IL-4 receptor targeting as an effective immunotherapy against triple-negative breast cancer

Sadiya Parveen¹, Sumit Siddharth², Laurene S Cheung¹, Alok Kumar¹, John R Murphy^{1*}, Dipali Sharma^{2*}, and William R Bishai^{1*}

¹Department of Medicine, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD 21231.

²Department of Oncology, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Johns Hopkins University School of Medicine and the, Baltimore, MD 21231, USA.

*Correspondence to be addressed to: john.murphy@jhmi.edu, dsharma7@jhmi.edu, wbishai@jhmi.edu

ABSTRACT

In many solid tumors including triple-negative breast cancer (TNBC), IL-4 receptor (IL-4R) upregulation has been shown to promote cancer cell proliferation, apoptotic resistance, metastatic potential and a Th2 response in the tumor microenvironment (TME). Immunosuppressive cells in the TME including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) also express the IL4-R. We hypothesized that selective depletion of IL4-R bearing cells in TNBC may have dual cytotoxic and immunotherapeutic benefit. To selectively target IL-4R⁺ cells, we genetically constructed, expressed and purified DABIL-4, a fusion protein toxin consisting of the catalytic and translocation domains of diphtheria toxin fused to murine IL-4. We found that DABIL-4 has potent and specific cytotoxic activity against TNBC cells *in vitro*. In murine TNBC models, DABIL-4 significantly reduced tumor growth, splenomegaly and lung metastases, and this was associated with reductions in MDSC, TAM and regulatory T-cells (Tregs) populations with a concomitant increase in the proportion of IFN γ ⁺ CD8 T-cells. The anti-tumor activity of DABIL-4 was absent in IL-4R KO mice directly implicating IL-4R directed killing as the mechanism of anti-tumor activity. Moreover, NanoString analysis of DABIL-4 treated TNBC tumors revealed marked decline in mRNA transcripts that promote tumorigenesis and metastasis. Our findings demonstrate that DABIL-4 is a potent targeted antitumor agent which depletes both IL-4R bearing tumor cells as well as immunosuppressive cell populations in the TME.

STATEMENT OF SIGNIFICANCE

In solid tumors like breast cancer, Interleukin-4 receptor (IL-4R) expression in the tumor microenvironment aids tumor growth and metastasis. IL-4R expression upon host immune cells further dampens antitumor immunity. In this study, we have genetically constructed a fusion protein toxin, DABIL-4, composed of the catalytic and translocation domains of diphtheria toxin and murine IL-4. DABIL-4 showed specific cytotoxicity against triple-negative breast cancer (TNBC) cells in vitro. DABIL-4 also markedly inhibited TNBC tumor growth and metastasis in vivo. The primary activity of DABIL-4 was found to be depletion of IL-4R+ immune cells in combination with direct elimination of tumor cells. In conclusion, DABIL-4 targeting of both tumor and immunosuppressive host cells is a versatile and effective treatment strategy for TNBC.

INTRODUCTION

Interleukin 4 (IL-4) is an important pleiotropic cytokine primarily secreted by activated Th2 lymphocytes, basophils, mast cells, and eosinophils. IL-4 promotes differentiation of Th2 cells, upregulates the expression of major histocompatibility complexes and the IL-4 receptor, and regulates immunoglobulin class switching, especially IgE production, making it an important cytokine in allergic responses (1). IL-4 functions by binding to three different classes of IL-4 receptors (IL-4R). The Type I receptor is primarily found on T-cells, basophils, mast cells, NK cells, and most B cells and consists of the IL4R α and common gamma C (γ c) subunits. The Type II receptor, comprised of the IL-13R α 1 and IL-4R α subunits, is expressed on tumor cells. And the Type III receptor present on B-cells and monocytes consists of the IL-4R α , IL-13R α 1 and γ c subunits (2).

Upregulation of the IL-4R (CD124) has been shown in multiple human and murine malignancies including glioma, lung, breast, pancreatic, bladder, colon and ovarian cancers (2). The binding of IL-4 to the IL-4R recruits and phosphorylates tyrosine kinases Jak1/2 and Tyk2, which then activate the PI3K/AKT, MAPK, and Jak/STAT6 signaling pathways (3). Upon activation, these pathways promote cancer cell proliferation, resistance to apoptosis, and metastatic potential (3, 4). In the TME, IL-4 elicits increased Th2 responses and activates myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs)—both of which express the IL-4R—to promote immunosuppression and angiogenesis (2). Therapeutic targeting of the IL-4/IL-4R interaction and its consequences has led to effective antitumor strategies which include pharmacological inhibition of the downstream pathways (AKT, ERK, JAK/STAT6, and mTOR), monoclonal antibody blockade of IL-4 or the IL-4R; engineered IL-4R antagonists, and IL-4 fused with cytotoxic payloads (2, 3).

