Flt3-ligand as a Vaccine Adjuvant: Results in a Study of Flt3-ligand Plus Tetanus Toxoid Immunization

Stephen Chui, MD*
Timothy M. Clay, PhD†
Paul J. Mosca, MD, PhD†
Amy C. Hobeika†
Takuya Osada, MD†
Laurent Galibert, PhD‡
Dania Caron, MD‡
H. Kim Lyerly, MD†§
Michael A. Morse, MD*

*Department of Medicine, †Department of Surgery, Duke University Medical Center, Durham, North Carolina
‡Amgen Corporation, Seattle, Washington
§Departments of Immunology and Pathology, Duke University Medical Center, Durham, North Carolina

ABSTRACT

Dendritic cells (DC) efficiently process and present antigens to the effector arm of the immune system, thereby stimulating immunity against antigens of both foreign and self origin. Administration of Flt3-ligand (FL) has been reported to increase dendritic cell (DC) numbers in mice and humans. As a result, FL has attracted interest as an adjuvant for vaccine immunotherapy. To investigate whether FL might increase the immune response to a model recall antigen, we administered FL 25µg/kg/d subcutaneously to six healthy volunteers followed by a standard injection of intramuscular tetanus toxoid (TT). A control cohort of six healthy volunteers received tetanus toxoid alone. Compared to subjects who received only TT, subjects who received Flt3L and TT had greater TT-specific DTH reactivity. In contrast, FL did not augment peripheral blood mononuclear cell proliferative responses or antibody responses to TT. FL resulted in inconsistent TT-specific T cell responses as measured by interferon-gamma ELISPOT and cytokine flow cytometry. We conclude that while FL mobilization of DC may enhance in vivo immune responses to a known immunogenic recall antigen, there are inconsistent effects on immune response detected by in vitro assays. Further study will be required to determine which individuals might experience augmentation of the immune response with FL.

INTRODUCTION

The specific and nonspecific compartments of the immune system function in concert to generate effectors and responses against peptides and proteins identified as "foreign." It is now recognized that dendritic cells (DC) function

as the most potent antigen presenting cell in the body. Dendritic cells possess the unique capacity to travel through and sample the extracellular matrix. Dendritic cells subsequently take up, process, and present antigens to naïve T cells. Recognition of peptides in the context of the appropriate co-stimulatory molecules and cytokines induces T cells to differentiate and assume effector functions.[1] Because of the powerful ability of DC to generate antigen specific immune responses and their potential capacity to stimulate immunity against tumor-associated self antigens, DC have formed the basis of many novel immunotherapy strategies attempting to enhance immunity to tumor antigens.

Since DC make up less than 1% of circulating white blood cells, a variety of methods have been utilized to generate and expand DC ex vivo prior to antigen loading and then delivery as a vaccine.[2] Unfortunately, these ex vivo culture techniques are laborious, time-consuming, expensive, and generate cells whose functional characteristics may differ from the DC found intrinsically within the body. As a result, in vivo cytokine-stimulated mobilization and antigen loading of DC has been proposed as a vaccination strategy to overcome the difficulties associated with ex-vivo DC production.

FMS-like tyrosine kinase 3 ligand (Flt3L, FL) is a cytokine growth factor that induces the proliferation and survival of primitive hematopoietic progenitor and stem cells.[3] When FL has been administered systemically to humans, the numbers of DC circulating in the peripheral blood has increased up to 40-fold.[4-7] Studies have shown that DC mobilized by human FL administration possess the capacity to stimulate T cells in vitro.[4] Flt3-ligand has attracted interest as an adjuvant intended to increase the efficacy of vaccination therapies by expanding DC numbers, which would in turn induce enhanced immune responses. Animal models that have evaluated various schedules of FL and antigen immunization produced conflicting results regarding the augmentation of antigen-specific immunity.[8-17]

A handful of human studies have been reported using FL as a vaccine adjuvant.[18-20] Results from these studies were variable: some of these studies reported no increase in T cell or antibody responses,[18,19] whereas others suggested amplification in antigen specific immunity as measured in some assays.[20] These inconsistent results could be attributed to differences in the immunogenicity of the antigens tested, the method of FL administration, and the disease status of the subjects. Furthermore, interpretation of these early clinical studies is limited by the small numbers of subjects and analysis of different facets of the immune response.

