Supplemental Tables and Figures

Auto-inhibitory regulation of S100A8/A9-alarmin activity locally restricts sterile inflammation

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Table S1. Confirmation of S100A9 array data by qRT-PCR data

		gene array, n = 3		**qRT-PCR,** n = 6	
gene	**ENTREZ gene ID**	**n-fold**	***Detection P***	**n-fold**	***P***
IL-6	3569	55.26	< .0001	1055.1 ± 261.2	0.0157
CXCL10/IP10	3627	2.15	< .0001	68.3 ± 52.7	ns
SOCS1	8651	7.53	< .0001	15.2 ± 4.0	0.0162
CCL5	6352	3.15	< .0001	28.8 ± 8.1	0.0263
SOCS3	9021	6.21	< .0001	7 ± 2.1	0.0363
TNFAIP6	7130	10.38	< .0001	170.9 ± 48.3	0.0246
IL-10	3586	4.96	< .0001	8.2 ± 1.8	0.0061
IL-1α	3552	5.08	< .0001	39.1 ± 20.6	0.0033
CD80	941	4.13	< .0001	18.6 ± 2.8	0.016
SOCS2	8835	3.5	< .0001	3.7 ± 1.0	0.0475
NFκB1	4790	2.51	< .0001	4.2 ± 0.9	0.0197
TNF-α	7124	2.39	< .0001	14.6 ± 4.2	0.0325

Genes regulated by 4h S100A9 stimulation of human monocytes (gene aray: n=3; qRT-PCR: n=6, mean ± SEM and paired t-test).

