Ubiquitin-conjugating Enzyme Ubc13 is a Critical Component of
TRAF-mediated Inflammatory Responses

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Abbreviations: TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor;
TAK1, transforming growth factor β-activated kinase 1; IL, interleukin; JNK, c-Jun N-
terminal kinase; MAPK, mitogen-activated protein kinase
Summary

Ubc13 is an ubiquitin-conjugating enzyme responsible for non-canonical ubiquitination of TRAF-family adapter proteins involved in Toll-like Receptor (TLR) and TNF-family cytokine receptor signaling. Gene ablation was used to study the function of Ubc13 in mice. While homozygous ubc13 gene disruption resulted in embryonic lethality, heterozygous ubc13+/− mice appeared normal without alterations in immune cell populations. Haploinsufficient ubc13+/− mice were resistant to lipopolysaccharide (LPS)-induced lethality, and demonstrated reduced in vivo ubiquitination of TRAF6. Macrophages and splenocytes isolated from ubc13+/− mice exhibited reduced LPS-inducible cytokine secretion and impaired activation of TRAF-dependent signal transduction pathways (NF-κB, JNK, p38 MAPK). These findings document a critical role for Ubc13 in inflammatory responses, and suggest that agents reducing Ubc13 activity could have therapeutic utility.
Introduction

Ubc13 is an Ubiquitin-conjugating enzyme (E2) that catalyzes the attachment of unusual polymers of Ubiquitin (E1) onto target proteins, where the Ubiquitin chain is linked via non-canonical lysine 63 (1-3) instead of canonical lysine 48 (4). Unlike K48-linked Ubiquitin polymers, these K63-linked Ubiquitin chains are not substrates for the proteasome (5, 6). Instead, K63-linked poly-Ubiquitination plays roles in protein activation, protein interactions, and subcellular targeting of proteins (7-9).

Ubc13 has been implicated in two cellular processes. First, in collaboration with cofactor Uve1A, it binds the RING domains of TRAF-family adapter proteins and promotes activation of protein kinases involved in signaling by Tumor Necrosis Factor (TNF)-family cytokine receptors (TNFRs) (10, 11) and by Toll-like Receptors (TLRs) (12). Second, in collaboration with cofactor Mms2, Ubc13 participates in DNA replication and repair pathways, probably by modifying PCNA, Rad5, Pol30 (13-15).

With regards to the role of Ubc13 in TRAF-mediated signaling, cytokines that activate TRAF2 result in its non-canonical ubiquitination and subsequent activation of the kinase JNK. Ubc13 modification of TRAF2 was linked to the translocation of TRAF2 to insoluble membrane domains (9), suggesting a role for non-canonical ubiquitination in targeting TRAFs to specified signaling compartments. Ubc13 was also associated with the activation of NF-κB, via its effect on TRAF6, resulting in activation of the kinase TAK1 (8).

Genes encoding six different TRAF-family adapter proteins are found within the human genome. TRAFs are multidomain proteins that contain a N-terminal RING domain (with the exception of TRAF1), followed by a series of zinc-finger domains, and
then the TRAF domain – a β-strand fold that binds several TNFRs or other proteins that become recruited to TNFR and TLR receptor complexes (16). Binding of Ubc13 to TRAFs depends on the RING domain, and mutants version of TRAF2, 5, or 6 that lack the RING domain function instead as dominant-negative inhibitors of signaling by the TNFRs and TLR/ILRs that bind them (7, 9, 17, 18). Reducing Ubc13 expression using RNA interference (RNAi) has demonstrated its role in the regulation of stress kinases and the NF-κB pathway (9).

To explore the cellular functions of Ubc13, we employed the method of targeted gene ablation in mice. We show here that while homozygous ablation of the gene encoding Ubc13 results in embryonic lethality, heterozygous mice have normal phenotypes, despite reduced levels of Ubc13 protein. However, ubc13+/ mice exhibit marked reductions in responsiveness to challenge with LPS, which was employed as a representative TRAF-dependent stimulus. Macrophages and lymphocytes cultured from these mice also display blunted responses to LPS and TNF with respect to cytokine secretion and various TNFR/TLR-mediated signal transduction events. The findings therefore document a rate-limiting role for Ubc13 in signaling by TNFRs and TLRs, and furthermore suggest that Ubc13 may be an attractive target for design of new therapeutic agents for treatment of certain inflammatory and autoimmune disorders.