In order to specifically address whether FL could function as a potent adjuvant for augmenting antigen-specific immune responses in humans, we chose to test the effects of FL on the immune response to vaccination using a model antigen known to consistently elicit antibody and T cell responses in healthy individuals. Tetanus toxoid was chosen as the model antigen because it has been shown to both activate T cell[21] and antibody[22] responses. Additionally, most individuals have previously been immunized against tetanus toxoid and would be expected to generate recall responses to re-challenge. In order to characterize and quantify both cellular and humoral immune responses, we evaluated T cells responses using several methodologies: the interferon-gamma ELISPOT, cytokine flow cytometry, and proliferation assays; and we evaluated the antibody response by a standard hemagglutination assay.

METHODS

Subjects

This protocol was approved by the Duke University Medical Center Institutional Review Board. Subjects were healthy volunteers, age ≥ 18 years and < 50 years (to avoid an effect on the study due to diminution in immunity with older age[23]), who could safely discontinue current prescription and nonprescription medications at least 7 days prior to receiving the first dose of the study drug and for the duration of the study. They were asked to abstain from the use of alcohol or illicit drugs for at least 1 week before initiating the study and for the duration of the study. Subjects were excluded if they had any medical condition requiring physician care, were pregnant or lactating, or had any history of autoimmune disease. No steroids were permitted in the 4 weeks prior to study initiation.

Protocol Treatment

Standard intradermal skin testing with recall antigens (Candida (1:1000 dilution), Mumps (undiluted), tetanus toxoid (0.1 mL of 1.6 Lf/mL solution) was performed according to our Clinical Allergy Lab standards and the diameter of erythema and induration was measured 48 hours later. Subjects underwent a 1 to 2 hour leukapheresis to obtain enough peripheral blood mononuclear cells for immune analysis. Subsequently, injections of FL 25 μg/kg/d subcutaneously (rotating sites on the arms, thighs, and abdomen) were performed for 10 days. Premedication with acetaminophen and diphenhydramine was permitted. The day after completing the FL, they underwent a repeat leukapheresis (1-2 hours), repeat DTH skin testing with the same 3 recall antigens, and then received 1 standard dose of TT (Connaught Laboratories, Swiftwater, Pa) intramuscularly into the deltoid. One week and four weeks after the vaccination, periph-eral blood was drawn for immunologic analysis. Also, at four weeks following the TT vaccination, they had repeat intradermal skin testing with the recall antigens.

The control cohort did not receive FL prior to TT vaccination. This cohort underwent the same procedures described above, except they underwent a single leukapheresis prior to the tetanus toxoid immunization and received no FL injections.

Enumeration of Peripheral Blood DC

Aliquots of PBMC from before and after the Flt3L administration were analyzed for percentage of DC by fluorescence-activated cell sorting (FACS). Cells were incubated within a blocking buffer (PBS + 1 mg/mL huIgG + anti-CD32 (IV-3) + anti CD64 (32.2) antibodies) 15 minutes prior to and during the entire labeling procedure. Dendritic cell subsets were identified using fluorochrome or biotin-conjugated BDCA1, BDCA2, BDCA3 antibodies (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's specifications. Alternatively, DC were first identified as lineage negative cells using antibody cocktail composed of FITC-conjugated anti-CD3, CD14, CD16, CD19, CD20, CD34, CD94, CCR3 antibodies (all from Pharmingen, San Diego, Calif) except CCR3 from (R&D systems, Minneapolis, Minn). Using this antibody cocktail, all Lineage-cells appeared HLA-DR+ as assessed with PE-conjugated anti-HLA-DR antibodies (TU36, Pharmingen). Within lineage negative cells, Plasmacytoid pre-DC and myeloid DC were then identified by using PE conjugated anti-BDCA2 and biotinylated anti-BDCA1 antibodies + streptavidin-APC (Molecular Probe Inc, Eugene, Ore) respectively. Results were compared to those generated with isotype matched negative controls after appropriate compensations. Greater

than 30,000 events were collected on a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson).

