A CD47-blocking TRAIL fusion protein with dual pro-phagocytic and pro-apoptotic anticancer activity

The expedient removal of dying, damaged or altered cells by phagocytosis is essential for homeostasis. However, cancer cells can evade such phagocytic elimination by cell surface-upregulation of phagocyte-inhibitory signals, such as CD47. CD47 is a prominent 'don't eat me' signal that binds to signal-regulatory protein alpha (SIRPα/SIRPA) expressed on phagocytes (Oldenborg *et al*, 2001). The CD47-SIRPα interaction triggers phosphorylation of the immunoreceptor tyrosine-based inhibition motif (ITIM) of SIRPα and thereby potently inhibits phagocyte activity. Both solid and haematological malignancies hijack this inhibitory pathway by overexpression of CD47 (Chao *et al*, 2010, 2011; Zhao *et al*, 2011; Willingham *et al*, 2012).

Recent studies indicated that blocking of CD47-SIRPα interaction promotes phagocytic elimination of CD47 overexpressing tumour cells (Chao *et al*, 2010; Kim *et al*, 2012). For instance, treatment of human B-cell non-Hodgkin lymphomas (B-NHL)-engrafted mice with CD47-blocking monoclonal antibody (MAb) B6H12 reduced lymphoma burden, improved survival and inhibited extranodal dissemination (Chao *et al*, 2010, 2011). Further combination of this CD47-blocking antibody with the therapeutic antibody rituximab (RTX; a chimeric anti-CD20 IgG1) triggered synergistic anticancer activity *in vivo* (Chao *et al*, 2010). In addition, inhibition of CD47-SIRPα interaction enhanced the killing of trastuzumab-opsonized breast cancer cells (Zhao *et al*, 2011). Thus, CD47-SIRPα blocking strategies can enhance the efficacy of anticancer antibodies.

Phagocytosis induced by RTX was also enhanced by F(ab') 2 fragments of MAb B6H12 (Chao *et al*, 2010). This finding opens up the possibility for design of immunotherapeutics that combine CD47 blockade with alternate effector moieties. Here, we explored this possibility by genetic fusion of a CD47-blocking antibody fragment (scFv) to the pro-apoptotic immune effector molecule TRAIL (tumour necrosis factor [TNF]-related apoptosis-inducing ligand). TRAIL is a death ligand of the TNF-ligand superfamily that has pronounced tumour-selective pro-apoptotic activity (reviewed in (Bremer *et al*, 2009)). In phase I clinical trials, TRAIL treatment triggered minimal toxicity and, when combined with RTX, produced clinical responses in B-NHL patients (Fox *et al*, 2010). This new fusion protein, designated anti-CD47:TRAIL, was designed to **1)** block CD47-SIRPα interaction and hereby potentiate phagocytosis induced by RTX, and **2)** concurrently trigger CD47-restricted apoptotic cell death in malignant B-cells.

To assess the effect of anti-CD47:TRAIL on RTX-induced phagocytosis, we performed mixed culture experiments with B-NHL cells and granulocytes as phagocytic effector cells as they are one of the most prevalent population of professional phagocytes. To this end, 1,10 -dioctadecyl-3,3,30,30 –tetramethylindodicarbocyanine (DiD)-labelled $CD20^+/CD47^+$ B-NHL cells (Fig. S1A–B) were mixed with granulocytes and incubated in the presence of RTX, MAb B6H12 or anti-CD47:TRAIL and combinations thereof. Subsequently, phagocytosis was determined by flow cytometry (see Fig. S1C for gating strategy). Treatment with RTX induced rapid phagocytosis of $CD20^+$ B-NHL cells, whereas treatment with anti-CD47:TRAIL alone did not (Fig. 1A). However, co-treatment with RTX and anti-CD47:TRAIL significantly increased tumour cell phagocytosis compared to RTX alone (Fig. 1A, $P < 0{\cdot}05$). These flow cytometry data were corroborated by microscopy data, which revealed prominent tumour cell engulfment by granulocytes upon co-treatment (Fig. 1B). The potentiating effect of anti-CD47:TRAIL on RTX-mediated phagocytosis was dose-dependent and apparent at low ng/ml concentrations of anti-CD47:TRAIL (Fig. 1C). Importantly, anti-CD47:TRAIL also enhanced phagocytic removal of primary patient–derived B-NHL cells (Fig. 1D).

