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Supplemental Information

Extracellular ATP Activates the NLRP3 Inflammasome and Is an Early Danger Signal of Skin Allograft Rejection

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human clinical samples. All samples were obtained under informed consent. This study was approved by the ethical committee of the "*Hospital Clínico Universitario Virgen de la Arrixaca*" (Murcia, Spain), according to the ethical guidelines of the 1975 Declaration of Helsinki. Eight liver transplant patients were subjected to progressive immunosuppressive drug withdrawal (10% decrements monthly). Candidacy criteria for the inclusion of these patients were (1) liver recipients with a functioning allograft for >2 years; (2) one year or longer without a documented rejection episode; (3) absence of cancer or autoimmune liver disease before liver transplantation. Four of these patients suffered rejection during the immunosuppression withdrawal process and were treated by reintroducing the immunosuppressive therapy that they were receiving before the beginning of the weaning protocol (non-tolerant patients), while the other four patients became tolerant, and are living free of immunosuppression, for no less than 1 year (tolerant patients). Liver rejection was determined by high serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase or gamma glutamyl transpeptidase (GGT) and by liver biopsy. The diagnosis of cellular rejection was based on the presence of predominantly mononuclear portal infiltrate plus non-suppurative ductal cholangitis with or without endothelitis. Demographic characteristics of patients are summarized in Supplementary Table 1. Peripheral blood mononuclear cells (PBMCs) and plasma were obtained at different points for each patient. Samples were stored frozen until their use.

Skin transplantation. The donor was sacrificed according to the Animal Health Service recommendations, the base of the ear was washed with alcohol and after excising the ear, the skin was harvested and placed in sterile PBS on ice. Recipient mouse was anesthetized with a mix of Xylacin-Ketamine (10 mg/kg and 80 mg/kg respectively) intraperitoneally administrated. The dorsal back of the mouse was then shaved and washed with alcohol, skin was tent with a rat-tooth forceps and cut below for making a ~1 cm incision. Donor skin was placed on the injury, removing excess of PBS and the excess of donor skin. A sterile bandage was placed to protect the skin graft and secured with a single 2-0 silk suture. Mice were then placed in a clean sterile cage and monitored daily. Transplantations were made using C57BL/6 wild-type, *Nlrp3*$^{-/-}$, *Casp1/11*$^{-/-}$, *P2rx7*$^{-/-}$ *Il1r1*$^{-/-}$ or *Il18*$^{-/-}$ (all in C57BL/6 background) mice as receptors. BALB/c mice were used as donors in allogeneic transplantations, while C57BL/6 were used as donors in isogeneic transplantations. Alternatively, BALB/c mice were used as recipient and allogeneic skin from C57BL/6 or *P2rx7*$^{-/-}$ (in C57BL/6 background) was used as donors when indicated in the text. Wild-type animals were treated with A438079 (100 μmol/kg) on the skin wound at the surgical moment and in some experiments then treated intraperitoneally daily. Some *P2rx7*$^{-/-}$ mice receiving allogeneic transplants were supplemented with 10^6 bone marrow differentiated macrophages (BMDM) under the transplanted skin the same day the transplant was performed.

Reagents and antibodies. D-Luciferin was obtained from Perkin-Elmer laboratories.-Mitomycin and apyrase were from Sigma-Aldrich. The specific P2X7 receptor antagonist A438079 was from Tocris. ACK lysing buffer (Lonza) and collagenase A (Roche). For the anaesthetic procedure, mice were treated with Xilacin (Xilagesic® Calier Laboratories) and with Ketamin (Ketolar® Pfizer Laboratories). Isoflorane was obtained from Inibsa laboratories. Commercial neutral buffered 4% formalin (Panreac Química) was used for fixation of the tissue samples. For histopathological staining, commercial Harris hematoxylin (acidified), alcoholic eosin and xylene substitute (Clear Rite) were purchased from Thermo and absolute ethanol from VWR. For immunohistochemical staining, high pH demasking antigen, washing buffer, antibody diluent and 3-3´-Diaminobencidine (DAB) solutions were purchased from Dako. Primary antibodies used were: polyclonal rabbit-anti CD3 (#A0452, Dako) and monoclonal rat anti-F4/80 clone CL:A3-1 (#MCA49RT, AbdSerotec). For CD3-lymphocyte immunostaining, a labelled polymer commercial system was used (EnVision System, Dako). For F4/80 immunostaining, Polyclonal biotinylated rabbit anti-rat antibody was used (#E0468, Dako), and an avidin-peroxidase complex system (Vectastain Elite ABC kit) from Vector Labs.

