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Supplementary Information for

DNA threads released by activated CD4$^+$ T lymphocytes provide autocrine costimulation

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Supplementary Materials and methods

EAE. $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) and $PLP_{139-151}$ (HSLGKWLGHPDKF) were synthesized using standard 9-fluorenylmethoxycarbonyl chemistry (Applied Biosystems, Foster City, CA) and purified as previously described (1). For EAE induction 8-12 weeks old female C57BL/6 mice were immunized with $MOG_{35–55}$ peptide dissolved in PBS and emulsified with an equal volume of Complete Freund's Adjuvant (CFA) (IFA containing 8 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra, Difco Laboratories) (1). Mice were injected s.c. in their flanks with 0.1 ml of the peptide emulsion (for a total of 100 μg $MOG_{35–55}$ and 400 μg *M. tuberculosis*/mouse) and, on the same day and 48 h later, were injected i.v. with 0.2 ml containing 200 ng *Bordetella pertussis* toxin (List Laboratories) dissolved in PBS. For T cell-priming experiments *in vivo*, EAE mice were treated intraperitoneally with vehicle (PBS with 5% DMSO) or SKQ1 (1250 nmoli/kg) every day from day 0 to day 7, when they were sacrified for *in vitro* experiments (n =5 mice/group). For the evaluation of therapeutic efficacy of mtROS inhibition, after disease onset EAE mice were randomized in two groups with similar disease score and were injected intraperitoneally with vehicle (PBS with 5% DMSO) or SKQ1 (1250 nmoli/kg) every day, until the end of the experiment. Mice were daily assessed for neurologic signs of EAE according to the following five-point scale: 0, healthy; 1, tail weakness or paralysis; 2, paraparesis (incomplete paralysis of one or two hind limbs/plegia of one hind limb); 3, paraplegia extending to the thoracic level; 4, forelimb weakness or paralysis with hind limbs paraparesis or paraplegia; and 5, moribund or dead animal. The wire-hang test was performed as described (2), to assess the ability of

EAE mice to hang to a wire cage lid when inverted (up to a maximum of 60 seconds). The longest hang duration of three tests/mouse was recorded. During pharmacological treatments, experimenters were blinded to the treatment regimen.

Pathological studies. At day 31 post-immunization, EAE mice were perfused with 4% paraformaldehyde and spinal cords and brains were removed and post-fixed o.n. in 10% formalin. Two μm paraffin-embedded sections were used for routine Haematoxylin and Eosin (H&E) staining to assess basic histopathological changes. The degree of inflammation was quantified by measuring the number of inflammatory infiltrates per mm^2. For immunohistochemistry studies, sections were stained with rat anti–myelin basic protein (MBP) antibody (1/50 dilution; Millipore, Temecula, CA) for myelin MBP and revealed by a rat-on-mouse HRP-polymer kit (Biocare Medical, Concord, CA, USA) using 3,3′-diaminobenzidine tetrahydrocloride (DAB, 0.05%) as a chromogen. Demyelination was quantified with a morphometric method using ImageJ software by measuring the percentage of demyelinated area over the total white matter area for each single spinal cord section. Neuropathological findings were quantified on an average of at least 7-8 complete cross-sections of spinal cord per mouse unaware of the identity of individual sections (n=3 mice for vehicle group; n=4 mice for SKQ1-treated group). Images are from 4X and 40X original magnification (scale bars: 500 μm and 100 μm respectively). Graphs (mean ± SEM) show comparative analysis of amount of infiltration (number of infiltrates per mm^2) and percentage of demyelinated areas in spinal cord.

Supplementary References.

1. Costanza M, Musio S, Abou-Hamdan M, Binart N, & Pedotti R (2013) Prolactin is not required for the development of severe chronic experimental autoimmune encephalomyelitis. *J Immunol* 191(5):2082-2088.

2. Lewis KE*, et al.* (2014) Microglia and motor neurons during disease progression in the SOD1G93A mouse model of amyotrophic lateral sclerosis: changes in arginase1 and inducible nitric oxide synthase. *J Neuroinflammation* 11:55.

Supplementary Figures and Table.

