

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Activation of KIT modulates the function of tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) in mast cells

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Keywords

apoptosis; KIT; mast cells; mastocytosis; tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R).

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Abstract

Background: Mastocytosis is characterized by the accumulation of mast cells (MCs) associated with activating mutations of *KIT*. Tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL-Rs) are preferentially expressed on neoplastic cells and induce the extrinsic apoptotic pathway. Recent studies reported on the expression of TRAIL-Rs and TRAIL-induced apoptosis in cultured human MCs, which depend on stem cell factor (SCF)-induced or constitutive KIT activation.

Material and methods: We sought to further define the impact of TRAIL-Rs on MCs *in vivo* and *in vitro*. Using Cre/loxP recombination, we generated mice with MC-specific and ubiquitous knockout of TRAIL-R. In these mice, anaphylaxis and numbers of MCs were investigated. We also explored the expression and function of TRAIL-Rs in cultured murine and human MCs upon activation of KIT. By conducting immunofluorescence staining, we analyzed the expression of TRAIL-Rs in MCs infiltrating the bone marrow of patients with mastocytosis.

Results: MC-specific deletion of TRAIL-R was associated with a slight, but significant increase in anaphylaxis. Numbers of MCs in MC-specific knockouts of TRAIL-R were comparable to controls. Whereas cultured IL-3-dependent murine MCs from wild-type mice were resistant to TRAIL-induced apoptosis, SCF-stimulated MCs underwent apoptosis in response to TRAIL. Interestingly, activating *KIT* mutations also promoted sensitivity to TRAIL-mediated apoptosis in human MCs. In line with these findings, MCs infiltrating the bone marrow of patients with mastocytosis expressed TRAIL-R1.

Conclusions: Activation of KIT regulates the function of TRAIL-Rs in MCs. TRAIL-R1 may represent an attractive diagnostic and therapeutic target in diseases associated with *KIT* mutations, such as mastocytosis.

Abbreviations

ActD, actinomycin D; BMMC, bone marrow-derived mast cell; Cpa3, carboxypeptidase A3; CTMC, connective tissue-type mast cell; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; MALP-2, macrophage-activating lipopeptide-2; MC, mast cell; Mcpt5, mast cell protease-5; MMC, mucosal mast cell; PCMC, peritoneal cell-derived mast cell; PI, propidium iodide; SCF, stem cell factor; TRAIL-R, tumor necrosis factor-related apoptosis-inducing ligand receptor.

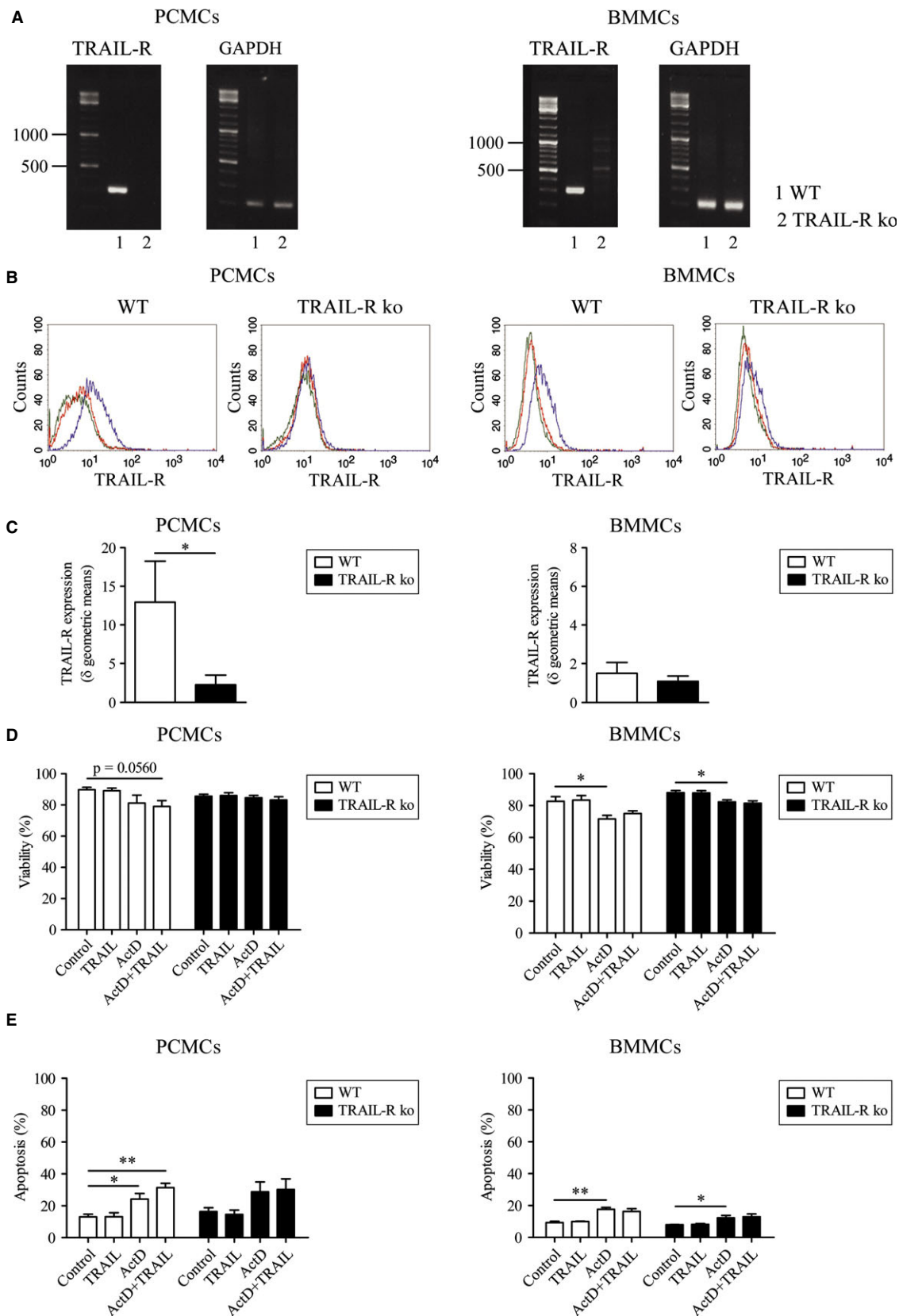


Figure 1 Characterization of TRAIL-R knockout PCMCs and BMMCs. (A) RT-PCR was performed for TRAIL-R and GAPDH in PCMCs and BMMCs from TRAIL-R^{ko} mice and wild-type controls, $n = 3$. (B) TRAIL-R surface expression was determined on PCMCs and BMMCs by flow cytometry, blue lines: receptor staining, red lines: isotype controls, and green lines: unstained controls. (C)

TRAIL-R expression was calculated as difference of mean fluorescence intensity between TRAIL-R-labeled cells and isotype controls; *indicates $P < 0.05$, mean $\pm$ SEM, $n = 3$ –4. Viability (D) and apoptosis (E) of PCMCs and BMMCs were analyzed upon incubation with TRAIL and ActD; *indicates $P < 0.05$, and **indicates $P < 0.01$, mean $\pm$ SEM, $n = 3$.

Mast cells (MCs) play crucial roles in allergies and exert major functions in innate and adaptive immune responses (1–4). They are long-lived cells, which arise from pluripotent hematopoietic stem cells of the bone marrow, circulate in blood, and undergo maturation after migration into tissues (5). Human MCs are subdivided into MCs expressing the proteases tryptase and chymase (MC_{TC}) and MCs expressing just tryptase (MC_T), while murine MCs are categorized into connective tissue-type mast cells (CTMCs) and mucosal mast cells (MMC). CTMCs are predominantly located in dermis, peritoneal cavity, and submucosa of the gastrointestinal tract, whereas MMCs exclusively reside in mucosal epithelia of the gastrointestinal tract and in the airways.

