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Research Article
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Long Term Follow Up: Phase I Trial of "bi-shRNA furin/GMCSF DNA/Autologous Tumor Cell" Immunotherapy (FANG™) in Advanced Cancer

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Abstract

Study Background: Previously, we demonstrated safety and correlated induced immune response with survival in a Phase I study of FANG immunotherapy in advanced cancer patients. We now report long term follow-up (FU) of Phase I treated patients including assessment of relationships of dose, γIFN-ELISPOT response, and patient demographics to safety and survival.

Methods: Safety, γIFN-ELISPOT response, and survival have been followed through 3+ years in advanced cancer patients who received ≥ 2-12 intradermal monthly injections of 1×10^7 or 2.5×10^7 cells/injection. Clinical and serological assessments were performed monthly, radiographic evaluations bimonthly, and γ-IFN-ELISPOT at baseline, and start of Cycle 2, 4, 6, 9, 12 then sequentially at FU.

Results: Previously, we reported results on 45 patients with successful FANG construction followed for 1 year (28 treated (designated FANG); 17 not treated based on availability of other alternative treatments or failed manufacturing (designated No FANG)). We now report FU results through year 3 on those patients and an additional 29 patients (7 FANG, 22 No FANG) subsequently entered into Phase I study (total N=35 FANG; total N=39 No FANG). The median survival of the current expanded Phase I trial population is 562 days vs. 122 days (p=0.00001). This is similar to the originally published data from two years earlier. The γ-IFN-ELISPOT reaction was positive in 14 of the current FANG treated patients and negative in 12 FANG treated patients at Month 3 or less post first injection. Survival correlated with γ-IFN-ELISPOT reaction; median 836 days vs. 440 days with positive and negative ELISPOT respectively, (p=0.04). No long term adverse toxicity has been seen and there was no significant correlation of immune response or survival with either dose or demographics.

Conclusions: Treatment with FANG vaccine continues to show long term safety and evidence of benefit in patients with many types of advanced cancer thereby justifying further efficacy testing.

Keywords: Cancer; RNA interference; Vaccine; Clinical

Introduction

The autologous whole tumor cell FANG™ vaccine, the use of which provides the afferent arm of the immune system the full and relevant tumor antigen matrix, is comprised of a plasmid which encodes both GMCSF and bi-shRNA furin DNA, and is transfected into the harvested tumor cells via electroporation [1,2]. By design, this vaccine is a combinatorial immune therapeutic producing the intra- and extra cellular adjuvant GMCSF protein which simultaneously expresses an innovative RNA interference (RNAi) moiety [3], bifunctional short hairpin RNA-furin (bi-shRNA-furin).Targeting the proproteinconvertase furin (which activates both TGF-β1 and β2) was previously shown to result in marked knockdown (>90%) of both TGF-β1 and β2 [2] in the Phase I clinical trial without interfering with GMCSF expression encoded by the same plasmid.

The motivating rationale of this "triad" vaccine was to overcome a core critical hurdle as defined at the recent "Immunotherapy Summit" of the Society for Immunotherapy of Cancer (SITC): the "complexity of cancer, tumor heterogeneity and immune escape [4]." The existence of genetic and, presumably, phenotypic antigenic intra- and inter-tumoral heterogeneity has now been convincingly established [5] and accounts for one of the reasons for the limited effectiveness of peptide-based vaccines. Despite immune escape due, in part, to the development of tolerance, tumor cells can retain their intrinsic immunogenicity and tolerance can be antigen-specific [6]. TGFβ allows for the development of regulatory functionality in indoleamine 2,3-dioxygenase (IDO) competent plasmacytoiddendritic cells and human epidermoid Langerhans cells [7] which underlies the conversion into and maintenance of the tolerogenic pathway [8,9].