In breast cancer, up-regulation of IL-4/IL-4R signaling has been associated with poor prognosis in both humans and murine models (5). Additionally, IL-4 blockade has been shown to effectively down-regulate the mitogen-activated protein kinase (MAPK) pathway and reduce the growth and invasion of breast cancer cells (4, 6). Interestingly, TNBC cells were shown to secrete higher levels of IL-4 in the tumor milieu, compared to estrogen-receptor (ER)-positive breast cancer cells, contributing to their proliferation and metastatic potential (4), and recently an IL-4 mediated boost in glucose and glutamine metabolism was identified as a driver of TNBC cell growth (7). As TNBC tumors are difficult to treat owing to their inherent molecular heterogeneity and lack of targetable receptors (estrogen, progesterone and HER2) (8-10), we sought to interrupt the IL-4/IL4R signaling axis in TNBC using a toxin-based approach.

We recently reported the development of a second-generation version of denileukin diftitox (a diphtheria toxin-related IL-2 fusion protein) that significantly decreased the growth of melanoma tumors when tested as monotherapy or in sequential combination with checkpoint blockade (11). In the present study, we describe the construction, expression, purification and action of IL-4R targeted fusion protein toxin: s-DAB₁₋₃₈₉-mIL-4 (referred to as DABIL-4 hereafter). DABIL-4 consists of the catalytic and translocation domain of diphtheria toxin contained in residues 1-389 genetically fused to murine IL-4 (mIL-4). We demonstrate that this fusion protein toxin specifically targets IL-4R⁺ murine TNBC cells *in vitro*. *In vivo* administration of DABIL-4 in murine 4T1 adenocarcinoma model showed significant reductions in tumor growth, splenomegaly and metastases to lung. We also observed a marked decline in the population of MDSCs, TAMs and Tregs with a concomitant increase in proportion of effector CD8⁺ T-cells in TME. In summary, we demonstrate that DABIL-4 is a potent cytotoxic agent against IL-4R⁺ cells that also depletes

immunosuppressive cell populations in both the TME and spleen and thereby promotes anti-tumor immunity.

RESULTS

Genetic construction, expression, and purification of DABIL-4

For the genetic construction and expression of DABIL-4, we used a strategy recently developed in our laboratory to re-construct the structural gene encoding denileukin diftotox with the native diphtheria *tox* signal sequence and a mutated *tox* operator (11). Using a *Corynebacterium diphtheriae* expression system, we achieved constitutive expression of the fusion protein toxin and its secretion into culture medium as a fully folded, biologically active, monomeric recombinant protein (**Fig 1A**). We constructed a recombinant plasmid (pKN2.6Z-LC128) to express DABIL-4. This plasmid was generated by replacing the IL-2 coding sequence in pKN2.6Z-LC127 (11) with a synthetic gene encoding murine interleukin 4 (mIL-4) that was codon-optimized for *C. diphtheriae* within an *E. coli* / *C. diphtheriae* shuttle vector (**Fig. S1**). For expression and purification of the fusion protein toxin, pKN2.6Z-LC128 was transformed into the non-lysogenic, non-toxigenic *C. diphtheriae* strain C7_s(-)^{tox} strain. The fusion protein toxin, which is secreted into the medium with this expression system, was concentrated, adsorbed onto a Ni²⁺-NTA affinity column, eluted and further purified by gel permeation chromatography to achieve >98% purity (**Fig 1B**). The chimeric nature of the fusion toxin was confirmed by immunoblot with anti-DT, anti-mIL-4, and anti-His₆ antibodies. As shown in **Fig 1C**, the 58 kDa band reacted with all the three antibodies confirming that the fusion protein toxin retained the expected antigenic determinants.

DABIL-4 selectively targets IL-4 receptor-expressing cancer cells in vitro

To evaluate whether DABIL-4 was cytotoxic for IL-4R-bearing cancer cells, we performed a dose-response analysis using 4T1 cells, a murine IL-4R⁺ triple-negative breast cancer cell line (6). Upon incubation with varying concentrations of DABIL-4 for 48 hours, 4T1 cells exhibited sensitivity to DABIL-4 in a dose-dependent manner with an IC₅₀ of 2 nM (**Fig 2A**), while heat-inactivated DABIL-4 was completely non-toxic (**Fig 2B**). Another TNBC cell line, E0771 (12), also showed sensitivity to the fusion toxin albeit with a slightly higher IC₅₀ value of 4 nM, a difference potentially attributable to different IL-4R expression levels and/or growth rates (**Fig S2**). 4T1 also exhibited sensitivity to DABIL-4 in a trypan blue dye exclusion assay with a calculated IC₅₀ of 0.026 nM (**Fig 2C**). This discrepancy in IC₅₀ values is likely due to the higher sensitivity of the dye exclusion assay compared to the colorimetric MTS assay. To demonstrate that the activity of DABIL-4 is mediated through the IL-4R, we examined the competitive inhibition of increasing concentrations of recombinant murine IL-4 upon the cytotoxicity of 25 nM DABIL-4. As shown in **Fig. 2D**, recombinant IL-4 completely blocked the cytotoxic activity, despite the presence of higher concentration of DABIL-4, demonstrating that the action of the fusion protein is mediated via the IL-4R/IL-4 interaction.

