Dimethylarginine metabolism during acute and chronic rejection of rat renal allografts

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Materials and methods-online Supplement

Quantification of ADMA, SDMA and L-arg

Hundred µl rat plasma were adjusted to a final volume of 1 ml with PBS (PAA Laboratories, Pasching, Austria) and subjected to crude fractionation on Oasis MCX solid-phase extraction (SPE) cartridges (30 mg, 1 ml; Waters, Eschborn, Germany). All conditioning, washing, and elution steps were performed on a vacuum manifold with a capacity for 20 columns (Waters) at a flow rate of ~ 0.5 ml/min. The SPE cartridges were conditioned with 2 ml of methanol/water/ammonia (50:45:5, vol/vol/vol) followed by 2 ml of PBS before sample application. Samples were passed through SPE cartridges, and contaminating components were removed with 2 ml 0.1 M HCl followed by 2 ml methanol. Basic compounds were eluted with 1 ml of methanol/water/ammonia (50:45:5, vol/vol/vol). Samples were dried in a vacuum centrifuge and stored at -20° C until further analysis. Eluates were re-dissolved in 230 µl distilled water and centrifuged at 14 000 *g* for 2 min to remove particles before derivatisation for HPLC.

Ortho-phthaldialdehyde (OPA) reagent was freshly prepared in potassium borate buffer (both Grom, Rottenburg-Hailfingen, Germany) according to the manufacturer's instructions. Samples (115 µl) were combined with 50 µl of OPA reagent, immediately transferred to the auto sampler, and injected after exactly 2 min. Quantification of amino acids was performed on an HPLC system consisting of an ASI-100 auto sampler, a P680 gradient pump, a RF-2000 fluorescence detector, and a data acquisition system (Chromeleon, version 6.60; all Dionex,

Idstein, Germany). Separation was carried out according to the method described by Teerlink T *et al.* [1] with slight modifications. Fluorescent amino acid derivatives were separated on a SunFire C18 column (4.6 x 150 mm; 3.5-µm particle size; 100 Å pore size) with a µBondapak C18 guard column at 30° C and a flow rate of 1.1 ml/min (all columns from Waters). After sample injection (125 µl), separation was performed under isocratic conditions with 8.8% (vol/vol) acetonitrile in 25 mM potassium phosphate buffer (pH 6.8) as solvent. The isocratic conditions were maintained for 24 min. To elute strongly bound compounds, the column was flushed with acetonitrile/water (50:50, vol/vol) for 5 min and reequilibrated under isocratic conditions for 15 min before the next injection. Fluorescent derivatives were detected at excitation and emission wavelengths of 330 and 450 nm, respectively. Quantification of L-arg, ADMA and SDMA was performed by external calibration.

DDAH activity

Hundred mg renal tissue were homogenized in 1 ml 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, Complete proteinase inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), and 1 mM Na_3VO_4, incubated for 1 h on ice and centrifuged, 15 min, 16 000 g. Fifty µl tissue extract were combined with 6 µl standard solution containing 500 pmol/µl ADMA and 100 pmol/µl SDMA. Internal standards exceeded endogenous methylarginine levels. Samples were adjusted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer, pH 6.5. After 2 h at 37°C, dimethylarginine concentrations were measured.

Immunoblotting

The protein concentrations of the extracts were determined using Micro BCA™ protein Assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein lysate (40 µg per sample)

were resolved on 8%, 10% or 12% reducing SDS-polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non fat milk powder dissolved in 50 mM Tris-HCl buffer, pH 7.6 containing 0.9% NaCl. Blots were incubated with with primary antibodies to iNOS (1:250, BD Biosciences Franklin Lakes, NJ), eNOS (1:4 000, Santa Cruz Biotechnology, Heidelberg, Germany), PRMT1 (1:2 000, Abcam, Cambridge, MA). ASYM24 antibodies (1:500, Millipore) were used to detect proteins containing asymmetric dimethylarginine-glycine repeats. Antibodies to eNOS, PRMT1 and asymmetric dimethylarginine-glycine repeats were diluted in blocking solution. Antibodies to iNOS were diluted in 10% Rotiblock (Roth, Karlsruhe, Germany) in PBS. To ensure equal protein loading, the membranes were incubated with a mAb to glyceraldehyde-3-phosphate (GAPDH) (1:20 000, Novus Biologicals, Littleton, CO). Immune complexes were visualized with horseradish peroxidase-conjugated secondary antibodies (1:5 000, Dako, Glostrup, Denmark) using the chemiluminescent reagent Lumi-Light Western blotting substrate (Roche, Mannheim, Germany). Densitometric analyses of protein bands were performed using a digital gel documentation system (Quantity One, Biorad, Germany).