We previously published [1] results of a non-randomized, Phase

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I FANG vaccine trial in late stage, refractory cancer patients with expected survival of 6 months or less who received 1 of 2 dose levels (1×10^7 or 2.5×10^7 cells/injection), by intradermal injection, once a month for a maximum of 12 months. Safety was confirmed and there was no evidence of a dose-response relationship; thus, the 1×10^7 cell dose per injection was identified as the Phase II recommended dose. Preliminary evidence of a survival trend with γ-IFN-ELISPOT positive versus γ-IFN-ELISPOT negative patients was noted. We now present results through 3 years FU that confirm safety and provide additional evidence of benefit related to FANG-induced immune response.

Materials and Methods

The construction and cGMP manufacturing of FANG have been described [1,2]. Following protocol specific informed consent, tumor is excised, placed in sterile media, and brought to the Gradalis, Inc. manufacturing facility (Carrollton, TX). Under cGMP conditions, the harvested autologous tumors are mechanically and enzymatically dissociated into a single cell suspension followed by a count of viable cells. The FANG vector is then electroporated into the autologous tumor cells using a Bio-Rad electroporator (Bio-Rad Laboratories, Hercules, CA). The cells are incubated overnight to allow transcription of the bi-shRNA^{furin} and expression of the GMCSF protein. The following day the tumor cells are irradiated (10,000 cGy), then aliquoted and cryopreserved until the time of injection. The total processing time for vaccine manufacturing is less than 48 hours. Each vaccine is subjected to a quality control testing regimen (less than 4 weeks duration). Release criteria include minimum GMCSF expression (≥ 30 pg/10^6 cells/ml) and TGFβ1 and TGFβ2 knockdown (≥ 30% from baseline) [1,2].

Patient population

Study design has been previously published [1]. All eligible patients were treated in the outpatient facilities of MCCRC, Dallas, TX and Texas Cancer Center, Abilene, TX. Inclusion criteria have been previously published [1]. Following completion of previously published analysis, an amendment was established which enabled continued accrual of subset populations of patients fulfilling the same inclusion criteria with advanced NSCLC, hepatocellular cancer, triple negative breast cancer and Ewing's sarcoma.

Imaging and lab assessment

Within 2 weeks prior to therapy, a complete medical evaluation, as previously described [1], was performed. Evaluations performed every 28 ± 3 days during therapy included: physical examination; ECOG assessment; CBC with differential and platelet count; serum chemistry and electrolytes; toxicity assessment; and clinical assessment of tumor response. Radiological assessments of tumors were obtained at months 2,4,6 and then quarterly as long as the patient remained alive and was under approved consent.

ELISPOT assay

ELISPOT (Enzyme-Linked Immunospot) assay was performed using Enzyme-Linked Immunospot Assay for Interferon Gamma (BD Biosciences, San Jose, CA) as previously described [1,10]. A value of >10 spots and 2x baseline was considered a positive response. Quantitation provided by ZellNet Consulting, Fort Lee, NJ.

Statistics

Survival was analyzed using SPSS to generate Kaplan Meier curves and included all patients procured as part of the clinical protocol with a malignant pathology. Survival of patients still alive was censored using the date of last follow-up. ELISPOT analysis was performed on patients receiving at least 2 vaccines and the response status at baseline and Month 3 or earlier from treatment start was compared using a paired t-test.