Pathologic accumulation of MCs is observed in mastocytosis, a heterogeneous disease with MC infiltrates in skin, bone marrow, and other tissues (6–9). Due to increased release of MC mediators, patients with mastocytosis suffer from anaphylaxis, pruritus, flushing, headache, abdominal cramping, and diarrhea (10). Some patients develop advanced categories, such as aggressive systemic mastocytosis and MC leukemia, presenting with cytopenia, hepatosplenomegaly, ascites, malabsorption, and weight loss. Usually, mastocytosis is associated with somatic *KIT* mutations, which induce constitutive activation of the receptor KIT leading to enhanced proliferation and activation of MCs (11). The most frequent mutation is *KIT* D816V, located in exon 17 of *KIT*. Current treatment options include avoidance of MC mediator release, H1 antihistamines, IFN- α , cladribine (2-chlorodeoxyadenosine), and tyrosine kinase inhibitors, which target the constitutive KIT activation. However, treatment is often not sufficient to control all symptoms.

Tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL-Rs) belong to the TNF receptor superfamily and can induce the extrinsic apoptosis pathway upon stimulation with the ligand TRAIL (12–14). TRAIL-Rs are primarily expressed on neoplastic cells and therefore represent ideal targets for cancer therapy (15–17). In fact, a series of preclinical studies with TRAIL-R-targeting agents demonstrated cancer cell-specific pro-apoptotic activity in various tumors. Moreover, phase I and phase II single-agent and combination studies on TRAIL-R-targeting agents showed only mild side-effects (18). In humans, there are two apoptosis-inducing TRAIL-Rs, TRAIL-R1 (DR4) and TRAIL-R2 (KILLER/DR5), and three non-apoptosis-inducing TRAIL-Rs, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and the soluble receptor osteoprotegerin (OPG). In mice, there is only one apoptosis-inducing TRAIL-R, mTRAIL-R (mDR5), and three non-apoptosis-inducing TRAIL-Rs that are members of the TNF receptor superfamily, but not homologues of the human non-apoptosis-inducing TRAIL-Rs (12).

Several recent studies reported on the expression of TRAIL-Rs and TRAIL-induced apoptosis in cultured human

MCs (19–21). These studies also demonstrated that IgE-dependent activation is able to enhance the expression of TRAIL-Rs and sensitivity toward TRAIL-mediated apoptosis (19, 21). In addition, human MCs were found to express the ligand TRAIL, which may enable autocrine induction of apoptosis via TRAIL-Rs (22). Furthermore, intracellular expression of TRAIL in MCs was increased after stimulation with lipopolysaccharide (LPS) and interferon- γ (IFN- γ). These findings suggested a prominent role for TRAIL-Rs in homeostasis of MCs.

In this study, we aimed to further investigate the function of TRAIL-Rs in MCs. Analysis of conditional mouse models with MC-specific and ubiquitous deletion of TRAIL-R revealed that TRAIL-R has no impact on MC numbers under physiologic conditions. However, anaphylaxis was extended in MC-specific TRAIL-R knockout mice. Cultured IL-3-dependent murine MCs were resistant to TRAIL-induced apoptosis, but showed susceptibility after KIT activation. In line, activating *KIT* mutations promoted TRAIL-R function in human MCs and MCs infiltrating the bone marrow of patients with mastocytosis expressed TRAIL-R1.

Material and methods

Generation of conditional TRAIL-R knockout mice

TRAIL-R^{fl/fl} mice (23) were bred to the Mcpt5Cre (24,25) (Mcpt5Cre/TRAIL-R^{fl/fl}) and DeleterCre strains (26) (TRAIL-R^{ko}). Germline deletion of the loxP-flanked fragment yielded TRAIL-R^{ko} mice on a C57BL/6 background. Genotyping for Mcpt5Cre, DeleterCre, TRAIL-R flox, and deletion of TRAIL-R was performed as described (23, 24, 26). The experiments were carried out in accordance with institutional and state guidelines (approval AZ 84-02.04.2011.A302).

Cell culture

Cell culture conditions of peritoneal cell-derived mast cells (PCMCs), bone marrow-derived mast cells (BMMCs) (27,28), HMC-1 (29,30), and TF1 cells (31) are described in the Appendix S1.

Expression of TRAIL-Rs

Expression of TRAIL-Rs was analyzed by RT-PCR and flow cytometry as detailed in the Appendix S1.

Viability and apoptosis upon incubation with TRAIL

Viability and apoptosis upon incubation with TRAIL were analyzed as described in the Appendix S1.

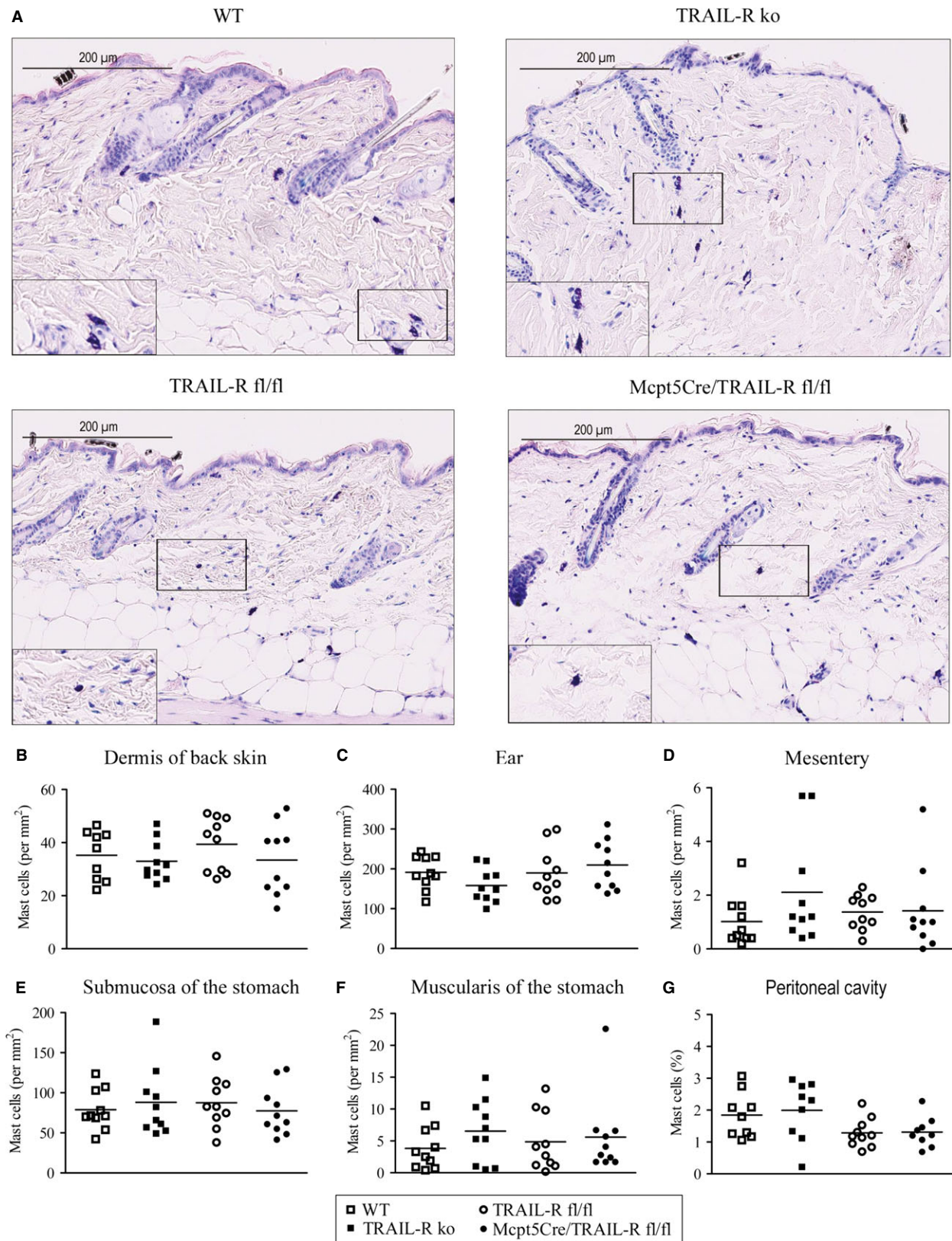


Figure 2 Enumeration of mast cells in different tissues of conditional TRAIL-R knockout mice. (A) Giemsa-stained back skin of wild-type, TRAIL-R^{ko}, TRAIL-R^{fl/fl}, and Mcpt5Cre/TRAIL-R^{fl/fl} mice is depicted (magnification: 260x, insert: 400 x). MC numbers in (B)