Results

Ubc13+/ mice are born normally and exhibit no abnormalities in immune cell populations.
To test the in vivo function of Ubc13 in mice, we turned to a commercially available collection of murine ES cell clones that contain single retroviral insertions into the mouse genome, identifying a clone in which a retroviral provirus is integrated into the first intron of the *ubc13* gene in reverse orientation (Fig. 1A). These ES cells were used to generate heterozygous mice, and the retrovirus-targeted *ubc13* gene was confirmed by polymerase chain reaction (PCR) analysis of genomic DNA using specific primers (Fig. 1B). Breeding together of heterozygous *ubc13*+/- never resulted in any homozygous *ubc13*+/- progeny, in over 36 matings. Analysis of embryos from timed-pregnancies suggested that *ubc13*+/- embryos die or fail to progress very early, probably before E5.5 days. In contrast, heterozygous *ubc13*+/- were born with normal Mendelian frequencies and appeared normal at birth, showing no gross developmental abnormalities or differences in size or weight compared to wild-type *ubc13*+/- littermates. Organ sizes were also not different, including thymus and spleen, which contained similar numbers of mononuclear cells in *ubc13*+/- and *ubc13*+/- mice (Fig. 1C).

We assessed various lymphocyte populations in *ubc13*+/- mice by immunofluorescence-based detection of surface markers, in conjunction with flow cytometry analysis. No differences between wild-type *ubc13*+/- and heterozygous *ubc13*+/- mice were observed with respect to the proportions of pre-B-cells in bone marrow (B220/sIgM) (Fig. 1D), thymocytes subtypes defined by CD4, CD8, CD25, and CD44 surface markers (Fig. 1E), and mature T-cells (CD3) and B-cells (IgM/IgD) in spleen (Fig. 1F). We conclude therefore that *ubc13* haploinsufficiency does not appear to alter normal immune system development. Furthermore, *ubc13*+/- mice had normal lifespan, and remained healthy typically for ≥ 2 years, displaying no tendencies toward
increased incidence of infection, autoimmunity or malignancy, compared to wild-type littermates.

**Ubc13 haploinsufficient mice are resistant to LPS challenge.**

TRAF6 plays an essential role in signaling by several TLRs, including the LPS receptor, TLR4 (19, 20). Because Ubc13 is required for TRAF6 activation (7), we compared the effects of LPS challenge on age-matched female ubc13+/+ and ubc13+/− mice, using a dose of LPS reported to be lethal for normal mice (21). These experiments showed that heterozygous ubc13+/− mice are significantly more resistant to LPS-induced lethality, with approximately half as many mice dying following challenge with 250 mg/kg LPS compared to wild-type mice (< 30% ubc13+/− vs > 60% ubc13+/+; p <0.001 by ANOVA) (Fig. 2). Thus, ubc13 heterozygosity was associated with resistance to the lethal effects of LPS.

**Ubc13+/− cells secrete less cytokines in response to LPS.**

LPS induces massive cytokine production contributing to its lethal effects in vivo. We therefore assessed the effects of LPS on cytokine production in cultures of primary splenocytes (Fig. 3A) and primary macrophages (Fig. 3B) differentiated in vitro from bone marrow of ubc13+/+ and ubc13+/− mice. LPS induced significantly less TNF, IL-6 and INFγ production in cultures of splenocytes and macrophages derived from heterozygous ubc13+/− mice compared to homozygous ubc13+/+ animals. Differences in cytokine production were more striking for splenocytes than for macrophages, but statistically significant in both cases.
Impaired activation of signal transduction pathways in ubc13\(^{+/-}\) mice stimulated with LPS.

Because Ubc13 collaborates with TRAFs in activation of a variety of downstream kinases (8, 9), we compared the status of the JNK and p38 MAPK in ubc13\(^{+/-}\) and ubc13\(^{+/+}\) mice after LPS challenge. For these experiments, mice were injected i.p. with LPS, then sacrificed 6 hrs later and spleens were recovered for preparation of protein lysates, which were analyzed by immunoblotting using phospho-specific antibodies specific for phospho-JNK and phospho-p38 MAPK. Compared to wild-type ubc13\(^{+/+}\) mice, spleens of haploinsufficient ubc13\(^{+/-}\) mice contained less phospho-JNK and phospho-p38 MAPK after LPS challenge (Fig. 4A). Total levels of JNK and p38 MAPK proteins however were similar in ubc13\(^{+/-}\) and ubc13\(^{+/+}\) mice, as shown by immunoblots using antibodies that react with phospho-independent epitopes on these proteins. Note that immunoblotting also revealed lower levels Ubc13 protein in the spleens of heterozygous ubc13\(^{+/-}\) mice compared to homozygous, wild-type ubc13\(^{+/+}\) mice (Fig. 4A), as expected.