Of note, at these concentrations MAb B6H12 did not potentiate RTX-induced phagocytosis (Fig. 1A-D), which is in apparent contrast with a previous report in which MAb B6H12 did synergize RTX-mediated phagocytosis (Chao *et al*, 2010). However, in our experiments we used significantly lower concentrations of both RTX and MAb B6H12 (RTX; 2·5 μg/ml vs. 10 μg/ml, MAb B6H12; 250 ng/ml vs. 10 μg/ml, respectively). Further, we used granulocytes as phagocytic effector cells, whereas Chao *et al* (2010) used macrophages. Third, TRAIL forms a stable homotrimer in scFv:TRAIL proteins (Bremer *et al*, 2004). Hence, trivalent binding by anti-CD47:TRAIL may result in a significantly higher CD47 blocking capacity compared with the bivalent blocking capacity of MAb B6H12.

Phagocytosis induction by RTX and anti-CD47:TRAIL was abrogated at 0°C, indicating that tumour cells were eliminated by active phagocytosis (Fig. 1E). Furthermore, co-treatment of $CD20^-$ Namalwa cells with RTX and anti-CD47:TRAIL did not enhance phagocytosis. Likewise, co-treatment of B-cell lines with anti-CD47:TRAIL and cetuximab (CTX; a chimeric anti-epidermal growth factor receptor IgG1) failed to enhance phagocytosis (Fig. 1F). Thus, anti-CD47:TRAIL selectively enhanced antibody-mediated phagocytosis of B-NHL cells by RTX in a target antigen-restricted manner.

Previously, we and others demonstrated that scFv:TRAIL fusion proteins have target antigen-restricted pro-apoptotic activity towards cancer cells (reviewed in (Bremer *et al*, 2009)). In line with this, anti-CD47:TRAIL triggered apoptosis in $CD47^+$ B-cell lines and in 4 of 5 primary malignant B-NHL samples (Fig. 2A, 2B). Importantly, normal blood cells were