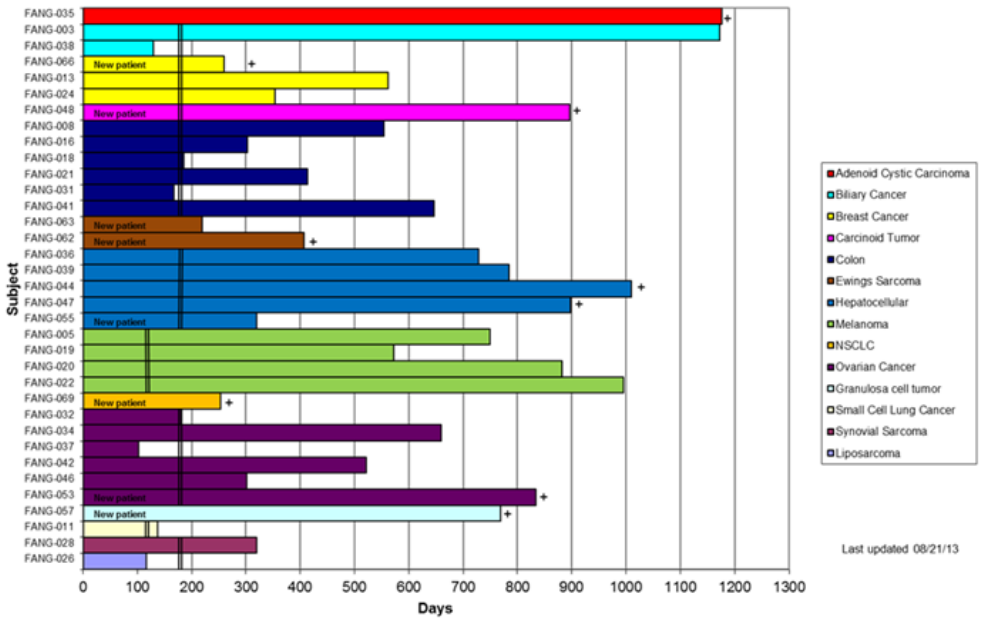
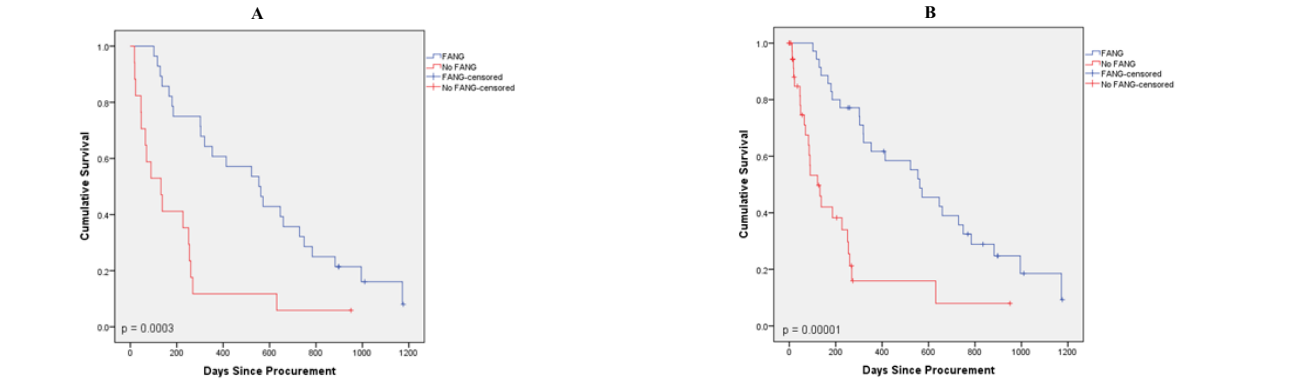


Figure 1: Survival of adult treated patients since procurement in the FANG™ Phase I protocol (n=35). + = alive, || = historical expected survival, new patient designates patients treated with FANG following initial publication.

Results

Long term response

Twenty-eight treated and 17 non-treated patients were described in our earlier report [1]. Including these total of 176 vaccines have now been administered to 35 patients. No long term toxic events have been reported or observed related to FANG. With long term follow up, individual survival durations of FANG-treated patients are shown in Figure 1. Long term survival comparison of the initial Phase I core patient population (FANG vs. No FANG) is also shown in Figure 2A. Further long term analysis incorporating 7 newly entered FANG treated patients (new total N=35) and 22 No FANG patients (new total N=39) are shown in Figure 2B. A total of 468 successful vaccines involving 56 patients (FANG and No FANG) have been constructed during Phase I trial production. Of the 39 No FANG patients who elected not to receive FANG, 24 patients had other treatment options and 15 patients did not as a result of failed vaccine manufacturing. Survival results between the original FANG and updated FANG populations are consistent and continue to demonstrate an advantage of FANG over No FANG and confirm safety. Long term FU also revealed no correlation of survival to dose, GMCSF expression, TGFβ1, and/or β2 knockdown, and/or furin knockdown or other demographic variables represented in Table 1.