Immunohistochemistry

The grafts were fixed by immersion in 4% buffered paraformaldehyde and embedded in paraffin. Sections (5-7 µm) were dewaxed, rehydrated and pretreated with 0.5 mg/ml Protease Type XIV (Sigma-Aldrich, Taufkirchen, Germany) in 50 mM Tris-HCl buffer pH 7.6, 0.9% NaCl for 15 min at room temperature followed by 1% H_2O_2 in PBS for 30 min. For staining with mAb ED1, protease pretreatment was omitted. After washing in PBS pH 7.2, the sections were incubated for 30 min with PBS, pH 7.2, 1% BSA (Serva, Heidelberg, Germany), 0.1% NaN_3 (p.a. Merck, Darmstadt, Germany) followed by over-night incubation at 4° C with

mouse anti-PRMT1 antibodies (1:2000, Abcam), mAb ED1 (directed to a rat CD68-like antigen; 1:500 Serotec, Düsseldorf, Germany) or mAb R73 (directed to the β-chain of the rat α/β T cell receptor; 1:500, Serotec) diluted in PBS/BSA/NaN_3. Bound primary antibodies were detected using the anti-mouse EnVision™ peroxidase system (DAKO, Hamburg, Germany) and 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) followed by a light counterstaining with hemalum. As a control, experiments omitting the primary antibodies were performed.

Quantitative RT-PCR

Total RNA was extracted from ~20 mg renal tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reversely transcribed using the M-MLV H- Reverse Transcriptase and 1 µg random hexamer primers (Promega, Mannheim, Germany), 1 h, 40°C. DDAH1 and DDAH2 mRNA expression was measured by quantitative RT-PCR (qRT-PCR) in an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using Platinum SYBR green qPCR Super Mix-UDG (Invitrogen, Karlsruhe, Germany) (n=4 per group, samples were run in duplicates). ΔCt values of the genes of interest were compared to the porphobilinogen deaminase (PBGD) gene. All primers (MWG Biotech, Ebersberg, Germany) were intron-spanning: DDAH1, 5´-CGCAGGAAGGAGGTTGACAT-3´ (sense), 5´-CGTCCCCACCATCTAAGGTT-3´ (antisense); DDAH2, 5´-AGTGAGGCTGCCCAAAAAG-3´ (sense), 5´-CGTCAGAGAGCTTTTGCAGAG-3´ (antisense); PBGD; 5´-GGCGCAGCTACAGAGAAAGT-3´ (sense), 5´-AGCCAGGATAATGGCACTGA-3´ (antisense). PCR conditions included denaturation, 5 min, 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 60°C, and 10 s at 72°C.

Quantification of nitrite/nitrate (NO_x)

Renal tissue was homogenized in ice-cold PBS (1 mg/ml) and centrifuged, 14 000 g, 10 min. Plasma or tissue extracts were diluted 1:2 in PBS, deproteinized by adding 15 g/l zinc sulfate, mixed for 1 min, and centrifuged, 10 000 g, 10 min, RT. To reduce nitrate to nitrite, 100 µl of the supernatant were mixed with 100 µl vanadium (III) chloride (8 mg/ml) and 100 µl Griess reagent containing 1% sulfanilamide, 0.025% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine (NEDD). After 30 min at 37°C, absorbance was read at 540 nm. Samples were assessed in duplicates, for blank values sulfanilamide and NEDD were omitted, and a linear standard curve of 0-100 µmol/l potassium nitrate reduced by vanadium (III) chloride was used.