In vitro experiments have shown that TRAFs become polyubiquitinated in a Ubc13-dependent manner upon ligation of TNFRs and TLRs (7, 22). To determine whether Ubc13 regulates the polyubiquitination of TRAF6 in vivo, we immunoprecipitated TRAF6 from spleen lysates prepared from LPS-treated ubc13\(^{+/-}\) and ubc13\(^{+/+}\) mice and then analyzed the immunoprecipitated proteins by immunoblotting using anti-Ubiquitin antibody. Indeed, the extent of ubiquitin conjugation of TRAF6 was greater in lysates prepared from wild-type ubc13\(^{+/+}\) compared to haploinsufficient ubc13\(^{+/-}\) mice (Fig. 4B). Immunoblot analysis revealed similar total levels of TRAF6 in
spleen tissue of these mice. These data thus provide the first evidence that Ubc13 modulates polyubiquitination of TRAF6 in vivo.

Experiments using cultured cells from $ubc13^{+/\text{-}}$ mice reveal selective impairment in signal transduction initiated by members of the TNFR and TLR family.

To further define the signaling defect in immune cells of $ubc13^{+/\text{-}}$ heterozygous mice, we performed experiments using primary splenocytes and primary macrophages, stimulating these cells in vitro with ligands that engage TRAF-dependent TNFRs (e.g. TNF) and TLRs (e.g. LPS). Comparisons were made with the TRAF-independent ligands – namely, γ-TriDAP (H-Ala-D-γ-Glu-diaminopimelic acid), a component of peptidoglycan that stimulates the NLR-family protein Nod1 (23), and UV-irradiation, a potent inducer of JNK and p38 MAPK activation (24). Signal transduction events examined in cultured cells included assessment of phosphorylation of JNK and p38 MAPK using phospho-specific antibodies (as above) and determination of levels of IκBα, a suppressor of p65/p50 NF-κB that becomes degraded in the context of TNFR/TLR signaling as a result of IκB kinase (IKK) activation (25-29).

Splenocytes and macrophages from heterozygous $ubc13^{+/\text{-}}$ mice showed clear reductions in one or both of these signal transduction end-points compared to wild-type $ubc13^{/+}$ cells, following stimulation with LPS or TNF. Macrophages, for example, displayed a striking difference in IκBα degradation following LPS treatment, while differences in phosphorylation of JNK and p38 MAPK were less robust (Fig. 5A). In contrast, cultured splenocytes exhibited a clear reduction in phosphorylation of JNK and p38 MAPK, but less striking differences in LPS-inducible IκBα degradation (Fig. 5B).
Nevertheless, LPS-stimulated splenocytes from $ubc13$ haploinsufficient mice produced less NF-κB DNA binding activity than $ubc13$ wild-type animals, based on electromobility gel-shift assays (EMSAs) (Fig. 6, which is published as supporting information on the PNAS web site). Exploration of a range of concentrations of LPS potentially could reveal doses at which these differences in signal transduction events are emphasized. For TNF, reductions in both TNF-induced degradation of IκBα and in TNF-stimulated phosphorylation of JNK and p38 MAPK were clearly evident in both macrophages and splenocytes derived from heterozygous $ubc13^{+/-}$ compared to wild-type $ubc13^{+/+}$ mice (Fig. 5C and D). Immunoblot analysis demonstrated comparable reductions in Ubc13 protein levels in macrophages and splenocytes of $ubc13^{+/+}$ compared to $ubc13^{+/-}$ mice.

In contrast to LPS and TNF, stimulation of splenocytes from $ubc13^{+/-}$ and $ubc13^{+/+}$ mice revealed little difference in induction of JNK and p38 phosphorylation by the Nod1 agonist γTriDAP or by UV-irradiation (Fig. 5E and F). The decline in IκBα levels induced by γTriDAP was also not substantially different in $ubc13^{+/-}$ compared to $ubc13^{+/+}$ cells. UV-irradiation caused little decline in IκBα levels in either $ubc13^{+/-}$ or $ubc13^{+/+}$ cells. Taken together, these data demonstrate that $ubc13$ haploinsufficiency selectively blunts signaling in the context of TLR and TNFR activation.