dermis of back skin, (C) ear, (D) mesentery, (E) submucosa of glandular stomach, (F) muscularis of glandular stomach, and (G) peritoneal cavity were determined in MC-specific and ubiquitous TRAIL-R knockout mice and controls, mean $\pm$ SEM, $n = 9-10$.

Enumeration of mast cells in different tissues

For enumeration of MCs in different tissues, paraffin-embedded sections of 9–10 animals of each genotype were stained with Giemsa and MCs were counted in 25 medium-power fields (200 $\times$, dermis of back skin, mesentery) or high-power fields (400 $\times$, ear, glandular stomach) using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) with Diskus software (Hilgers, Königswinter, Germany). The percentage of MCs in peritoneal cavity was analyzed as described (24). Peritoneal cells were isolated by peritoneal lavage and directly labeled with 660 ng/ml PE-conjugated anti-mouse Fc ϵ RI α (clone MAR-1, eBioscience, Frankfurt, Germany) and 400 ng/ml allophycocyanin (APC)-conjugated anti-mouse CD117 (c-Kit) (clone 2B8, eBioscience) for 30 min at 4°C. After washing twice, 1×10^4 cells were analyzed flow cytometrically and the percentage of double-positive cells was determined.

β -Hexosaminidase assay

β -Hexosaminidase assay was performed as described in the Appendix S1.

Passive systemic anaphylaxis

Passive systemic anaphylaxis was performed as described (32). Mice were injected i.p. with 12.5 μ g DNP-specific IgE (clone SPE7, Sigma-Aldrich, Steinheim, Germany) in 150 μ l saline solution. Twenty-four hours later, rectal body temperature was taken and mice were challenged i.p. with 200 μ l DNP-HSA solution (1 μ g/ μ l). The extent of anaphylaxis was analyzed by measuring rectal body temperature every 10 min after DNP injection for 120 min.

Immunofluorescence staining

Sections of paraffin-embedded bone marrow biopsies from 5 patients with indolent systemic mastocytosis, classified according to established World Health Organization criteria (6–9), were analyzed by double immunofluorescence staining using the Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA, USA). Sections were pretreated for antigen retrieval and permeabilization (Vector Antigen Unmasking Solution, Vector Laboratories, Inc) before incubation with anti-TRAIL-R1 (antibodies-online GmbH) and anti-TRAIL-R2 antibodies (antibodies-online GmbH) or mast cell tryptase mAb (Chemicon, Bedford, MA, USA) overnight at 4°C followed by incubation with biotinylated anti-mouse IgG and labeling with Fluorescein Avidin DCS or Texas Red Avidin DCS (Vector Laboratories). Analysis was carried out with Zeiss AxioCam and documented with Axiovision software (Carl Zeiss, MicroImaging GmbH, Göttingen, Germany).

Statistical analysis

Results are expressed as mean $\pm$ SEM of the indicated number of experiments. Means were compared using Student's *t*-test for unpaired values. Two-way ANOVA was used to compare time courses in anaphylaxis experiments. A *P* value of <0.05 was considered significant.

Results

Generation of mast cell-specific and ubiquitous TRAIL-R knockout mice

To investigate the impact of TRAIL-R on MCs, we generated a conditional mouse model with MC-specific knockout of TRAIL-R. We crossed our recently generated mouse strain Mcpt5Cre (24, 25), which expresses Cre recombinase under the control of the MC-specific promoter mast cell protease-5 (Mcpt5) allowing for gene inactivation in CTMCs, with the previously described TRAIL-R flox line (23) (Mcpt5Cre/TRAIL-R^{fl/fl}). For comparison, we also generated mice with ubiquitous knockout of TRAIL-R by crossbreeding Deleter-Cre (26) with the TRAIL-R flox strain (TRAIL-R^{ko}).

To examine TRAIL-R function, we first cultured peritoneal cell-derived mast cells (PCMCs) and bone marrow-derived mast cells (BMMCs) from wild-type and TRAIL-R^{ko} mice and analyzed deletion efficiency by RT-PCR and flow cytometry (Fig. 1A–C). As expected, wild-type controls expressed TRAIL-R, whereas BMMCs and PCMCs derived from TRAIL-R^{ko} mice failed to show TRAIL-R expression. Expression of TRAIL-R on wild-type PCMCs was more pronounced compared to wild-type BMMCs.

To investigate TRAIL-induced apoptosis, PCMCs and BMMCs were stimulated with TRAIL and ActD for 72 h and viability and apoptosis were evaluated by flow cytometric analysis of PI uptake and annexin-V staining, respectively (Fig. 1D,E). Wild-type PCMCs underwent apoptosis (Fig. 1E) and showed a trend toward reduced viability (Fig. 1D) in response to TRAIL in conjunction with ActD. On the other hand, wild-type BMMCs failed to show susceptibility to exposure with TRAIL. This observation was unexpected because human MCs were previously reported to undergo apoptosis in response to TRAIL (19, 21).

Impact of TRAIL-R on turnover of murine mast cells *in vivo*

To explore whether TRAIL-R affects turnover of MCs under physiologic conditions, we analyzed the numbers of MCs in dermis of back skin, ear, mesentery, and glandular stomach by histology (Fig. 2A–F) and in peritoneal cavity by flow cytometry (Fig. 2G). MC numbers in mice with ubiquitous and MC-specific knockout of TRAIL-R were comparable to

controls. Moreover, there was no significant difference in growth and viability upon growth factor deprivation between wild-type and TRAIL-R^{ko} BMMCs (Fig. S1). Together, these findings demonstrate that TRAIL-R does not participate in the physiologic homeostasis of MCs.

Effect of TRAIL-R on mast cell activation

To define the impact of TRAIL-R on the activation of MCs via FcεRI and TLRs, we next investigated degranulation of PCMCs upon stimulation with IgE/DNP, LPS, macrophage-activating lipopeptide-2 (MALP-2), and calcium ionophore (Fig. 3A). Using the β-hexosaminidase assay, we observed significant degranulation in wild-type and TRAIL-R^{ko} PCMCs in response to incubation with IgE/DNP and calcium ionophore, whereas degranulation upon stimulation with LPS and MALP-2 was comparable with unstimulated controls (Fig. 3A). Furthermore, there was a trend toward increased degranulation in TRAIL-R^{ko} PCMCs compared to wild-type PCMCs upon incubation with IgE/DNP and calcium ionophore (not significant). To also explore whether TRAIL-R affects MC activation *in vivo*, we analyzed IgE-dependent passive systemic anaphylaxis (Fig. 3B). Interest-