Assessment of rat renal function

Plasma creatinine measurement was performed based on Jaffe's method [2], and plasma urea content was assessed based on the enzymatic reaction described by Roch-Ramel [3]. Both parameters were measured with the ADVIA Chemistry System, Bayer (Siemens Healthcare Diagnostics, Deerfield, IL, USA) by the Laboratory of Clinical Chemistry, University Hospital Giessen and Marburg, Germany.

References

1. Teerlink T, Nijveldt RJ, de Jong S *et al.*, Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem* 2002; 303: 131-7.
2. Slot C, Plasma creatinine determination. A new and specific Jaffe reaction method. *Scand J Clin Lab Invest* 1965; 17: 381-7.
3. Roch-Ramel F, An enzymic and fluorophotometric method for estimating urea concentrations in nanoliter specimens. *Anal Biochem* 1967; 21: 372-81.

Supplementary Table 1: *Densitometrical analyses of individual protein arginine methylation in healthy control kidneys, LEW to LEW isografts (iso) and DA to LEW allografts on day 4 after transplantation*

	Median [min-max] *(p-value)*, ASYM24/GAPDH					
	P1	P2	P3	P4	P5	P6
C (n=4)	0.9 [0.4-1.7]	1.0 [0.5-1.3]	0.3 [0.0-3.3]	0.9 [0.4-1.6]	1.0 [0.4-1.5]	0.6 [0.0-2.8]
iso (n=4)	3.1 [0.9-5.6] *(0.114)*	0.6 [0.3-1.2] *(0.343)*	1.1 [0.7-2.5] *(0.343)*	0.6 [0.4-1.6] *(0.886)*	2.7 [0.8-3.8] *(0.200)*	9.2 [0.0-14.3] *(0.200)*
allo (n=4)	1.8 [1.2-6.5] *(0.886)*	0.2 [0.1-0.6] *(0.114)*	1.9 [1.2-3.7] *(0.343)*	0.1 [0.1-0.8] *(0.200)*	2.0 [1.1-4.4] *(0.886)*	54 [41-112] *(0.029)*

The bands of proteins P1-P6 are labeled in the blots depicted in Supplementary Figure 4; *isograft versus healthy control kidney, #allograft versus isograft.

Supplementary Table 2: *Densitometrical analyses of individual protein arginine methylation in healthy control kidneys, LEW to LEW isografts (iso) and F344 to LEW allografts on day 9 after transplantation*

	Median [min-max] *(p-value)*, ASYM24/GAPDH					
	P1	P2	P3	P4	P5	P6
C (n=4)	1.1 [0.6-1.1]	0.8 [0.7-1.5]	0.0 [0.0-4.0]	0.9 [0.5-1.5]	0.8 [0.5-1.7]	0.0 [0.0-4.0]
iso (n=4)	9.8 [3.6-15.0] *(0.029)*	2.0 [1.4-2.6] *(0.057)*	n.d. *(0.689)*	0.6 [0.0-1.8] *(0.886)*	2.4 [1.1-4.0] *(0.057)*	0.0 [0.0-7.1] *(0.886)*
allo (n=4)	2.5 [1.0-3.0] *(0.029)*	0.9 [0.4-1.1] *(0.029)*	49 [46-63] *(0.029)*	2.7 [1.8-3.1] *(0.057)*	4.1 [2.2-4.6] *(0.343)*	16 [10-29] *(0.029)*