As previously reported, once internalized by the target cell, the diphtheria toxin catalytic domain ADP-ribosylates elongation factor 2 (EF-2) which leads to the inhibition of cellular protein synthesis, followed by induction of apoptosis and cell death within 48 -72 hours (13). Using a chemiluminescence based assay, we demonstrated that DABIL-4 treatment led to 3-fold induction of caspase 3/7 activity in 4T1 cells which was comparable to the cytotoxic agent doxorubicin (Dox)(**Fig 2E**). Immunoblot analysis further demonstrated apoptosis induction in DABIL-4 treated cells revealing cleavage of both PARP and caspase3 (**Fig 2F**). These results confirm that DABIL-4

is a potent cytotoxic agent which induces apoptosis in the targeted cancer cells expressing the IL-4R.

DABIL-4 inhibits tumor growth and prevents lung metastases in a murine TNBC model

The 4T1 mammary tumor model in syngeneic BALB/c mice shares many characteristic features with human breast cancer including progressive growth of primary tumors and the ability to metastasize to lungs, liver, bone and brain. Following orthotopic injection of as few as 10,000 cells into the mammary fat pads, palpable tumors appear within 7 days and metastases are observed by 18-28 days (14). We utilized the orthotopic 4T1 murine model to evaluate the cytotoxicity of DABIL-4 against 4T1 cells *in vivo* (**Fig 3A**). Tumor bearing mice were treated by intraperitoneal injection on alternate days with either 10 µg DABIL-4 or PBS alone. As shown in **Figs. 3B** and **3C**, we observed significant reductions in tumor volume and weight in mice treated with DABIL-4. To rule out the strain specificity, we tested the efficacy of DABIL-4 in the orthotopic E0771 TNBC model generated in C57BL/6 strain background and observed that DABIL-4 significantly reduced E0771 tumor growth (**Fig S3**).

4T1 tumors are also known to have high metastatic potential (14). To evaluate the impact of DABIL-4 upon metastatic progression, we harvested lungs from 4T1 tumor-bearing mice from both treated and untreated mice on day 17, isolated metastatic cancer cells and performed clonogenic assays. We observed a 12-fold reduction in the number of metastatic tumor colonies cultured from the lungs of DABIL-4 treated mice compared to the PBS-treated group (**Fig 3D** and **3E**). To further characterize the *in vivo* effect of DABIL-4 upon the metastatic potential, we analyzed total RNA isolated from the primary tumors using the NanoString PanCancer Immune Profiling Panel Assay. We noted significant declines in the level of several transcripts associated with metastasis (Cd36 (15), Egr3 (16-18) and Maf (19)) and tumorigenesis (Ccr2 (20), Ccr6 (21))

in many solid tumors including breast cancer (**Fig 4** and **Table 1**). We also observed an increased levels of the C200r1 transcript which is associated with inhibition of metastasis and tumorigenesis (22, 23)(**Fig 4** and **Table 1**). These observations indicate that DABIL-4 not only prevents primary tumor growth but also inhibits metastatic dissemination of 4T1 cells to secondary sites.

DABIL-4 depletes MDSCs and TAMs in vivo thereby reducing immunosuppression in the TME

One of the pathologic hallmarks of the 4T1 model is splenomegaly (up to 8-fold increases in spleen weight) mostly due to granulocytic hyperplasia (24). We found that DABIL-4 treatment led to a 20% reduction in the spleen weights in 4T1 bearing mice suggesting that this fusion protein toxin also affects granulocytosis and tumor-associated leukemoid reactions in addition to tumor growth inhibition (**Fig 5A**). It is well known that IL-4R is not only expressed on 4T1 tumor cells but also upon immune cells, specifically myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). Both of these immune cell populations contribute to immunosuppression within the TME as well as metastasis (25-28). To investigate whether the observed inhibition of tumor growth was due to direct targeting of IL-4R⁺ tumor cells, to depletion of IL-4R⁺ MDSCs and TAMs, or to a combination of the two, we orthotopically implanted IL-4R⁺ 4T1 cells in an IL-4R knockout mouse strain (IL4Rα^{-/-}) and treated with either PBS or DABIL-4 thrice weekly starting on day 7. We observed a delayed progression of 4T1 tumors in the IL-4R knockout mice. Moreover, treatment of tumor-bearing IL-4R knockout mice with DABIL-4 reduced spleen weights by 9% (vs 20% in WT) and tumor weights by 18% (vs 52% in WT), reductions that were less impressive than those we observed in wild type mice (**Fig 5B, 5C and 5D**). These results in IL-4R knockout mice indicate that direct 4T1 cell killing by DABIL-4 contributes modestly to delayed tumor expansion. Further, they suggest that depletion of IL-4R⁺

immunosuppressive host cell populations is likely a key second mechanism of DABIL-4's antitumor efficacy.