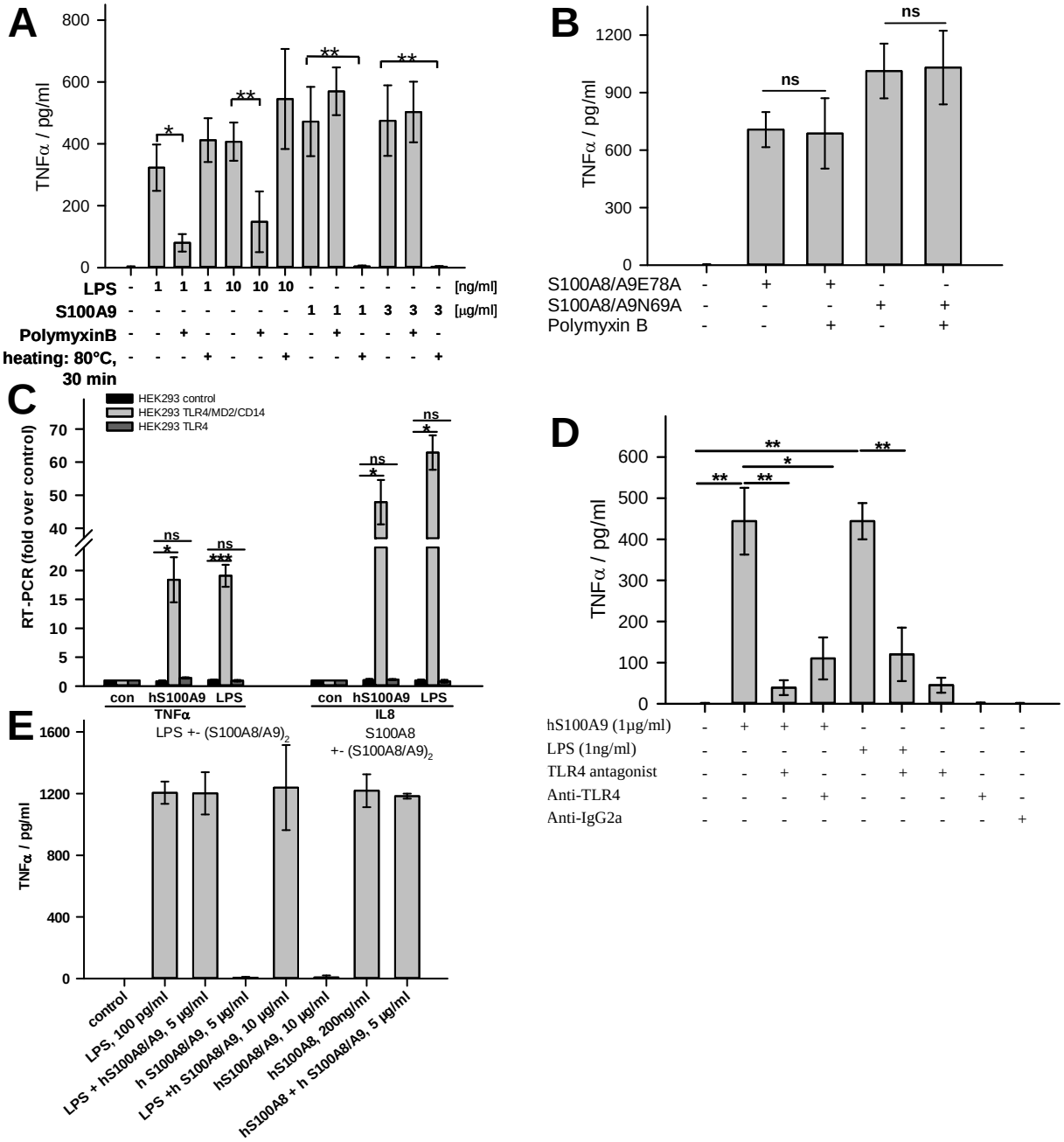

Figure S1. Activity of endotoxin free S100 preparations depends on TRL4/MD2 signaling pathway
(A,B) Human monocytes were stimulated with 1 or 3 µg/ml S100A9 and 1 or 10 ng/ml LPS (A) or 1 µg/ml rhS100A8/S100A9N69A or rhS100A8/S100A9E78A (B) in the presence or absence of polymyxin B for 4 h, or stimulated with S100A9 and LPS that was pre-incubated at 80° C for 30 min prior stimulation (A). TNFα levels in the supernatants were determined by ELISA. Polymyxin B (25 µg/ml) had no inhibitory effect on S100-induced TNFα expression, whereas no heating effects were observed for LPS. (C) Stably transfected HEK293 cells (TLR4/CD14/MD2, TLR4 or empty vector) were left untreated or stimulated with 5 µg/ml S100A9 or 10 ng/ml LPS for 4 h. qRT-PCR analysis was performed for TNFα and IL8 mRNA expression. Results are shown as relative to baseline expression in unstimulated cells and RPL was used as a housekeeping control gene. These results indicate that the co-receptor MD2 is necessary for cell activation by S100A9. (D) Human monocytes were pre-incubated for 30 min with 1 µg/ml of TLR4 antagonist RS-LPS or 1 µg/ml antibody to TLR4 (clone HTA125) or isotype-matched control antibody (IgG2a), followed by 4 h stimulation with LPS or S100A9. TNFα release was quantified by ELISA and confirmed the role of TLR4/MD2 for S100A9. (E) Human monocytes were prestimulated with 5 or 10 µg/ml hS100A8/A9 tetramers for 30 min followed by 100 pg/ml LPS or 200 ng/ml hS100A8 for 4h. TNFα levels in the supernatants were determined by ELISA. hS100A8/A9 tetramers had no inhibitory effect on LPS or S100A8-induced TNFα expression. Data represent mean ± s.d. from three independent experiments. Statistically significant differences were assessed by two tailed t-test, *P < 0.05, **P < 0.01, ***P < 0.001 and ns = not significant.

