Redirected T-Cell Killing of Solid Cancers Targeted with an Anti-CD3/Trop-2 Bispecific Antibody is Enhanced in Combination with Interferon-α

Edmund A. Rossi,1,2 Diane L. Rossi,1 Thomas M. Cardillo,1 Chien-Hsing Chang1,2 and David M. Goldenberg1,2,3

1Immunomedics, Inc., Morris Plains, NJ 07950; 2IBC Pharmaceuticals, Inc., Morris Plains, New Jersey 07950, USA; 3Garden State Cancer Center, Center for Molecular Medicine and Immunology, Morris Plains, New Jersey 07950, USA

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Abbreviations: 19-3 BiTE, biosimilar of blinatumomab; (19)-3s, anti-CD19 Fab dimer fused to an scFv of Okt3; 20-2b, immunocytokine fusion protein comprising veltuzumab and IFN-α; (20)-3s, anti-CD20 Fab dimer fused to an scFv of Okt3; AUC, area under the curve; BiTE, bispecific T-cell engager; bsAb, bispecific antibody; CRS, cytokine release syndrome; DNL, DOCK-AND-LOCK; (E1)-3s, anti-Trop-2 Fab dimer fused to an scFv of Okt3; EC50, 50% effective concentration; EpCAM, epithelial cell adhesion molecule; GAM, goat anti-mouse second antibody; IC50, concentration resulting in 50% cell lysis; IFN, interferon; IL, interleukin; lysismax, maximal cell lysis; MST, median survival time; PBMCs, peripheral blood mononuclear cells; scFv, single-chain variable domain fragment; TAA, tumor-associated antigen; TF12, anti-Trop-2/anti-histamine succinyl glycine bispecific Tri-Fab; TNF, tumor necrosis factor

Corresponding Author: Edmund A. Rossi, Immunomedics, Inc. 300 The American Road, Morris Plains, New Jersey 07950, USA; E-mail: erossi@immunomedics.com.

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Abstract

Trop-2 is highly expressed in diverse epithelial cancers with limited presence on normal tissues. (E1)-3s is a T-cell-redirecting trivalent bispecific antibody (bsAb), comprising an anti-CD3 scFv covalently linked to a stabilized dimer of a Trop-2-targeting Fab using DOCK-AND-LOCK™. We show for the first time that a bsAb-mediated bi-directional trogocytosis occurs between target and T cells and involves immunological synapses. We studied the effects of interferon-α on (E1)-3s-mediated T-cell killing of human gastric and pancreatic cancer cell lines. T-cell activation, cytokine induction and cytotoxicity were evaluated ex vivo using PBMCs or T cells with NCI-N87 gastric cancer as target cells. In vivo activity was assayed with NCI-N87 and Capan-1 (pancreatic) xenografts. In presence of target cells and PBMCs, (E1)-3s did not cause excess cytokine production. When combined with (E1)-3s, peginterferonalfa-2a, which alone did not increase T-cell activation or raise cytokine levels over baseline, increased CD69 expression, but did not significantly increase cytokine induction. (E1)-3s mediated a highly potent T-cell lysis of NCI-N87 target cells in vitro. Inclusion of peginterferonalfa-2a, or a more potent form of interferon-α, 20*-2b, significantly potentiated the activity of (E1)-3s by more than 2.5- or 7-fold, respectively. In vivo, combining peginterferonalfa-2a with (E1)-3s delayed Capan-1 growth longer than each single agent. Similarly, combination therapy delayed tumor proliferation of NCI-N87 compared to (E1)-3s or peginterferonalfa-2a single-treatment groups. (E1)-3s effectively induced T-cell-mediated killing of Trop-2-expressing pancreatic and gastric cancers, which was enhanced with interferon-α.
Introduction

Various formats of bispecific antibodies (bsAbs) engineered to redirect cytotoxic T cells, most often via CD3 binding, have demonstrated therapeutic efficacy in preclinical and clinical settings by also binding tumor-associated antigens (TAA) (1). The potential of this modality has been demonstrated preclinically for both hematopoietic (2,3) and solid cancers (4-8). Tandem scFvs consisting of an anti-CD3 and an anti-TAA domain, termed bispecific T-cell engagers (BiTEs), have advanced the furthest, with two agents under clinical investigation (9). The CD19xCD3 BiTE (blinatumomab or MT103) has been studied in Phase I trials of lymphoma and leukemia (10), and in Phase II trials for B-acute lymphoblastic leukemia (11). MT110, the EpCAM (epithelial cell adhesion molecule) antibodyxCD3 BiTE (12), is currently undergoing a Phase I study in various solid tumors, including lung, gastric, colorectal, breast, prostate, and ovarian cancers (ClinicalTrials.gov NCT00635596).

Trop-2 [or EGP-1 (epithelial glycoprotein-1), GA733-1, or M1S1], is another TAA that could be highly effective for targeting various epithelial cancers. However, it has yet to be investigated in any bsAb format for T-cell-rediredcted therapy. Trop-2 is a 35 kDa transmembrane glycoprotein that is overexpressed relative to normal tissues in a variety of human cancers, including pancreatic and gastric carcinomas, where increased expression correlates with poor prognosis (13-17).

