gastroenterol. 105:14-32; quiz 33.

Purohit, V., Gao, B., and Song, B.J. 2009. Molecular mechanisms of alcoholic fatty liver. Alcohol. Clin. Exp. Res. 33:191-205.

Rongey, C., and Kaplowitz, N. 2006. Current concepts and controversies in the treatment of alcoholic hepatitis. World J. Gastroenterol. 12:6909-6921.

Sarphie, T.G., D'Souza, N.B., and Deaciuc, I.V. 1996. Kupffer cell inactivation prevents lipopolysaccharide-induced structural changes in the rat liver sinusoid: an electron-microscopic study. Hepatology 23:788-796.

Tilg, H., Jalan, R., Kaser, A., Davies, N.A., Offner, F.A., Hodges, S.J., Ludwiczek, O., Shawcross, D., Zoller, H., Alisa, A. et al. 2003. Anti-tumor necrosis factor-alpha monoclonal antibody therapy in severe alcoholic hepatitis. J. Hepatol. 38:419-425.