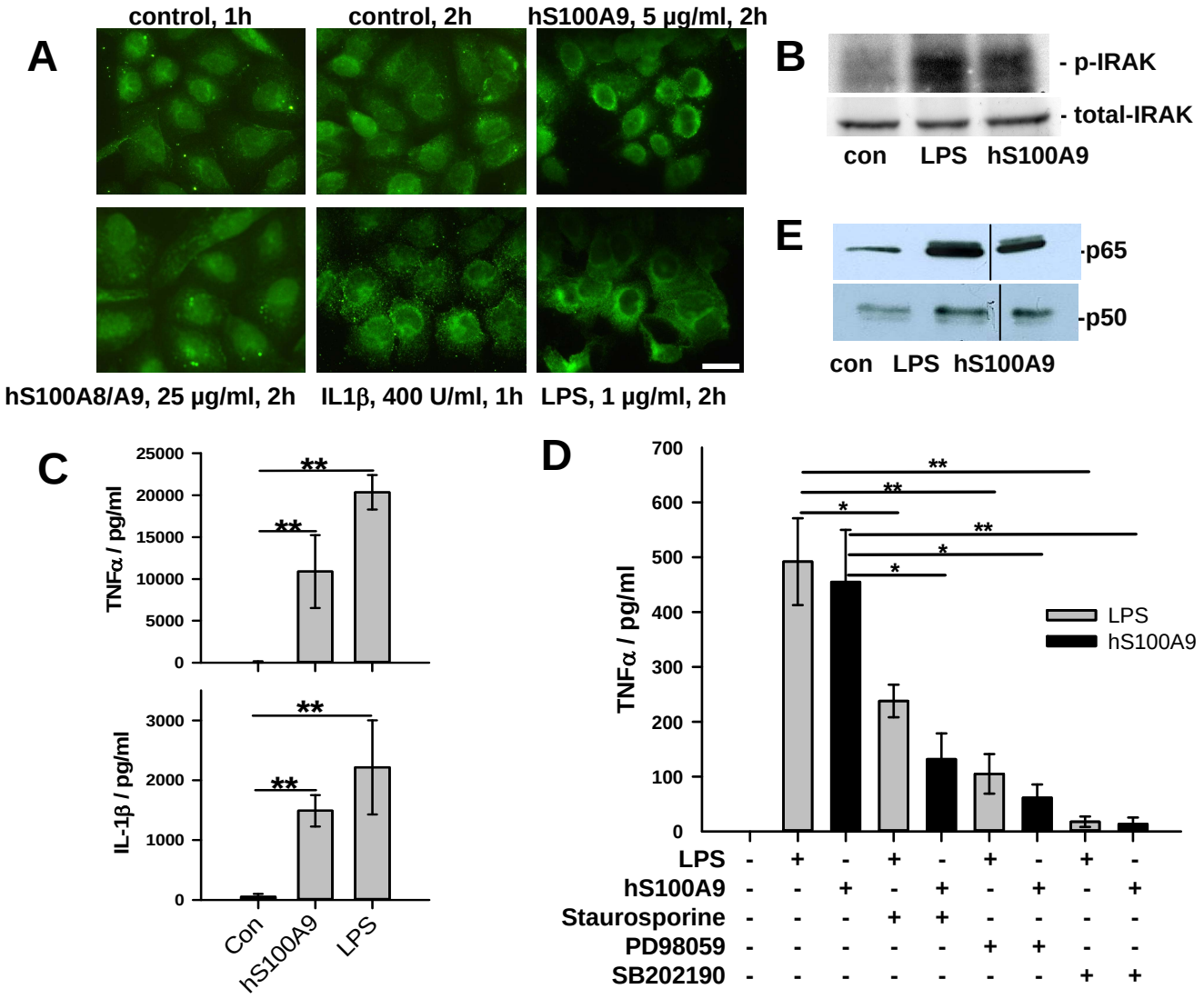

Figure S2. S100A9 induces NF-κB activation via TLR4-MD2

(A) MyD88 translocation from the cytosol to the plasma membrane in human alveolar epithelial cells (A549 cells) cultured to confluence on fibronectin-coated LabTec chamber slides. Cells were left untreated or exposed to S100A9 (5 µg/ml, 2 h), S100A8/S100A9 (25 µg/ml, 2 h), IL1β (400 U/ml, 1 h) or LPS (1 µg/ml, 2 h) and processed for immunofluorescence staining using a polyclonal anti-MyD88. Scale bar = 25 µm. (B,C) IRAK activation (B) and cytokine production (C) by human monocytes primed overnight with IFN-γ (500 U/ml) and subsequently exposed to either LPS (10 ng/ml) or S100A9 (5 µg/ml) for 45 min. In (B) cells were lysed and IRAK hyperphosphorylation (P-IRAK, autoradiography) was determined compared to loading controls (total-IRAK, western blot). In (C) cell supernatants from parallel experiments were analyzed by ELISA 4 h after stimulation for secreted TNFα and IL1β. (D) S100A9 and LPS signaling are dependent on MAP kinase p38, ERK1/2 and PKC activation. TNFα levels as determined by ELISA in human monocyte supernatants after 4 h of stimulation with LPS (10 ng/ml) or S100A9 (5 µg/ml) in the absence or presence of protein kinase inhibitors: PKC inhibitor staurosporine (10 nM), ERK1/2 inhibitor PD98059 (20 µM) or p38 inhibitor SB202190 (7 µM). (E) Human monocytes were left untreated or stimulated with S100A9 (5 µg/ml) or LPS (1 ng/ml) for 30 min. Nuclear cell extracts were prepared for subsequent western blotting for p65 and p50 subunits of NF-κB, indicating NF-κB translocation into the nucleus. One representative western blot of three independent experiments is shown. The lanes/samples were run on the same gel but were noncontiguous. The results confirmed activation of the classical TLR4 signal transduction pathway by S100A9-stimulation. (C, D) Data represent the mean ± s.d. from three independent experiments. Statistically significant differences were assessed by two tailed t-test, *p < 0.05, **p < 0.01.