Cells and treatments. BMDM were obtained from wild-type or *P2rx7*$^{-/-}$ mice using 25% of L-cell conditioned media as previously described (Baroja-Mazo et al., 2014). pmeLuc cells were maintained in DMEM:F12 (1:1) supplemented with 10% foetal calf serum (FCS), 2mM glutamax 1% and G418 0.2 mg/ml (Acros Organic). Extracellular ATP was measured in supernatants of BMDM after exposition to complete C57BL/6 or Balb/c ear skin or 10 mg/ml of OVA (Sigma), using the ATP Bioluminescent Assay kit from Sigma-Aldrich and following manufacturer's instructions. BMDMs incubated or not for 2 h with 10 mg/ml of OVA or 1 ¦ g/ml of LPS were incubated for 5 min with 25 ¦ M of YoPro-1 (Life Technologies) and then stimulated with ATP (3 mM). Fluorescence of DNA-bound Yo-Pro-1 was measured in a Synergy Mx plate reader (BioTek) every 30 sec at 485±9 nm excitation and 515±9 nm emission.
One-way mixed lymphocytic reaction was performed with 2 x 10^5 lymphocytes obtained from axillar nodes 3 or 7 days after transplantation. They were cultured in the presence of 2 x 10^5 mitomycin treated-splenocytes from

C57BL/6 or BALB/c for 4 days in RPMI supplemented with 10% FCS, 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich), Penicillin/Streptomycin (50 U/ml and 5000 µg/ml respectively), 10 mM HEPES, 1x non-essential amino acid cocktail (Sigma-Aldrich) and 50 ¦ M 2-mercaptoethanol (Sigma-Aldrich).

ATP and liver enzymes determination. Concentration of ATP in the plasma of human samples or supernatants of stimulated BMDMs was determined by luminescence by means of the ATP assay mix (Sigma-Aldrich) following the manufacturer's instructions. Liver enzymes (AST, ALT, alkaline phosphatase and GGT) were determined in the plasma of human samples a COBAS® 8000 modular analyser series (Roche).

Humoral allogeneic response. Determination of the different IgGs titter against allogeneic antigens was performed incubating serum from transplanted mice (day 7 post-transplant) with thymocytes from mice with the same haplotype than the graft. After 30 min of incubation, cells were washed and then incubated with anti-IgG1, IgG2a, IgG2b and IgG3 biotinylated antibodies in separate tubes. At the end, cells were incubated with streptavidin-FITC and subjected to flow cytometry as explained above.

Quantitative reverse transcriptase-PCR analysis. Detailed methods used for qRT-PCR have been described previously (López-Castejón et al., 2011). Briefly, transplanted skin was collected from mice at 3 and 7 days after transplantation and tissue was homogenised in RLT buffer (Qiagen) using Omni Tip^{TM} plastic generator probes (Omni). RNA was purified using the RNAesy kit (Qiagen) according to manufacturer's recommendations and quantified on a nanodrop 2000 (Thermo Fisher). Reverse transcription was realized using iScript^{TM} cDNA Synthesis kit (BioRad). qPCR was performed in an iQ^{TM} 5 Real Time PCR detection System (BioRad) with a SYBR Green mix (Takara) and primers used were obtained from Qiagen (Quantitec primer Assay, Qiagen). The presented relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalizing to *Gapdh* expression as endogenous control and the fold increase in expression was relative to healthy mice skin.

SUPPLEMENTAL TABLE:

Table S1. Baseline and demographic characteristics of liver transplant patients, Related to Figure 3.

Variables	Non-tolerant (rejection)	Tolerant
Number of patients (*n*)	4	4
Age [Mean ± SE; median (range)]	[70.5 ± 2.1; 70 (66-76)]	[69.5 ± 3.9; 69.5 (60-79)]
Age at transplantation [Mean ± SE; median (range)]	[62.8 ± 1.3; 64 (59-64)]	[58.5 ± 2.8; 58.5 (52-65)]
Years from transplant to rejection/tolerance [Mean ± SE; median (range)]	[6.3 ± 1; 6 (4-9)]	[9.5 ± 2.1; 9 (6-14)]
Gender (*n*; %)		
Male	(3; 75)	(3; 75)
Female	(1; 25)	(1; 25)
Diseases (*n*; %)		
Alcoholic cirrhosis	(3; 75)	(3; 75)
HBV	(1; 25)	
Wilson disease		(1; 25)
Drug-based IS (*n*; %)		
Cyclosporine A		(1; 25)
Tacrolimus	(4; 100)	(2; 50)
Cyclosporine A + Everolimus		(1; 25)

Abbreviations: HBV: Hepatitis B virus; IS: immunosuppression;

SUPPLEMENTAL FIGURES:

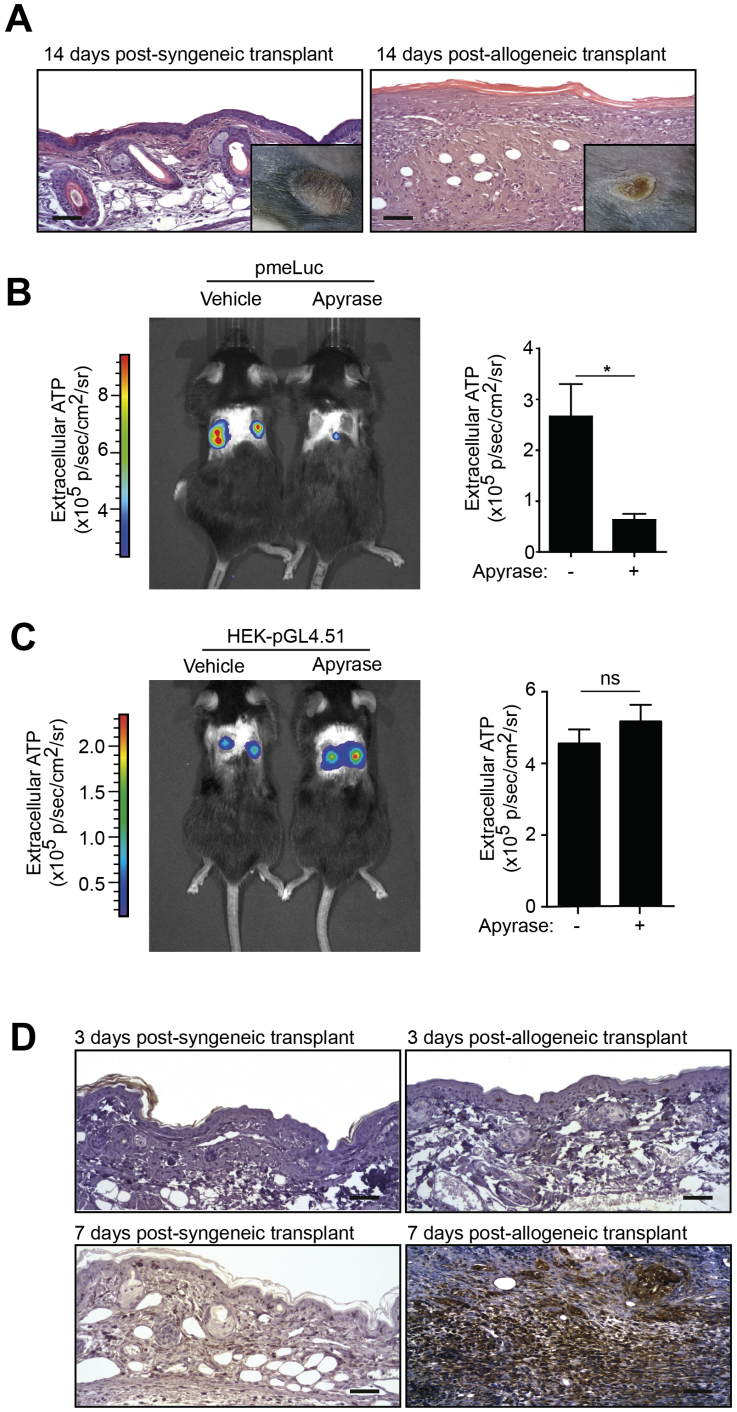

Figure S1. Early release of ATP is associated to allografts, Related to Figure 1. (**A**) Representative image of haematoxylin and eosin stained syngeneic or allogeneic skin grafts on wild-type mice collected 14 days after transplantation; scale bar 50 µm; inserts show macroscopic view of the transplanted graft. (**B**) Extracellular ATP detected in allografts with the pmeLuc biosensor 3 days after transplantation in wild-type mice treated under the transplant with vehicle or apyrase (4 IU). Left, luciferase signal of one representative mouse per group. Right, average radiance (p/s/cm^2/sr) of *n*= 7 transplants. (**C**) Allografts injected with HEK293 cells expressing an intracellular luciferase (HEK-pGL4.51) 3 days after transplantation in wild-type mice treated under the transplant with vehicle or apyrase (4 IU). Left, luciferase signal from one representative mouse before (vehicle) and after apyrase treatment. Right, average radiance (p/s/cm^2/sr) of *n*= 5-6 transplants. (**D**) Representative picture of CD3 immunostaining on syngeneic or allogeneic skin grafts 3 or 7 days after transplantation in wild-type mice; scale bar 50 µm. Data are presented as mean ± sem from the indicated number of independent experiments. *p< 0.05; *ns*, no significant difference (p> 0.05); *t*-Student for **B**,**C**. For **B,C** data are represented as mean ± SEM.