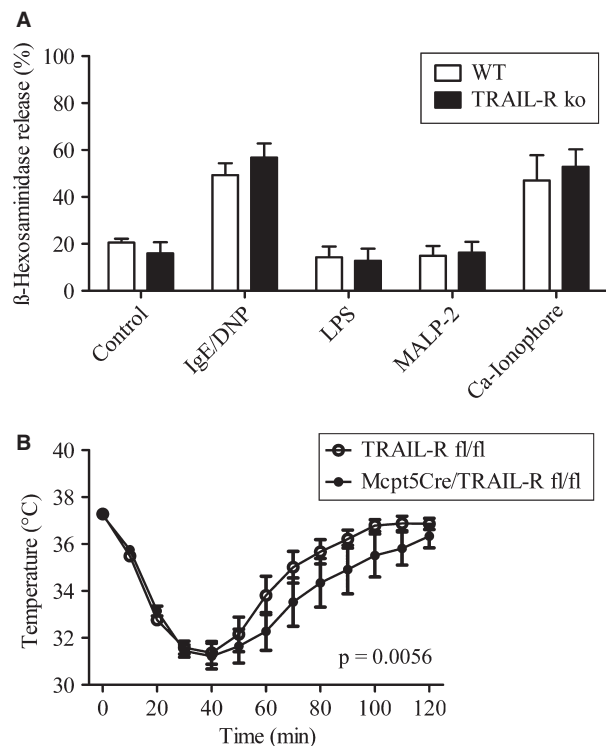


Figure 3 Effect of TRAIL-R on functions of murine mast cells. (A) Degranulation of PCMCs from TRAIL-R^{ko} mice and wild-type controls was analyzed by β-hexosaminidase assay upon stimulation with IgE/DNP, LPS, MALP-2, and calcium-ionophore for 30 min, mean ± SEM, *n* = 3. (B) Temperature profile during passive systemic anaphylaxis was monitored in 8–9 mice of each genotype, mean ± SEM. *P* = 0.0056 was calculated by two-way ANOVA for the temperature course in Mcpt5Cre/TRAIL-R^{fl/fl} mice compared to TRAIL-R^{fl/fl} controls.

ingly, we observed a significantly delayed return to the baseline body temperature in Mcpt5Cre/TRAIL-R^{fl/fl} mice compared to TRAIL-R^{fl/fl} controls.

Impact of SCF-induced KIT activation on the expression of TRAIL-R and TRAIL-induced apoptosis

As mentioned above, previous studies had demonstrated TRAIL-mediated apoptosis in primary and cultured human MCs, which are all dependent on SCF-induced or constitutive activation of KIT (19, 21). In contrast, murine BMMCs, which were resistant to TRAIL (Fig. 1D), primarily depend on IL-3 and are able to proliferate without SCF (28). In line, in our initial experiments, we had observed more pronounced TRAIL-R expression and TRAIL-induced apoptosis in PCMCs, which are cultured in SCF-containing medium, compared to IL-3-cultured BMMCs (Fig. 1B–D). We therefore hypothesized that incubation with SCF affects the expression and/or the function of TRAIL-R. Hence, we compared TRAIL-R expression and TRAIL-mediated apoptosis in wild-type and TRAIL-R^{ko} BMMCs cultured with or without SCF (Fig. 4). Interestingly, incubation with SCF for 24 h slightly upregulated the expression of TRAIL-R on wild-type BMMCs (Fig. 4A,B). Furthermore, stimulation with TRAIL in the presence of ActD decreased the survival of wild-type BMMCs cultured with SCF (Fig. 4C,D).

Effect of activating KIT mutations on the expression of TRAIL-Rs and TRAIL-induced apoptosis

To explore whether constitutive KIT activation, which is a feature of mastocytosis and several other cancers, also alters the expression of TRAIL-Rs and TRAIL susceptibility, we next compared two clones of the human MC line HMC-1, which exhibit different activating KIT mutations (Fig. 5) (29,30). The clone HMC-1.1 carries the KIT mutation V560G, while HMC-1.2 exhibits KIT V560G as well as KIT D816V, resulting in more pronounced KIT activation. HMC-1.2 cells expressed significantly higher levels of TRAIL-R1 and showed a tendency toward increased expression of TRAIL-R2 compared to HMC-1.1 (Fig. 5A,B). Both subclones failed to express the decoy receptor TRAIL-R3.

In line with the enhanced expression of TRAIL-R1 on HMC-1.2, TRAIL treatment significantly induced cytotoxicity and apoptosis after 24 and 48 h in HMC-1.2, but not in HMC-1.1 cells (Fig. 5C,D). To test this observation for the KIT D816V mutation alone, we also analyzed the effect of KIT D816V using a transfection model of the human erythroleukemia cell line TF1 (Fig. S2) (31). TF1 cells were transfected either with wild-type KIT or KIT D816V. Again, apoptosis in response to TRAIL in combination with ActD was significantly increased in TF1 cells transfected with KIT D816V compared to wild-type KIT (Fig. S2D), confirming that constitutive activation of KIT induces susceptibility toward TRAIL-mediated apoptosis. In line with these findings, the tyrosine kinase inhibitor PKC412 significantly reduced the expression of TRAIL-R1 and TRAIL-R2 in HMC-1.1 cells, but not in HMC-1.2 cells (Fig. S3).

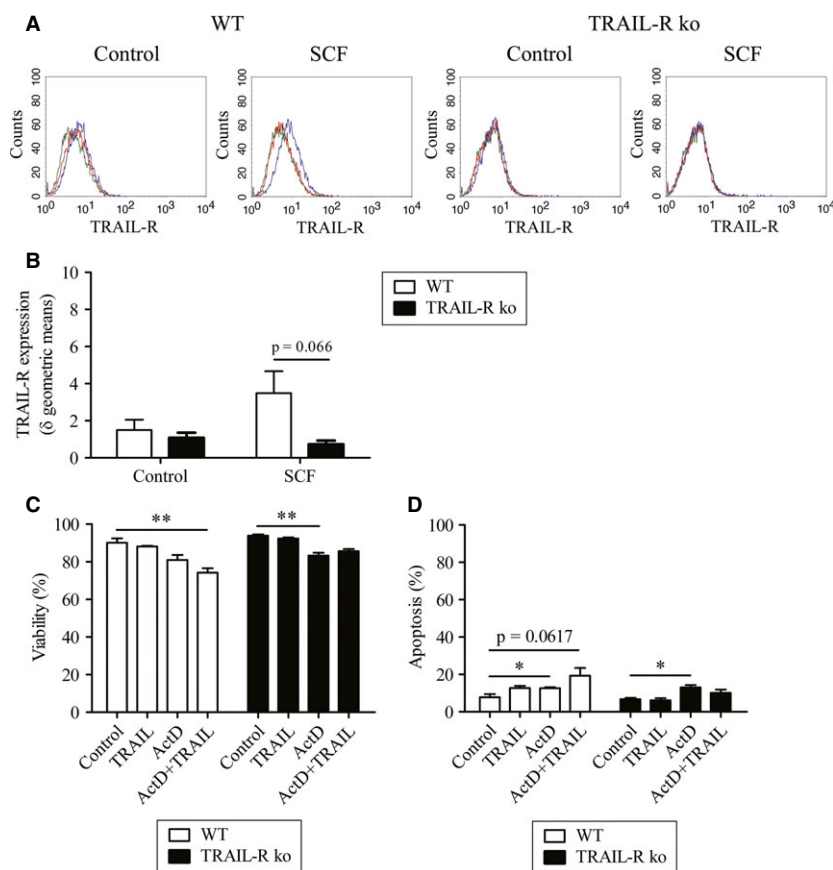


Figure 4 Expression of TRAIL-R and response to TRAIL in BMMCs cultured in SCF. (A) Expression of TRAIL-R was analyzed by flow cytometry on BMMCs from TRAIL-R^{ko} mice and wild-type controls after pre-incubation with 100 ng/ml SCF for 24 h, blue lines: receptor staining, red lines: isotype controls, and green lines: unstained controls. (B) TRAIL-R expression was calculated as difference of mean

fluorescence intensity between TRAIL-R-labeled cells and isotype controls, mean $\pm$ SEM, $n = 3-4$. Viability (C) and apoptosis (D) of BMMCs were measured upon incubation with 30 ng/ml SCF, 500 ng/ml TRAIL, and 5 ng/ml ActD for 72 h; *indicates $P < 0.05$, and **indicates $P < 0.01$, mean $\pm$ SEM, $n = 3$.