Cell Preparation for Immunologic Analysis

Fresh peripheral blood mononuclear cells (PBMC) were prepared from whole blood or leukapheresis products by the density-gradient method using Ficoll Paque Plus (Amersham Pharmacia AB, Uppsala, Sweden). Assays assessing T cell function directly on whole blood or PBMC were initiated as soon as possible after blood collection, and blood remained at room temperature (RT) until use (no more than six hours).

Lymphocyte Proliferation Assay

For each sample, PBMC were cultured at 1×10^5 cells per well of a 96 well plate in the presence of tetanus toxoid, preservative-free recombinant hepatitis B surface antigen (HBsAg; kind gift from Merck Research Laboratories, West Point, Pa), phytohemagglutinin (PHA; Sigma Chemicals, St. Louis, Mo) or medium alone for 4 days. The following antigens were assayed in triplicate in a total volume of 200 µL: no antigen; hepatitis B surface antigen (HBsAg) at 0.04, 0.2 or 1 µg/mL; TT (preservative-free; Connaught Laboratories Willowdale, Ontario, Canada) at 1, 5, or 25 Lf/mL; and PHA at 0.25, 0.5, or 2.5 µg/mL. After 4 days, 3H-thymidine (1 µCi; NEN Life Science Products, Boston, Mass) was added to each well for 18 to 20 hours and the DNA was harvested onto filter paper using a TOMTEC automated cell harvester (Wallac, Turku, Finland). ^{3}H-thymidine incorporation was determined using a MicroBeta Plus Scintillation counter (Wallac). Data are presented as mean stimulation index (S.I.), which was determined by subtracting the mean cpm of media alone wells from cpm of wells incubated with antigen divided by the mean cpm of media alone.

Interferon Gamma ELISPOT Analysis

Immulon-2 HB 96-well microtiter plates (Dynex Technologies, Chantilly, Va) were coated with 2 µg/mL Anti-Human interferon gamma (IFN-α) monoclonal antibody (mAb; Endogen, Woburn, Mass) 16 to 20 hours at 4°C. PBMC in complete medium (RPMI 1640 with 10% fetal bovine serum) were added to the blocked plate at 2×10^5 cells/well in quadruplicate for each antigen. 1:3 serial dilutions of PBMC were prepared. Antigens or mitogen diluted in complete medium were added to make up a total volume of 100 µL/well and included the following: HBsAg, 1 µg/mL; TT, 5 Lf/mL; PHA, 2.5 µg/mL; or complete medium alone. The plates were incubated at 37°C in 5% CO_2 for 50 hours. Plates were washed ten times with 0.01% Tween-20 in PBS. Anti-human IFN-γ biotinylated antibody (1 µg/mL; Endogen) was added at 50 µL/well and incubated over night at 4°C. Plates were washed as above, and 50 µL/well streptavidin-conjugated alkaline phosphatase (1:200; Jackson Immunoresearch, West Grove, Pa) was added and incubated for 2 hours at RT. Plates were washed, and 50 µL/well BCIP/NBT substrate (Kirkgaard-Pettigrew Laboratories, Gaitherburg, MD) was added and incubated for 30 to 60 minutes. Plates were washed three times with deionized water and inverted on paper towels until dry. Spots were counted manually, with each spot assumed to represent one IFN-γ secreting cell.