We recently introduced a new platform for producing trivalent bsAbs for T-cell-directed therapy (18). These constructs, which are assembled using the DOCK-AND-LOCK™ (DNL™) method (19), are designated (X)-3s, where the code “(X)” denotes a stabilized TAA-specific Fab dimer that is fused site-specifically to an scFv of Okt3,
indicated as “3s” (Figure 1A). The (X)-3s were shown to mediate the formation of conjugates of T cells and cognate target cells, induce T-cell activation and proliferation in the presence of target cells, redirect T-cell-mediated killing of target cells in vitro, and inhibit growth of human tumor xenografts in vivo. One of the constructs exemplified in the report, (E1)-3s, for which (X) was derived from the humanized version of the original murine anti-Trop-2 mAb, RS7 (17), exhibited therapeutic efficacy in a human pancreatic cancer xenograft model (18).

Interferon-alpha (IFN-α) has received approval for treatment of several neoplastic diseases (20). In oncology, the main indication of IFN-α is for patients with resected stage II and III melanoma, in whom IFN-α prolongs disease-free survival and shows a trend toward increased overall survival (21). For some tumors, IFN-α can have a direct and potent anti-proliferative effect through activation of STAT1 (22). IFN-α has a pleiotropic influence on immune responses through effects on myeloid cells, NK cells, DCs, B cells, and T cells, and influences the production of numerous other cytokines, including IFN-γ, TNF-α, IL-1, IL-6, IL-12, IL-15, and IL-18 (23). Several reports identify CD8+ T cells as direct targets of IFN-α, affecting their IFN-γ production, survival, activation, clonal expansion, and memory differentiation (24-31). IFN-α provides a strong and direct signal to human CD8+ T cells, thereby resulting in upregulation of critical genes for cytotoxic T-cell activity, and is absolutely critical in the case of human-naïve CD8+ T cells for effector function acquisition (26).

This study confirms our prior observations that (E1)-3s effectively induces T-cell-redirected killing of pancreatic and gastric cancer cell lines (18), validating Trop-2 as a choice candidate TAA for therapy of various epithelial-derived cancers using this
modality. Here, we show for the first time transfer of membrane proteins by trogocytosis (32,33) between target and T cells through immunologic synapses induced by a bsAb. Further, we demonstrate that IFN-α enhanced the therapeutic efficacy of (E1)-3s without increasing the production of other cytokines to levels that could induce cytokine release syndrome (CRS). The concept of adjuvant therapy with IFN-α, or other cytokines, might be universally applicable for enhanced efficacy of T-cell immunotherapy.

**Materials and Methods**

**Cell Lines**

Human cell lines of Capan-1 (pancreatic cancer), BxPC3 (pancreatic cancer), NCI-N87 (gastric cancer), and Raji (Burkitt lymphoma) were purchased from the ATCC (Manassas, VA). Cryopreserved vials from an early passage (≤5), were thawed and used within 50 passages for experiments. Samples of the cell lines were authenticated by the ATCC by Promega short tandem repeat authentication testing, where each was identical with the respective database profile. Peripheral blood mononuclear cells (PBMCs), T cells and cell lines were maintained in RPMI-1640 containing 10% FBS (20% for Capan-1), 1% L-glutamine, 1% penicillin-streptomycin, and 1% MEM non-essential amino acids. All cell culture media and supplements were purchased from Life Technologies.

**Reagents**
Peginterferonalfa-2a was obtained commercially. TF12, 20*-2b and hRS7 were supplied by Immunomedics. Preparation of (E1)-3s, (19)-3s and (20)-3s (Figure 1A) were described previously (18). Details of the biochemical methods used for preparation and characterization of (E1)-3s are provided as Supplementary Methods online. The bsAbs, (19)-3s (CD19XCD3) and (20)-3s (CD20XCD3), have the same design as (E1)-3s, with the stabilized (Fab)2 to Trop-2 replaced with a (Fab)2 to CD19 and CD20, respectively. TF12 (Figure 1B), which is a bispecific Tri-Fab generated by DNL to comprise two Trop-2 (hRS7)-binding Fabs fused to a third Fab that binds the hapten, histamine succinyl glycine, and not to any human cells/tissue, has been described previously (34). The 19-3 BiTE, which has the identical deduced amino acid sequence as blinatumomab (CD19XCD3 BiTE), was produced recombinantly from a stable SpESF transfectant cell line, and purified from culture supernatant fluids with Ni-Sepharose.

**Flow Cytometry**

Flow cytometric studies were performed on a FACSCalibur flow cytometer (BD Bioscience) with the data analyzed with FlowJo software (Tree Star). All antibody cell staining steps were for 30 min on ice, followed by two washes with 0.5% BSA-PBS. Fluorochrome-conjugated antibodies included anti-CD4-FITC (fluorescein isothiocyanate), anti-CD4-APC (allophycocyanine), anti-CD8-PE (phycoerythrin), and anti-CD69-APC, all from Biolegend; and, goat anti-mouse IgG Fc-FITC (GAM-FITC; Santa Cruz). Detection of Trop-2 with MABC518 (Millipore) was not blocked by any of the bsAbs. Other cell-staining agents were PKH26 Red and PKH67 Green Fluorescent
Cell Linker Kits from Sigma and 7-AAD (amino-actinomycin D). The number of cell-surface Trop-2 molecules/cell was measured using anti-human Trop-2 PE, clone MR54 (eBiosciences) and QuantiBRITE PE beads (BD Bioscience), following the manufacturer’s protocol. Each cell line was measured on three separate occasions.

**Isolation of PBMCs and T cells**

Human PBMCs were prepared from heparinized whole blood (buffy coat) of healthy donors purchased from The Blood Center of New Jersey (East Orange, NJ) using UNI-SEPMAXI tubes (Novamed Ltd., Jerusalem, Israel), under a protocol approved by the New England Institutional Review Board. Total T cells or CD8+ T cells were isolated from PBMCs by negative selection using Pan T Cell or CD8+ T Cell Isolation Kits (Miltenyi Biotec, Auburn, CA), respectively, according to the manufacturer’s protocol.