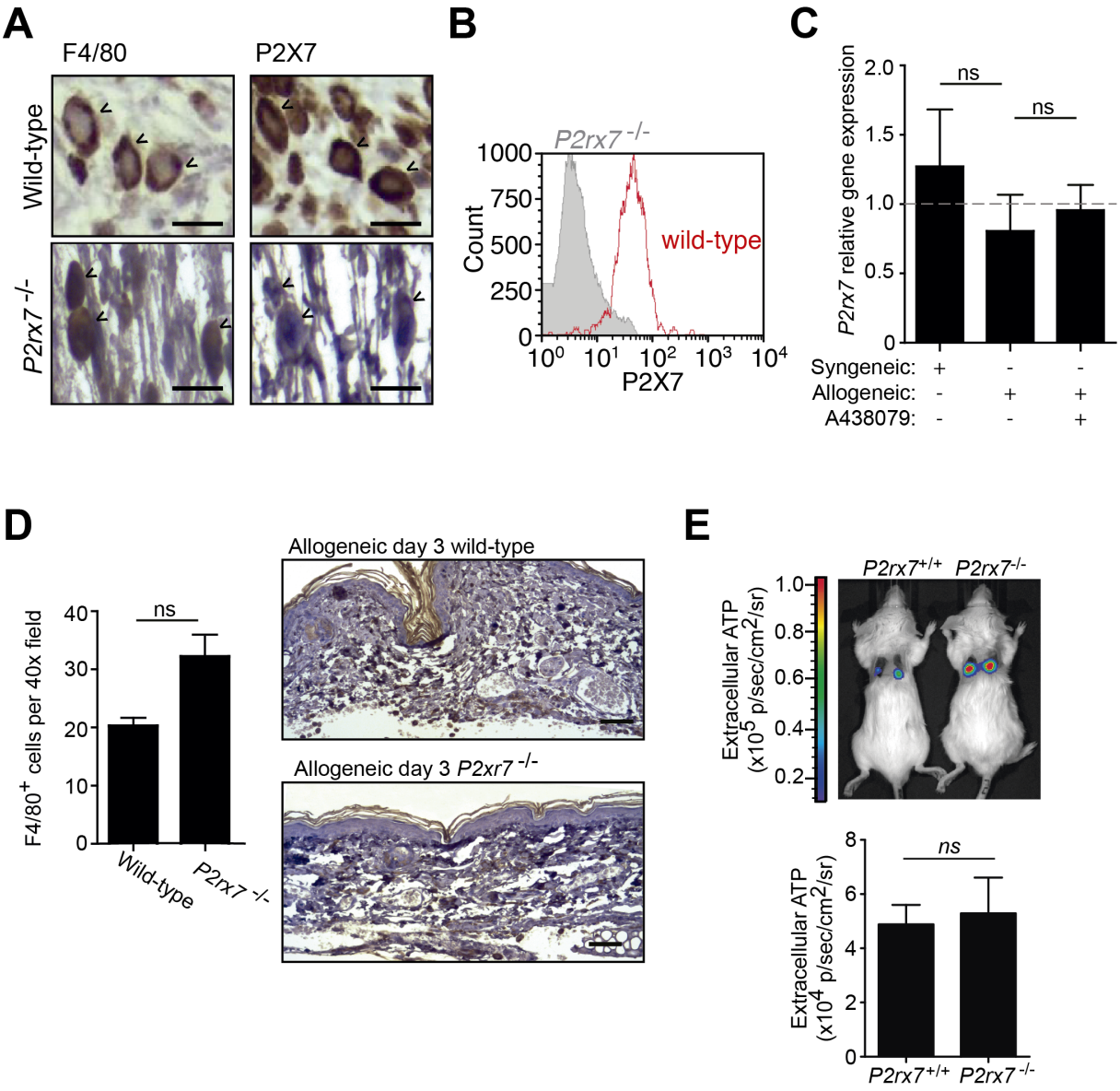

Figure S2. Expression of P2X7 receptor in allografts, Related to Figure 2. (**A**) Representative serial sections of allografts stained for P2X7 receptor (right) and macrophage F4/80 antigen (left) in wild-type or *P2rx7*$^{-/-}$ mice; scale bar 25 µm; arrowheads denotes F4/80^{+} macrophages. (**B**) Flow cytometry staining for surface P2X7 receptor from F4/80^{+} splenic macrophages isolated from wild-type (red) or P2X7 receptor deficient mice (grey). (**C**) *P2rx7* gene expression determined by quantitative RT-PCR in syngeneic or allogeneic grafts 3 days after transplantation in wild-type mice untreated or i.p. treated with A438079 (100 µmol/kg/day) (*n*= 3); *Gapdh* expression was used as endogenous control and the relative expression was compared with healthy skin from non-transplanted mice. (**D**) Amount of F4/80-positive cells per 40x field in skin allografts 3 days after transplantation from wild-type or *P2rx7*$^{-/-}$ mice (*n*= 20 different field of view from 3 independent transplants). Right: representative immunostained sections for F4/80, scale bar 50 µm. (**E**) Extracellular ATP detected in allografts with the pmeLuc biosensor 3 days after transplantation in wild-type BALB/c recipient mice with C57BL/6 donor skin from wild-type or P2X7-deficient mice as indicated. Top, luciferase signal of one representative mouse per group. Bottom, average radiance (p/s/cm2/sr) of n= 4 to 6 transplants. Data are presented as mean ± sem from the indicated number of independent experiments. *ns*, no significant difference (p> 0.05); ANOVA with Bonferroni's post-test for **C** and and *t*-Student for **D,E**. For **C-E** data are represented as mean ± SEM.