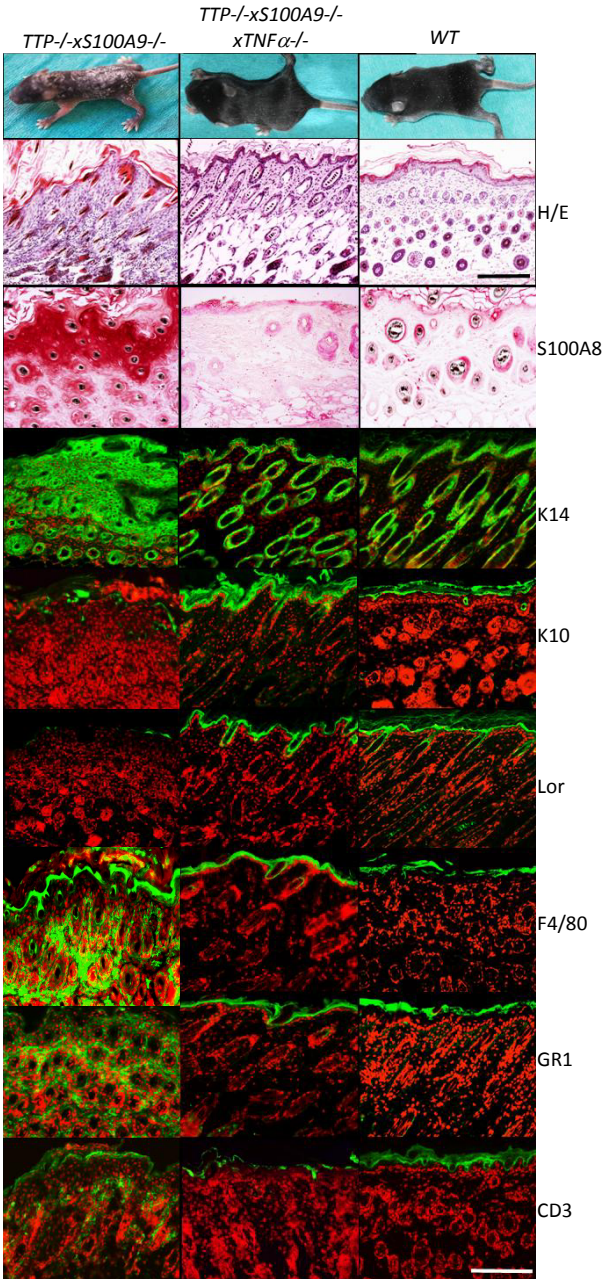

Figure S3. Knock-out of TNFα protects TTP-/-xS100A9-/- mice from a psoriasis-like phenotype
TTP-/-xS100A9-/-xTNFα-/- mice do not develop inflammatory skin disease (top panel). Skin sections were stained as described in Figure 2. Red counterstaining shows nuclei.
Scale bar: 200 µm (H&E); 50 µm (immunostaining).