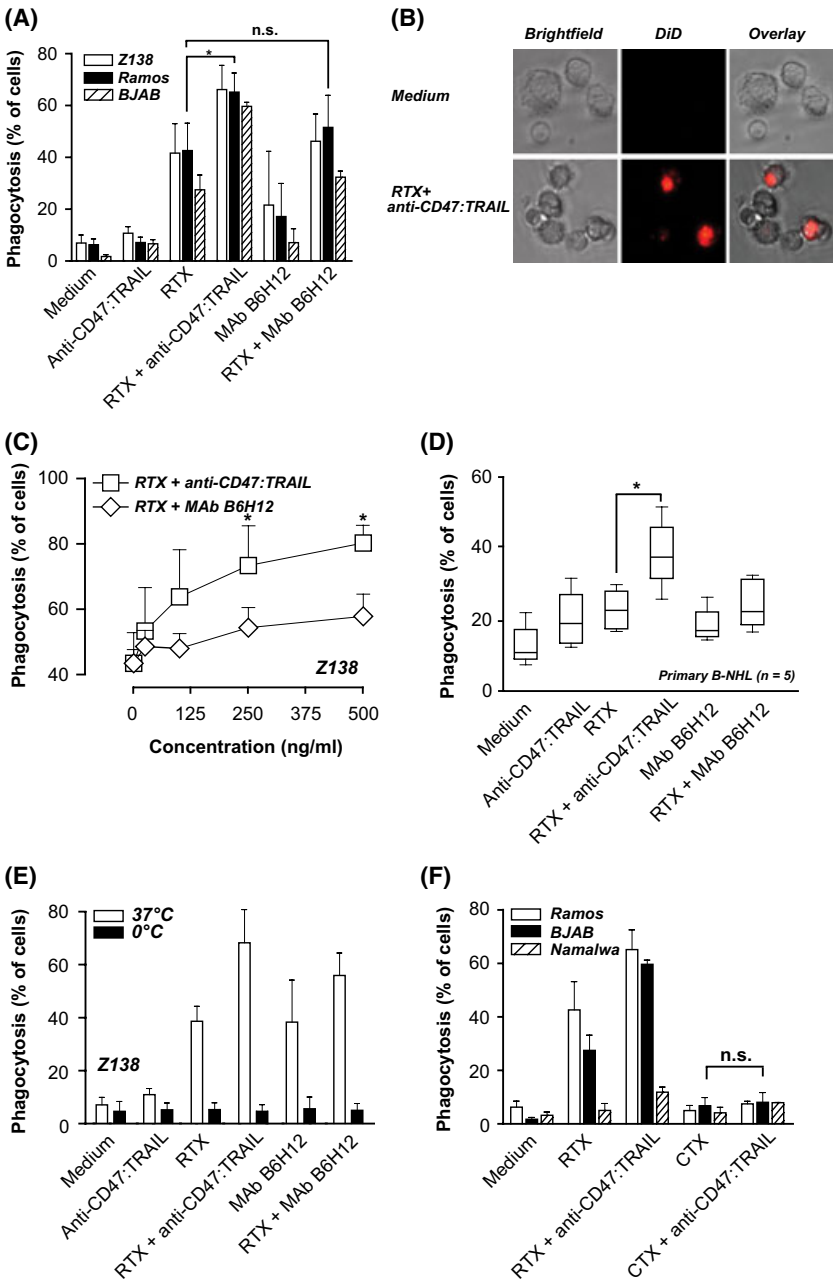

Fig 1. Anti-CD47:TRAIL enhances the phagocytic activity of Rituximab. (**A**) B cell non-Hodgkin lymphoma (B-NHL) cell lines (1,10 -dioctadecyl-3,3,30,30–tetramethylindodicarbocyanine [DiD]-labelled) were mixed with human granulocytes (1:1 ratio) pre-activated for 2 h with 50 ng/ml γ-interferon (IFN-γ) and 10 ng/ml granulocyte colony-stimulating factor. Subsequently, mixed cultures were incubated for 2 h at 37°C in the presence of medium, rituximab, (RTX, 2·5 μg/ml), monoclonal antibody (mAb) B6H12 (250 ng/ml), anti-CD47:TRAIL (250 ng/ml) or combinations thereof. Granulocyte-mediated phagocytosis of tumour cells was determined by flow cytometry. (**B**) Fluorescent picture of the phagocytosis assay showing engulfed DiD-labelled tumour cells inside the granulocytes. (**C**) Phagocytosis induced by RTX in the presence of increasing concentrations of anti-CD47:TRAIL or MAb B6H12 as determined by flow cytometry. (**D**) Phagocytosis induced by RTX treatment in the presence or absence of anti-CD47:TRAIL or MAb B6H12 in primary patient-derived malignant B-cells ($n = 5$) as determined by flow cytometry. (**E**) Phagocytosis experiment as in (**A**), but performed at 37°C and 0°C to demonstrate elimination by active phagocytosis. (**F**) Phagocytosis in $CD20^+$ and $CD20^-$ B-cell lines induced by RTX in the presence or absence of anti-CD47:TRAIL. In a control experiment, anti-CD47:TRAIL was combined with Cetuximab (CTX), a chimeric human IgG1 MAb directed toward the human Epidermal Growth Factor Receptor (EGFR). *$P < 0{\cdot}05$.

fully resistant to treatment with anti-CD47:TRAIL (Fig. 2B). Furthermore, anti-CD47:TRAIL potentiated RTX-mediated pro-apoptotic activity in the presence of granulocytes (Fig. 2C).