Expression of TRAIL-Rs on MCs infiltrating the bone marrow of patients suffering mastocytosis

To further investigate the physiologic relevance of these findings, we next analyzed the expression of TRAIL-Rs on MCs infiltrating the bone marrow of patients with mastocytosis. For this purpose, we performed double immunofluorescence staining of bone marrow specimens using an anti-tryptase antibody in combination with antibodies against either TRAIL-R1 or TRAIL-R2 (Fig. 6). Here, we observed prominent expression of TRAIL-R1 in infiltrating MCs. In contrast, expression of TRAIL-R2 was restricted to only few MCs in the bone marrow infiltrates. Consistent with our *in vitro* data (Fig. 5B), these findings show that patients with mastocytosis, who carry activating *KIT* mutations, express TRAIL-R1 in mast cell infiltrates of the bone marrow.

Discussion

In most cell populations, TRAIL induces apoptosis in transformed neoplastic cells, but not in normal cells (15–17). In

contrast, several recent studies reported on susceptibility of cultured human MCs, which depend on KIT activation, toward TRAIL-mediated apoptosis *in vitro* (19, 21). To explore the function of TRAIL-R *in vivo*, we generated conditional mouse models with MC-specific and ubiquitous deletion of TRAIL-R. In these mice, we observed that TRAIL-R deletion does not affect numbers of MCs in different tissues. However, MC-specific deletion of TRAIL-R was associated with a slight, but significant increase in anaphylaxis. Cultured IL-3-dependent MCs from control mice were resistant to TRAIL-induced apoptosis, despite expression of TRAIL-R. On the other hand, activation of KIT by SCF or mutations induced sensitivity of murine and human MCs to TRAIL-mediated apoptosis. Furthermore, MCs infiltrating the bone marrow of patients with mastocytosis expressed TRAIL-R1. Thus, our study demonstrates that the activation of KIT regulates the function of TRAIL-R and provides an explanation for the discrepant findings in human compared to murine MCs.

Unlike suggested by the studies in human MCs (19, 21), numbers of MCs in mice with MC-specific and ubiquitous

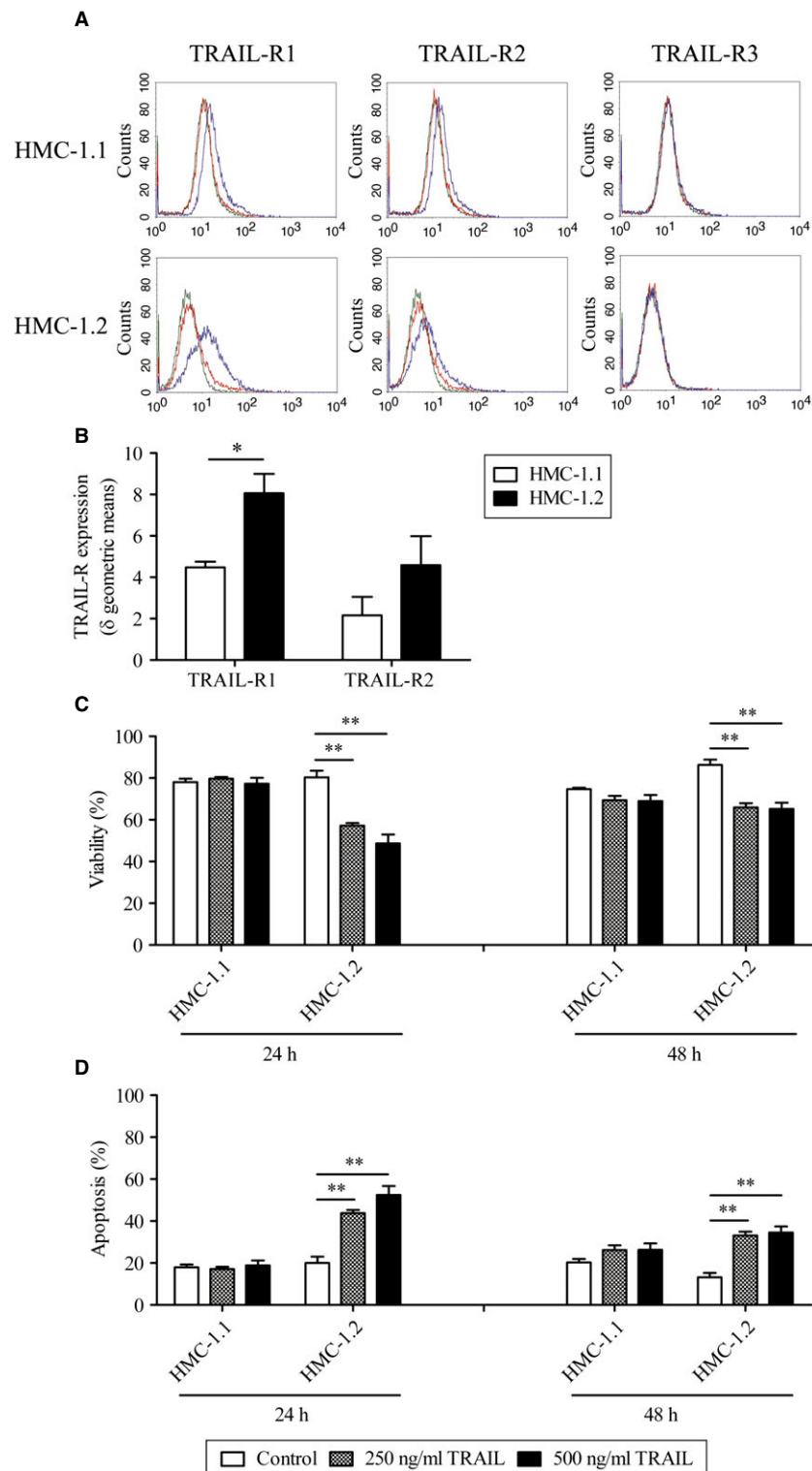


Figure 5 Expression of TRAIL-R1-3 and TRAIL-induced apoptosis in HMC-1 cells. (A) Expression of TRAIL-R1-3 was analyzed on HMC-1.1 and HMC-1.2 cells by flow cytometry, blue lines: receptor staining, red lines: isotype controls, and green lines: unstained controls. (B) TRAIL-R expression was calculated as the difference of mean

fluorescence intensity between TRAIL-R-labeled cells and isotype controls; *indicates $P < 0.05$, mean $\pm$ SEM, $n = 3$. Viability (C) and apoptosis (D) of HMC-1.1 and HMC-1.2 cells were assessed after incubation with 250 ng/ml and 500 ng/ml TRAIL; **indicates $P < 0.01$, mean $\pm$ SEM, $n = 3$.