Intracellular Cytokine Detection Assay

A modification of a previously described intracellular cytokine assay was employed[24] and was performed as follows. Seven mL of blood fresh blood collected in sodium-heparin tubes was

immediately placed in a 50 mL conical polypropylene tube, and a sterile 10% (w/v) glucose solution was added to a final concentration of 0.2% (w/v). Anti-CD28 mAb (BD, San Jose, Calif) was added at a final concentration of 2 μg/mL, and the blood was gently mixed and incubated at room temperature (RT) for 15 minutes. 2 mL of blood was aliquoted into each of three 50 mL conical tubes. Next, Dulbecco's phosphate-buffered saline (PBS, Gibco-BRL) alone or 5 Lf/mL TT was added. Tubes were gently agitated and were incubated upright in a humidified incubator containing 5 % CO_2, with continuous rocking and occasional manual agitation. After two hours, brefeldin A (5 μg/mL; Sigma) was added, and the incubation was continued for another three hours. Cold 20 mM EDTA in PBS was then added to a final concentration of 2 mM, and tubes were incubated for 15 minutes at RT. Tubes were then vortexed briefly, 20 mL cold PBS was added to each, and tubes were centrifuged at 400 x *g* for 7 minutes. The supernatant was discarded, 30 mL of 1X FACS Lysing Solution (BD) was added to each, and tubes were incubated at RT for 10 minutes. Samples were then centrifuged at 400 x *g* for 7 minutes, and the supernatant was discarded. Pellets were re-suspended in 10 mL cold PBS, and tubes were centrifuged for 7 minutes at 400 x *g*. The supernatants were again discarded, and pellets were re-suspended in cold 10% DMSO in PBS containing 1% BSA. Samples were aliquoted into 2 mL polypropylene cryopreservation tubes and were frozen at -80°C in a freezing chamber. Samples were stored at -80°C until the end of the monitoring period (28 days), and all samples were then thawed simultaneously for staining and fluorescence-activated cell sorting (FACS) analysis.

Samples were stained as follows: 100 μL of each sample (equivalent to 200 μL whole blood) was aliquoted into each of four wells of a 96-well microtiter dish. Samples were centrifuged at 500 x *g* for 5 minutes, were washed once with 200 μL 1% BSA in PBS, and were centrifuged again. Fluorophor-conjugated mAb cocktails were prepared with the following volumes of reagents per 25 μL: 7 μL 1% BSA (in PBS) plus 10 μL FITC, 5 μL PerCP, and 3 μL PE conjugated monoclonal antibodies. Each cocktail contained monoclonal antibodies against CD4- or CD8-PerCP or –APC, tumor necrosis factor α (TNF-α) or IFN-γ-FITC, and CD69-PE. 25 μL of antibody cocktail (BD) was added to each well, and mixed well using a multi-channel micropipette. Samples were incubated for 30 minutes at RT in the dark with gentle rocking. 150 μL of 1% BSA was then added to each well, and the plate was centrifuged. The supernatant was removed, and cells were washed again with 200 μL per well of 1% BSA. Cells were centrifuged, supernatants were removed, and samples were re-suspended in 200 μL 1% BSA and transferred to polypropylene tubes for FACS analysis.

Samples were analyzed by multi-parameter flow cytometry (FACSCaliber, BD) immediately following the completion of staining. Instrument settings were established using isotype-matched negative and positive controls, and quadrant markers were set at the first decade on both the x (FL-1) and y (FL-2) axes during instrument setup and calibration. Gates were defined so as to include CD4+ or CD8+ lymphocytes. Approximately 5,000 gated cells were analyzed from each sample; we were able to analyze at least 3,000 gated events in > 98% of cases.