The differences in signal transduction events observed in heterozygous $ubc13^{+/-}$ cells are not the secondary result of a difference in cell proliferation or survival. In this regard, IκBα, phospho-JNK, and phospho-p38 MAPK levels were measured within 1 hr after stimulation, before changes in cells growth of survival. Also, analysis of the percentage of viable B220⁺ splenocytes by FITC-annexin V binding at 24 hr post-stimulation with LPS revealed only a modest (< 10%) difference in the percentage of
surviving cells (Fig. 7A, which is published as supporting information on the PNAS web site). Similarly, determination of the percentage of cycling splenocytes as determined by bromo-deoxyuridine (BrdU) incorporation after 24 hrs of LPS stimulation revealed only ~10% difference, suggesting that ubc13 haploinsufficiency only slightly alters LPS-induced proliferation of lymphocytes (Fig. 7B, which is published as supporting information on the PNAS web site).

**Discussion**

Here, we provide evidence that Ubc13 plays a rate-limiting role in vivo in signal transduction mediated by TRAF-dependent receptors of the TNFR and TLR families. Using haploinsufficient ubc13+/− mice, we observed striking decrements in responses to LPS compared to wild-type ubc13+/+ mice with respect to lethality, polyubiquitination of TRAF6, and activation of protein kinases that operate as downstream mediators of inflammatory signaling. Cells derived from heterozygous ubc13+/− mice also displayed blunted responses with respect to LPS and/or TNF-induced cytokine secretion, phosphorylation of JNK and p38 MAPK, and IκBα degradation. In contrast, signaling induced by TRAF-independent stimuli was not altered by ubc13 haploinsufficiency.

The heterozygous ubc13+/− mice employed here show a clear reduction in levels of Ubc13 in macrophages and splenocytes compared to their wild-type ubc13+/+ littermates. The extent of the decrement in Ubc13 protein levels in other types of cells in these haploinsufficient mice remains to be determined. Cell type-specific or developmental stage-specific differences in Ubc13 protein levels could impact the phenotype of animals.
In our mice in which retroviral insertional mutagenesis inactivates one *ubc13* allele, however, *ubc13* haploinsufficiency did not cause apparent aberrations in lymphocyte differentiation or homeostasis.

We found that homozygous disruption of *ubc13* resulted in embryonic lethality at a very early stage of development. The explanation for this early lethality is unknown, but it could be related to the alternative role that Ubc13 plays in DNA replication and repair (14). The rapid cell division that characterizes the early embryo may necessitate that Ubc13 is available during development. Though *ubc13*+/− splenocytes demonstrated only a modest decline in S-phase entry, the residual levels of Ubc13 protein in these haploinsufficient cells presumably are sufficient to sustain proliferative responses. This notion is also supported by the observation that *ubc13*+/− mice contain normal numbers of immune cells, suggesting that any role of Ubc13 in DNA replication and repair is adequately preserved in the haploinsufficient state.

Though additional studies are required before firm conclusions can be reached, the phenotype of haploinsufficient *ubc13*+/− mice suggests that the unusual E2 encoded by this gene may represent an attractive target for drug discovery efforts aimed at producing new therapies for inflammatory or autoimmune diseases. However, given the involvement of TRAFs in receptor-mediated signaling pathways important for innate immunity, the anti-inflammatory benefits of Ubc13-targeted therapeutics must be weighed against risk of infection. Use of *ubc13* haploinsufficient mice in clinically relevant models of acute and chronic inflammatory and autoimmune diseases may reveal the best scenarios for exploiting Ubc13 as a therapeutic target.
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Materials and Methods

Generation of ubc13 Haploinsufficient Mice.

Searching the Omni Bank database (http://www.lexgen.com) for ES cell clones with retrovirus insertions in the ubc13 gene, we found a clone (OST374154) in which the integration site for the retrovirus was located in the first intron of mouse ubc13 gene. This ES cell clone was used to derive mice on a C57Bl6/129SV background. The position of retrovirus insertion was confirmed by PCR using primers distinguishing the germline and mutant genes: The primers used to detect ubc13 germline were: forward 5’- AACTACAGGTGTTATCCATCACAC-3’ and reverse 5’- TACCAAAAGATTCTCTTGCC-3’. Targeted ubc13 was detected using the same reverse primer described above and the forward primer 5’- GGCGTTACTTAAGCTAGCTTGC-3’. Female mice of 4-6 month-old were injected i.p. with LPS (Escherichia coli 026:B6, L8274, Sigma) and monitored hourly to assess time of death.
Flow Cytometry.

