

Age-related response to an acute innate immune challenge in mice: proteomics underline a telomere maintenance-related cost.

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Electronic Supplementary Material 1: Methods

Proteomics

Frozen spleen samples (N=6 per group) were grinded under liquid nitrogen for 1 minute at 25Hz using a Mixer Mill MM400 (Retsch, Eragny sur Oise, France), and proteins were extracted from the resulting powder using 4 µl of extraction buffer per mg of tissue. Extraction buffer was composed of 8 M urea, 2 M thiourea, 4% Chaps, and protease inhibitors (Sigma-Aldrich, Lyon, France). After sonication on ice (3 x 10 seconds, 135 watts), seven volumes of cold acetone were added to samples that were kept at -20°C overnight. Precipitated proteins were pelleted by centrifugation (20 minutes, 4°C, 13500 x g), and dissolved in Laemmli buffer (10 mM Tris pH 6.8, 1 mM EDTA, 5% β-mercaptoethanol, 5% SDS, 10% glycerol) after discarding supernatants. Total protein concentrations were determined using the RCDC Protein Assay kit from Bio-Rad (Hercules, CA, USA). At this stage, a sample pool comprising equal amounts of all protein extracts was made, to be injected regularly during the whole experiment and thus allowing QC-related measurements.

20 µg of proteins of each sample were electrophoresed on SDS-PAGE gels (12% polyacrylamide) for 75 minutes at 50 V. After protein fixation (50% ethanol, 3% phosphoric acid), gels were stained overnight using colloidal Coomassie Blue. For each lane, six 2 mm bands were excised, and proteins were in-gel digested with trypsin (Promega, Madison, WI, USA; 67 ng/band) at 37°C overnight after de-staining, reduction (10 mM DTT), alkylation (55 mM iodoacetamide), and dehydration using a MassPrep station (Micromass, Waters, Milford, MA, USA). Tryptic peptides were extracted using 60% acetonitrile, 0.1% formic acid in water. Organic solvent was then eliminated using a vacuum centrifuge (SpeedVac, Savant, Thermoscientific, Waltham, MA, USA), and peptides were re-suspended in 90 µL of 1% acetonitrile, 0.1% formic acid in water. A set of reference peptides (iRT kit; Biognosys AG, Schlieren, Switzerland) was finally added to the pooled sample prior to LC-MS/MS analyses.

Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to an Impact HD Q-TOF mass spectrometer equipped with Captive Spray source and nano booster (Bruker Daltonik GmbH, Bremen, Germany). Both instruments were controlled by Hystar (v 3.2) and OtofControl (Rev 3.4, Bruker Daltonics). One µl of each sample was first loaded on a Symmetry C18 pre-column (0.18 x 20 mm, 5 µm particle size; Waters) using a mobile phase composed of 99% of solvent A (0.1% formic acid in water) and 1% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 5 µl/min for 3 minutes, and peptides were then eluted at a flow rate of 450 nL/min using a UPLC separation column (BEH130 C18, 200 mm x 75 µm, 1.7 µm particle size; Waters) maintained at 60 °C with the following gradient: from 1% to 5% of B in 1 minute, then from 5% to 35% of B in 59 minutes. Samples were injected using a randomized sequence within block injections, each of them consisting of one biological replicate of

each group plus sample pool. To reduce carry-over, both a column wash program (50% ACN during 20 min) and a solvent blank were included in between each sample. Peak intensities and retention times of reference peptides were monitored in a daily fashion.

The Impact HD was operated in positive ion mode using the following settings: source temperature was set to 150°C while dry gas flow was 3 L/min and spray voltage was optimized to -1300 V. Acetonitrile was used as dopant in the nanobooster and nebulizer pressure was set to 0.2 bar. Spectra were acquired by automatic switching between MS and MS/MS modes in the mass range of 150-2200 m/z with a fixed cycle time of 3 seconds. MS acquisition rate was set to 2 Hz and MS/MS acquisition rate ranged from 3 to 25 Hz depending on the precursor intensity. Preferably, ions with a charge of 2 to 5 were selected for CID fragmentation using nitrogen as collision gas. Ions were excluded from fragmentation after the acquisition of 1 MS/MS spectrum and exclusion was released after 1 minute, except when precursor intensity exceeded the value during the first selection by a factor of at least 3. Online correction of TOF calibration was performed using hexakis(2,2,3,3,-tetrafluoropropoxy)phosphazine ([M+H]⁺ 922.0098 m/z) as lock-mass.

MS raw data processing was performed using MaxQuant (v 1.5.2.8). Peak lists were searched against a Swiss-Prot-derived protein database created using the MSDA software suite [1]. The database contained *Mus musculus* (TaxID 10090) protein sequences (November 2014; 16688 sequences) to which sequences of common contaminants (247 entries) were added using the Andromeda search engine implemented in MaxQuant. Identifications were filtered to obtain false discovery rates (FDR) below 1% for both peptide spectrum matches (minimum length of 7 amino acids) and proteins, a maximum number of one missed cleavage was tolerated, and carbamidomethylation of cysteine residues and oxidation of methionine residues were considered as variable modifications. The search window was set to 10 ppm for precursor ions and 40 ppm for fragment ions. For all identified proteins, corresponding annotations from the GO databases (see in ESM2) were automatically extracted using the Annotation Explorer module of the MSDA software [1].