Treatment Status	Total n	Mean (days)	Median (days)	No. Surviving
No FANG	17	205	132	1
FANG	28	576	554	4
Overall	45	440	302	5

Treatment Status	Total n	Mean (days)	Median (days)	No. Surviving
No FANG	39	228	122	15
FANG	35	604	562	9
Overall	74	442	302	24

Figure 2: Survival comparison of FANG vs. no FANG patients since procurement (A): (update to original manuscript patients). Note: 1 patient grouped in the No FANG patient at the time of manuscript submission, has since been treated and is now reflected in the FANG group. Survival comparison of all current FANG vs. no FANG patients since procurement(B).

Characteristic	No FANG™ (n=39)	FANG™ (n=35)	All (n=74)
Age (years)			
Mean	56	56	56
Median	56	60	56
Range	19-84	18-84	18-84
Gender			
Male	21	11	32
Female	18	24	42
Ethnicity			
African American	1	2	3
Asian	2	0	2
Caucasian	35	30	65
Hispanic/Latino	1	3	4
Dose Level			
1.0×10^7 cells/ml	15	16	31
2.5×10^7 cells/ml	11	19	30
Vaccine failure	6	N/A	6
Insufficient dose/cells	7	N/A	7

*All patients required prior surgical debulking

Table 1: Demographic Data of Evaluable Patients (n=74).

Comparison to prognostic score

Wheler et al. [11] recently identified 5 critical risk factors related to survival of a group composed of unselected Phase I trial patients. These include low albumin (<3.5 g/dL), high LDH (>ULN IU/L), increased number of metastasis (>2 anatomical sites), GI tumor type, and ECOG performance status ≥ 1). Using these same risk variables we assigned a score to each of the FANG treated patients for whom data were available (n=31) excluding LDH, insofar as it was not included as a standard laboratory assessment in this Phase I trial. As a consequence a conservative comparison, with reservation, of FANG treated patients to the 1,181 Phase I cancer patients described by Wheler is provided in the analysis shown in Table 2. Results suggest FANG treated patients achieve survival duration greater than prognostic score prediction.

Comparison of MDACC prognostic score between FANG and no FANG patients in Figure 2A revealed a score of 2.2 (higher risk) for former and 1.6 (lower risk) for the latter suggesting that biased patient entry (insofar as this Phase I study was not randomized) based on prognostic factors associated with survival of Phase I patient population are not responsible for the survival difference between FANG and No FANG treated patients.