**Cell Binding**

PKH67 green-labeled NCI-N87 cells (1.25 x 10^5 cells/well) were added to 8-well chamber slides (ThermoWaltham) and allowed to attach overnight. The following day, media was removed, and PKH26 red-labeled CD8+ T cells (1 x 10^6 cells/well) were added in media containing 0.1 μg/mL of (E1)-3s, (19)-3s or TF12. After 30 min at 37°C, slides were dipped in PBS to remove any unbound cells. Fluorescent images were captured with an Olympus BX66 microscope (Shinjuko, Tokyo, Japan) equipped with a Mercury-100W laser (Chiu Technical Corp., Kings Park, NY), using an Olympus 20X/0.75 air objective lens and a Kodak DC290 Camera (Rochester, New York) with
115X zoom. A WB filter allowed simultaneous fluorescence of both red (appears as orange) and green fluorochromes. Images were captured and processed using Adobe Photoshop CS3 v.10 software with a Kodak Microscopy Documentation System 290 plug-in application.

**Trogocytosis**

BxPC3 cells were detached with trypsin (does not affect Trop-2) and mixed with purified T cells. Cell mixtures were treated with 0.1 nM bsAbs at 37°C for 1 h. Cells were stained with either: 1) anti-Trop-2 MABC518 followed by GAM-FITC, or 2) anti-Trop-2-PE clone MR54 and anti CD4-APC. Single BxPC3 and T cells were gated from cell conjugates by forward and side scattering, as well as Trop-2 and CD4 fluorescence.

**In-vitro cytotoxicity**

Freshly-isolated CD8+ T cells were incubated for 24 h with 0.1 nM peginterferonalpha-2a, 0.1 nM 20*-2b (Figure 1C), or media only. Treated or untreated T cells and PKH67 green fluorescent-labeled NCI-N87 cells were combined at a 5:1 ratio (5 x 10^4 target cells and 2.5 x 10^5 effector cells/well) in 48-well plates containing serial dilutions of (E1)-3s in triplicate. Peginterferonalpha-2a or 20*-2b were maintained at 0.1 nM in the appropriate cell mixtures. Plates were incubated for 48 h at 37°C. Suspension cells were removed and the attached cells were detached with trypsin-EDTA and combined with the corresponding suspension. Cells were washed and resuspended in 1% BSA-PBS containing 30,000 CountBright Absolute Counting Beads (Life Technologies) and 1 μg/mL of 7-AAD. Total live target cells (7-AAD-/PKH67+) were
counted by flow cytometry. For each sample, 8,000 CountBright beads were counted as a normalized reference. The specific lysis (%) was calculated using the formula: \[1 - \frac{(A_1/A_2)}{x 100}, \] where A₁ and A₂ represent the number of viable target cells in the test and untreated samples, respectively. Statistical significance (\(P \leq 0.05\)) was determined for IC₅₀ (the concentration resulting in 50% lysis), EC₅₀ (50% effective concentration) and lysis\(^{\text{max}}\) (maximal target cell lysis) by F-test on non-linear regression (sigmoidal dose-response) curves with Prism software.

**Cytokine release**

Cytokine release was measured ex vivo using 5 x 10⁵ cells/0.5 mL/well of either NCI-N87, which were allowed to attach overnight, or Raji. Freshly-isolated PBMCs (5 x 10⁶ cells/0.4 mL/well) were added to each well. Treatments (100 µL, 10X) comprising (19)-3s, 19-3 BiTE, (E1)-3s, peginterferonalpha-2a, or (E1)-3s plus peginterferonalpha-2a were added to 0.1 nM for each reagent. Alternatively, titrations ranging from 1 pM to 10 nM were used for dose-response studies. Following a 20-h incubation at 37°C with gentle shaking, the supernatant fluid was diluted 1:2 (or greater when necessary) and the concentrations of TNF-α, IFN-γ, IL-2, IL-6, and IL-10 measured using Single-Analyte ELISAArray kits (Qiagen), following the manufacturer’s protocol.

**In vivo studies**

All animal studies were approved by the Rutgers School of Biomedical and Health Sciences Institutional Animal Care and Use Committee (IACUC). Female 4-8-week old NOD/SCID mice (Charles River, Wilmington, MA) were injected s.c. with a mixture of 5
x 10^6 tumor cells (Capan-1 or NCI-N87) and T cells (2.5 x 10^6) combined with an equal volume of matrigel. Therapy began 1 h later by i.v. injection, as per the BiTE methodology (35). Treatment regimens, dosages, and number of animals in each experiment are described in the figure legends. Tumor volume was determined twice weekly by measurements in two dimensions using calipers, with volumes defined as: L x w^2/2, where L is the longest dimension of the tumor and w the shortest.

Statistical analysis of tumor growth was based on area under the curve (AUC). Profiles of individual tumor growth were obtained through linear-curve modeling. An F-test was employed to determine equality of variance between groups prior to statistical analysis of growth curves. A Critical Z test on the survival data identified any outliers within a given treatment group with P≤0.05 censored from the final data analysis. A two-tailed t-test was used to assess statistical significance between the various treatment groups and controls, except for the untreated control, where a one-tailed t-test was used. Additionally, efficacy was determined by log-rank using Prism software on Kaplan-Meier curves using survival surrogate endpoints as time for tumor progression to 1.0 cm^3. Significance was considered at P≤0.05 for all comparisons.

