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Chapter 4

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

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CD47-blocking TRAIL-induced phagocytosis

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) 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 hematologic malignancies hijack this inhibitory pathway by overexpression of CD47 (Willingham et al, 2012; Chao et al, 2010). Recent studies highlight that blocking of CD47-SIRPα interaction promotes phagocytic elimination of CD47 overexpressing tumor 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 MAb B6H12 reduced lymphoma burden and improved survival. 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). 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 (TNF-related apoptosis-inducing ligand). TRAIL is a death ligand of the TNF-ligand superfamily that has pronounced tumor-selective pro-apoptotic activity (reviewed in (Bremer et al, 2009)). In phase I clinical trials, TRAIL treatment triggered minimal toxicity and, in combination with RTX, produced clinical responses in B-NHL patients (Fox et al, 2010). This new fusion protein, designated antiCD47: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 antiCD47: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, DiD-labeled CD20+/ CD47+ B-NHL cells (Fig. S1A-B) were mixed with granulocytes and incubated in the presence of RTX, MAb B6H12 or antiCD47:TRAIL and combinations thereof. Subsequently, phagocytosis was determined by flow cytometry (see Fig.S1C for gating strategy).
**Figure 1.** AntiCD47:TRAIL enhances the phagocytic activity of Rituximab (RTX). A. B-NHL cell lines (DiD labeled) were mixed with human granulocytes (1:1 ratio) pre-activated for 2h with 50ng/ml IFN-γ and 10ng/ml G-CSF. Subsequently, mixed cultures were incubated for 2h at 37°C in the presence of medium, RTX (2.5μg/ml), mAb B6H12 (250ng/ml), antiCD47:TRAIL (250ng/ml), or combinations thereof. Granulocyte-mediated phagocytosis of tumor cells was determined by flow cytometry. B. Fluorescent picture of the phagocytosis assay showing engulfed DiD-labeled tumor cells inside the granulocytes. C. Phagocytosis induced by RTX in the presence of increasing concentrations of antiCD47:TRAIL or MAb B6H12 as determined by flow cytometry. D. Phagocytosis induced by RTX treatment in the presence or absence of antiCD47:TRAIL or MAb B6H12 in primary patient-derived malignant B-cells.
Treatment with RTX induced rapid phagocytosis of CD20+ B-NHL cells, whereas treatment with antiCD47:TRAIL alone did not (Fig.1A). However, co-treatment with RTX and antiCD47:TRAIL significantly increased tumor cell phagocytosis compared to RTX alone (Fig.1A, p<0.05). These flow cytometry data were corroborated by microscopy data, which revealed prominent tumor cell engulfment by granulocytes upon co-treatment (Fig.1B). The potentiating effect of antiCD47:TRAIL on RTX-mediated phagocytosis was dose-dependent and apparent at low ng/ml concentrations of antiCD47:TRAIL (Fig.1C). Importantly, antiCD47:TRAIL also enhanced phagocytic removal of primary patient–derived B-NHL cells (Fig.1D).

Of note, at these low ng/ml 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 vs. 10 ug/ml, mAb CD47; 250ng vs. 10 ug/ml, respectively). Further, we used granulocytes as phagocytic effector cells, whereas Chao et al used macrophages. Third, TRAIL forms a stable homotrimer in scFv:TRAIL proteins (Bremer et al, 2004). Hence, trivalent binding by antiCD47: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 tumor cells were eliminated by active phagocytosis (Fig.1E). Furthermore, co-treatment of CD20- Namalwa cells with RTX and antiCD47:-TRAIL did not enhance phagocytosis. Likewise, co-treatment of B-cell lines with antiCD47:TRAIL and cetuximab (CTX; a chimeric anti-EGFR IgG1) failed to enhance phagocytosis (Fig.1F). Thus, antiCD47: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, antiCD47: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 antiCD47:TRAIL (Fig.2B). Furthermore, antiCD47:TRAIL potentiated RTX-media-
Figure 2  AntiCD47:TRAIL induces apoptosis in B-NHL tumor cells via TRAIL-R signaling and CD47 cross-linking. A. Direct apoptosis inducing activity was investigated by incubating B-NHL cell lines with antiCD47:TRAIL (250ng/ml) and MAb B6H12 (250ng/ml) in a 48-well plate (3x10^4/well) for 20h at 37οC in the absence of granulocytes. Apoptosis was determined by flow cytometry using an Annexin-V/Propidium Iodide kit. B. Primary tumor 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 antiCD47:TRAIL was investigated in the presence or absence of the pan-caspase inhibitor zVADfmk (40μM) or the TRAIL neutralizing monoclonal antibody 2E5 (2μg/ml). KillerTRAIL was used as a positive control (1μg/ml). E. Schematic representation of the proposed mode of action of antiCD47:TRAIL. 1. binding of antiCD47:TRAIL to CD47 blocks interaction between CD47 and SIRPα and thereby enhances the phagocytic activity of granulocytes as induced during treatment with RTX. 2. antiCD47:TRAIL binding to CD47 cross-links CD47, which triggers caspase-independent cell death signaling in malignant B-cells. 3. binding of antiCD47: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.
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ted 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 antiCD47:TRAIL was previously shown to trigger CD47-mediated apoptosis in B-NHL cells (Kikuchi et al, 2004). Thus, antiCD47:TRAIL may have dual pro-apoptotic signaling capacity via CD47 cross-linking (caspase-independent) and via target-antigen restricted cross-linking of agonistic TRAIL-receptors (caspase-dependent). In line with this, apoptotic activity of antiCD47: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 antiCD47:TRAIL (Fig.2D). Thus, antiCD47:TRAIL appears to concurrently trigger apoptosis via CD47-crosslinking and TRAIL-receptor signaling.

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 phagocytosis. In contrast, antiCD47:TRAIL inhibits CD47-SI-RPα interactions without this potential risk for Fc-mediated toxicity.

In conclusion, antiCD47:TRAIL effectively blocks CD47-mediated “don’t eat me” signaling, 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 antiCD47:sTRAIL 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.
References
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