To directly address the effects of DABIL-4 on host immune cell populations, we analyzed tumor/spleen from 4T1 tumor-bearing wild type mice using multicolor flow cytometry. As in **Fig. 3A**, mice began thrice weekly DABIL-4 or PBS treatment on day 7 after 4T1 tumor implantation. In spleen homogenates derived from DABIL-4 treated mice, we noted a 26% reduction in total myeloid cell population (CD11b⁺) (**Fig 6A**). This observation prompted us to investigate the drug's impact upon two major myeloid cell subsets, MDSCs and macrophages. We first considered MDSCs and observed a marked decline in the total MDSC population (**Fig S4A**). Additionally, evaluation of two distinct MDSCs subsets; polymorphonuclear MDSCs (PMN-MDSCs; CD11b⁺ Ly6G⁺ Ly6C^{low} CD124⁺) and monocytic-MDSCs (M-MDSCs; CD11b⁺ Ly6G⁻ Ly6C^{high} CD124⁺) revealed a 60% reduction in IL-4R⁺ PMN-MDSCs population in DABIL-4 treated mice (**Fig 6B**), while the level of IL-4R⁺ M-MDSCs remained the same (**Fig S4B**). We also noted a sharp decline in the population of PMN-MDSCs subset expressing IL-10 (**Fig 6C**). This cytokine is known to skew anti-tumor T-cell responses towards a Th2 phenotype promoting tumor growth (29). Second, we evaluated macrophages and observed a marked increase in the overall population of macrophages (CD11b⁺ F4/80⁺) in spleen homogenates (**Fig S4C**). Upon further investigation, we found that DABIL-4 treatment had opposite effects upon M1- and M2-like macrophages. While we observed a significant decline in the population of M2-like macrophages (IL-4R⁺ CD206⁺) (**Fig 6D**), the population of pro-inflammatory CD86⁺ and TNFα⁺ M1-like macrophages increased (**Fig 6E and 6F**).

Similarly, in tumor homogenates, we noted reduction in IL-4R and CD206 expressing macrophages (**Fig S4D**) and an enrichment of TNFα⁺ macrophages (**Fig S4E**). These results

suggest that DABIL-4 targets and depletes IL-4R⁺ MDSCs and macrophages thereby reducing their ability to suppress host mounted immune responses, resulting in a more robust anti-tumor response.

DABIL-4 increases effector T cell population and their cytotoxic potential in vivo

Next, we investigated the effect of DABIL-4 administration upon both CD4⁺ and CD8⁺ lymphocytes in 4T1 bearing mice. As in **Fig. 3A**, mice began thrice weekly DABIL-4 or PBS treatment on day 7 after 4T1 tumor implantation and homogenates from spleens and tumors were analyzed using multicolor flow cytometry. In spleens, we detected enrichment of both CD4⁺ and CD8⁺ lymphocytes in DABIL-4 treated mice (**Fig S5A and S5B**). Both subsets also exhibited reduced expression of CD39 (**Fig S5C and S5D**), a phenotype associated with improved anti-tumor activity (30).

In the tumors, despite no apparent changes in total CD4⁺ and CD8⁺ T cells, we found a significant increase in CD44 and IFN γ expressing CD8⁺ lymphocytes in DABIL-4 treated mice (**Fig 7A and 7B**). We also detected increased IFN γ and decreased CD39 expressing CD4⁺ T cells. (**Fig S6A and S6B**). There was also a significant decrease in the population of activated Treg cells (**Fig 7C**). These changes in lymphocyte subsets indicate that DABIL-4 treatment mice elicit enhanced anti-tumor responses in the TME. Accordingly, we detected significantly elevated levels of GzmK mRNA transcript in total RNAs isolated from these tumors (**Fig 7D and Table 1**) which may indicate either enhanced cytotoxic potential of lymphocytes or an overall increase in GzmK expressing lymphocytes population in the TME. The same analysis also revealed decline in IL-10 receptor expression in tumors from DABIL-4 treated mice (**Fig 7E and Table 1**).

We also found an increase in frequency of an unconventional lymphocyte population, double-positive (DP) CD4⁺ CD8⁺ T-cells, in tumors isolated from DABIL-4 treated mice (p = 0.07)

(**Fig S6C**). There was, however, a statistically significant increase in CD44 expressing DP lymphocytes (**Fig S6D**). Compared to CD4 and CD8 mono-positive T cells, DP lymphocytes exhibit characteristics similar to memory T lymphocytes, have a higher capacity to produce cytokines, and improved lytic potential (31, 32). Based on these observations we infer that DABIL-4 mediated depletion of immunosuppressive cell populations facilitates significant increases in the population of activated effector T cells in the TME.

DISCUSSION

Here, we report the genetic construction, expression and purification of a diphtheria toxin-based fusion protein, DABIL-4, which targets the cell killing capability of diphtheria toxin to IL-4R-bearing cells. Using a *C. diphtheriae*-based expression host described earlier by Cheung et al. in 2019 (11), we expressed/purified DABIL-4 and demonstrated that the fusion toxin exhibits potent and specific cytotoxicity against IL-4R bearing TNBC cells *in vitro* and *in vivo*. The fusion toxin reduced tumor growth and lung metastases in a murine syngeneic TNBC adenocarcinoma model primarily by depleting IL-4R⁺ immune cell populations such as MDSCs thereby facilitating a robust anti-tumor T cell response, but it also showed modest depletion of the 4T1 tumor cells themselves.