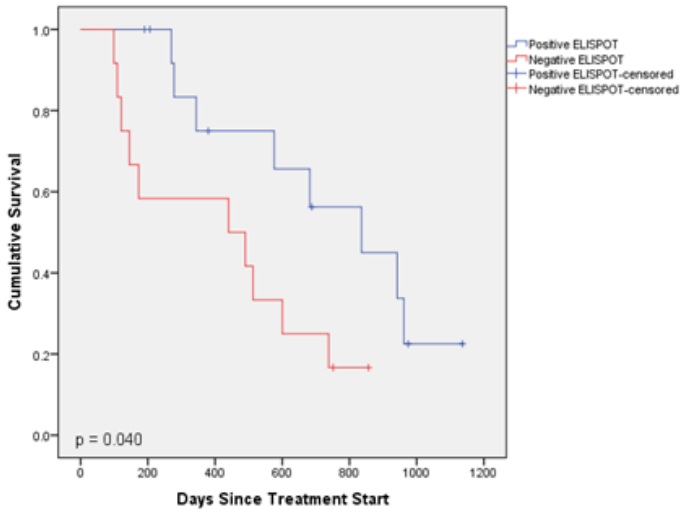
Immune response advance to survival

In the initial Phase I publication [1], we described a correlation of FANG-induced γ-IFN-ELISPOT response with patient survival. At longer term FU with additional patients, correlation of survival with the month 3 γ-IFN-ELISPOT responses persists as shown in Figure 3. Individual γ-IFN-ELISPOT responses over time are shown in Figure 4. As seen, one patient had a baseline γ-IFN-ELISPOT of 10 spots that did not double (as per criteria for positive response) after FANG vaccination and two patients demonstrated a positive γ-IFN-ELISPOT response after month 3 with continued vaccination. These 3 patients were included in the γ-IFN-ELISPOT negative group for survival assessment in Figure 3 per protocol definition.

MDACC Prognostic Score	FANG Phase I Months Survival n = 31*	MDA Months Survival (Wheler, et al.) n = 1,181
0 Low Risk	34.7	24.0
1 Low-intermediate Risk	25.0	15.2
2 Intermediate Risk	11.8	8.4
3 High-Intermediate Risk	26.1	6.2
4 High Risk	6.2	4.1

*4 patients did not have complete medical records access in long term follow up

Table 2: Survival of FANG Phase I patients by MDACC Prognostic Score.



ELISPOT Status	Total n	Mean (days)	Median (days)	No. Surviving
Negative	12	428	440	2
Positive	14	756	836	6
Overall	26	610	576	8

Figure 3: FANG Phase I survival relationship to immune response. ELISPOT(+) vs. ELISPOT(-) patients since treatment start: Results show survival comparison of adult patients, who had an ELISPOT assessment done at 3 months post treatment start, and showed positive ELISPOT immune response to FANG™ (blue) vs. those with a negative ELISPOT immune response to FANG™ (red).

The median number of vaccines administered was 5.5. As is noted in Figure 4, patients continued to demonstrate ELISPOT responsiveness after discontinuation of FANG administration for as long as they could be evaluated under protocol. Thus far, no patient with a positive γ-IFN-ELISPOT response has experienced a persistent decrease in response after initial stimulation. Survival comparison of γ-IFN-ELISPOT positive response patients, negative response patients, and No FANG patients is shown in Figure 5. Interestingly, FANG treated patients with γIFN-ELISPOT negativity also demonstrated survival advantage over No FANG patients suggesting the possibility that immune mechanisms activated by FANG but not reflected in the γ-IFN-ELISPOT assay used may be at play.

Discussion

The continued evidence of safety of FANG over a prolonged time frame in conjunction with a maintained survival advantage correlating

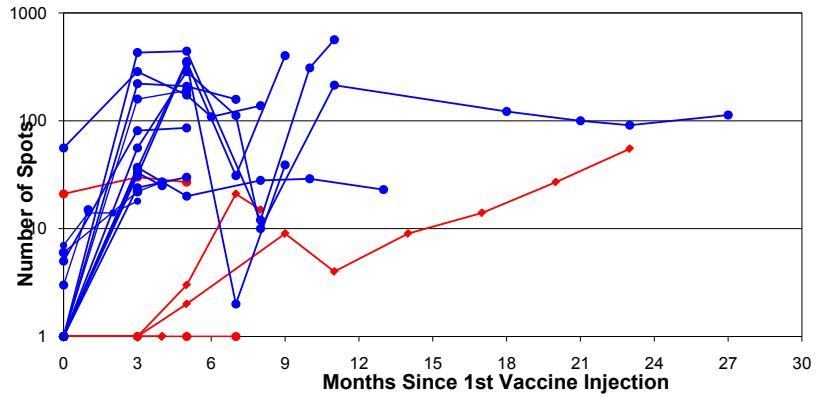
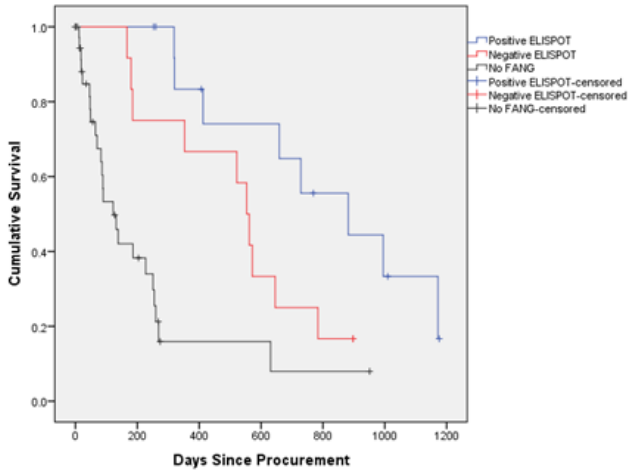