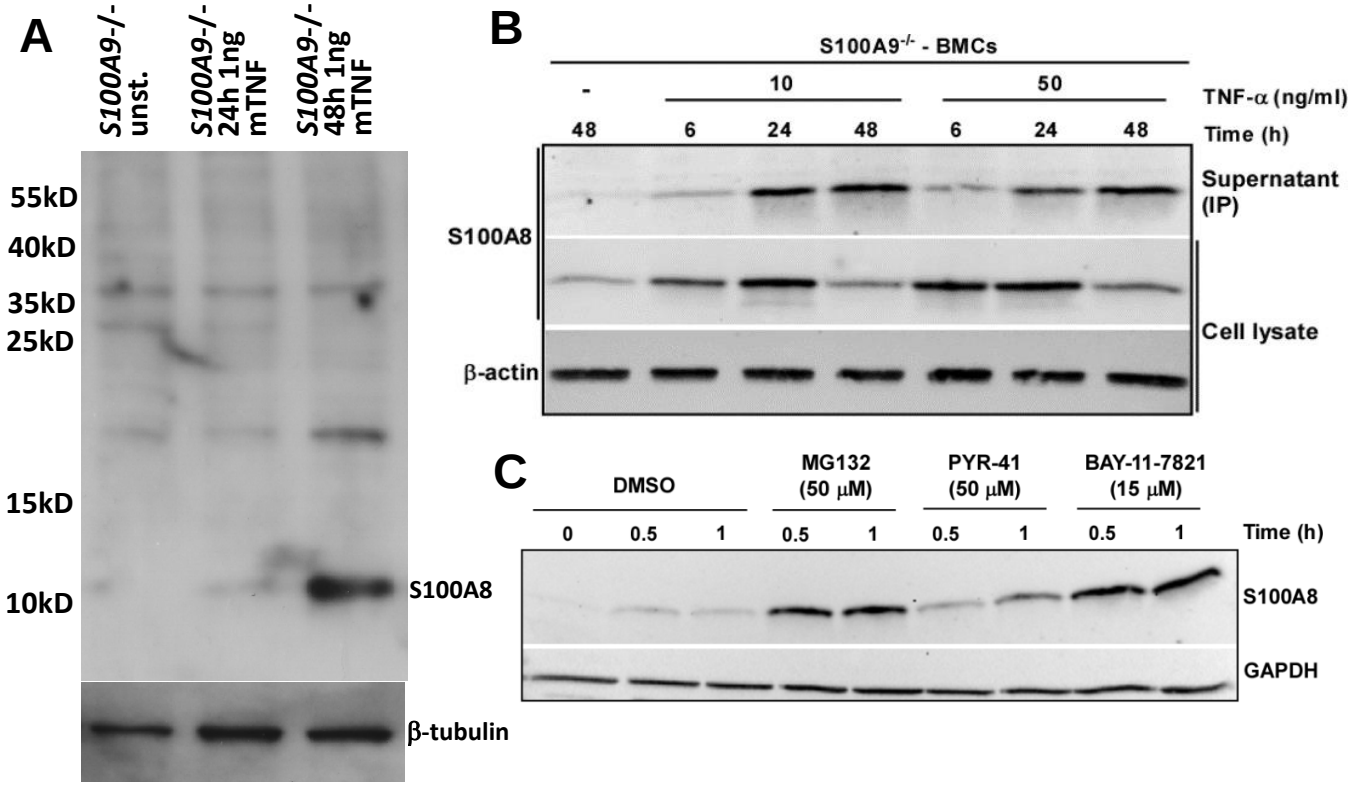

Figure S4. Recovery of S100A8 protein in isolated S100A9-/- keratinocytes (A) or bone marrow cells (B, C) after mTNFα stimulation for 2 days (A,B) or proteasome blocking (C)
Western blot analysis shows expression of S100A8 in mTNFα treated compared to untreated S100A9-/- keratinocytes isolated from newborn mice (A) or bone marrow cells from adult mice (B) at time points indicated in the figure. Shown are representative western blots of three independent experiments. (C) The figure shows immunoblotting analysis of S100A8 expression after treatment of S100A9-/- BMCs with proteasome inhibitor MG-132 (50 μM) or inhibition of enzymes responsible for ubiquitination E1 (PYR-41, 50 μM) or E2 (BAY-11-7821, 15 μM) at 0.5 h and 1 h time points compared to DMSO treated control S100A9 -/- BMCs. A long exposure time of 300 seconds was necessary to visualize faint S100A8 bands (quantification of S100A8 protein by ECL). Shown are representative western blots of three independent experiments.

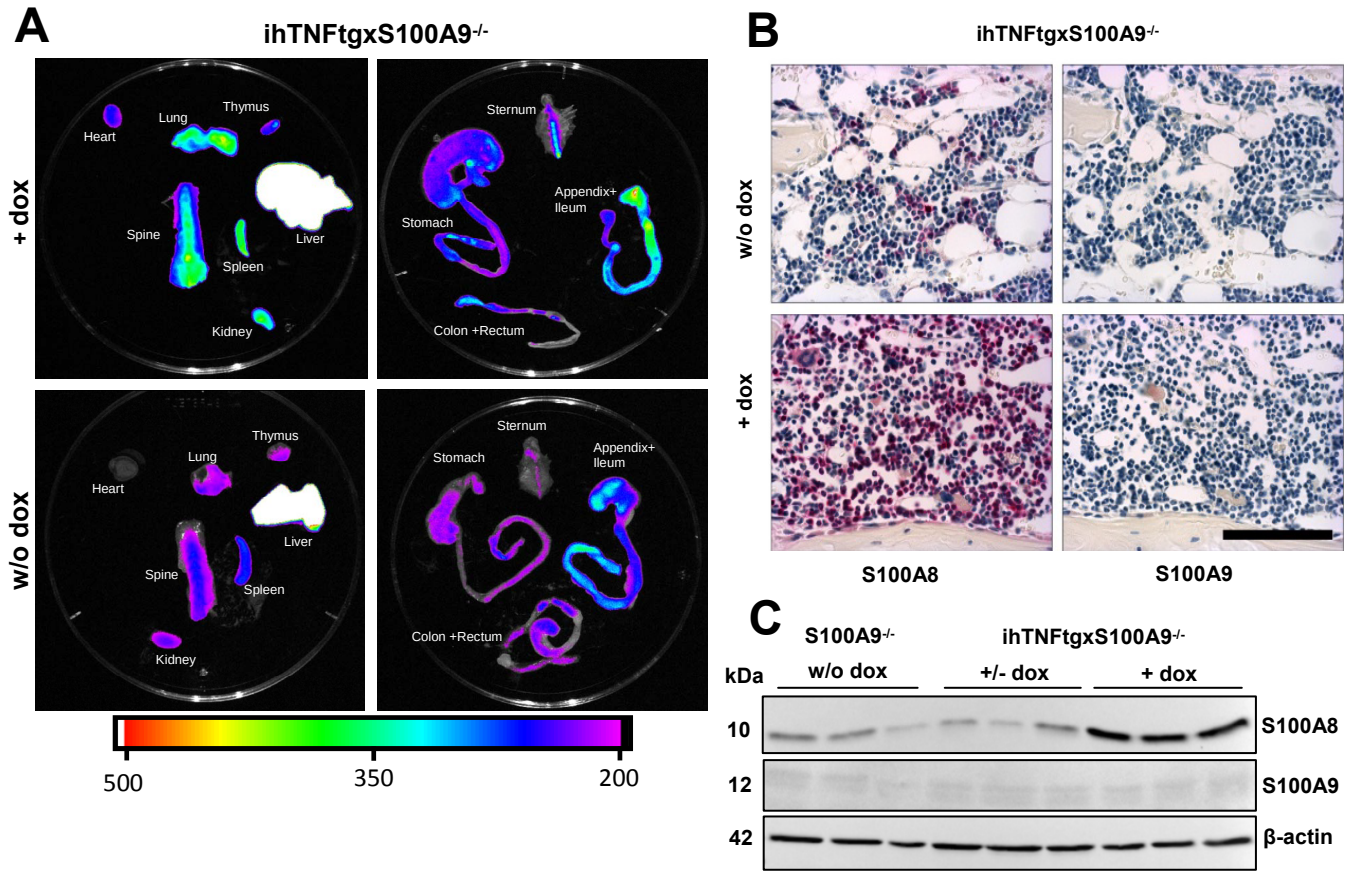

Figure S5. S100A8 protein recovery in various organs of ihTNFtgxS100A9-/- mice after TNFα induction