Lakkis et al. reported a similar IL-4R⁺ diphtheria toxin-based fusion protein toxin from an *Escherichia coli*-based system and demonstrated targeted cytotoxicity to IL-4R bearing eukaryotic cells *in vitro* and a marked reduction in delayed type hypersensitivity *in vivo* (33). However, the therapeutic efficacy of the Lakkis et al. protein was not evaluated in preclinical animal tumor models and hence its effect upon host immune cells remained unknown. A similar immunotoxin, IL-4(38-37)-PE38KDEL, comprised of a circularly permuted human IL-4 fused with *Pseudomonas aeruginosa* exotoxin A, was found to be cytotoxic against the human breast

cancer cell line, MDA-MB231, both *in vitro* and murine xenograft models (34). It was also tested in pre-clinical models of various human malignancies including breast cancer (2, 34). These studies with IL-4(38-37)-PE38KDEL were carried out in immunocompromised mice without an assessment of its effect on IL-4R⁺ immune cells, and thus its antitumor efficacy was largely attributed to direct killing of IL-4R-bearing tumor cells (34). In the present study, we demonstrated that DABIL-4 has a potent and direct cytotoxic activity against IL-4R⁺ positive TNBC cells *in vitro*. To assess the degree of direct tumor cell killing by DABIL-4, we tested it in IL-4R KO mice and found only modest reductions in tumor progression (Fig 5B-5D) suggesting the IL-4R⁺ immune cell depletion accounts for a significant degree of its antitumor efficacy. Indeed, we further demonstrated significant DABIL-4-mediated depletion of MDSCs and TAMs with concomitant increases in effector T cell populations and reductions in Tregs.

In the present study, we observed a selective depletion of splenic PMN-MDSCs while the population of splenic M-MDSCs remained the same in DABIL-4 treated mice. Interestingly, in spleen of 4T1 tumor bearing mice, PMN-MDSCs constituted the major MDSCs subset and exhibited higher proliferation rate compared to M-MDSCs (35, 36). This higher proliferation rate could be responsible for the increased susceptibility of PMN-MDSCs to DABIL-4 which inhibits translation, the major machinery required by the rapidly growing cells (37). In many cancers, M-MDSCs are shown to be abundant in the TME and tend to rapidly differentiate into TAMs (35). This dynamic conversion could be one of the reasons why we detected depletion of TAMs but not M-MDSCs in tumor. However, considering our limited ability to distinguish M-MDSCs from macrophages (38), the observed depletion of TAMs may also arise from targeting of both TAMs and M-MDSCs. Further investigation will be required to reach to a conclusion.

In 4T1 tumor bearing mice, the spleen acts as the primary site of MDSCs proliferation and sequestration (24, 36) from where these cells are then recruited to the TME in response to tumor-secreted chemokines (39). Indeed, evidences suggests that MDSCs-mediated suppression of T cells promote immunosuppression in the TME (40) (22). Accordingly, splenectomy has been shown to reduce the amount of tumor-infiltrating MDSC and to cause tumor regression in murine cancer models (41) suggesting that splenic MDSCs may also contribute to tumorigenesis (42), which explains why DABIL-4-mediated depletion of splenic MDSCs caused a robust anti-tumor response in the present study.

In this study, we demonstrated therapeutic efficacy of DABIL-4 as a monotherapy in murine model of triple-negative breast cancer. We propose that the therapeutic efficacy of DABIL-4 may be further potentiated by combining it with other effective immunotherapeutic approaches such as checkpoint inhibitors. While our study focused on breast cancer, there are several reasons to anticipate that DABIL-4 may offer a valuable immunotherapeutic approach against other solid tumors. First, we observed DABIL-4 mediated anti-tumor activity in both PDL1-negative (4T1) and positive (E0771) tumors models. Second, DABIL-4 showed efficacy even against well-established 4T1 murine tumors. The fusion toxin was active when therapy began either on day 7 (**Fig 3B**) or day 11 (**Fig S3**) post tumor implant. Third, IL-4R upregulation on immune and tumor cells and its role in tumorigenesis and metastasis is well-established in case of multiple solid tumors (2, 3), and DABIL-4 targets these tumor adaptations. In summary, DABIL-4 is a promising therapeutic that demonstrates both direct killing of IL-4R-positive tumor cells combined with depletion of IL-4R-bearing immunosuppressive cells such as MDSCs and TAMs. As such, It holds potential as dual cytotoxic and immunotherapeutic agent for the treatment of multiple cancers including triple-negative breast cancer and warrants further study.

FIGURE LEGENDS

Fig 1. Genetic construction and purification of DABIL-4 fusion toxin using *C. diphtheriae*.

