Molecular Medicine

Supplemental Data

Dichotomy between RIP1- and RIP3-Mediated Necroptosis in Tumor Necrosis Factor-α–Induced Shock

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SUPPLEMENTAL MATERIALS AND METHODS

Reagents and antibodies

The purified rat α-mouse CD16/CD32 antibody and the α-FADD and α-RIP1 were purchased from BD Biosciences (Heidelberg, Germany). The α-caspase-8 monoclonal antibody was from Cell Signaling (Frankfurt, Germany). Antibodies LEAF™ purified α-mouse Ly-6G, α-mouse CD49b-PE (pan-NK cells), α-mouse Ly-6G/Ly-6C (Gr-1) and α-mouse F4/80-PE were purchased from BioLegend (Uithoorn, Netherlands). The rabbit α-asialo GM1 lyophilisate was acquired from Wako Chemicals (Neuss, Germany). Clodronate liposomes, termed Clodrolip, and empty control liposomes were freshly prepared as described previously [1]. The small unilamellar Clodrolip liposomes for the *in vivo* studies contained 18 mg clodronate/ml. The inactive necrostatin-1 (Nec-1i) was purchased from Calbiochem (Darmstadt, Germany).

Cloning, expression and purification of recombinant proteins

Bacterial expression and purification of recombinant TAT-crmA fusion protein were slightly modified from the previously described procedure [2]. Briefly, after induction of the TAT-crmA transformation of bacteria for 3 hours at 37°C with 0.4 mM IPTG, cells were harvested by centrifugation (6,500 x g, 10 min, 4°C) followed by sonification in binding buffer (500 mM NaCl, 20 mM Tris-HCl and 5 mM imidazole, pH 7.9). Suspensions were clarified by centrifugation (14,000 x g, 20 min, 4°C), and supernatants containing TAT proteins were purified under native conditions using pre-equilibrated nickel-nitrilotriacetic acid columns (QIAGEN, Hilden, Germany). To remove background of contaminating bacterial proteins, columns were washed by stepwise additions of increasing imidazole concentrations. Finally, target proteins were eluted with an elution buffer containing 500 mM NaCl, 20 mM Tris-HCl and 100 mM imidazole (pH 7.9). Removal of salt was performed using a disposable PD-10 (Sephadex G-25) desalting column equilibrated with RPMI medium supplemented with 0.026 % albumin. Fusion protein was either used immediately after purification or stored at -20°C. To control quality and confirm quantification, protein samples were analyzed by gel electrophoresis (data not shown).

A TAT-construct containing the crmA sequence mutated in amino acid position 291 (named mut.TAT-crmA) is identified as a loss-of-function crmA [3] and served as a specific negative control for unspecific effects mediated by the TAT domain and as negative control for the protease targeting region of crmA. Thereby, this mutant lacks the ability to protect against PCD. This plasmid was generated by site-directed mutagenesis through replacement of the triplet ACA (threonine) at position 291 against CGC (arginine) using the QuickChangeII Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The resulting construct of a selected colony harbouring the cDNA of mut.TAT-crmA was transformed into competent *E.coli* bacteria, followed by induction of expression in LB media with IPTG as described for TAT-crmA.

The pEGFP-RIPK3 expression construct was a kind gift by John Silke, La Trobe University, Australia. Stable transfection of HEK-293 cells using PolyFect reagent (QIAGEN) was performed according to the manufacturer's instructions by using 4 μg DNA per 100-mm dish.

Animals and in vivo models

6 week old female Balb/c and *nu/nu* mice (average weight approx. 20 g) were purchased from Charles River (Sulzfeld, Germany). All mice were kept on a standard diet and a 12 hour day night rhythm. All *in vivo* experiments were performed according to the *Protection of Animals Act* after approval.

Depletion of immune cells in vivo

For the depletion of macrophages, NK-cells or granulocytes Balb/c mice were treated once 24 hours (macrophages) or 48 hours (NK-cells and granulocytes) prior TNFα injection with 100 mg clodronate/kg body weight [1], 100 μl of α-asialo GM1 serum [4] or 100 μg α-Ly-6G [5] in 200 μl total volume (supplemented with PBS) i.p., respectively (n = 9). Quantification of depletion was confirmed in each 3 of these mice by FACS analysis after the isolation of

splenocytes using α-mouse CD16/CD32 followed by α-mouse F4/80-PE (for macrophages) or after the isolation of whole blood using α-mouse CD49b (for NK-cells) or α-mouse Ly-6G/Ly-6C (for granulocytes). The remaining 6 mice from each group were used in the TNFα-mediated shock model.