In line with published data, treatment with MAb B6H12 alone did not induce apoptosis (Fig. 2A) (Chao *et al*, 2010). Nevertheless, CD47 cross-linking by other anti-CD47 antibodies was reported to trigger caspase-independent cell death. In this respect, a bivalent form of the antibody fragment used in anti-CD47:TRAIL was previously shown to trigger CD47-mediated apoptosis in B-NHL cells (Kikuchi *et al*, 2004). Thus, anti-CD47:TRAIL may have dual pro-apoptotic signalling capacity via CD47 cross-linking (caspase-independent) and via target-antigen restricted cross-linking of agonistic TRAIL-receptors (caspase-dependent). In line with this, the apoptotic activity of anti-CD47:TRAIL was only partly blocked by pan-caspase inhibitor zVAD-fmk, whereas the pro-apoptotic activity of a constitutively active TRAIL preparation was completely blocked (Fig 2D). Furthermore, TRAIL-neutralizing MAb 2E5 only partly inhibited apoptosis induction by anti-CD47:TRAIL (Fig. 2D). Thus, anti-CD47:TRAIL appears to concurrently trigger apoptosis via CD47-crosslinking and TRAIL-receptor signalling.

As CD47 is widely expressed, the use of therapeutic intact humanized or chimerized anti-CD47 antibodies of selected isotypes may trigger toxicity towards normal cells by antibody-dependent cellular cytotoxicity and/or antibody-dependent