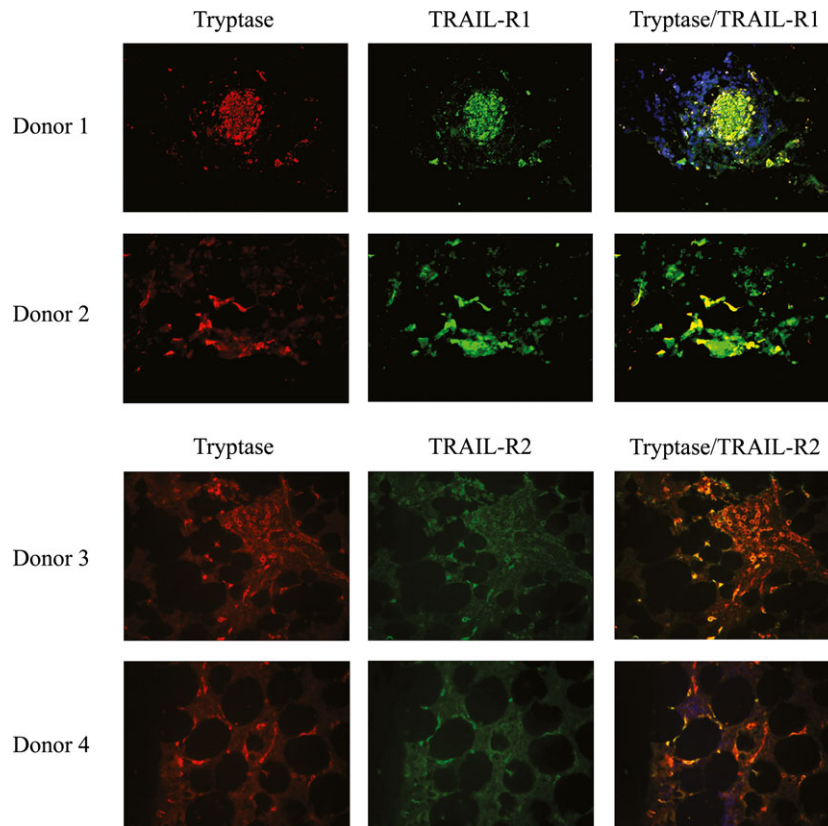


Figure 6 Expression of TRAIL-R1 and TRAIL-R2 on MCs infiltrating the bone marrow of patients with indolent systemic mastocytosis. Double immunofluorescence staining of bone marrow specimens of

patients with indolent systemic mastocytosis was performed using antibodies for tryptase (red) and TRAIL-R1 or TRAIL-R2 (green). Colocalization resulted in yellow color, $n = 5$.

TRAIL-R deletion were not altered (Fig. 2). Moreover, wild-type PCMCs, cultured in SCF-containing CHO medium, showed higher expression of TRAIL-R compared to IL-3-cultured BMMCs (Fig. 1B,C) and underwent apoptosis upon incubation with TRAIL and ActD (Fig. 1E). These results suggested an interaction between KIT activation and TRAIL-R function. In fact, incubation of control BMMCs with SCF enhanced the expression of TRAIL-R (Fig. 4A,B) and promoted TRAIL-induced cell death in the presence of ActD (Fig. 4C). Interestingly, similar findings have been described for human MCs activated via FcεRI (19, 21). Compound 48/80, however, which shows similar activity on mediator release as anti-IgE, has been reported not to affect TRAIL-induced apoptosis (21). Thus, TRAIL sensitivity in MCs is regulated by different mechanisms, including stimulation of KIT and FcεRI. It is not known whether other KIT-expressing cells, such as hematopoietic stem cells, germ cells, interstitial cells of Cajal, or melanocytes, also show increased TRAIL-induced apoptosis upon activation of KIT.

We observed expression of TRAIL-R1 on MCs infiltrating the bone marrow of patients with mastocytosis (Fig. 6). Due to enhanced release of MC mediators, some of these patients suffer from anaphylaxis. Unexpectedly, however, IgE-mediated anaphylaxis was slightly extended in Mcpt5Cre/TRAIL-R^{fl/fl} mice compared to TRAIL-R^{fl/fl} controls (Fig. 3B). These

findings are controversial and cannot be explained at the moment. On the other hand, as mentioned above, the effect on anaphylaxis in Mcpt5Cre/TRAIL-R^{fl/fl} mice was only marginal and only a limited number of patients were investigated in our study.

Our new finding on modulation of TRAIL-R function by KIT activation might have various clinical implications. First, as activating *KIT* mutations are a hallmark of the pathogenesis of mastocytosis (6–11) and our data demonstrate that MCs in the bone marrow of patients with mastocytosis express TRAIL-R1, TRAIL-R1 might serve as diagnostic marker and therapeutic target specific for KIT-activated neoplastic MCs. Moreover, it might be worthwhile to explore expression and function of TRAIL-Rs in other diseases associated with activating *KIT* mutations, such as gastrointestinal stromal tumors, melanoma, seminoma, and acute myeloid leukemia. In melanoma, *in vitro* studies suggested anticancer activity of agonistic TRAIL-R antibodies (33); however, to our knowledge, the impact of *KIT* mutations on TRAIL sensitivity has not been investigated in this disease.

In addition to TRAIL-R regulation by activating *KIT* mutations, it is also conceivable that SCF exerts specific functions of MCs by modulating the activity of TRAIL-Rs. For example in inflammation, one could assume that SCF,

beyond its well-described function in recruitment of MCs (34), serves to limit inflammation by promoting TRAIL-R-mediated apoptosis of MCs. Interestingly, stimulation of human MCs with LPS and INF- γ *in vitro* has been found to promote the expression of the ligand TRAIL, enabling auto-crine apoptosis (22). In case TRAIL-R function in MCs is altered in inflammation, targeting this mechanism might be useful for the treatment of inflammatory diseases.

Taken together, our data demonstrate that deletion of TRAIL-R has only a minor impact on homeostasis and activation of mast cells. Furthermore, we show that SCF-induced and constitutive KIT activation induces the expression of TRAIL-Rs and TRAIL-mediated apoptosis in MCs. These findings provide a rationale for exploiting TRAIL-Rs as diagnostic and therapeutic targets in mastocytosis and other diseases associated with *KIT* mutations.

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Conflicts of interest

All authors declare that they have no relevant conflict of interests.

Author contributions

SPG and KH designed the study. AF, SPG, JMS, ARa, MG, and SL performed the experiments. AF, SPG, ARa, UR, PD, HK, HW, ARo, and KH analyzed the data. AF and KH wrote the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplemental methods.

Figure S1. Proliferation and viability upon growth factor deprivation of BMMCs.

Figure S2. Expression of TRAIL-R1-3 and TRAIL-induced apoptosis in TF1 cells transfected either with wild-type *KIT* or the *KIT* mutation D816V.

Figure S3. Effect of PKC412 on expression of TRAIL-Rs on HMC-1 cells.

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Supplemental Methods

Cell culture

PCMCs were isolated by peritoneal lavage from wild-type and TRAIL-R^{ko} mice and cultured in RPMI 1640 medium (Gibco, Invitrogen, Darmstadt, Germany) supplemented with 10 % FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Biochrom AG, Berlin, Germany), 30 % WEHI-3B conditioned medium (as a source of IL-3) and 20 % CHO-conditioned medium (as a source of SCF).

BMMCs were generated from the femoral and tibial bone marrow of wild-type and TRAIL-R^{ko} mice and cultured in DMEM medium (high glucose, Gibco, Invitrogen) supplemented with 20 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Biochrom AG) and 30 % WEHI-3B-conditioned medium (as a source of IL-3) for at least four weeks. To assess the purity of BMMC cultures, expression of KIT and FcεRI was analyzed by flow cytometry as described (1).

The human MC line HMC-1, originally derived from a patient with MC leukemia, was maintained in Iscove's Modified Dulbecco's Medium (Gibco, Invitrogen) containing 10 % FCS, 4 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µmol/l 2-mercaptoethanol (Gibco, Invitrogen).

The human erythroleukemia TF1 cell line, transfected with wild-type KIT or the KIT mutation D816V, was cultured in RPMI 1640 medium supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom AG). Additionally, TF1 wild-type KIT cells were incubated with 5 ng/ml hGM-CSF.