SUPPLEMENTAL REFERENCES

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2. Krautwald S, Ziegler E, Rolver L, Linkermann A, Keyser KA, SteenP, Wollert KC, Kunzendorf U. (2010) Effective blockage of both the extrinsic and intrinsic pathways of apoptosis in mice by TAT-crmA. *J.Biol.Chem.* 285: 19997-20005.
3. Ekert PG, Silke J, Vaux DL. (1999) Inhibition of apoptosis and clonogenic survival of cells expressing crmA variants: optimal caspase substrates are not necessarily optimal inhibitors. *EMBO J.* 18: 330-338.
4. Bodhankar S, Woolard MD, Sun X, Simecka JW. (2009) NK cells interfere with the generation of resistance against mycoplasma respiratory infection following nasal-pulmonary immunization. *J.Immunol.* 183: 2622-2631.
5. Stegemann S, Dahlberg S, Kroger A, Gereke M, Bruder D, Henriques-Normark B, Gunzer M. (2009), Increased susceptibility for superinfection with Streptococcus pneumoniae during influenza virus infection is not caused by TLR7-mediated lymphopenia. *PLoS.One.* 4: e4840.

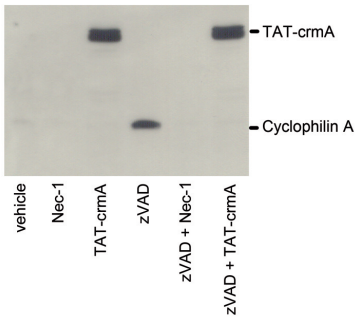

Figure S1. Cyclophilin A release in zVAD-treated L929 cells is prevented by Nec-1 or TAT-crmA
L929 cell were treated with or without 25 μM zVAD in the presence or absence of 30 μM Nec-1 or 500 μM TAT-crmA. Release of cyclophilin A into the supernatant was detected by western blot. Note the unspecific detection of TAT-crmA by the cyclophilin A antibody.

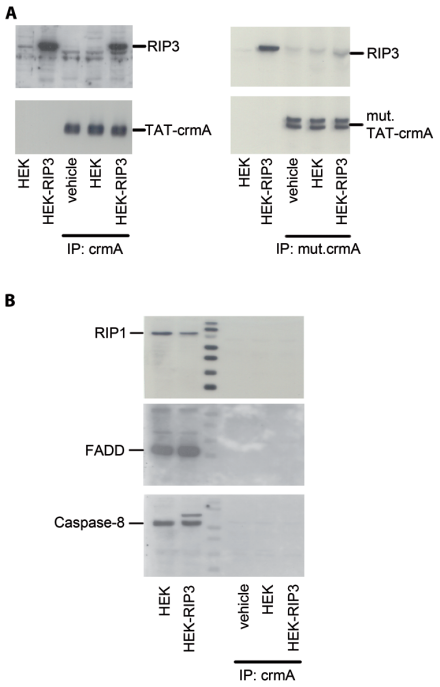

Figure S2. TAT-crmA coprecipitates RIP3 from overexpressing HEK cells
Parental HEK cells or RIP3-transfected HEK cells were used for co-immunoprecipitation assays as indicated. (A) TAT-crmA, but not mut.TAT-crmA precipitated RIP3. (B) The other components of complex II and/or the ripoptosome (RIP1, FADD and caspase-8) were not precipitated under these conditions.

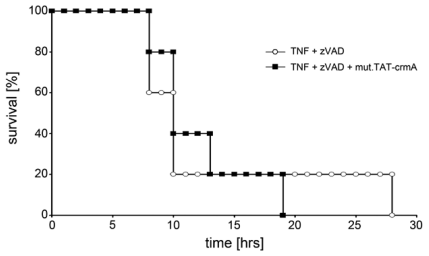

Figure S3. Mutated TAT-crmA does not influence survival following TNF /zVAD-mediated hyperacute shock
Mice were treated with TNFα + zVAD in the absence (open circles) or presence of mutated TAT-crmA (mut.TAT-crmA, black squares). Survival is presented as a Kaplan-Meyer plot. Whereas TAT-crmA accelerates death after TNFα/zVAD-application, not statistically significant difference is observed with the mutant (n = 5).