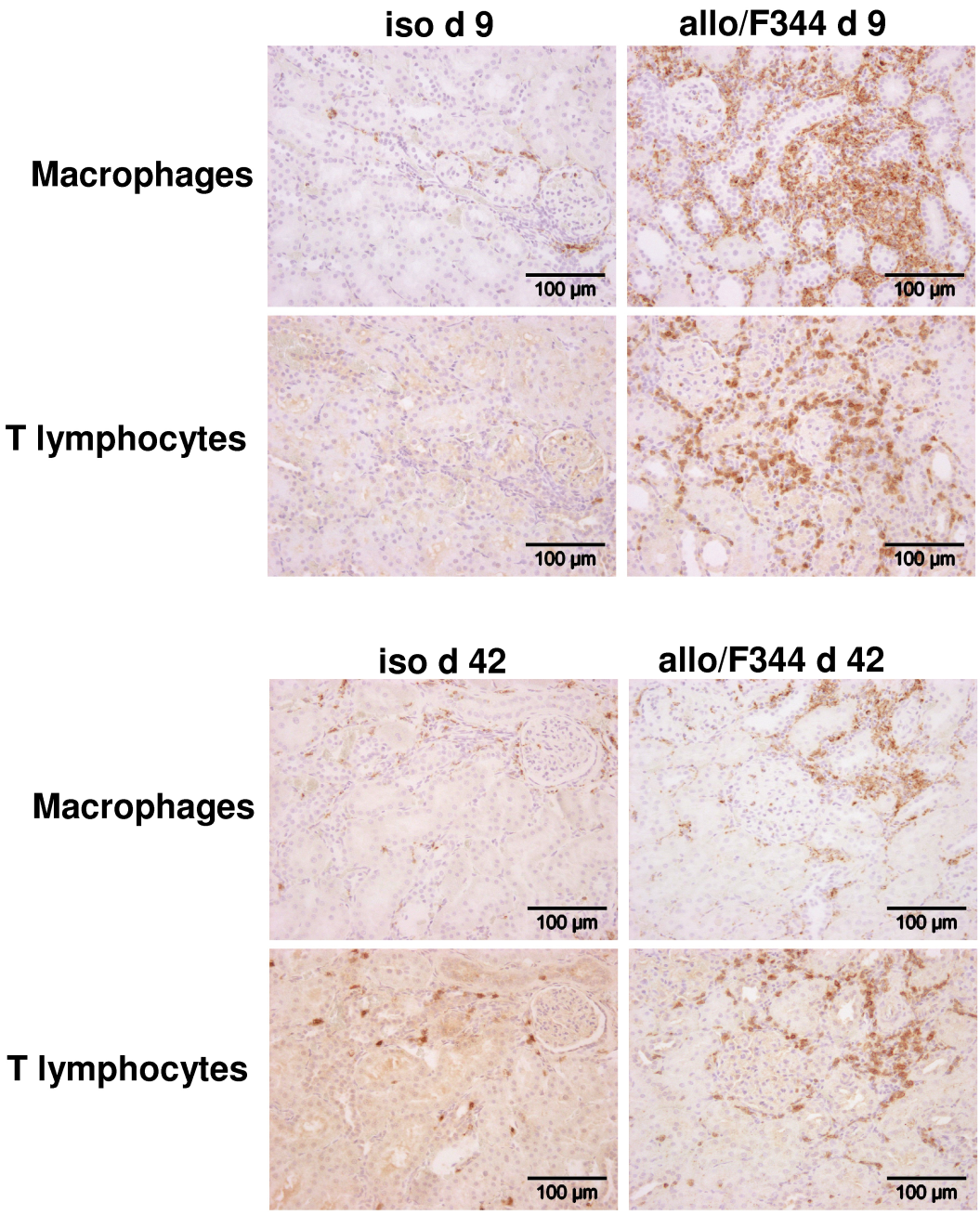
The bands of proteins P1-P6 are labeled in the blots depicted in Supplementary Figure 4; *isograft versus healthy control kidney, #allograft versus isograft, n.d. not detected.

Supplementary Table 3: *Densitometrical analyses of individual protein arginine methylation in healthy control kidneys, LEW to LEW isografts (iso) and F344 to LEW allografts on day 42 after transplantation*