Results

Biochemical Characterization

Affinity purification of (E1)-3s using KappaSelect and Ni-Sepharose resins produced a homogeneous product that resolved as a single protein peak of the expected retention time by size exclusion HPLC (Supplementary Figure S1) and comprising only the three
constituent polypeptides (hRS7 Fd-DDD2, hRS7 kappa and Okt3-scFv-AD2) by reducing SDS-PAGE (Supplementary Figure S2). LC-MS analysis of (E1)-3s identified a single RP-HPLC peak having a deconvoluted mass of 135,400.3 Da, which is within 25 ppm of the calculated mass of 135,396.9 Da from its deduced amino acid sequence, including the predicted amino-terminal pyroglutamates on the Okt3scFv-AD2 and each of the two CH1-DDD2-hRS7 Fd chains (Supplementary Figure S3).

Trop-2 binding affinity measured by surface plasmon resonance (Biacore) for (E1)-3s ($K_D = 1.03 \pm 0.19$ nM) was not significantly different ($P = 0.1995$) from hRS7 IgG ($K_D = 0.86 \pm 0.05$ nM) (Supplementary Figure S4).

**Cell Binding and Trogocytosis**

(E1)-3s induces the formation of immunologic synapses between T cells and target cells. This was shown previously using Capan-1 pancreatic carcinoma cells (18). Here, addition of 0.1 μg/mL (E1)-3s to a mixture of purified CD8+ T cells and NCI-N87 gastric carcinoma cells, which were membrane-labeled with red and green fluorescence, respectively, resulted in the formation of conjugates evident by fluorescence microscopy (Figure 2A and B). No conjugates were observed in the presence of (19)-3s (Figure 2C) or TF12 (Figure 2D), which bind only T cells or NCI-N87, respectively. Dunking the slides in saline washed off the vast majority of T cells in wells containing (19)-3s or TF12, whereas many T cells remained bound to the adherent NCI-N87 cells in the wells treated with (E1)-3s.
Treatment of BxPC3 (500,000 Trop-2/cell) and purified T cell-mixtures with (E1)-3s specifically induced trogocytosis, whereby Trop-2 was transferred from BxPC3 to T cells (Figure 2E and F). Whereas (E1)-3s treatment resulted in 40% Trop-2+ T cells, <5% of the T cells were counted in the Trop-2+ gate following treatment with control bsAbs binding only Trop-2 (TF12) or CD3 [(20)-3s], or with (E1)-3s in the absence of BxPC3 cells. The uptake of Trop-2 by T cells coincided with its reduction on BcPC3 cells (Figure 2G and H). During the short incubation time the T cells (97.5% live) and BxPC3 (94.5% live) remained at high viability, indicating that the T cells acquired the tumor antigens by trogocytosis and not by adhering to membrane fragments of dead cells (Supplementary Figure S5). Trogocytosis mediated by (E1)-3s was bidirectional, since T cell membrane components were transferred to BxPC3 cells, as shown for CD4 (Supplementary Figure S6).

**In-Vitro Cytotoxicity**

To evaluate redirected T-cell killing of Trop-2-expressing tumor cells, CD8+ T cells were mixed with NCI-N87 cells in the presence or absence of IFN-α2 (0.1 nM peginterferonalfa-2a or 20*-2b) along with titrations of (E1)-3s (Figure 3). Considerable variability in T-cell potency was observed among donors. With a donor of very active T cells, (E1)-3s mediated a highly potent (IC$_{50}$=0.37 pM; lysis$_{\text{max}}$=77.1%) T-cell lysis of NCI-N87 cells, and inclusion of peginterferonalfa-2a enhanced its activity, improving the IC$_{50}$ (0.14 pM; P=0.0001) by more than 2.5 fold and increasing lysis$_{\text{max}}$ (84.0%; P<0.0001) (Figure 3A). NCI-N87 was only weakly sensitive to the direct actions of IFN-α (peginterferonalfa-2a IC$_{50}$ = >10 nM, Supplementary Figure S7), and inhibited <10% by 0.1 nM peginterferonalfa-2a in the absence of (E1)-3s. The more potent form of IFNα,
20*-2b, consisting of 4 IFN-α molecules fused to a bivalent anti-CD20 mAb by DNL (36,37), enhanced the potency of (E1)-3s by more than 7-fold (IC$_{50}$=0.05 pM; $P<0.0001$). At 0.1 nM, 20*-2b inhibited NCI-N87 by 12.6% in the absence of (E1)-3s. The 20*-2b was included only to show enhanced activity with another (more potent) form IFN-α, and that the effect is not restricted to peginterferonalfa-2a. The anti-CD20 mAb moiety is not functional in this experiment. In a similar assay using very weak donor T cells, (E1)-3s was considerably less potent (EC$_{50}$=39 pM; lysis$^\text{max}$=21%); however, addition of peginterferonalfa-2a enhanced the potency by $>25$ fold (EC$_{50}$=1.4 pM; $P=0.0008$) (Figure 3B). Potent (E1)-3s-mediated T-cell killing also was observed for the human pancreatic cancer line, BxPC3 (IC$_{50}$=0.4 pM); however, the effect of adding IFN-α was not evaluated with this cell line (Supplementary Figure S8).