Regarding quantification, data normalisation and protein abundance estimation was performed using the MaxLFQ (label free quantification) option implemented in MaxQuant [2] using a “minimal ratio count” of one. “Match between runs” was enabled using a one minute time window after retention time alignment. Only unmodified peptides were considered for quantification (except those for which a modified counterpart was detected) while shared peptides were excluded. All other MaxQuant parameters were set as default. Only proteins quantified with a minimum of two unique peptides and with at least four valid values per group as well as the ones “absent” (i.e. 0 valid values) in samples from a given group were kept for further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [3] partner repository with the dataset identifier PXD009005.

Regarding QC-related measurements, retention times of iRT peptides remained very stable over the five repeated injections of the control pool sample, with a mean coefficient of variation (CV) of 0.7%, indicating decent and steady HPLC performance. Moreover, a mean CV of 15.8% was recorded for LFQ values all proteins identified in all five repeated injections of the control pool sample. These different values argue for the good stability of nanoLC-MS/MS system during the whole duration of analyses, and good reproducibility of protein abundance determination.

Telomere length measurements

Telomere length in splenocytes was measured on 20 mice: 5 young-PBS and 5 young-LPS mice, and 4 old-PBS and 6 old-LPS mice. DNA was extracted using Dneasy Tissue kit Qiagen, following manufacturer protocol, and DNA quality and quantity were checked using a nonodrop ND-1000 spectrophotometer (Labtech).

Using extracted DNA, individual telomere length at death was measured using a quantitative PCR (qPCR) approach initially developed on humans [4]. Shortly, we used (i) for the telomere sequence (named T) amplification, the same primer sequences and qPCR conditions as those given in [5], and (ii) for the single copy-number control gene (named S) the *recombination activating gene* (RAG)-1 (ID: 19373 Mus musculus RAG1 gene; GenBank, NIH). The qPCR was conducted on a final mix of 10 μ L with 5 μ L GoTaq RT-qPCR mix (Promega, Madison, WI, USA), 2 μ L containing 5 ng of DNA and 3 μ L of primers at a concentration of 200 nM, with the following amplification conditions: 95°C for 2 min, followed by 40 cycles of data collection for 45 s at 95°C, 30 s at 64°C, and 30 s at 72°C (RAG1) and 30 cycles of data collection for 15 s at 95°C, 30 s at 56°C, and 30 s at 72°C (telomere sequence). Specificity of the amplifications was checked in each run by ending the cycles with a melting step. qPCR runs were conducted on 96-wells MasterCycler RealPlex (Eppendorf AG, Germany), all samples being measured in duplicates on the same plate also containing a calibration curve (one random chosen sample serially diluted from 10 ng to 0.625 ng) for amplification efficiency calculation. Given that temperatures of annealing are different for the telomere and control gene, two distinct runs of amplification were necessary (one for the control gene and one for the telomere). Amplification efficiencies were of 100.1 % (S) and 99.4 % (T). Intra-plate coefficients of variation were of 0.69 ± 0.14 % for T Cq values, 0.35 ± 0.05 % for S Cq values, and 7.27 ± 0.96 % for the final T/S values used as a measure of mean individual telomere length. T/S is a relative telomere length calculated using one random sample (called golden sample) of a fixed value of 1 (see [4, 6]).

Western-Blot Analyses

Ten μ g of each sample were separated on 8–16% Bio-Rad Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, Hercules, CA). Gels were imaged after activation using the Bio-Rad ChemiDoc Touch Imaging System and transferred to nitrocellulose membranes (0.2 μ m) using the Bio-Rad Trans-Blot Turbo Transfer System. Blots were immediately imaged to check for proper transfer, they were blocked for 1 h at room temperature with a solution of TBS-T (Tris 25 mM, NaCl 137 mM, KCl 2.68 mM, 5% Tween 20) containing 4% of BSA, and they were then incubated overnight at 4 °C with a primary antibody targeting telomeric repeat-binding factor 1 (TRF1; sc-5596; Santa Cruz Biotechnology, Dallas, TX), or telomeric repeat-binding factor 2 (TRF2; sc-47693; Santa Cruz Biotechnology), or protection of telomeres protein 1 (POT1; sc-33789; Santa Cruz Biotechnology), telomeric repeat-binding factor 2-interacting protein 1 (RAP1; sc-53434; Santa Cruz Biotechnology), or TRF1-interacting nuclear factor 2 (TIN2; ab197894, Abcam, Paris, France). Antibodies against TRF1 and POT1 were used at 1/200 dilution in the blocking solution, those against TRF2 and RAP1 at 1/500, and the one against TIN2 at 1/1000. After three washings for 10 min each in TBS-T, blots were incubated for 1h at room temperature with a peroxidase-conjugated secondary antibody (sc-2004 or sc-2005, Santa Cruz Biotechnology, Dallas, TX) diluted (1/5000) in the blocking solution. After three washings for 10 min each in TBS-T, blots were incubated for 1 min in Luminata Classico Western HRP substrate (Merk Millipore, Molsheim, France) and then imaged for chemiluminescence using the ChemiDoc Touch Imaging System (Bio-Rad). Image analysis was done using Bio-Rad Image Lab software v5.1. Signals were normalized to total proteins, as measured on the stain-free gel image, and intensity values were expressed relative to those in young mice injected with PBS, which were assigned an arbitrary value of one.

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