Anti-Tetanus Antibody Titer
The titer was performed by the Duke University Medical Center Clinical Laboratories using a tanned cell hemag-

glutination assay, in which human Type O erythrocytes, treated with tannic acid to permit adsorption of tetanus, are mixed with patient serum and agglutination is measured.[25]

RESULTS
Administration of FL Is Well Tolerated and Increases Peripheral Blood Dendritic Cells
Six healthy individuals (4 male, 2 female, average age 39.8 ± 7.2 years) received the FL followed by the tetanus immunization. The FL was well tolerated, consisting primarily of grade 1 fatigue (3), grade 1, 2 lymphadenopathy (2), grade 1/2 injection site erythema (3). One individual experienced grade 1 left upper quadrant pain and a slightly enlarged spleen on CT scan. The total white blood cell count increased from $6.5 ± 2.0$ x $10^3/mm^3$ to $13.1 ± 4.4$ x $10^3/mm^3$ (P=0.006) and the percentage of monocytoid cells increased from $7.5 ± 1.5\%$ to $30.2 ± 8.9\%$. (P=0.0006). The percentage of dendritic cells (lineage negative, HLA DR positive) following FL was $12.9 ± 4.0$ % (n=5). In two subjects in whom pre- and post-FL DC were enumerated, the percentage increased 32 to 39 fold. Thus, the FL mobilized DC to levels similar to that reported in prior studies. The tetanus immunizations were tolerated well without any toxicity reported. The 6 healthy individuals in the comparison group (3 male, 3 female, average age 36.8 ± 5.4 years) received the tetanus immunizations alone and tolerated these well.

FL Effect on Skin Test Reactivity to TT
We have previously observed that FL increases reactivity to recall antigens, even in the absence of immunization.[5] In this current study (Table 1), we again observed a tendency for the area of induration and erythema at the Candida, mumps, and tetanus skin test sites to increase following FL, although the increases were not statistically significant.

Four weeks after FL administration and TT immunization, Candida and mumps DTH reactivity remained stable or returned to baseline. However, subjects who received FL continued to experience a further increase in the area of induration and erythema at the tetanus site, which neared but did not achieve statistical significance. For the subjects who received the TT immunization without FL, there was no increase in the skin site reactivity to the TT test dose.