(A) Six-week-old ihTNFtgxS100A9-/- mice that have received 1 mg/ml dox for 33 days (upper panel) or not (lower panel) were i.v. injected with Cy5.5-labeled anti-S100A8 antibodies (2 nmol dye/mouse) and organs were harvested 24h later, placed on petri dishes and scanned for fluorescence intensity. High fluorescence values in the lung, spine, spleen, kidney and sternum were observed only in dox-treated mice, indicating S100A8 protein expression. Liver tissue always showed a high non-specific fluorescence which is not specific for S100A8 protein expression but reflects non-specific enrichment and degradation of the Cy5.5 coupled antibodies in this organ (n = 5 for each group). (B) S100A8 and S100A9 immunostainings of bone marrow cells in metacarpal sections of ihTNFtgxS100A9-/- mice in the presence (6 weeks) and absence of doxycycline confirms S100A8 recovery, as detected by FRI, Bar = 100 µm. (C) Western blot analysis of bone marrow cell samples (3 for each group) as described in Figure 4H. The data confirm S100A8 protein expression in bone marrow cells of only ihTNFtgxS100A9-/- mice on dox treatment, which was abolished when mice were kept for an additional 7 days without doxycycline (+/-dox).

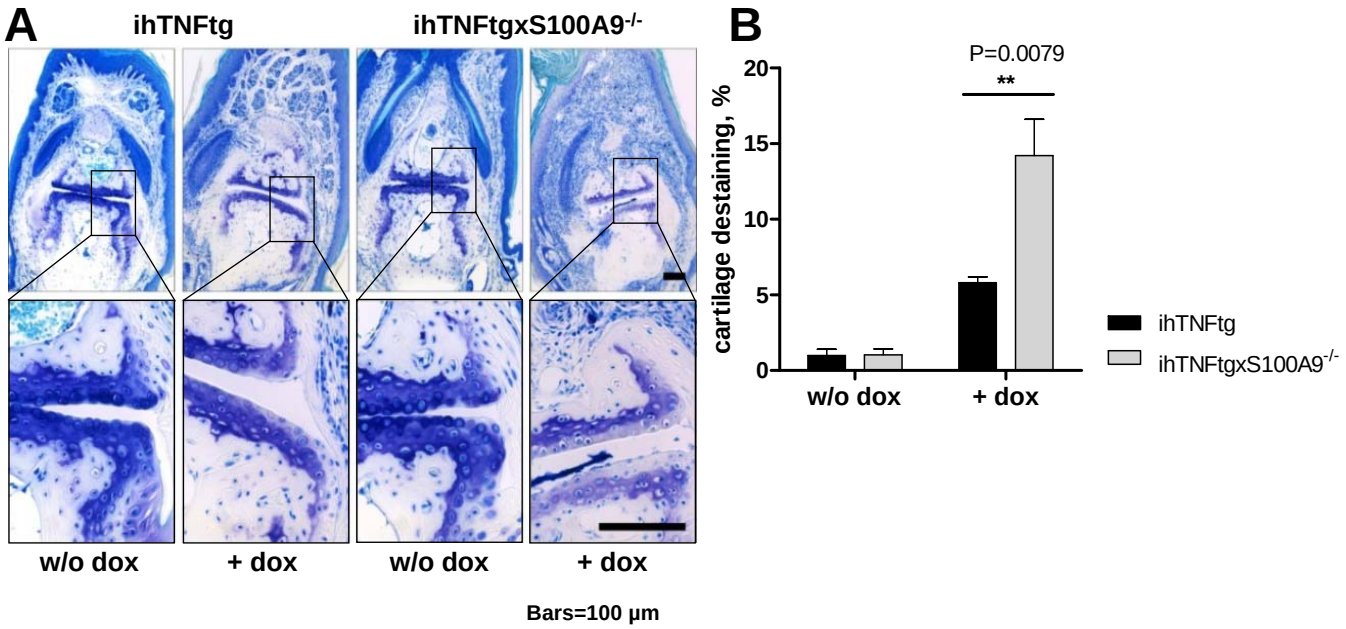

Figure S6. Cartilage is more severely affected in joints of dox-treated ihTNFtgxS100A9-/- mice compared to dox-treated ihTNFtg mice