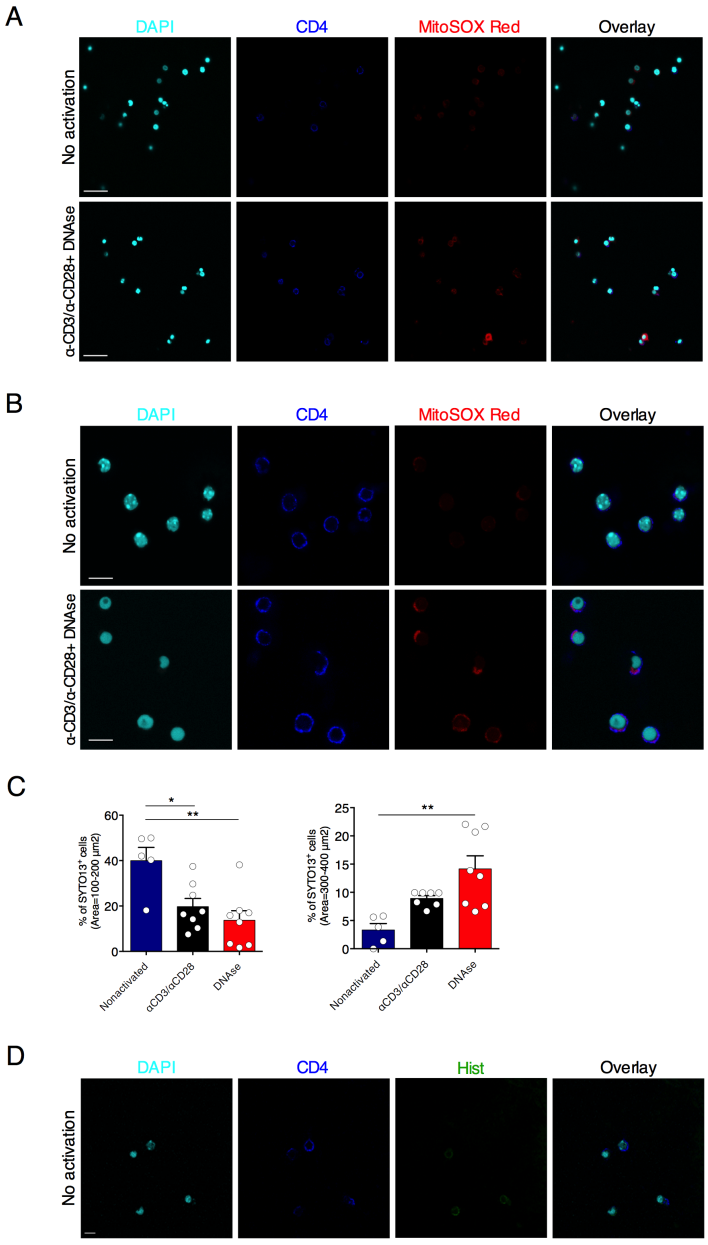

Fig. S1. Release of DNA threads by CD4$^+$ T cells *in vitro*. (A-B) Representative confocal fluorescence micrographs of human enriched CD4$^+$ T cells (A) or mouse naïve CD4$^+$ T cells (B) left nonactivated or activated with α-CD3 and α-CD28 Abs in presence of DNAse. After 24h of culture, T cells were fixed with 4 % paraformaldehyde and stained for DNA (DAPI, cyan) CD4 (blue) and MitoSOX Red; scale bars: 25 µm (A) and 10 µm (B). (C) Statistical analysis of software-assisted quantification of DNA threads released by naïve CD4$^+$ T cells left nonactivated or activated 24h with α-CD3 and α-CD28 Abs in absence or presence of DNAse. * $P < 0.05$; ** $P < 0.01$ by one-way ANOVA with Tukey's multiple comparisons test. (D) Representative confocal fluorescence micrographs of mouse naïve CD4$^+$ T cells left nonactivated for 24h, fixed with 4% paraformaldehyde and stained for DNA (DAPI, cyan), CD4 (blue) and histones (green). Scale bar: 10 µm. (A-D) Data are representative of three independent experiments.

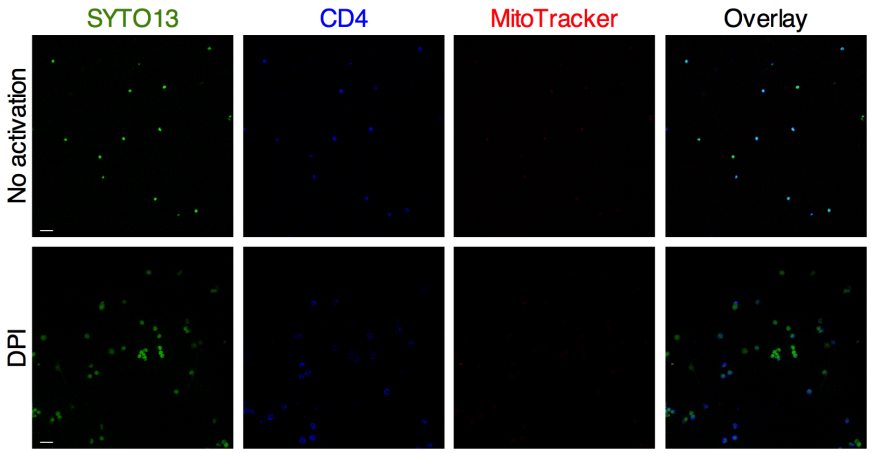

Fig. S2. DPI inhibits THREDs release. Mouse naïve $CD4^+$ T cells were left nonactivated or activated (24h) with α-CD3/α-CD28 Abs in presence of DPI (10 μM), a NAPDH inhibitor. Cells were stained for DNA (SYTO13, green), CD4 (blue) and MitoTracker (red); scale bars: 20 μm. Data are representative of three independent experiments.