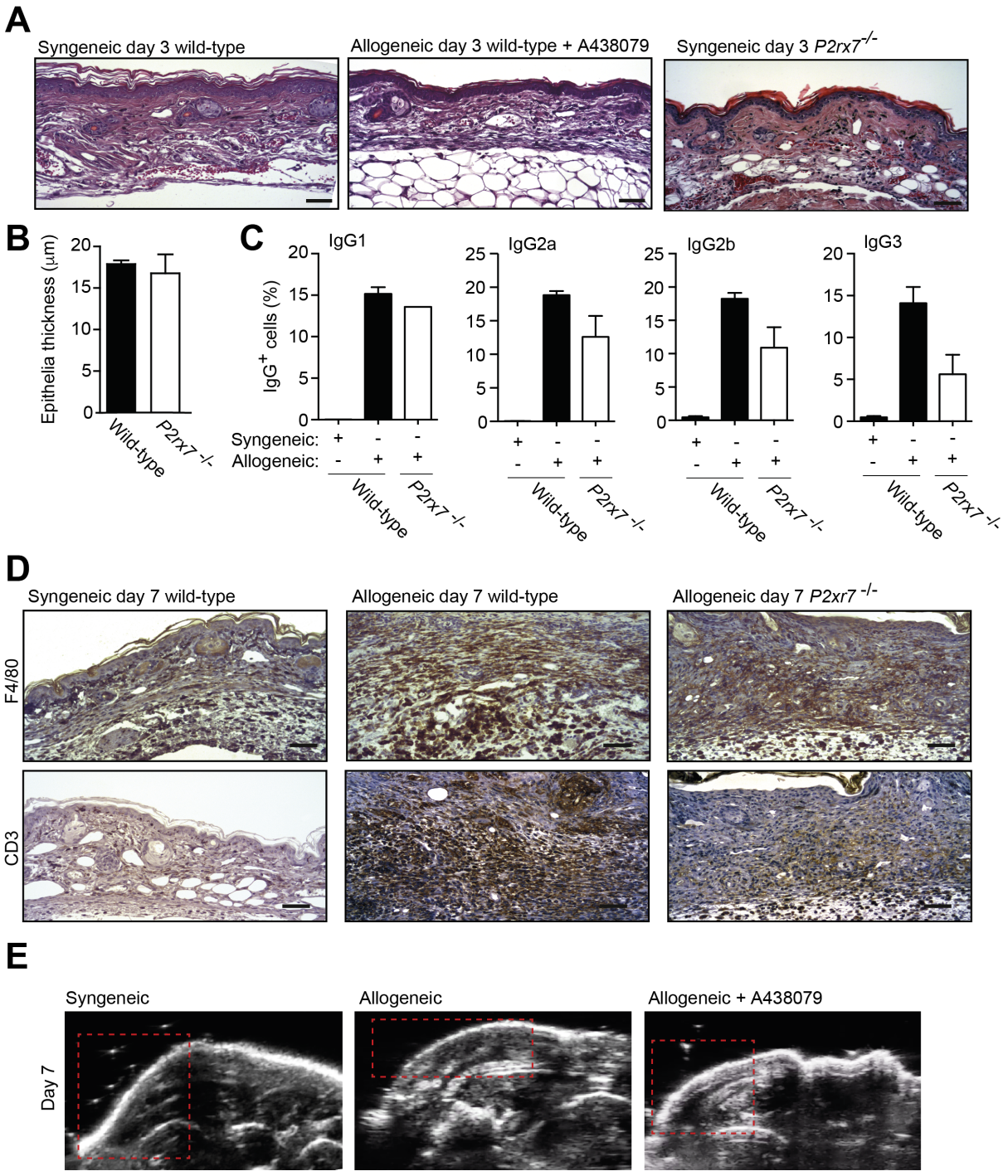

Figure S3. Allogeneic rejection response is impaired after P2X7 receptor blocking, Related to Figure 3. (**A**) Representative image of haematoxylin and eosin stained syngeneic skin grafts 3 days after transplantation from wild-type or *P2rx7*$^{-/-}$ mice; or from allogeneic skin grafts of wild-type mice treated with A438079; scale bar 50 µm. (**B**) Measurement of epidermal thickness from syngeneic transplanted skin on animal groups described in **A** (*n*= 40-74 different measurements from 4 independent transplants). (**C**) Plasma levels of allogeneic specific IgGs 7 days after transplantation (*n*= 2-11 independent animals). (**D**) Representative images of F4/80 or CD3 immunostaining on syngeneic or allogeneic skin grafts 7 days after transplantation in wild-type or *P2rx7*$^{-/-}$ mice; scale bar 50 µm. (**E**) Representative ultrasound images from syngeneic or allogeneic grafts in mice untreated or treated with A438079, red dotted box indicates the