Cells were prepared from thymus glands, bone marrow, and spleens of untreated mice, and then stained with fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), cyanine dye coupled peridinin chlorophyll protein- (PerCP-Cy5.5), or allophycocyanin- (APC) conjugated antibodies (BD Biosciences Pharmingen) as previously described (30). Cells were flow-sorted using a FACSCanto (BD Biosciences Pharmingen) and data were analyzed with FlowJo software (Tree Star).

Cell isolation and Culture.

Splenocytes were harvested from spleens of sacrificed 2-3 month-old Ubc13+/− and littermate control mice using mouse erythrocyte lysing kit (R&D systems). The resulting cells were cultured at 37°C with 5% CO2 in RPMI 1640 medium with 0.5 % FCS and 50 μM β-mercaptoethanol before LPS stimulation. Bone marrow cells were harvested from hind leg bones of sacrificed 2-3 month-old Ubc13+/− and littermate control mice. Bone marrow cells were cultured at 37°C with 5% CO2 in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS). CSF-1 (Sigma) was added to the medium for the first week to promote differentiation. Adherent macrophages were stimulated for 24 hrs with various amounts of LPS.

Immunoblotting.

Cell extracts were prepared with lysis buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM Na3VO4, 10 mM NaF, 10 mM Na2P2O7, 1 mM EGTA, 1 mM EDTA, 1% NP-40, and
protease inhibitor cocktail (Roche). Equal amounts of cell lysates were subjected to
immunoblot analysis. Anti-JNK, anti-phospho-JNK (Thr183/Thr185), anti-p38, anti-
phospho-p38 (Thr180/Tyr182) antibodies were obtained from Cell Signaling, Inc. Anti-
IκBα and anti-TRAF6 were purchased from Santa Cruz Biotechnology, Inc. Anti-Ubc13
was obtained from Zymed laboratories. Tissue extracts were obtained using tissue-
grinder (Knotes Glass co.) with the lysis buffer, and then centrifuged at 12,000 x g for 5
min. Equal amounts of the lysates were subjected to immunoblot analysis.

In vivo Ubiquitination Assay.

Mice were injected i.p. with 150 mg/kg LPS and sacrificed 6 hrs later. Spleen tissue
lysates were prepared and normalized for total protein content. Protein extracts (1 mg)
were subjected to immunoprecipitation with rabbit polyclonal anti-TRAF6 antibody
(Santa Cruz) conjugated to protein A Sepharose 4B. Beads-bound proteins were then
eluted in SDS-sample buffer and subjected to immunoblotting with anti-ubiquitin
antibody (COVANCE).

Cytokine Measurements.

Macrophages and splenocytes were cultured at 5 x 10^5 cells/ml with or without LPS for
24 hrs. Culture supernatants then were assayed for murine TNFα, IL-6, and INFγ using
enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems, Inc. Data were
normalized for cell numbers, and assays were performed in triplicate.
References


**Figure Legends**

**Fig. 1.** Targeted disruption of mouse *ubc13* gene does not alter immune cell populations.

(A) The targeting vector, wild-type *ubc13* allele, and targeted allele are depicted. The vector was targeted to the first intron of the *ubc13* locus. The closed rectangles denote exons of *ubc13*. (B) PCR analysis of genomic DNA extracted from mouse tails. Primers were designed to amplify the regions of wild-type and mutant alleles. PCR products of wild-type (WT) and mutated (KO) alleles are shown for two mice of each genotype. The genotypes of mice are presented above the lane. (C) Comparison of total cell numbers of the thymocytes and splenocytes in *ubc13*+/+ and *ubc13*+/- mice (mean ± SD; n = 5 female mice of age 2 to 3-month-old). Flow cytometry analysis of lymphocyte subpopulations in bone marrow (D), thymus (E), and spleen (F) of *ubc13*+/+ and *ubc13*+/- mice. (D) IgM and B220 expression of the total bone marrow population. (E) Thymocytes were stained with antibodies to CD4, CD8, TCRγδ, DX5, B220 (lineage marker, Lin), CD25, and CD44. Upper: CD4 and CD8 expression of the total thymocyte population. Lower: CD44 and CD25 expression (gated in Lin- cells). (F) Upper: CD3 and B220 expression of the total splenocyte population. Lower: IgM and IgD expression of the total splenocyte population. Numbers in quadrants indicate percentage of positive cells in that region. Data are representative of three different experiments using age- and sex-matched mice.
Fig. 2.  *Ubc13*+/− mice are resistant to LPS induced lethality.