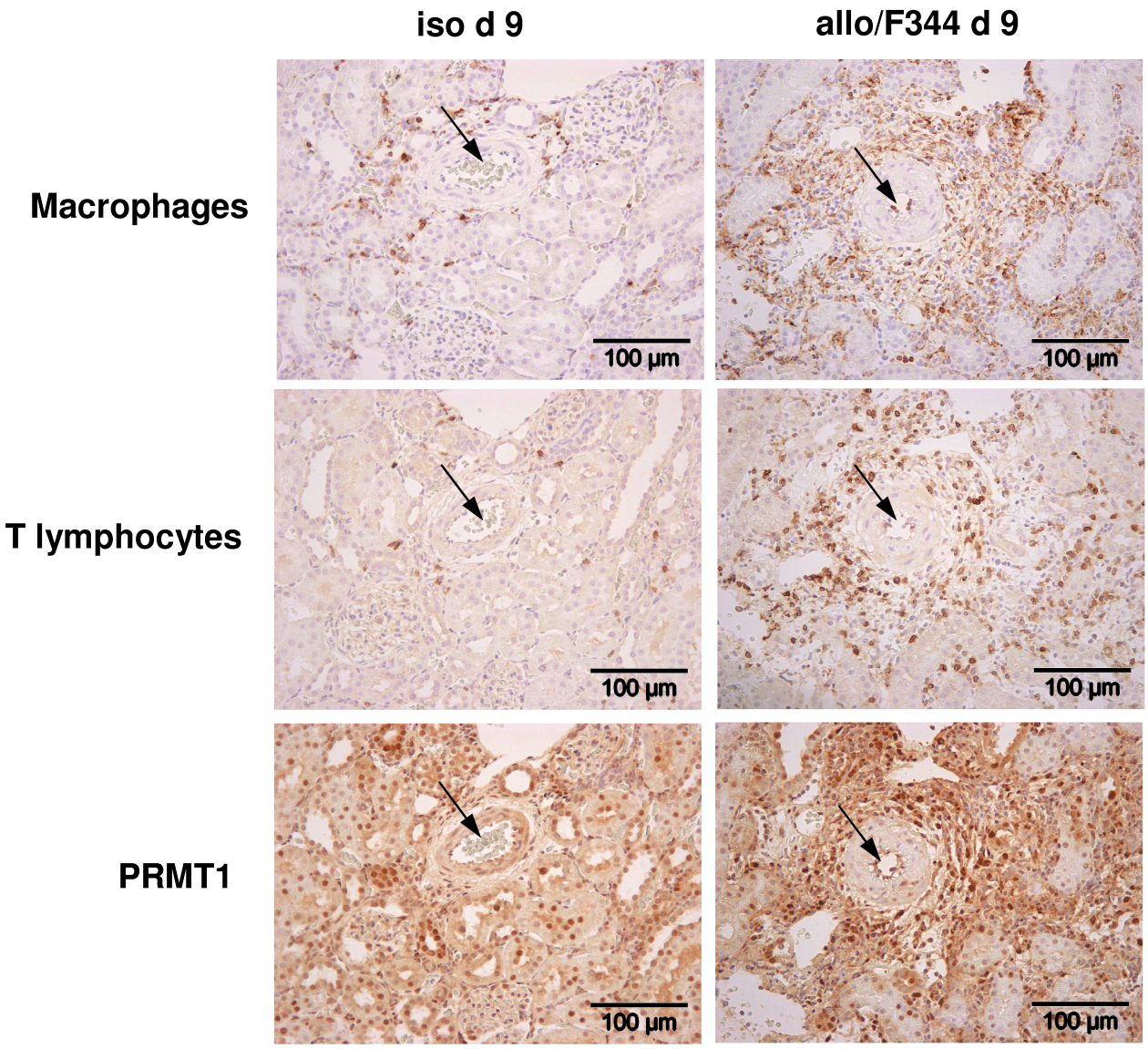
	Median [min-max] *(p-value)*, ASYM24/GAPDH					
	P1	P2	P3	P4	P5	P6
C (n=4)	1.0 [0.7-1.1]	1.0 [0.8-1.1]	0.7 [0.6-1.9]	0.5 [0.3-2.5]	0.9 [0.8-1.2]	0.8 [0.7-1.4]
iso (n=4)	3.7 [1.0-5.7] *(0.114)*	1.5 [1.1-1.7] *(0.029)*	2.2 [1.2-2.7] *(0.057)*	1.2 [0.6-1.4] *(0.343)*	1.3 [1.1-1.4] *(0.057)*	1.1 [0.7-1.1] *(0.687)*
allo (n=4)	1.4 [1.0-4.3] *(0.486)*	0.3 [0.1-0.5] *(0.029)*	3.0 [1.4-5.0] *(0.200)*	0.7 [0.6-0.9] *(0.200)*	1.3 [1.0-1.7] *(0.886)*	1.4 [1.3-2.8] *(0.029)*

The bands of proteins P1-P6 are labeled in the blots depicted in Supplementary Figure 4; *isograft versus healthy control kidney, #allograft versus isograft.