transplanted area. Data are presented as mean ± sem from the indicated number of independent experiments for **B,C**. For **B,C** data are represented as mean ± SEM.

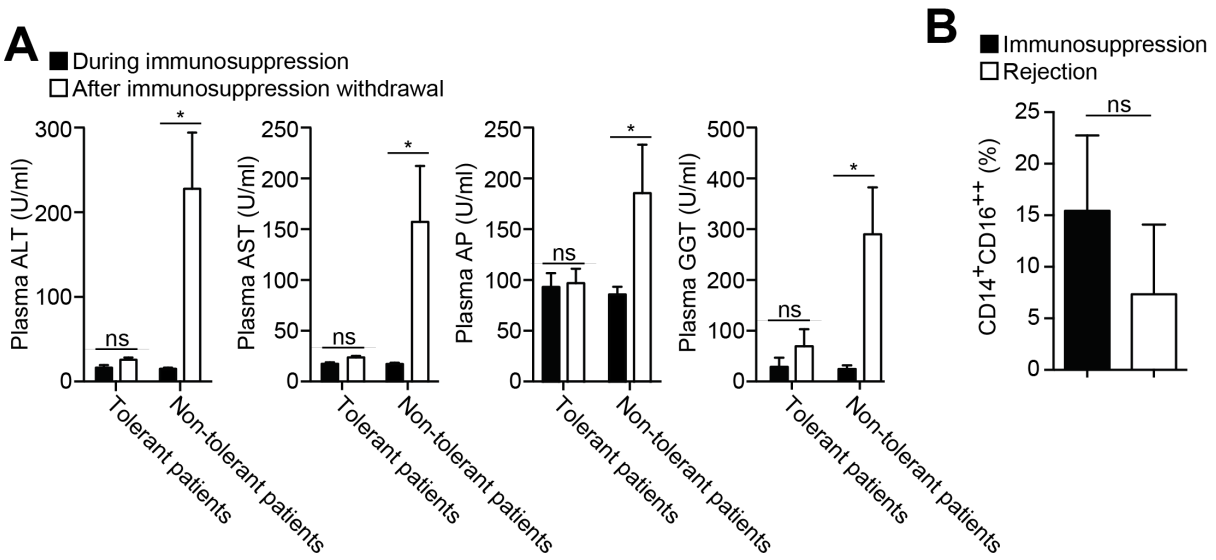

Figure S4. Plasma levels of hepatic enzymes in human liver transplantation, Related to Figure 3. (**A**) Activity of different plasma liver enzymes (ALT: alanine aminotransferase, AST: aspartate aminotransferase, AP: alkaline phosphatase, GGT: gamma glutamyl transpeptidase) in the serum from liver-transplanted patients (tolerant or non-tolerant) during immunosuppression and after immunosuppression withdrawal (n= 4 patients/group). (**B**) Percentage of inflammatory monocytes ($CD14^{+}CD16^{++}$) from non-tolerant liver-transplanted patients during immunosuppression and after immunosuppression withdrawal (rejection) (n= 4 patients). Data are presented as mean ± sem. *p< 0.05; ns: not significant (p> 0.05) ANOVA-two ways for **A**; *t*-Student for **B**. For **A,B** data are represented as mean ± SEM.