*(A,B) ihTNFtgxS100A9-/- and ihTNFtg mice were treated with 1 mg/ml dox for 6 weeks or left untreated (w/o dox). (A) Staining of fore paw sections with toluidine blue indicate a stronger extracellular matrix degradation in DIP joints of dox-treated ihTNFtgxS100A9-/- mice compared to ihTNFtg mice. Bars = 100 µm. (B) Quantification of cartilage destaining in DIP joints (shown in (A)) by morphometry (n = 5 mice per group). Statistical analysis was performed using the Mann-Whitney U-test, **p < 0.01.*

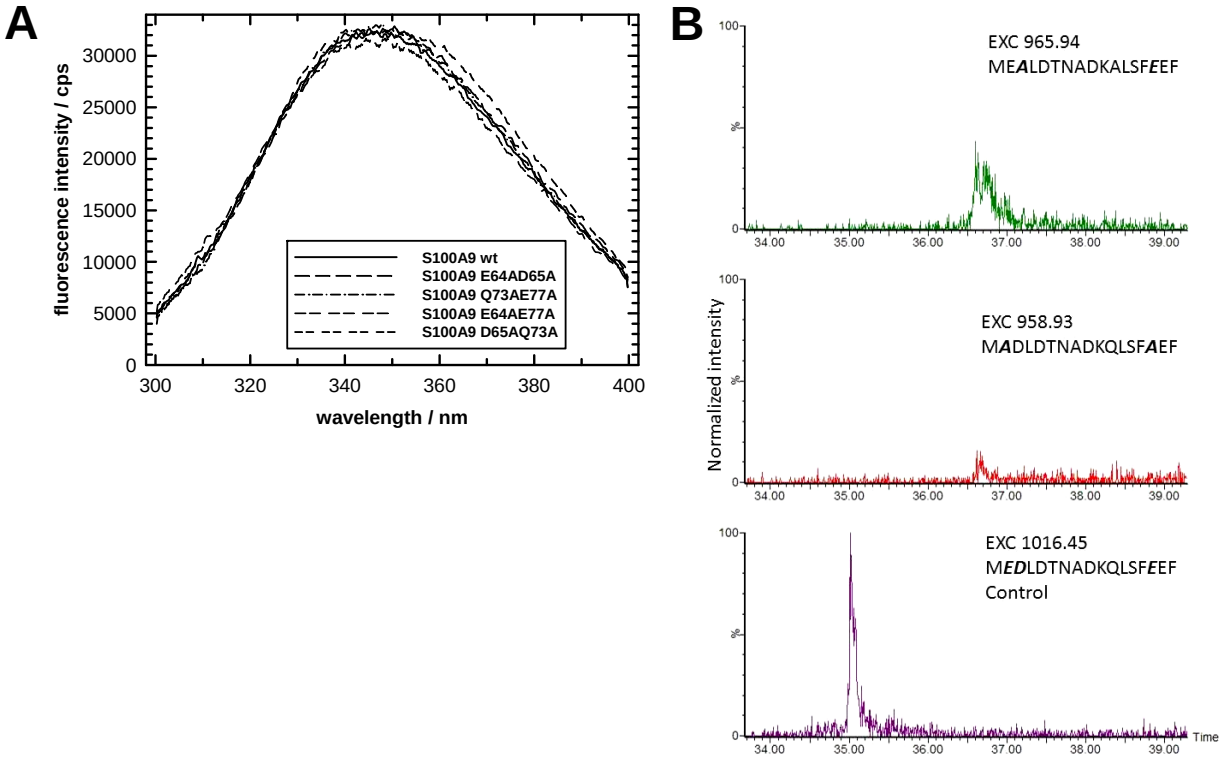

Figure S7. (A) Comparison of fluorescence emission spectra of wt-S100A9 and double mutants as indicated in the figure show no effects of the different mutations on intrinsic Trp and Tyr fluorescence properties of the proteins. Spectra were taken at 20° C in 20 mM Tris, 1 mM DTT, pH 7.5 at a protein concentration of 5 µg/ml. All spectra were buffer corrected. No structural changes could be detected in the presence of calcium indicating lack of oligomerization upon calcium binding. (B) Extracted ion chromatograms for doubly-charged ions of peptides with sequences as indicated in the figure normalized to 957 counts (as measured for the control peptide). Experiments were performed as described in Figure 5G and H and analysed by MClass reversed phase nanoliquid chromatography coupled to Synapt G2 Si mass spectrometer (Waters Corp.). Eluted peptides were confidently assigned by their retention time and their gas phase fragmentation spectra determined with pure peptide prior to the binding experiment. IP eluates were purified first using solid phase extraction (OmixTips), dried and redissolved in 5 µl 5 % acetonitrile, 0.1 % formic acid prior analysis.