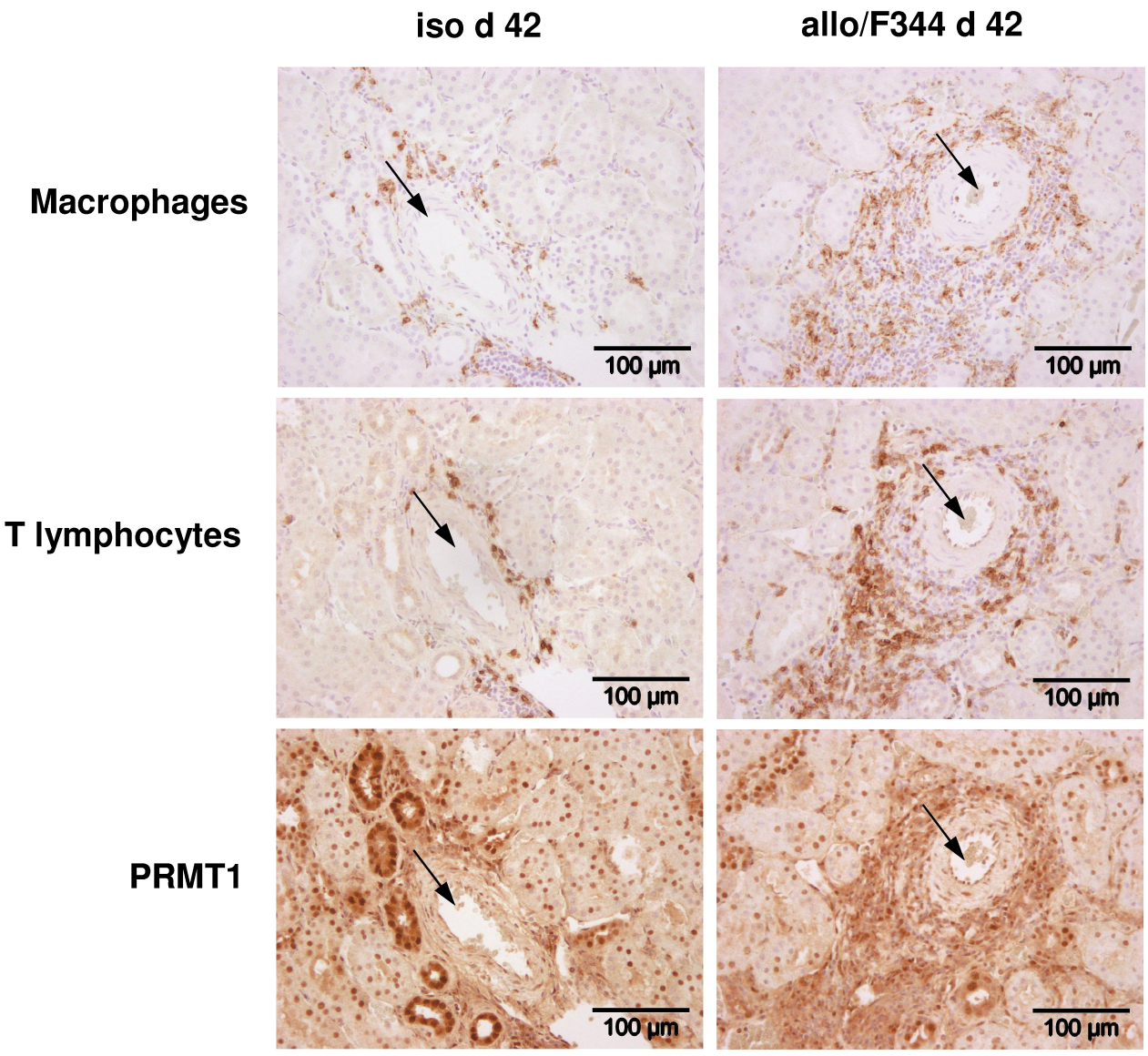
Supplementary Figure 1