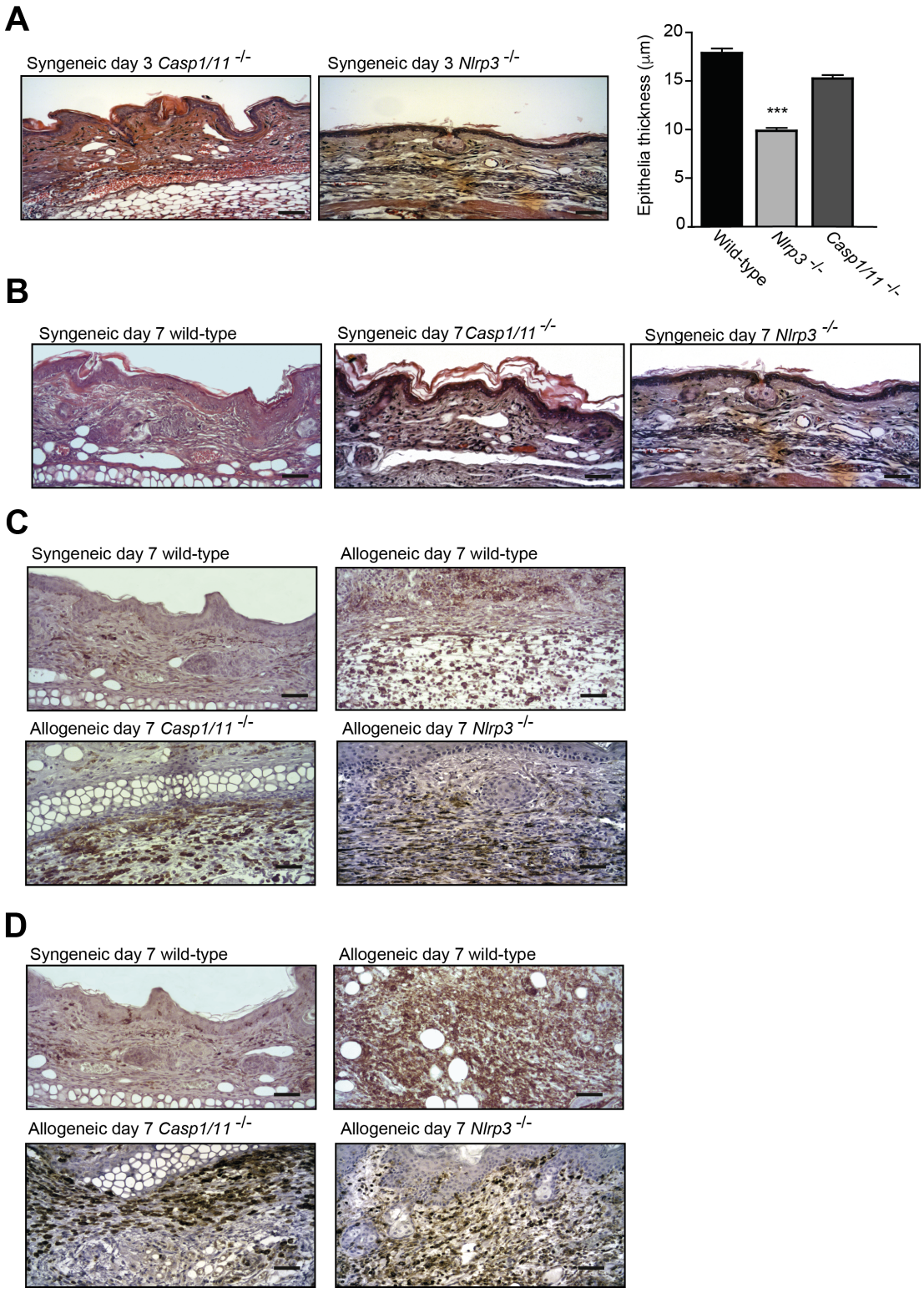

Figure S5. Allogeneic rejection response is diminished in inflammasome deficient mice, Related to Figure 6. (**A**) Representative image of haematoxylin and eosin stained syngeneic skin grafts 3 days after transplantation from *Casp1/11*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice; scale bar 50 µm (left); Measurement of epidermal thickness (*n*= 40-74 different measurements from 4 independent transplants) (right); Data are presented as mean ± sem. ***p< 0.001; ANOVA with Bonferroni's post-test. (**B**) Representative images of haematoxylin and eosin stained syngeneic skin grafts 7 days after transplantation in wild-type, *Casp1/11*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice; scale bar 50 µm. (**C,D**) Representative picture of F4/80 (**C**) or CD3 (**D**) immunostaining on syngeneic or allogeneic skin grafts 7 days after transplantation in wild-type, *Casp1/11*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice; scale bar 50 µm. For **A** data are represented as mean ± SEM.