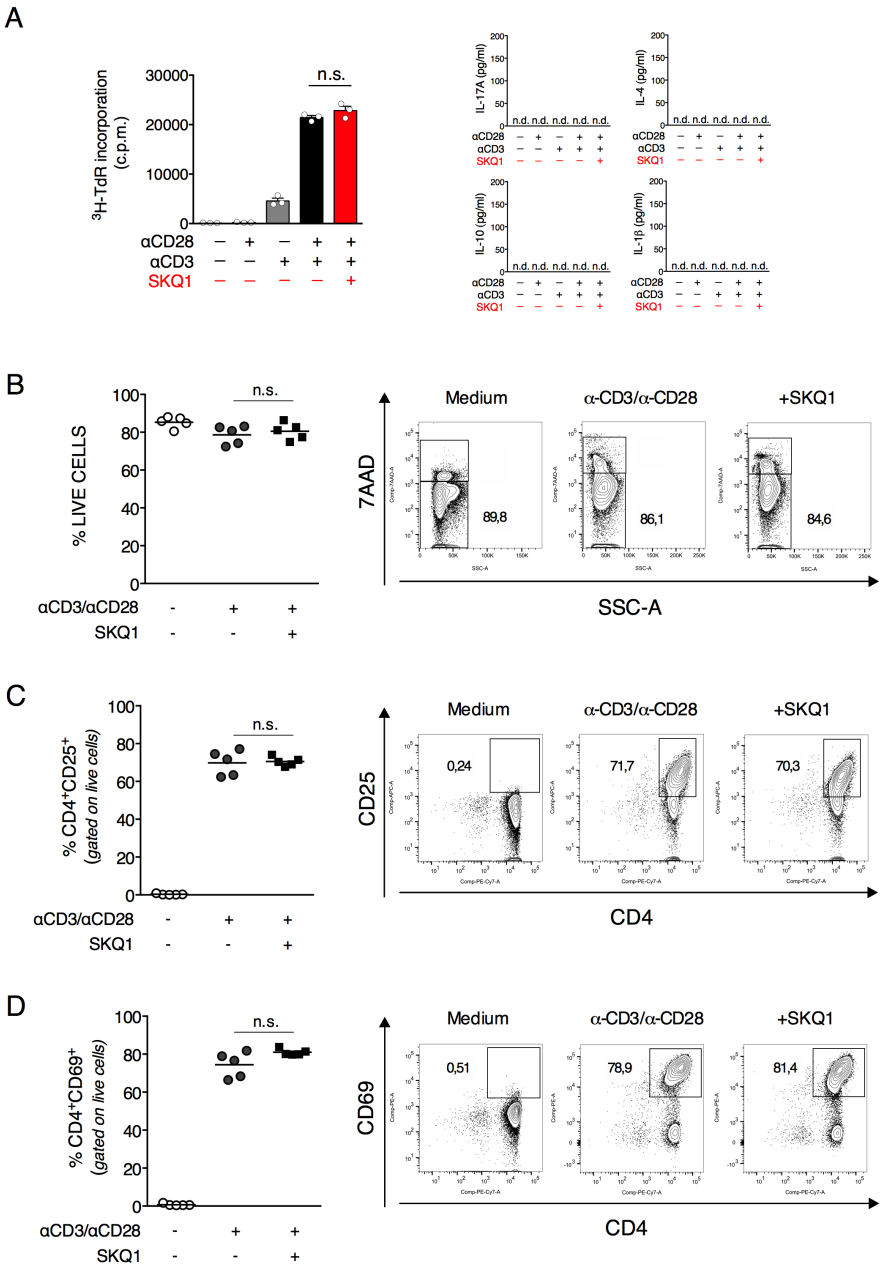

Fig. S3. **Analysis of the effects of mtROS inhibition on T cell functions.** (A) Mouse naïve CD4$^+$ T cells were left nonactivated or activated as depicted with or without SKQ1 (150 nM) and analyzed for proliferation rate by [^{3}H]-thymidine (^{3}H-TdR) incorporation assay (counts per minute, c.p.m.) (48h + 18h) and cytokine production by ELISA (72h). Data represent mean ± SEM of quadruplicate wells for proliferation and quintuplicate wells pooled and tested in duplicate for cytokine production. Cell viability and activation were evaluated after 24h of culture by staining with 7AAD (B) and Abs directed to surface markers CD25 (C) and CD69 (D). Statistical analysis by two-tailed unpaired *t* test (A) and Mann-Whitney *U* test (B-D). (A-D) Data are representative of n=3 independent experiments. (n.d., not detectable; n.s., not significant).