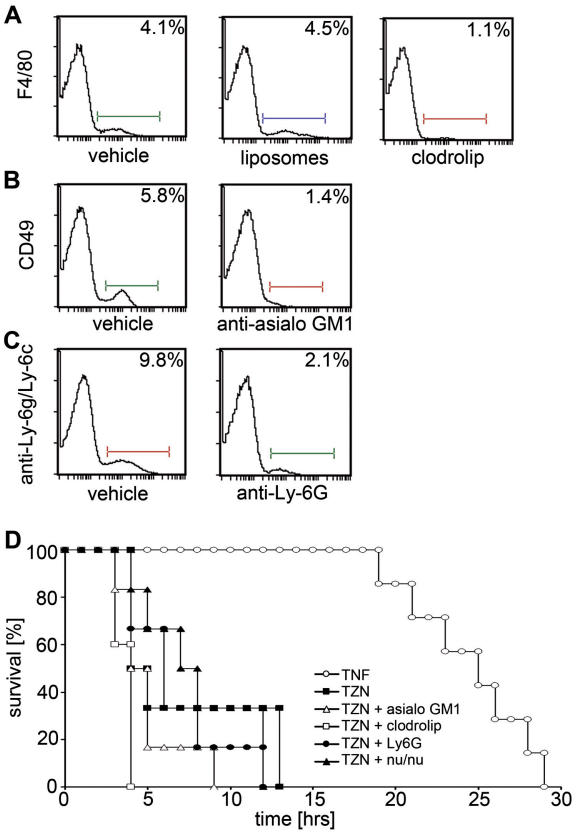

Figure S4. T-cell deficiency in Balb/c *nu/nu* mice or *in vivo* depletion of macrophages, NK-cells and granulocytes in Balb/c mice does not prolong survival after hyperacute TNFα/zVAD/Nec-1-mediated shock

(A - C) demonstrates successful depletion of the indicated immune cells *ex vivo* by using the indicated antibodies. (D) Control mice were treated with TNFα, only, as in Fig. 4A (open circles). All other mice were injected intravenously with TNFα and intraperitoneally with zVAD/Nec-1 (TZN, black squares). T-cell deficiency in Balb/c *nu/nu* mice (black triangles) or depletion of macrophages (open squares), NK-cells (open triangles) or granulocytes (black dots) before TNFα/zVAD/Nec-1 injection resulted in the indicated survival rates (n = 6).

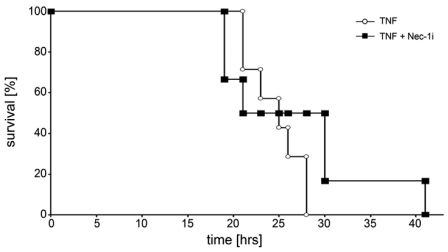

Figure S5. Inactive necrostatin-1 (Nec-1i) does not influence survival following TNFα-induced shock

Mice were treated with 25 μg TNFα in the absence (open circles) or presence of inactive necrostatin-1 (Nec-1i, black squares). Survival is presented as a Kaplan-Meyer plot. Nec-1i does not accelerates death after TNFα application (n = 6).

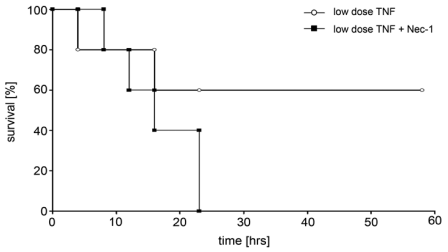

Figure S6. Nec-1 accelerates time to death after treatment with low dose TNFα

Mice were treated with low dose mouse TNFα alone (9 μg per mouse, open circles) or with the combination of low dose TNFα + Nec-1 (black squares). Survival is presented as a Kaplan-Meyer plot (n = 5, $p < 0.05$).