**Cytokine Release**

A Trop-2XCD3 BiTE (or equivalent) was not available for comparison with (E1)-3s. However, the availability of both (19)-3s, which has the same (X)-3s molecular configuration as (E1)-3s, and 19-3 BiTE, which has the identical amino acid sequence as the CD19XCD3 BiTE, blinatumomab, enabled a head-to-head comparison to evaluate the relative cytokine-inducing potency of the two bsAb formats. Initially, titrations of (19)-3s and 19-3 BiTE were added to mixtures of PBMCs (two independent donors), and Raji NHL cells and the levels of TNF-α, IFN-γ and IL-6 were measured after 20 h (Supplementary Figure S9). Minimal cytokine levels were detected from PBMCs alone, even with the addition of a bsAb. However, due to a mixed lymphocyte reaction occurring between Raji and the donor PBMCs (stronger for Donor A), cytokine levels in untreated cell mixtures were elevated for each TNF-α (200 pg/mL and 50...
pg/mL), IFN-γ (600 pg/mL and 200 pg/mL) and IL-6 (190 pg/mL and 220 pg/mL). The levels of TNF-α and IL-6 were increased above those of untreated only at ≥1 nM (19)-3s. Apparently, (19)-3s inhibited TNF-α and IL-6 production at lower concentrations. Alternatively, TNF-α and IL-6 were elevated to >1000 pg/mL at all concentrations of 19-3 BiTE tested (≥1 pM). The levels of IFN-γ were not increased significantly by (19)-3s, whereas 19-3 BiTE showed a dose-dependent increase to >2000 pg/mL. For all further comparisons, agents were tested at 0.1 nM, which is approximately what has been used in similar studies with BiTE (38). We compared the levels of TNF-α, IFN-γ, IL-2, IL-6, and IL-10 induced by 0.1 nM (19)-3s or 19-3 BiTE from Raji mixed with PBMCs, using four different donors (Figure 4A). With each of the four donors, the levels of each of the five cytokines was significantly higher with 19-3 BiTE, compared to (19)-3s. The mean TNF-α concentration with 19-3 BiTE (2284 ±1483 pg/mL) was 8-fold higher (P=0.0001) than that with (19)-3s (280 ±188 pg/mL). Treatment with 19-3 BiTE, compared to (19)-3s, resulted in levels of IFN-γ (3002 ±560 pg/mL vs 416 ±169 pg/mL), IL-2 (13,635 ±2601 pg/mL vs 1024 ±598 pg/mL), IL-6 (981 ±364 pg/mL vs 168 ±96 pg/mL), and IL-10 (4006 ±2520 pg/mL vs 493 ±242 pg/mL) that were 7-, 13-, 6- and 8-fold higher for 19-3 BiTE, respectively (P<0.0001 for each). These results indicate that the (X)-3s bsAb format is a considerably less potent inducer of cytokine release, compared to the BiTE format.

In general, (E1)-3s in the presence of PBMCs and target cells caused even less cytokine production than (19)-3s, because there is no mixed lymphocyte reaction to elevate the baseline levels (Figure 4B). Levels remained low for the pro-inflammatory cytokines INF-γ (<100 pg/mL), TNF-α (<100 pg/mL) and IL-2 (<250 pg/mL) with 4 of 5
donors. IL-6 was low (<400 pg/mL) in 3 of 5 donors, and moderate (800 – 1100 pg/mL) in donors D-2 and D-5. Donor D-2 also responded to the (E1)-3s more than the others for IFN-γ (1000 pg/mL) and TNF-α (190 pg/mL). IL-10, an anti-inflammatory cytokine, was significantly \( P<0.0001 \) elevated by (E1)-3s to >1200 pg/mL in 3 of 5 donors. Of note, donor D-2, who had a uniquely potent pro-inflammatory response, produced relatively low levels of IL-10 (230 pg/mL) after treatment with (E1)-3s. Peginterferonalfa-2a alone did not increase the level of any cytokine over background. Addition of peginterferonalfa-2a to (E1)-3s consistently increased IFN-γ (~1.5-3-fold) over (E1)-3s alone. For the rest of the cytokines, there was an apparent trend for a moderately increased production with the combination; however, a consistent effect was not observed.

**T-cell Activation**

Addition of 0.1 nM peginterferonalfa-2a increased CD69 upregulation on T cells treated with (E1)-3s moderately, but significantly. For (E1)-3s dose-response experiments measuring %CD69\(^+\) T cells, the EC\(_{50}\) was lowered from 26 pM to 16 pM \( P<0.0001 \) for CD4\(^+\) T cells, and from 11 pM to 6 pM \( P=0.0204 \) for CD8\(^+\) T cells in the presence of IFN-α (Figure 5A). Peginterferonalfa-2a combined with (E1)-3s resulted in more CD69\(^+\) cells (Figure 5B and C, \( P<0.0001 \)), and also, the activated cells had significantly higher CD69 expression with IFN-α (Figure 5B and D; MFI= 907 vs 726; \( P<0.0001 \)). Peginterferonalfa-2a induced minimal CD69 expression in the absence of (E1)-3s. Likewise, (E1)-3, either alone or in combination with peginterferonalfa-2a, did not activate T cells in the absence of target cells.
In Vivo Anti-Tumor Activity of (E1)-3s is Augmented with IFN-α