Female mice (age 4-6 month) were injected i.p. with 250 mg/kg LPS, then monitored hourly. The proportion of surviving *ubc13*+/− (white circles) and *ubc13*+/*+ (black circles) mice is indicated (n = 13-14 mice per group). Statistical significance was determined by ANOVA, *p* < 0.001.

Fig 3.  *Ubc13* is required for LPS-induced cytokine production.

Production of TNF, IL-6, and IFNγ in cultures of *ubc13*+/+ and *ubc13*+/− macrophages (A) and splenocytes (B) was analyzed 24 hr after stimulation with LPS (10 μg/ml). Data represent mean ± SD of triplicate samples, and are representative of 3 independent experiments. *, *p* < 0.05 and **, *p* < 0.005, compared with wild-type mice (Student’s *t*-test).

Fig. 4.  Reduced LPS stimulation of phosphorylation of JNK and p38 MAPK and diminished polyubiquitination of TRAFs in haploinsufficient *ubc13*+/− mice.

(A) Wild-type and *ubc13*+/− mice were injected i.p. with 150 mg/kg LPS and sacrificed 6 hrs later. Spleen tissue lysates were prepared, normalized for total protein content, and analyzed by SDS-PAGE/immunoblotting using phospho-specific or pan-specific JNK and p38 MAPK antibodies or anti-Ubc13 antibody. (B) Spleen lysates from 150 mg/kg LPS-treated (+) or untreated (-) *ubc13*+/+ or *ubc13*+/− mice were subjected to immunoprecipitation using anti-TRAF6 antibody. Immune-complexes (top) or lysates (bottom) were analyzed by immunoblotting using anti-Ubiquitin (top) and anti-TRAF6 antibodies (bottom).
Fig. 5. Impaired signal transduction in *ubc13* haploinsufficient cells treated with TRAF-dependent stimuli.

Macrophages (A) and splenocytes (B) of *ubc13*+/+ and *ubc13*+/- mice were stimulated with TNF (10 ng/ml) for various times. Degradation of IκBα and phosphorylation of JNK and p38 were detected by immunoblot analysis using antibodies to IκBα, phospho-JNK (p-JNK), and phospho-p38 (p-p38). Total amounts of JNK and p38 are also presented as loading controls. Levels of Ubc13 were determined using anti-Ubc13 antibody. (C and D) Macrophages (C) and splenocytes (D) of *ubc13*+/+ and *ubc13*+/- mice were stimulated with LPS (20 μg/ml) for various times. Degradation of IκBα and phosphorylation of JNK and p38 were detected by immunoblot analysis using antibodies to IκBα, phospho-JNK (p-JNK), and phospho-p38 (p-p38). Total amounts of JNK and p38 are also presented as loading controls. Levels of Ubc13 were determined using anti-Ubc13 antibody. (E and F) Splenocytes of *ubc13*+/+ and *ubc13*+/- mice were stimulated with γTriDAP (5 μg/ml) (E) and UV-irradiation (10 J/m²) (F) for various times. Normalized cell lysates were analyzed by immunoblotting for degradation of IκBα and phosphorylation of JNK and p38 using antibodies to IκBα, phospho-JNK (p-JNK), and phospho-p38 (p-p38). Total amounts of JNK and p38 are also presented as loading controls.
Time (hrs)

Survival (%)
A

- TNF (pg/ml): Ubc13+/+ vs. Ubc13+-
- IL-6 (pg/ml): Ubc13+/+ vs. Ubc13+-
- IFN-γ (pg/ml): Ubc13+/+ vs. Ubc13+-

B

- TNF (ng/ml): Ubc13+/+ vs. Ubc13+-
- IL-6 (ng/ml): Ubc13+/+ vs. Ubc13+-
- IFN-γ (pg/ml): Ubc13+/+ vs. Ubc13+-

* P< 0.05
** P< 0.005