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). In addition, inhibiting 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 -tetramethylendioxybenzocycanine (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 < 0.05). 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
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 cellular ADCC.
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).

References


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