In vivo efficacy with human pancreatic cancer was evaluated with Capan-1 xenografts. In the first study, treatment with a combination of (E1)-3s and peginterferonalfa-2a [median survival time (MST) >59 days] was superior to all other treatments ($P<0.0007$, log-rank), including (E1)-3s (MST = 50 days) or peginterferonalfa-2a (MST = 53 days) alone (Figure 6A). Even with the omission of T cells, peginterferonalfa-2a extended survival (MST = 45 days, $P=0.0059$ vs saline, log-rank), indicating direct action on the tumor cells. However, peginterferonalfa-2a was more effective in the presence of T cells ($P=0.0260$, AUC), suggesting stimulation of T cells by IFN-α. TF12, which binds target but not T cells, did not affect tumor growth or survival. A repeat experiment, using T cells from a different donor, confirmed the results of the first study (Figure 6B). The second study continued until all groups reached their MST. As in the initial experiment, the combination of (E1)-3s and peginterferonalfa-2a (MST = 119.5 days) was superior to all other groups in terms of both tumor growth inhibition and overall survival ($P=0.0475$ vs (E1)-3s alone; $P<0.0001$ vs all other groups; log-rank). (E1)-3s (MST = 68 days) was superior ($P=0.0373$, AUC over 29 days) to peginterferonalfa-2a with T cells (MST = 53 days) and to T cells alone (MST = 37.5 days; $P=0.0014$ log-rank).

For the NCI-N87 gastric cancer xenograft model (Figure 6C), the combination of (E1)-3s and peginterferonalfa-2a (MST>88 days) was superior to (E1)-3s alone (MST=49 days; $P=0.0007$, log-rank). Compared to the control group with only T cells (MST=32 days), peginterferonalfa-2a alone with T cells provided only a minor, but
significant, survival advantage (MST=35 days; \( P=0.0276 \)). (E1)-3s plus peginterferonalfa-2a without T cells did not improve survival significantly.

The antigen density measured for NCI-N87 [247,000(±65,000) Trop-2/cell] and Capan-1 [157,000 (±37,000) Trop-2/cell] was not significantly different. Compared to NCI-N87, Capan-1 cells were >5-fold more sensitive (IC\(_{50}=2\) nM vs. >10 nM) to direct inhibition by peginterferonalfa-2a \textit{in vitro} (Supplementary Figure S7). (E1)-3s does not cross-react with mouse Trop-2 or CD3 (not shown), and NOD-SCID mice are T-cell deficient.

Discussion

Previously, we described the potential of the (X)-3s bsAb format for redirecting T cell-mediated therapy of both hematopoietic and solid tumors using several example constructs, including (E1)-3s, (19)-3s and (20)-3s (18). In one \textit{in vivo} experiment from that study, where Capan-1 xenografts were treated with (E1)-3s, we included groups with peginterferonalfa-2a, because prior (unpublished) data showed that Capan-1 was inhibited by IFN-\(\alpha\). Since the aspect of IFN-\(\alpha\) was preliminary and beyond the scope of the initial report, we presented only the results for the groups without peginterferonalfa-2a. However, the striking enhancement observed with the addition of IFN-\(\alpha\) spurred further investigation, leading to the current report. The previously reported data, as well as three additional groups including peginterferonalfa-2a (not published), are included in this report in Figure 6A. Herein, we also include a new Capan-1 study, which used a different T-cell donor and was extended until all groups reached their MST, confirming
that IFN-α can enhance the in-vivo efficacy of T-cell killing of an IFN-α-sensitive cell line. We followed with a study showing IFN-α also can enhance T-cell-mediated killing of a cell line that is weakly sensitive to the direct actions of IFN-α. These in vivo studies were performed following methods, including dosing and schedules, typically used with BiTE constructs (35).

Flieger and colleagues demonstrated that in-vitro killing by CD3⁺CD56⁺ NK-T cells, which were expanded ex vivo and redirected with an EpCAMxCD3 BiTE (MT110), was enhanced with either IFN-α or IL-2 (39). However, even in the absence of the bsAb, IFN-α significantly inhibited the target cells. Since a control to evaluate potential direct effects of IFN-α on target cells was lacking, the extent to which the enhanced cytotoxicity was due to IFN-α stimulating NK-T cells, compared to direct inhibition of target cells, could not be determined. Therefore, we measured the sensitivity to IFN-α for both target cells and included groups with peginterferonalfa-2a only, both in the presence and absence of pan-T cells. For Capan-1 tumors, which were more sensitive to IFN-α in vitro, peginterferonalfa-2a improved survival in the absence of T cells, and even moreso with T cells, indicating that IFN-α acted on both Capan-1 as well as T cells in this model. In the absence of T cells, peginterferonalfa-2a did not improve survival of mice bearing NCI-N87 xenografts, which were weakly sensitive to IFN-α in vitro, indicating that the enhancement with IFN-α was due primarily to its actions on T cells. The mechanism of the observed T-cell enhancement by IFN-α is unclear. The increase in CD69 expression attributed to IFN-α was moderate, but significant, suggesting that the cytokine may potentiate T-cell activation induced with the bsAb. Additionally, IFN-α specifically increased (up to 3-fold) the release of IFN-γ, which is considered the chief
cytotoxic cytokine produced by cytotoxic T cells, whereas none of the other cytokines measured increased consistently.

To our knowledge, combination therapy with IFN-α and a T-cell-redirecting bsAb has not been investigated clinically, or even in animal models. However, IL-2 was combined with a F(ab’)2 fragment of an anti-CD3/EpCAM quadroma in a clinical trial (40), but treatment was limited due to considerable toxicity most likely caused by induction of secondary cytokines, known as CRS or cytokine storm. Systemic administration of IL-2 is known to induce a cytokine storm (41), and the severity of adverse events associated with CRS, such as with the TGN1412 catastrophic trial, are correlated with IL-2 release (42). Although it is not without side effects, immunotherapy with IFN-α, which is not produced by T cells, is not typically associated with cytokine storm.