(A) Schematic of DABIL-4 fusion toxin construct compared to DABIL-2 (B) Coomassie-stained SDS/PAGE gel after purification (lane 2). Lane 1 is molecular weight markers. Expected size of DABIL-4 is 58 kDa. (C) Immunoblot of the purified DABIL-2 and DABIL-4 fusion toxins. Staining with anti-DT antibody revealed diffuse bands for both DABIL-2 and DABIL-4 and arrow corresponds to the molecular weight of the fusion toxins.

Fig. 2. DABIL-4 exhibits cytotoxic activity against IL-4R⁺ 4T1 cells and induces apoptosis. (A)

Activity of DABIL-4 in an MTS assay showed an IC₅₀ of 2 nM while (B) post heat-inactivation, it showed no cytotoxicity. (C) DABIL-4 in trypan blue dye exclusion assay showed cytotoxicity with an IC₅₀ of 0.026 nM. (D) Recombinant IL-4, in a dose-dependent fashion, blocked the cytotoxic activity of 25 nM DABIL-4 in an MTS assay. (E) Caspase 3/7 activity luminescence-based assay (Promega) performed with 4T1 cells treated with 20 nM DABIL-4. Caspase activity was comparable to doxorubicin, an agent known to cause apoptosis in 4T1 cells. (F) Immunoblot analysis of 4T1 cells treated with varying concentration of DABIL-4 showed upregulation of apoptotic markers (cleaved PARP and cleaved Caspase-3). β -actin was used as the loading control.

Fig 3. DABIL-4 shows anti-tumor activity in 4T1 adenocarcinoma model in WT background.

(A) Schematic of the experiment. Treatment was given thrice weekly on days 7, 9, 12, 14, 16, 19, 21, 23 post tumor implantation. (B) DABIL-4 administration led to the decline in tumor volume (n=5), (C) tumor weight (n=5) and, (D) lung metastasis (n=3) both qualitatively and (E) quantitatively. Statistical significance for tumor growth and lung metastasis experiments was assessed by two-tailed unpaired student t-test considering an unequal distribution. Tumors were

measured by electronic Vernier calipers. Red arrows indicate the days when treatment was administered. Data are shown as mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig 4. DABIL-4 monotherapy modulates level of mRNA transcripts associated with metastasis and/or tumorigenesis in tumor microenvironment. Total RNA isolated from 4T1 tumors (n=3) from both PBS- and DABIL-4 treated mice on day 17 and were subjected to NanoString PanCancer Immune Profiling Panel analysis and solver software. Expression profiles of transcripts promoting metastasis (A-C) or tumorigenesis (D, E) and transcript inhibiting metastasis and tumorigenesis (F) are shown for both treatment groups. RNA expression values are given as log₂ and data are shown as mean $\pm$ SD. Statistically significant differences were defined by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig 5. DABIL-4 shows no anti-tumor activity against 4T1 adenocarcinoma model in IL-4R knockout mice. (A) DABIL-4 administration in 4T1 bearing Balb/c mice showed reduction in spleen weights. In IL-4R KO mice, 10,000 4T1 cells were orthotopically implanted and allowed to establish. Starting day 7, DABIL-4 was given via i.p. thrice a week on alternate days for total of 8 doses. Mice were sacrificed on day 27. The fusion toxin administration did not significantly reduce (B) spleen weights, (C) tumor volumes and, (D) tumor weights. Statistical significance for these experiments was assessed by two-tailed unpaired student t-test considering an unequal distribution. Tumors were measured by electronic Vernier calipers. Data are shown as mean $\pm$ SD. *P < 0.05, NS=non-significant. N=5 mice per group.

Fig 6. DABIL-4 administration depletes immunosuppressive myeloid cell population in spleen. As in Fig 3A, mice were treated with DABIL-4 thrice weekly beginning on day 7, and they were sacrificed on day 25. Single cell suspensions of spleens from both treatment groups were stained with specific antibodies and analyzed by flow cytometry (n=5). We found differences in the

population of (A) CD11b⁺ cells, (B) IL-4R⁺ PMN-MDSCs, (C) IL-10⁺ PMN-MDSCs, (D) IL-4R⁺ CD206⁺ M2 macrophages, and (E) CD86⁺ M1 macrophages and (F) TNFα⁺ macrophages. Data are represented as mean ± SD and as percentage of CD45⁺ population (except panel D which is represented as “percentage population of macrophages”). Spleen were analyzed on day 26 post tumor implant. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig 7. DABIL-4 treatment promotes infiltration of effector cell populations in tumor microenvironment, mediating anti-tumor response. As in Fig 3A, mice were treated with DABIL-4 thrice weekly beginning on day 7, and they were sacrificed on day 17 and 25. Single cell suspensions of the tumors isolated from both the groups were stained and were analyzed by flow cytometry (n=5). We found differences in the population of (A) activated CD44⁺ CD8⁺ T cells of CD45⁺, (B) IFNγ⁺ CD8⁺ T cells of CD3⁺, and (C) CD39⁺ Tregs of CD45⁺ cells. Total RNA isolated from these tumors were subjected to NanoString PanCancer Immune Profiling Panel analysis and nSolver software (n=3). Expression profiles of (D) Granzyme-K and, (E) IL-10 receptor transcripts are shown for both treatment groups. RNA expression values are given as log₂. All panels except panel B correspond to day 17 post tumor implantation while panel B corresponds to day 25. All data are shown as mean ± SD. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01.