Figure 4: ELISPOT FANGTM vaccine treated patient peripheral blood mononuclear cell response to non-transfected autologous tumor cells (n=26) overtime. Blue indicates multiple ELISPOT assessments of month 3 positive patients. Red indicates multiple ELISPOT assessments of month 3 negative response patients.



Treatment Status	Total n	Mean (days)	Median (days)	No. Surviving	p-value (in comparison to No FANG)
No FANG	39	228	122	15	
ELISPOT (-)	12	526	554	2	0.009
ELISPOT (+)	14	819	882	6	0.00002
Overall	65	460	319	23	

Figure 5: FANG Phase I adult survival relationship to treatment status and ELISPOT status. FANG/ELISPOT (+) vs. FANG/ELISPOT (-) vs. no FANG since procurement survival results are shown.

with tumor-specific immune response lends credence to the rationale underlying the use of the vaccine in advanced cancer patients. Evidence of a significant FANG induced γ-IFN-ELISPOT response in more than half of FANG treated patients (despite extensive prior treatment and the presence of bulky disease) supports the combinatorial mechanism of the GMCSF/bi-shRNA furin plasmid when transfected into autologous tumor cells and utilized as a monthly intradermal injection in advanced cancer patients. The persistence of the positive γ-IFN-ELISPOT responses suggests that the vaccine may contribute to the programming of long term effector-memory T cells [12,13]. Moreover, correlation between survival and elicited γ-IFN-ELISPOT response supports the contention that FANG vaccine can impact cancer patient survival. Analysis of prognostic indicators that correlate with survival of Phase I treated patients, particularly in the γ-IFN-ELISPOT responsive vs. non-responsive patients, adds further weight to the survival impact of the FANG vaccine in patients with advanced cancer. The MDACC prognosticscore comparison involved an expansive number of Phase I trial patients and long term follow up. It was interesting in comparison to the FANG data that differences held at every individual prognostic score level (0-4), although minimal advantage was demonstrated at the poorest prognostic score level (high risk, 4). Nevertheless, lacking a prospectively randomized control population, these results must remain inconclusive at this time.

Rejection of antigen-expressing tumor cells has been shown to be mediated by specific host cytolytic T cells (CD8+ CTL) [14,15], the presence of which correlate with survival. CD8+ CTL tumor infiltrating lymphocytes (TIL) have been shown to mediate durable regression of established tumors in mice with advanced tumor burdens [16,17]. In patients bearing metastatic tumors, a number of groups have demonstrated the existence of anti-tumor CTL responses. Peripheral blood mononuclear cells as well as TIL contain populations of cells and individual clones that demonstrate tumor specificity; they lyse autologous tumor cells, but not natural killer targets, allogeneic tumors cells, or autologous fibroblasts [18-22]. Therefore, despite the successful process of immune escape, generally manifested as immune tolerance [23], in established cancer, tumor-specific antigens do exist on human tumor cells which can retain their intrinsic antigenicity insofar as the tolerant state can be antigen specific [6],