Growth rate of BMMCs

To compare the growth rate of wild-type and TRAIL-R^{ko} BMMCs, 8 x 10⁵ cells per well were seeded in 1 ml medium and cells were counted after one week and again adjusted to 8 x 10⁵

cells per well. Counting was performed for three weeks in three independent cultures of wild-type and TRAIL-R^{ko} BMMCs.

Viability of BMMCs upon growth factor deprivation

To analyze viability upon growth factor deprivation, 3×10^5 wild-type and TRAIL-R^{ko} BMMCs per well were cultured in 1 ml medium supplemented either with 30 % WEHI-3B conditioned medium, 70 ng/ml mSCF (PeproTech, Hamburg, Germany) or both. After 24 h, cells were washed, incubated in medium without growth factors and viability was evaluated after 24, 48 and 72 h by flow cytometric analysis of PI uptake. Control BMMCs were again incubated in medium with growth factors. The percentage of viable cells after deprivation of growth factors compared to control cells was determined.

RT-PCR to detect TRAIL-R transcripts

RNA was isolated from 1×10^6 PCMCs or BMMCs of the different genotypes using the RNeasy Mini Kit (Qiagen, Hilden, Germany). 1 µg of RNA was applied for cDNA synthesis using the SuperScript VILO cDNA synthesis Kit (Invitrogen, Darmstadt, Germany). PCR was performed with 200 ng cDNA and primer sequences for TRAIL-R and GAPDH were chosen as described (2).

Surface expression of TRAIL-Rs

TRAIL-R surface expression on PCMCs, BMMCs (in some experiments, as indicated, cells were preincubated with 100 ng/ml mSCF (PeproTech) for 24 h), HMC-1 and TF1 cells was evaluated by flow cytometry. PCMCs or BMMCs were directly labeled with 125 ng/ml (PCMCs) or 500 ng/ml (BMMCs) PE-conjugated anti-mouse mTRAIL-R2 (eBioscience, Frankfurt, Germany) or PE-conjugated golden syrian hamster isotype control (eBioscience) for

30 min at 4°C. HMC-1 cells were pre-incubated with human Fc receptor binding inhibitor (eBioscience) for 20 min at 4°C and labeled with 5 µg/ml PE-conjugated anti-human TRAIL-R1, 2.5 µg/ml PE-conjugated anti-human TRAIL-R2 (both from eBioscience), 3 µg/ml PE-conjugated anti-human TRAIL-R3 (antibodies-online GmbH, Aachen, Germany) or PE-conjugated mouse IgG1 κ isotype control (eBioscience) for 30 min at 4°C. After washing twice, 1×10^4 cells were analyzed for receptor expression using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, Heidelberg, Germany). TRAIL-R expression was calculated as difference of mean fluorescence intensity between TRAIL-R-labeled cells and isotype controls.

Viability and apoptosis upon incubation with TRAIL

To analyze TRAIL-induced apoptosis, 1×10^5 cells per well (in some experiments, as indicated, BMNCs were also incubated with 30 ng/ml mSCF (PeproTech)) were incubated in 200 µl medium containing 250 ng/ml or 500 ng/ml rhTRAIL and 1 µg/ml or 2 µg/ml of the corresponding enhancer for ligand binding (both from Alexis Biochemicals, Enzo Life Sciences, Lörrach, Germany), respectively. Some samples were also co-incubated with different concentrations of actinomycin D (ActD) (PCMCs: 50 ng/ml ActD; BMNCs: 5 ng/ml ActD; TF1: 3 ng/ml ActD) (Sigma-Aldrich, Steinheim, Germany). The number of viable and apoptotic PCMCs, BMNCs, HMC-1 and TF1 cells was evaluated after 24 and 48 h by flow cytometric analysis of propidium iodide (PI) (Sigma-Aldrich) uptake and staining with annexin-V-FITC (BD Biosciences), respectively.

β-Hexosaminidase assay

To measure the β-hexosaminidase release, 10⁶ PCMCs per well were either pre-incubated for 24 h at 37°C with 1 µg/ml DNP-specific IgE (clone SPE7, Sigma-Aldrich), washed with tyrode buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM Hepes, 0.1 % BSA, 5 % FCS, pH 7.4) and stimulated for 30 min at 37°C in 500 µl tyrode buffer containing 100 ng/ml DNP-HSA (Sigma-Aldrich) or directly stimulated for 30 min with 1 µg/ml LPS (E. coli, Sigma-Aldrich), 250 ng/ml MALP-2 (Alexis Biochemicals) and 250 nM calcium ionophore (Sigma-Aldrich). Unstimulated cells were used as controls. After centrifugation, supernatants were separated, pellets were lysed in 0.5 % Triton X-100 (Roth, Karlsruhe, Germany) for 5 min at room temperature, and the substrate p-NAG (p-nitrophenyl-n-acetyl-β-D-glucosaminide, Sigma-Aldrich) was added to supernatants and lysed pellets. The activity of the enzyme β-hexosaminidase was analyzed by measuring the cleavage of the substrate photometrically at 405 nm. Degranulation was calculated as percentage of β-hexosaminidase activity in the supernatants compared with total β-hexosaminidase activity.

Supplemental References

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Figure S1

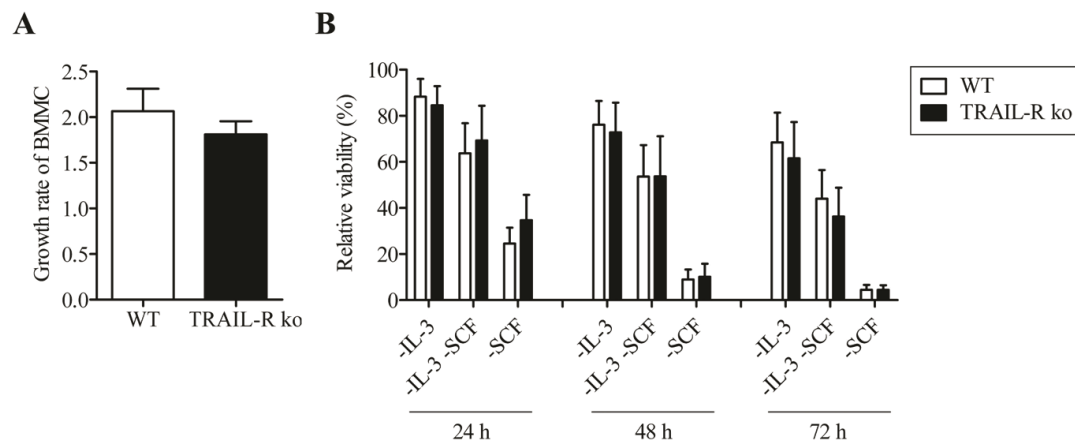


Figure S1 Proliferation and viability upon growth factor deprivation of BMMCs.

(A) The growth rate per week of wild-type (WT) and TRAIL-R^{ko} BMMCs was determined by counting BMMCs in three independent cultures for three weeks, mean $\pm$ SEM. (B) BMMCs were cultured in medium supplemented with 30 % IL-3-containing medium, 70 ng/ml SCF or both for 24 h. Then, growth factors were removed and viability was evaluated after 24, 48 and 72 h by flow cytometric analysis of PI uptake, mean $\pm$ SEM, n = 4.