AUTHOR CONTRIBUTIONS

SP, JRM, DS and WRB conceptualized the study and designed the research approach; SP, SS, AK performed research; LC, JRM contributed new reagents/analytic tools; SP, JRM, DS, WRB analyzed data; and SP, JRM, DS, WRB wrote the paper, SP, SS, LC, AK, JRM, DS, WRB critically reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

J.R.M., and W.R.B. hold positions in Sonoval, LLC, which holds rights to develop certain diphtheria toxin-based fusion proteins

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TABLE

Table 1: List of mRNA transcripts identified using NanoString PanCancer Immune Profiling Panel

mRNA Transcript	Expression (log ₂)		P-Value	Pathway	Reference
	PBS	s-DABIL-4			
Cd36	7.81	7.51	0.005	M	(15)
Egr3	7.90	7.52	0.01	M	(43)
Maf	8.45	8.10	0.01	M	(19)
Ccr2	11.23	9.98	0.04	T	(20)
Ccr6	5.04	4.43	0.04	T	(21)
Cd200r1	7.80	8.17	0.03	T/M	(22, 44)
Gzmk	5.06	6.42	0.04	I	(45)
Il10ra	8.85	8.18	0.04	I	(46)

FIGURES

Fig. 1

Fig 1. Genetic construction and purification of DABIL-4 fusion toxin using *C. diphtheriae*.

(A) Schematic of DABIL-4 fusion toxin construct compared to DAB IL-2. (B) Coomassie-stained SDS/PAGE gel after purification (lane 2). Lane 1 is molecular weight markers. Expected size of DABIL-4 is 58 kDa. (C) Immunoblot of the purified DAB IL-2 and DABIL-4 fusion toxins.

Fig. 2

Fig. 2. DABIL-4 exhibits cytotoxic activity against IL-4R⁺ 4T1 cells and induces apoptosis.

(A) Activity of DABIL-4 in an MTS assay showed an IC₅₀ of 2 nM while (B) post heat-inactivation, it showed no cytotoxicity. (C) DABIL-4 in trypan blue dye exclusion assay showed cytotoxicity with an IC₅₀ of 0.026 nM. (D) Recombinant IL-4, in a dose-dependent fashion, blocked the cytotoxic activity of 25 nM DABIL-4 in an MTS assay. (E) Caspase 3/7 activity luminescence-based assay performed with 4T1 cells treated with 20 nM DABIL-4. Caspase activity was comparable to doxorubicin (Dox), an agent known to cause apoptosis in 4T1 cells. (F) Immunoblot analysis of 4T1 cells treated with varying concentration of DABIL-4 showed upregulation of apoptotic markers (cleaved PARP and cleaved Caspase-3). β-actin was used as the loading control.

Fig 3.

Fig 3. DABIL-4 shows anti-tumor activity in 4T1 adenocarcinoma model in WT background.

(A) Schematic of the experiment. Treatment was given thrice weekly on days 7, 9, 12, 14, 16, 19, 21, 23 post tumor implantation. (B) DABIL-4 administration led to the decline in tumor volume (n=5), (C) tumor weight (n=5) and, (D) lung metastasis (n=3) both qualitatively and (E) quantitatively. Statistical significance for tumor growth and lung metastasis experiments was assessed by two-tailed unpaired student t-test considering an unequal distribution. Tumors were measured by electronic Vernier calipers. Red arrows indicate the days when treatment was administered. Data are shown as mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 4

Fig 4. DABIL-4 monotherapy modulates level of mRNA transcripts associated with metastasis and/or tumorigenesis in tumor microenvironment. Total RNA isolated from 4T1 tumors (n=3) from both PBS- and DABIL-4 treated mice on day 17 and were subjected to NanoString PanCancer Immune Profiling Panel analysis and solver software. Expression profiles of transcripts promoting metastasis (A-C) or tumorigenesis (D, E) and transcript inhibiting metastasis and tumorigenesis (F) are shown for both treatment groups. RNA expression values are given as log₂ and data are shown as mean $\pm$ SD. Statistically significant differences were defined by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig 5.

Fig 5. DABIL-4 shows no anti-tumor activity against 4T1 adenocarcinoma model in IL-4R knockout mice. (A) DABIL-4 administration in 4T1 bearing Balb/c mice showed reduction in spleen weights. In IL-4R KO mice, 10,000 4T1 cells were orthotopically implanted and allowed to establish. Starting day 7, DABIL-4 was given via i.p. thrice a week on alternate days for total of 8 doses. Mice were sacrificed on day 27. The fusion toxin administration did not significantly reduce (B) spleen weights, (C) tumor volumes and, (D) tumor weights. Statistical significance for these experiments was assessed by two-tailed unpaired student t-test considering an unequal distribution. Tumors were measured by electronic Vernier calipers. Data are shown as mean $\pm$ SD. *P < 0.05, NS=non-significant. N=5 mice per group.