CRS is a risk associated with immunotherapy using any T-cell directed mAb (e.g., Okt3) or bsAb, including BiTE (43). However, not all bsAb formats necessarily have the same risk. Brandl et al. reported cytokine induction with blinatumomab, where response levels of IL-2, IL-6, IFN-γ, and TNF-α were variable among donors and typically peaked at >1 ng/mL, with some donors reaching levels as high as 5 ng/mL (38). We lacked a suitable BiTE, or equivalent construct, for direct comparison with (E1)-3s. However, we were able to compare the relative cytokine-inducing potency between the (X)-3s and BiTE formats, using a CD19XCD3 BiTE (identical sequence as blinatumomab) and (19)-3s made by DNL. The 19-3 BiTE induced similar cytokine levels as reported by Brandl and colleagues under similar conditions (38). The levels of the five cytokines measured were 7-13-fold higher for 19-3 BiTE, compared to those of
(19)-3s. The use of foreign lymphoma cells (Raji) caused a mixed lymphocyte reaction, which increased the baseline cytokine levels, particularly for IL-2. BiTE, but not (19)-3s, increased the cytokine levels well above the mixed lymphocyte baseline level. Using NCI-N87 gastric carcinoma cells as the target for (E1)-3s did not increase baseline cytokine levels. We observed an expected variability in donor response to (E1)-3s; however, the resulting cytokine levels were even lower than those induced by (19)-3s, particularly for TNF-α and IFN-γ, which were <100 pg/mL. Nevertheless, one of five donors had elevated levels (~1 ng/mL) of IFN-γ and IL-6. Addition of IFN-α (peginterferonalfa-2a) to (E1)-3s increased IFN-γ 2-3-fold, but did not consistently affect the levels of the other cytokines. These results suggest that compared to other constructs, such as BiTE, the (X)-3s bsAb format may be less likely to induce CRS, and the addition of IFN-α to a therapeutic regimen is not likely to increase this risk. These experiments were designed only to compare the two bsAb formats and to evaluate the potentially added risk that might accompany combination therapies including IFN-α. These results are not predictive of actual serum levels expected in the clinic.

We observed considerable variability in the potency of donor T cells. The in vitro results shown in Figure 3 represent the most and least active T cells that we have tested, with a 100-fold difference in potency (IC₅₀ = 0.37 pM vs. 39 pM) for killing NCI-N87; however, an IC₅₀ = 1–5 pM is most representative (>10 donors) and the low-activity T cells was atypical. Notably, lysis with the weaker T cells was augmented by IFN-α more than with the potent T cells.

EpCAM is a widely exploited TAA that is overexpressed in many carcinomas. However, the heterogeneous expression of EpCAM in carcinomas and the fact that
EpCAM is not tumor-specific, since it is expressed on most normal epithelia, raise concerns that immunotherapy directed towards EpCAM could have severe side effects (44,45). Like EpCAM, Trop-2 is highly expressed in diverse carcinomas, but its expression in normal tissues is under debate. Several reports indicate that, in contrast to tumor cells, somatic adult tissues show little or no Trop-2 expression, which is invariably upregulated in tumors, regardless of baseline expression in normal tissues (46,47). However, recent evidence indicates expression of Trop-2 on epithelia of several normal tissues (48). Nonetheless, expression of Trop-2 in Cynomolgus monkeys did not result in toxicities after administrations of reasonably high doses of hRS7 (humanized anti-Trop-2) conjugated with SN-38 as an antibody-drug conjugate (ADC) (49). Further, in clinical studies with this anti-Trop-2 ADC, no increased normal organ toxicity other than manageable neutropenia and diarrhea, expected from the drug (a metabolite of irinotecan), was observed at therapeutic doses (50). Thus, immunotherapy, including T-cell-redirected therapy, using Trop-2 for tumor targeting, is expected to have a similar, or greater, therapeutic index compared to similar regimens targeting EpCAM.

To our knowledge, this is the first report of trogocytosis between target tumor and T cells mediated by a bsAb. This finding demonstrates that the target/T-cell conjugates induced with (E1)-3s have functional immunologic synapses. We observed a similar bi-directional trogocytosis between B cells and T cells, which was mediated by (19)-3s (unpublished data), and suspect that this is likely a common phenomenon with T-cell redirecting bsAbs that warrants further investigation.

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Figure Legends

Figure 1. Schematic representation of (E1)-3s (A), TF12 (B), and 20*-2b (C). Each conjugate was prepared by DNL, where AD2 and DDD2 peptides are depicted as yellow and blue helices, respectively, and locking disulfide bonds are indicated by red lines. Variable domains (V_H and V_L) were derived from hRS7 anti-Trop-2 (blue ovals), h679 anti-histamine-succinyl glycine (purple ovals) or veltuzumab anti-CD20 (orange ovals). The Okt3 scFv is displayed as overlapping green ovals. Antibody constant domains are shown as grey ovals. (19)-3s has a similar structure as (E1)-3s shown in panel A, with hA19 anti-CD19 variable domains replacing those of hRS7.