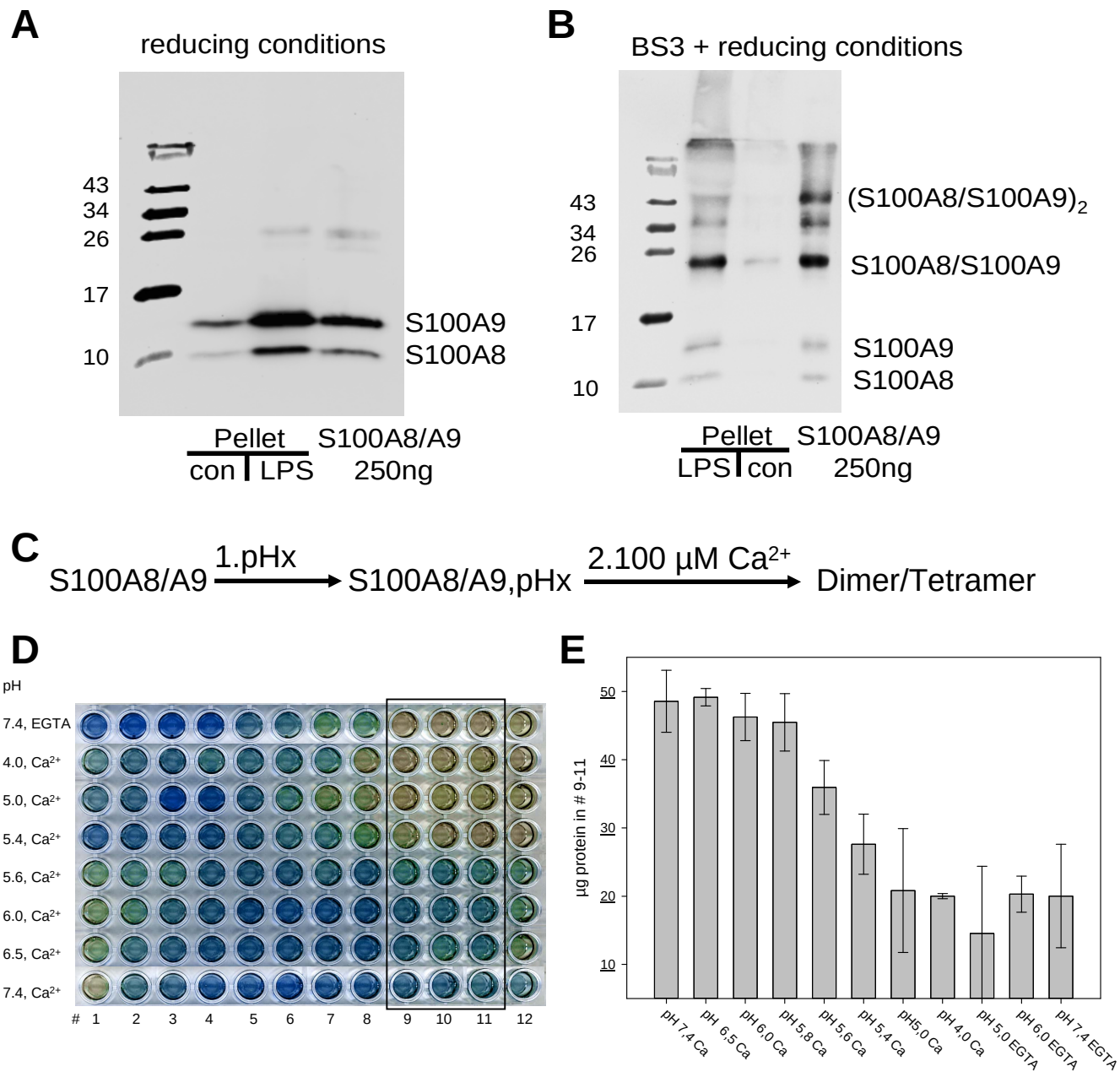

Figure S8. (A,B) Granuloma formation was induced by subcutaneous injection of 1 ml of polyacrylamide gel (PAG) in the flank regions of mice. To induce inflammation, LPS was added to PAG pellets (10 μ g of LPS/ml). The other pellet served as control (con). Implanted pellets were harvested after 24 h and analyzed for S100A8/S100A9 oligomeric forms released by immigrated phagocytes. A and B show western blot results of S100A8 and S100A9 of isolated pellets from mice in the presence and absence of LPS. (A) SDS-PAGE was run under reducing conditions to avoid artificial disulfide bridges in non-covalently associated S100A8/S100A9 complexes. Recombinant prepared and calcium bound S100A8/S100A9 served as control. In (B) the protein cross-linker BS3 was added directly after isolation of the pellets to prevent disassembly of non-covalently associated S100A8/S100A9 oligomers before SDS-PAGE separation. Purified recombinant calcium bound and BS3 cross linked S100A8/S100A9 served as control. (C) Schematic overview of pH dependency of S100A8/S100A9 tetramer formation. (D,E) Density centrifugation runs of S100A8/S100A9 at varyíng pH in the presence of either 100 µM Ca2+ or 1 mM EGTA. Complexes were loaded on top of a 20% glycerol solution and after centrifugation (24h, 35000RPM), successive collected fractions from top (#1) to bottom (#12) were analyzed by Bradford assay (D). Fractions 9-11 were chosen for analyzing dimer/tetramer moieties (E).