Transforming growth factor β (TGF-β) comprises a family of multi-functional proteins that regulate the growth and function of many normal and neoplastic cell types [24-27]. TGF-β2 signal transduction has been found to affect the expression of more than 20 different genes [28-31]. TGF-β exerts a wide range of effects on a variety of cell types and has been shown to stimulate or inhibit cell growth, induce apoptosis and increase angiogenesis [32-36]. Many tumors including, but not limited to, breast, ovarian, colon, esophageal, gastric, hepatocellular, pancreatic, SCLC and NSCLC produce high levels of active TGF-β isoforms [37-45]. Furthermore, overexpression of TGF-β has been correlated with tumor progression and poor prognosis [38,39]. Elevated TGFβ2 levels have also been linked with immunosuppression in both the afferent and efferent limbs of the immune response network [24-26,39,46-48]. Tumor-derived TGF-β1 induces the upregulation of PD-L1 in immunocompetent splenic dendritic cells and are causally related to the shift in dendritic cell phenotype from immunostimulatory to immunosuppressive transgenic in the LSL-K-ras^{G12D}/+p53$^{loxP/loxP}$ murine model of induced metastatic ovarian cancer [49]. TGFβ2 inhibits T cell activation in response to antigen stimulation as well as targeting cytotoxic T cell cytolytic pathways [50]. Additionally, TGF-β2 has antagonistic effects on the Natural Killer (NK) cells as well as the induction and proliferation of the lymphokine-activated killer (LAK) cells [51-56].

The immune suppressor functions of TGF-β are likely to play a major role in modulating the effectiveness of cancer cell vaccines. TGF-β inhibits GMCSF induced maturation of bone marrow derived dendritic cells (DCs) [57] as well as expression of MHC Class II and co-stimulatory molecules [58]. It has been shown that antigen presentation by immature DCs result in T cell unresponsiveness [59]. TGFβ also inhibits activated macrophages [60] which not only includestheir antigen presenting function [61,62] vis-à-vis the adaptive immune pathway but, in addition, via its contribution to IL-4 induced M2 polarization [63] may also undercut activation of the innate immune pathway [64]. Therefore, both the ubiquitous expression and multifunctionality of the TGF-β isoforms, including the inhibitory effects of these isoforms on GMCSF immune modulatory function (see below), provide the basis fora combinatorial TGF-β-suppressed / GMCSF-expressing immune modulating therapeutic.

Tumor cells genetically modified to secrete GMCSF have demonstrated potent induction of anti-tumor immunity compared to other cytokines [65,66]. Limited clinical results also suggest that treatment with recombinant GMCSF protein or use of GMCSF DNA vectors [67] may translate into clinical advantage, through immune stimulation [68-70]. GMCSF is involved in the augmentation of tumor antigen presentation [65,71].

In one study immunologic effects of B16 melanoma cells engineered to secrete either GMCSF or Flt-3-Ligand (FL) immunologic effects were reported [66]. Three profound differences between the 2 cytokines were described. First, GMCSF induced a subset of DCs that were superior for the phagocytosis of apoptotic tumor cells [72-74]. Second, compared to FL, GMCSF evoked higher levels of costimulatory molecules, which may have induced more efficient T cell stimulation, thereby broadening the arsenal to include lymphocyte effector mechanisms [75]. Third, GMCSF promoted uniformly high levels of CD1d on DCs, in contrast to FL, which triggered a more heterogeneous expression [76]. CD1d is a non-classical MHC Class I molecule that presents lipid antigens [77]. The CD1d lipid complex activates natural killer T cells (NKT) cells [78]. NKT cells play a pivotal role in therapeutic tumor responsiveness [79].

Based on the current results, the FANG vaccine has advanced to Phase II evaluation designed to 1) gain further evidence of effectiveness in a randomized study, 2) evaluate effectiveness in the adjuvant setting in patients with minimal residual disease, and 3) determine the feasibility of concurrent immune modulating therapeutic doses of chemotherapy and vaccine administration. These studies will hopefully also shed light on methods of optimizing the use of this combinatorial immunotherapy in patients with different therapeutic requirements contingent on tumor type and stage.

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