Figure 2. Immunological synapse formation and bi-directional trogocytosis mediated by (E1)-3s. (A-D) Fluorescence microscopy showing conjugates of T cells and NCI-N87 cells mediated by (E1)-3s. Red fluorescence-labeled (appear orange) CD8+ T cells were combined with green fluorescence-labeled NCI-N87 at a 4:1 ratio in the presence of 0.1 µg/mL (E1)-3s (A + B), (19)-3s (C) or TF12 (D). Fluorescent images were captured after 30-min incubation at 37°C. (E-H) Purified T cells were mixed 5:1 with BxPC3 cells and incubated for 60 min with 0.1 nM of the indicated bsAb, before staining with anti-Trop-2 MAb C518 and GAM-Fc-FITC. The cells were analyzed by flow cytometry, with non-conjugated T cells and BxPC3 cells first gated by forward vs side scattering. Trogocytosis of Trop-2 from BxPC3 cells to T cells was evident by detection of Trop-2 on T cells, specifically in cell mixtures with (E1)-3s, shown as percent Trop-2-positive unconjugated T cells (E) and as histograms of Trop-2 fluorescence intensity on T cells.
Trogocytosis resulted in a reduction of Trop-2 on BxPC3 cells, shown as the geometric MFI (G) and histograms (H). *P<0.0001 vs all others.

**Figure 3.** In-vitro cytotoxicity. Purified CD8+ T cells isolated from two different donors (A and B) were pre-treated for 24 h with 0.1 nM peginterferonalfa-2a (▲, dashed), 0.1 nM 20*-2b (●, grey) or media (■, black) before combining with PKH-67 green fluorescent labeled NCI-N87 cells at a 5:1 ratio. The cell mixtures were treated with titrations of (E1)-3s for two days before counting the number of live NCI-N87 cells by flow cytometry. Non-linear regression analysis (sigmoidal dose-response) of the percent lysis, which was calculated for each sample using the formula: \([1-(A_1/A_2)] \times 100\), where \(A_1\) and \(A_2\) represent the number of viable target cells in the test and untreated samples, respectively, vs the log of the molar concentration of (E1)-3s.

**Figure 4.** Cytokine induction. (A) PBMCs (6 x 10^6 cells/well) were combined with Raji (5 x 10^6 cells/well) and treated for 20 h with 0.1 nM 19-3 BiTE (checkered), (19)-3s (black), or incubated without bsAb (white, not tested for D-5). (B) NCI-N87 cells (5 x 10^5 cells/0.5 mL/well) were cultured overnight in 24-well plates to allow cell attachment. PBMCs were added to wells containing attached NCI-N87 cells (10:1 ratio) and treated for 20 h with 0.1 nM of (E1)-3s (black), peginterferonalfa-2a (white), (E1)-3s plus peginterferonalfa-2a (checkered), or untreated (gray). Concentrations of TNF-α, IFN-γ, IL-2, IL-6, and IL-10 in the supernatant fluids were determined using commercial ELISA kits. D-1 through D-8 are independent blood donors, where only D-5 was used in both A and B at the same time.

**Figure 5.** T-cell activation. Purified T cells were mixed 5:1 with NCI-N87 cells and treated for 18 h with (E1)-3s before measuring CD69 expression by flow cytometry. (A)
Non-linear regression analysis (sigmoidal dose-response) of the percent CD69-positive CD4$^+$ (●) or CD8$^+$ (■) T cells vs the log of the molar concentration of (E1)-3s, in the presence (dashed line) or absence (solid line) of 0.1 nM peginterferonalfa-2a. (B) Histogram showing anti-CD69-APC staining of CD8$^+$ T cells following treatment with 0.1 nM (E1)-3s (dotted), 0.1 nM peginterferonalfa-2a (gray), or a combination of both agents (black), in the presence of NCI-N87 cells. (C + D) Percent CD69-positive CD8$^+$ T cells (C), and the geometric mean fluorescence of the CD69$^+$ cells (D), after incubation with 0.1 nM (E1)-3s (E) and/or 0.1 nM peginterferonalfa-2a (P), in the absence or presence of NCI-N87 target cells (T). Each treatment was assayed in triplicate. Error bars, S.D. *, $P<0.001$.

**Figure 6.** *In-vivo* efficacy with human pancreatic and gastric cancer xenografts. Groups of 8 mice inoculated with human T cells and Capan-1 pancreatic (A & B) or NCI-N87 gastric (C) cancer cells were treated daily for five days with 50 µg of (E1)-3s (▲, solid black) or 60 µg TF12 (▼, gray), once weekly for four weeks with 0.6 µg of peginterferonalfa-2a (★, solid black), a combination of (E1)-3s and peginterferonalfa-2a regimens (●, solid black) or with saline (●, dashed black). An additional group was inoculated with Capan-1, but not T cells, and treated with peginterferonalfa-2a (□, dashed black). A, B and C are independent studies. Top panels, Kaplan-Meyer survival plots. Bottom panels, mean tumor volumes (±S.D.) vs days. Data marked with an asterisk were adapted from Fig. 6C in reference 18.
Figure 6

| Median Survival (Days) | Capan-1 | NCI-N87 |
|------------------------|---------|---------|
| **A**                  |         |         |
| (E1)-3s + Peginterferonalpha-2a | >59     | 119.5   |
| (E1)-3s                | 50*     | 68      |
| Peginterferonalpha-2a (with T cells) | 53      | 53      | 35   |
| Peginterferonalpha-2a (no T cells) | 45      | -       | 35   |
| TF12                   | 28*     | -       | -     |
| Untreated (Target + T cells only) | 24*     | 37.5    | 32   |

Note: * denotes statistical significance.
Molecular Cancer Therapeutics

Redirected T-Cell Killing of Solid Cancers Targeted with an Anti-CD3/Trop-2 Bispecific Antibody is Enhanced in Combination with Interferon-alpha

Edmund A Rossi, Diane L Rossi, Thomas M. Cardillo, et al.

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