Figure S2

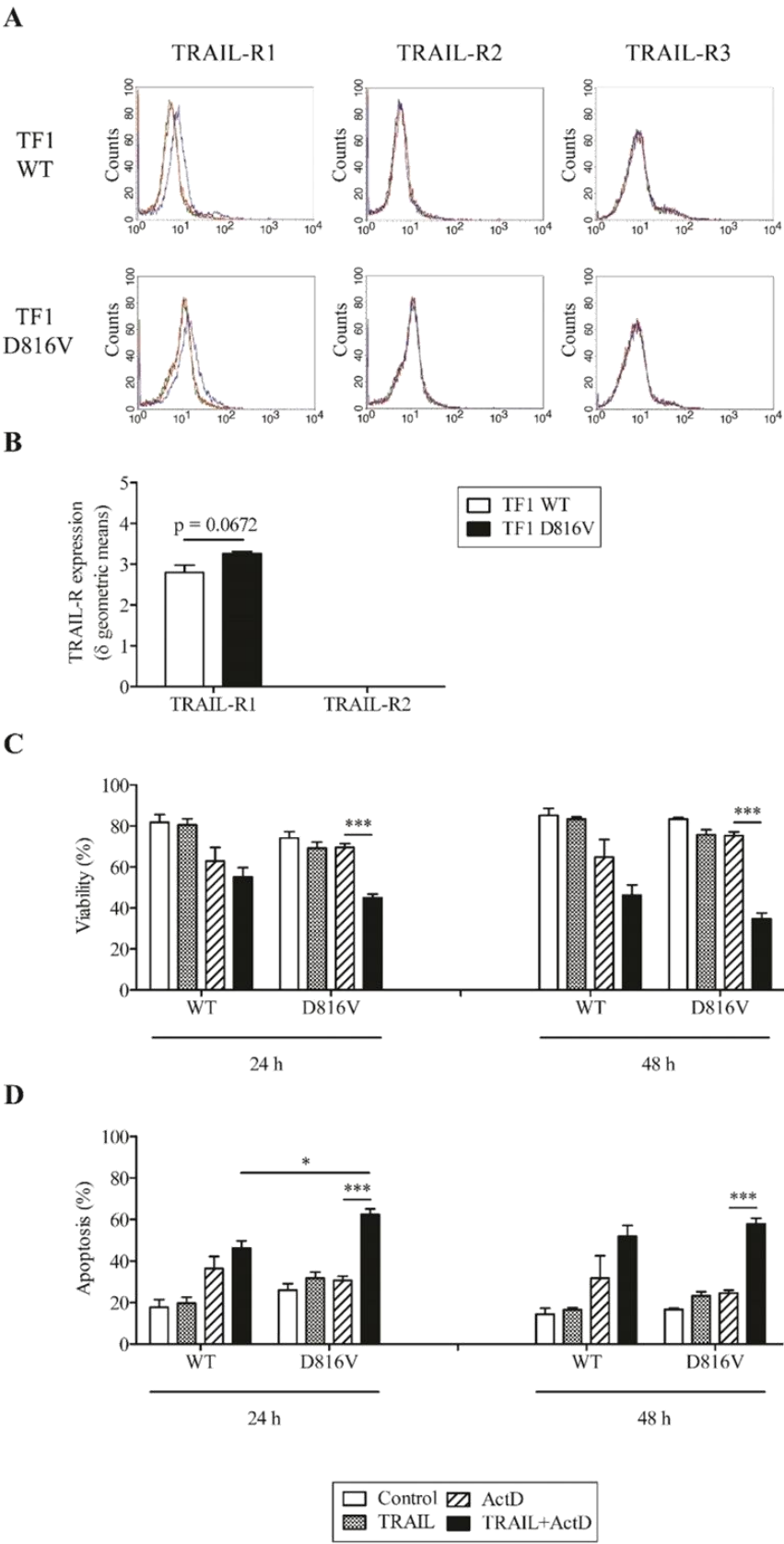


Figure S2 Expression of TRAIL-R1-3 and TRAIL-induced apoptosis in TF1 cells transfected either with wild-type *KIT* or the *KIT* mutation D816V.

(A) Expression of TRAIL-R1-3 was analyzed on TF1 cells by flow cytometry, blue lines: receptor staining, red lines: isotype controls, green lines: unstained controls. (B) TRAIL-R expression was calculated as difference of mean fluorescence intensity between TRAIL-R-labeled cells and isotype controls, $n = 3$. Viability (C) and apoptosis (D) of TF1 cells were determined after incubation with 500 ng/ml TRAIL and 3 ng/ml ActD, * indicates $p < 0.05$ and *** indicates $p < 0.001$, mean $\pm$ SEM, $n = 3$.

Figure S3

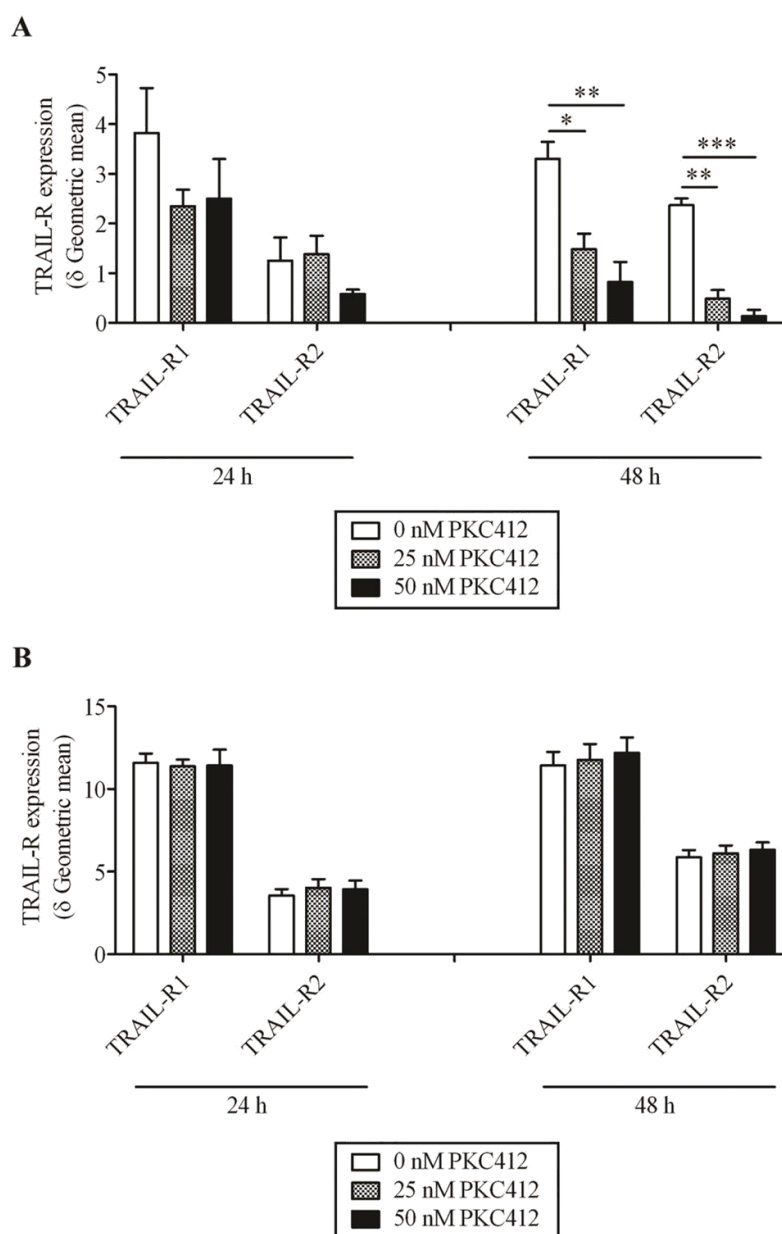


Figure S3 Effect of PKC412 on expression of TRAIL-Rs on HMC-1 cells.

HMC-1.1 (A) and HMC-1.2 cells (B) were incubated with subtoxic concentrations of PKC412 and expression of TRAIL-R1 and TRAIL-R2 was assessed by flow cytometry after 24 and 48 h. TRAIL-R expression was calculated as difference of mean fluorescence intensity between TRAIL-R-labeled cells and isotype controls, * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$, mean $\pm$ SEM, $n = 3$.