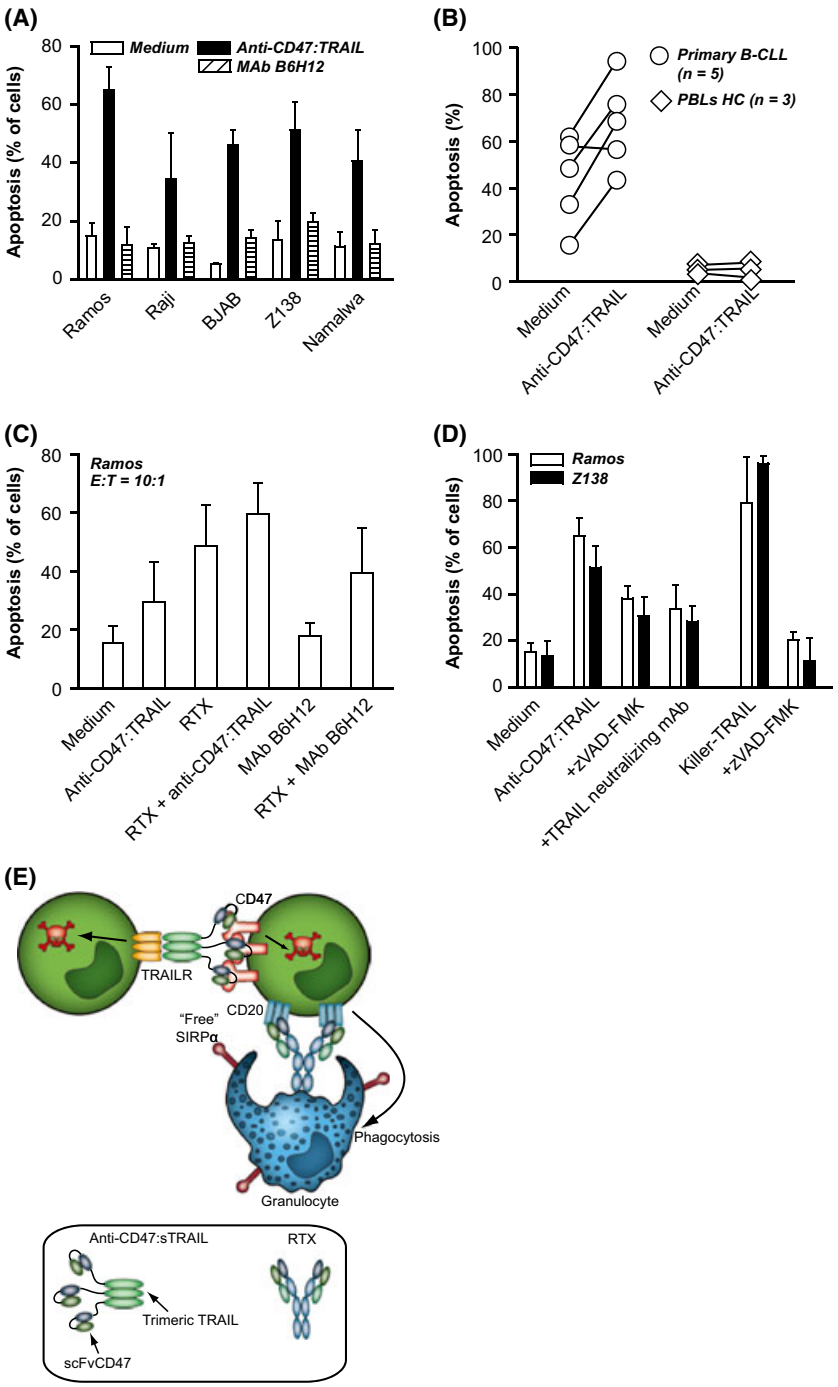

Fig 2. Anti-CD47:TRAIL induces apoptosis in B-NHL tumour cells via TRAIL-R signalling and CD47 cross-linking. (**A**) Direct apoptosis inducing activity was investigated by incubating B cell non-Hodgkin lymphoma (B-NHL) cell lines with anti-CD47:TRAIL (250 ng/ml) and MAb B6H12 (250 ng/ml) in a 48-well plate (3×10^4/well) for 20 h at 37°C in the absence of granulocytes. Apoptosis was determined by flow cytometry using an Annexin-V/Propidium Iodide kit. (**B**) Primary tumour cells derived from B-NHL patients ($n = 5$) and peripheral blood lymphocytes from healthy volunteers were treated as in (**A**). (**C**) Granulocytes and Ramos cells were mixed (E:T ratio of 10:1) and treated with the different agents to determine induction of apoptosis in the presence of granulocytes. (**D**) Direct pro-apoptotic activity of anti-CD47:TRAIL was investigated in the presence or absence of the pan-caspase inhibitor zVADfmk (40 μM) or the TRAIL neutralizing monoclonal antibody 2E5 (2 μg/ml). KillerTRAIL was used as a positive control (1 μg/ml). (**E**) Schematic representation of the proposed mode of action of anti-CD47:TRAIL. **1.** binding of anti-CD47:TRAIL to CD47 blocks interaction between CD47 and SIRPα and thereby enhances the phagocytic activity of granulocytes as induced during treatment with RTX. **2.** anti-CD47:TRAIL binding to CD47 cross-links CD47, which triggers caspase-independent cell death signalling in malignant B-cells. **3.** binding of anti-CD47:TRAIL to CD47 leads to cell surface accretion of TRAIL, which allows for CD47-restricted activation of TRAIL/TRAIL-receptor caspase-dependent apoptotic cell death of $CD47^+$ malignant B-cells.

cellular phagocytosis. In contrast, anti-CD47:TRAIL inhibits CD47-SIRPα interactions without this potential risk for Fc-mediated toxicity.

In conclusion, anti-CD47:TRAIL effectively blocks CD47-mediated 'don't eat me' signalling, promotes RTX-induced phagocytosis by granulocytes and triggers CD47-restricted apoptosis in malignant B-cells (for schematic see Fig. 2E). This multifunctional therapeutic activity of anti-CD47:TRAIL may be of general use for optimizing antibody-based cancer therapy and serves as proof of concept for combining CD47-blockade with alternate effector principles that may further synergize anticancer activity.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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Supporting Information

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

Fig S1. (A) Expression of CD20 and CD47 in the used cell lines as determined by flow cytometry. (B) Expression of CD20 and CD47 in patient derived primary B-NHL cells as determined by flow cytometry. (C) Gating strategy of the phagocytosis assay analyzed by flow cytometry. The granulocytes were gated (gate P1) based on forward/ sideward scatter (left). Within the granulocyte population/ gate P1, the DiD-positivity was determined as depicted for four experimental coditions (right).

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