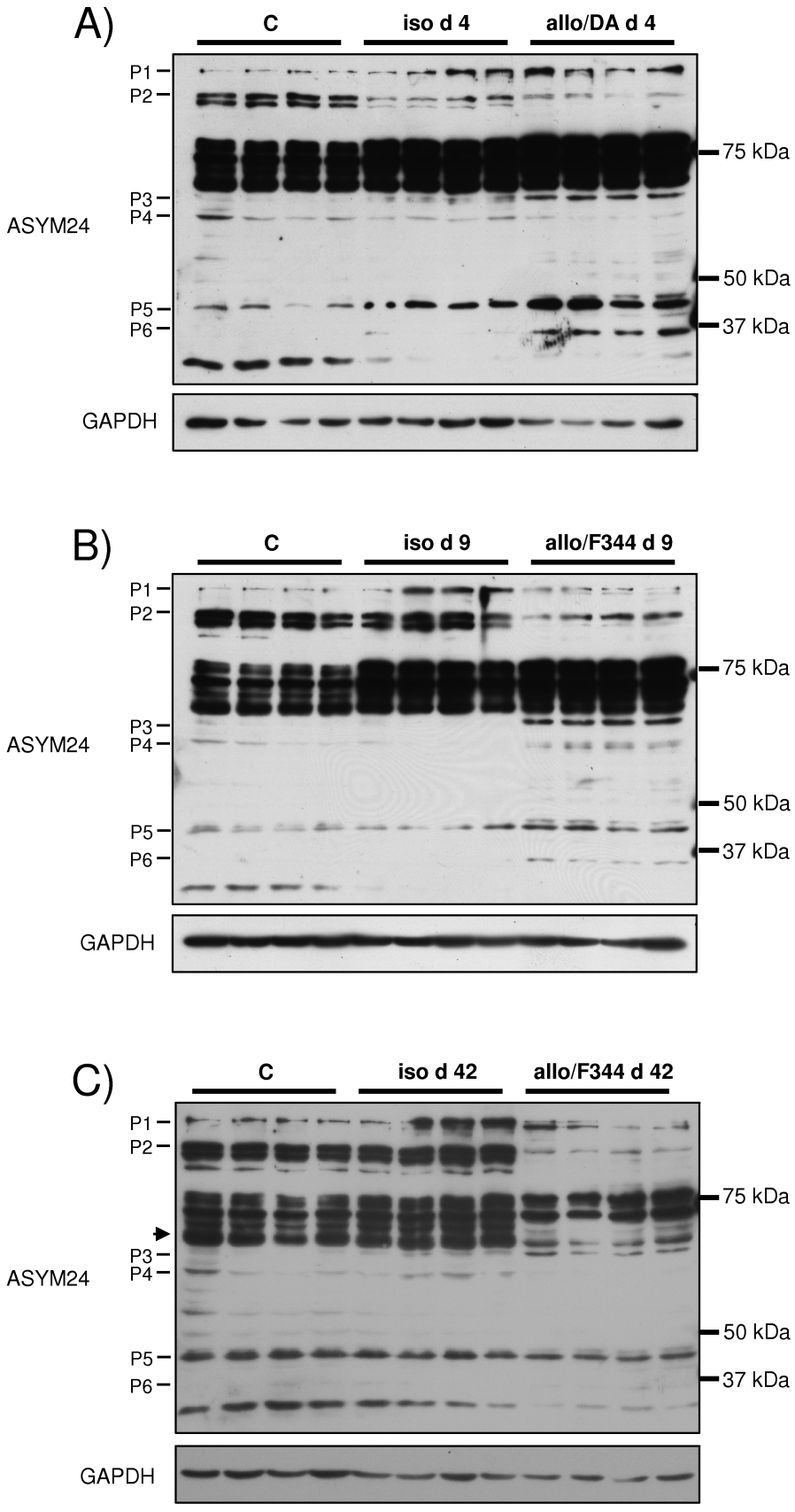
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5