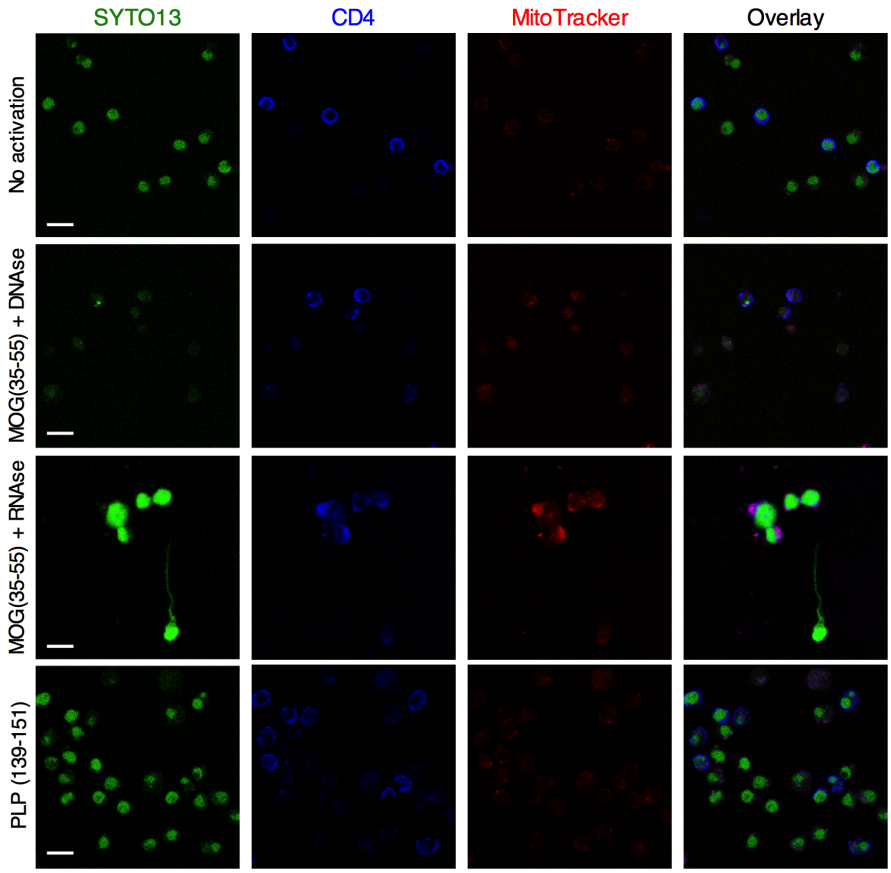

Fig. S4. THREDs extrusion by MOG_{35-55}-primed T cells. Total $CD4^+$ T cells purified from MOG_{35-55}-immunized C57BL/6 mice (n=5) at 7 dpi were re-stimulated with mitomycin C-treated syngenic splenocytes in absence or presence of $PLP_{139-151}$ or MOG_{35-55} plus DNAse or RNAse. After 72h cells were analyzed by confocal microscopy for DNA (SYTO13, green) CD4 (blue) and MitoTracker (red); scale bar: 20 μm.

Group	N° of mice	EAE score at treatment start	Cumulative Disease Score	Recovery to score 0-1 (%)[†]
Experiment 1				
Vehicle	8	2,2 ± 0,42	66,8 ± 2,4	0/8 (0)
SKQ1	9	2,3 ± 0,17	40,1 ± 3,9 **	7/9 (78)
Experiment 2				
Vehicle	6	2,3 ± 0,42	48,7 ± 3,8	0/6 (0)
SKQ1	6	2,0 ± 0,26	32,3 ± 4,5 *	4/6 (67)

Table S1. EAE in vehicle- and SKQ1-treated mice.
C57BL/6 mice with chronic EAE were i.p. injected with vehicle or SKQ1 daily after the onset of the disease until the end of the experiment (from 9 to 31 d.p.i. in experiment 1 and from 13-16 to 30 d.p.i. in experiment 2) and monitored for clinical signs of the disease. Data are mean ± SEM. ** $P = 0.0012$, * $P = 0.026$ by Mann-Whitney U test. [†] Number of mice showing clinical score of 0 or 1 for at least two consecutive days at the end of the experiment.

Legends for Supplementary videos.

Supplementary Video 1. Representative naïve female C57BL/6 mouse subjected to the wire-hang test.

Supplementary Video 2. Representative chronic EAE mouse treated with vehicle from 9 to 31 d.p.i. and subjected to the wire-hang test.

Supplementary Video 3. Representative chronic EAE mouse treated with SKQ1 from 9 to 31 d.p.i. and subjected to the wire-hang test.