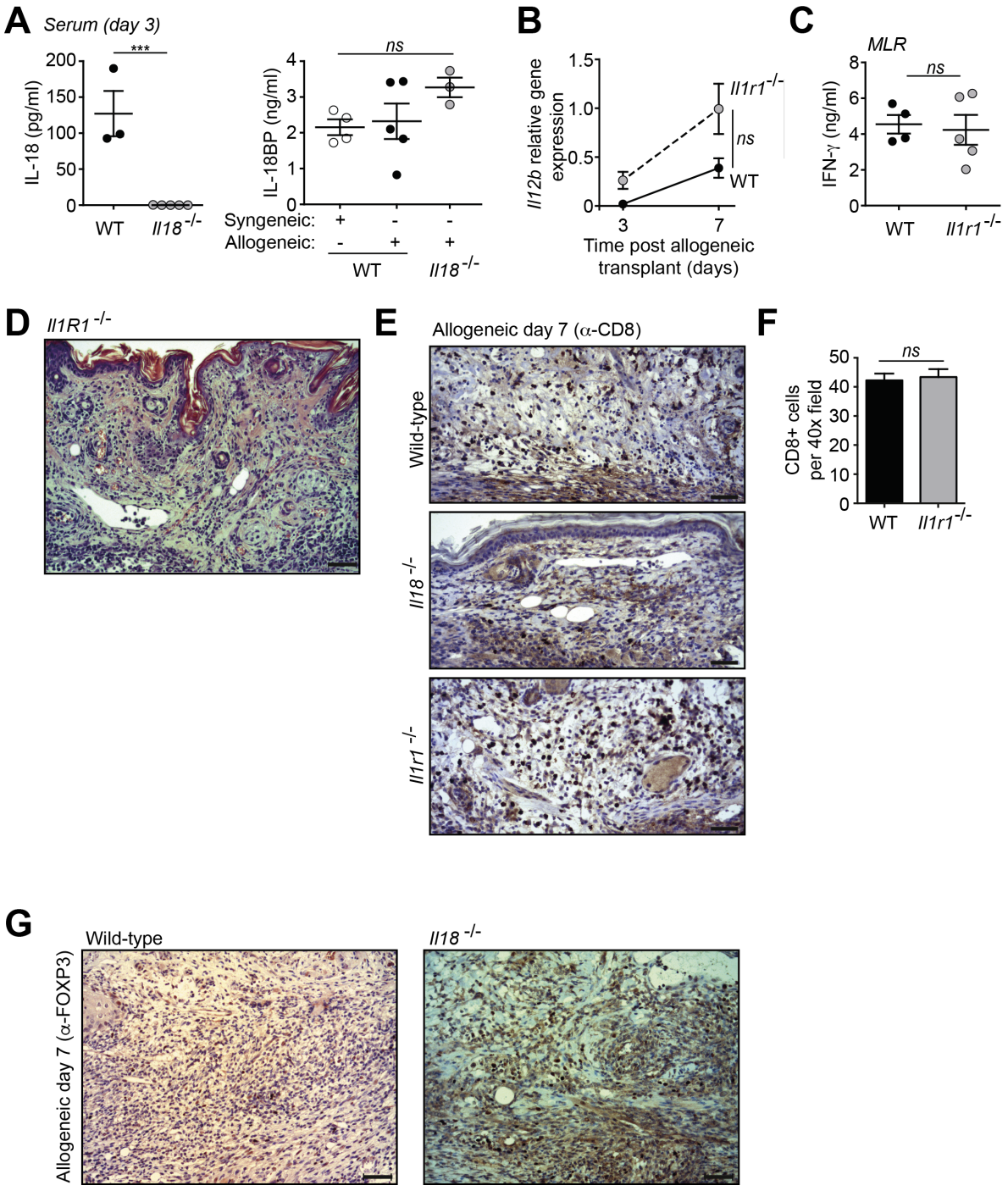

Figure S6. IL-1 signalling is not involved in inflammasome alloimmune response, Related to Figure 7. (**A**) IL-18 (left) and IL-18 binding protein (IL-18BP) (right) in serum 7 days after transplantation (n= 3-5) using C57BL/6 wild-type or *Il18*$^{-/-}$ as recipient and C57BL/6 (syngeneic) or BALB/c (allogeneic) skin as donor. (**B**) Relative gene expression for *Il12b* in grafts 3 or 7 days after transplantation using C57BL/6 wild-type or *Il1r1*$^{-/-}$ as recipient and BALB/c skin as donor (n= 4-5). (**C**) IFN-γ ELISA produced by axillar lymph node lymphocytes collected 7 days after transplantation and subjected to mixed lymphocytic reaction (MLR) with allogeneic splenocytes (n= 4-5). (**D**) Representative images of haematoxylin and eosin stained skin grafts, using C57BL/6 *Il1r1*$^{-/-}$ as recipient and BALB/c skin as donor; scale bar 50 µm. (**E**) Representative picture of CD8 immunostaining on allogeneic skin grafts 7 days after transplantation BALB/c skin in C57BL/6 wild-type, *Il18*$^{-/-}$ or *Il1r1*$^{-/-}$ mice; scale bar 50 µm. (**F**) CD8^{+} cells 7 days after transplantation using C57BL/6 wild-type or *Il1r1*$^{-/-}$ as recipient and BALB/c skin as donor (n= 24-51 field of views from 3 transplants). (**G**) Representative picture of FOXP3 immunostaining on allogeneic skin grafts 7 days after transplantation BALB/c skin in C57BL/6 wild-type or *Il18*$^{-/-}$ mice; scale bar 50 µm. For **A**,**C** each circle represents an individual mouse. *t*-Student for **A-C,F**. For **A-C,F** data are represented as mean ± SEM.