Fig. 6

Fig 6. DABIL-4 administration depletes immunosuppressive myeloid cell population in spleen.

As in **Fig 3A**, mice were treated with DABIL-4 thrice weekly beginning on day 7, and they were sacrificed on day 25. Single cell suspensions of spleens from both treatment groups were stained with specific antibodies and analyzed by flow cytometry (n=5). We found differences in the population of (A) CD11b⁺ cells, (B) IL-4R⁺ PMN-MDSCs, (C) IL-10⁺ PMN-MDSCs, (D) IL-4R⁺ CD206⁺ M2 macrophages, and (E) CD86⁺ M1 macrophages and (F) TNF α ⁺ macrophages. Data are represented as mean $\pm$ SD and as percentage of CD45⁺ population (except panel D which is represented as “percentage population of macrophages”). Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. 7

Fig 7. DABIL-4 treatment promotes infiltration of effector cell populations in tumor microenvironment, mediating anti-tumor response. As in Fig 3A, mice were treated with DABIL-4 thrice weekly beginning on day 7, and they were sacrificed on day 17 and 25. Single cell suspensions of the tumors isolated from both the groups were stained and were analyzed by flow cytometry (n=5). We found differences in the population of (A) activated CD44⁺ CD8⁺ T cells of CD45⁺, (B) IFN γ ⁺ CD8⁺ T cells of CD3⁺, and (C) CD39⁺ Tregs of CD45⁺ cells. Total RNA isolated from these tumors were subjected to NanoString PanCancer Immune Profiling Panel analysis and nSolver software (n=3). Expression profiles of (D) Granzyme-K and, (E) IL-10 receptor transcripts are shown for both treatment groups. RNA expression values are given as log₂. All panels except panel B correspond to day 17 post tumor implantation while panel B corresponds to day 25. All data are shown as mean $\pm$ SD. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. *P < 0.05, **P < 0.01.

SUPPLEMENTRY FIGURES

Fig. S1

Fig S1. pKN2.6Z-LC128 shuttle vector plasmid map.

Fig. S2

Fig S2. DABIL-4 shows cytotoxic activity against IL-4R⁺ E0771 TNBC cell line. Activity of DABIL-4 in MTS assay showed an IC₅₀ of 4 nM.

Fig. S3

Fig S3. DABIL-4 exhibits anti-tumor activity in E0771 adenocarcinoma model in C57BL/6 mice.

50,000 E0771 cells were orthotopically implanted in C57BL/6 mice. Starting day 11, DABIL-4 was given i.p. on alternate days for total of 5 doses. Tumor volumes were measured using electronic Vernier Calipers. Statistical significance was calculated by two-tailed unpaired student t-test considering an unequal distribution. Data are shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Fig. S4

Fig S4. DABIL-4 treatment modulates myeloid cell populations in spleen and tumor

microenvironment. Single cell suspensions of spleen and tumors were stained and analyzed by flow cytometry (n=5). We evaluated differences in the population of (A) IL-4R⁺ MDSCs of CD45⁺, (B) IL-4R⁺ M-MDSCs of CD45⁺, (C) macrophages of CD45⁺. (D) IL-4R⁺ CD206⁺ expression on macrophages and, (E) TNFα⁺ macrophages of CD45⁺. All panels correspond to day 25 post tumor implantation. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. Data are represented as mean ± SD. *P < 0.05, NS=non-significant.

Fig. S5

Fig S5. DABIL-4 administration promotes accumulation of lymphoid cell populations in spleen.

Single cell suspensions of spleen were stained and analyzed by flow cytometry (n=5). We evaluated differences in the population of (A) CD4⁺ and (B) CD8⁺ T-cells of CD45⁺ cells. We also noted differences in (C) CD39 expression upon CD4⁺ and (D) CD8⁺ T-cells. All panels correspond to day 25 post tumor implantation. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. Data are represented as mean $\pm$ SD. *P < 0.05, **P < 0.01.

Fig. S6

Fig S6. DABIL-4 promotes accumulation of activated effector cell population in tumor

microenvironment. Single cell suspensions of tumors were stained and analyzed by flow cytometry (n=5). We evaluated differences in the population of (A) IFN γ production by CD4⁺, (B) CD39⁺ CD4⁺ of CD45⁺, (C) double-positive (DP) CD4⁺ CD8⁺ of CD3⁺ and (D) CD44⁺ DP of all lymphocytes. All panels except panel A correspond to day 17 post tumor implantation while panel A corresponds to day 25. Statistical significance between the groups was assessed by two-tailed unpaired student t-test considering an unequal distribution. Data are represented as mean $\pm$ SD. *P < 0.05, NS=non-significant.