FL Does Not Augment the Proliferative Response of Peripheral Blood Mononuclear Cells to TT
PBMC from each time point were assessed for their proliferation in response to TT by calculating a stimulation index (SI) (Figures 1A and B). All individuals enrolled had a pre-existing response to TT (SI at least 2.0) at baseline. Following FL, the proliferative response to TT decreased in 5 of 6 subjects and increased in 1 of 6 subjects. At week 1 following the TT injection, only 1 of those who experienced a decrease in proliferation had a TT-specific SI appreciably greater than their baseline (Figure 1A). Furthermore, only the subject who had an increase in the proliferative response with FL experienced a further increase in the proliferative response at week 4. In comparison, the proliferative response increased by week 1 following the TT immunization in all 6 who had not received FL (Figure 1B). Three of these subjects had further increases in their proliferative response at week 4. These data suggest that FL did not augment the in vitro proliferative response to TT, and may have instead dampened the TT proliferative response.
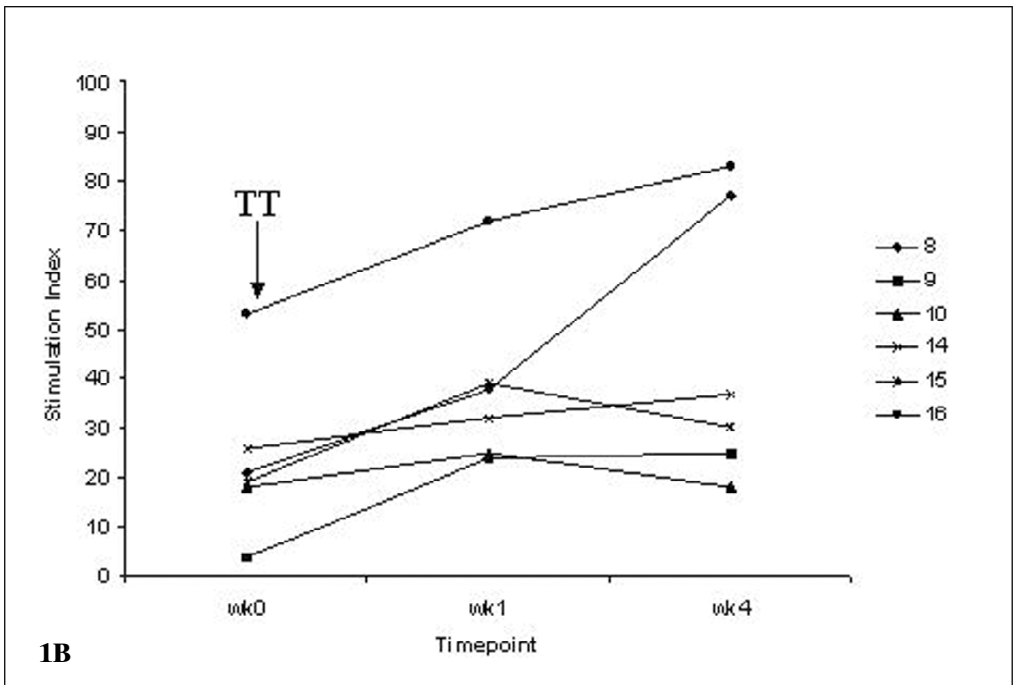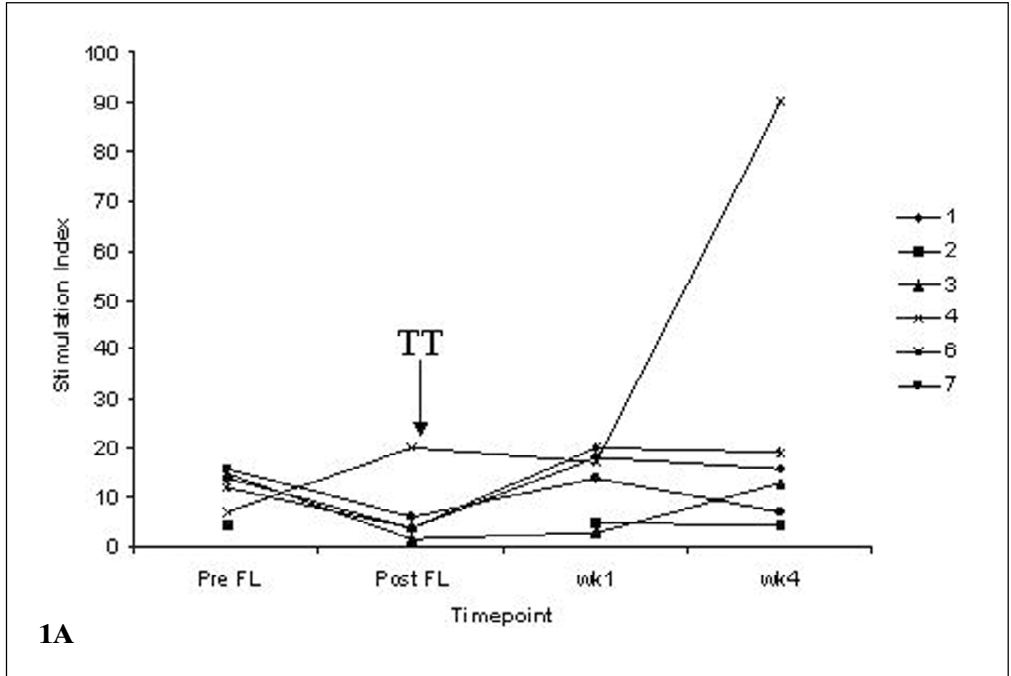
1A

1B

Figure 1. Proliferative response of normal donor peripheral blood mononuclear cells to TT prior to and following tetanus vaccination. Donors either received FL (1A) or did not receive FL (1B) before tetanus vaccination (denoted by the arrow). Proliferation in response to TT was measured at 96 hours as ^{3}H-thymidine incorporation in PBMC. Responses are represented as a stimulation index, calculated by dividing the number of counts per minute for the cells exposed to TT by the number of counts per minute in cells incubated without antigen as a control.
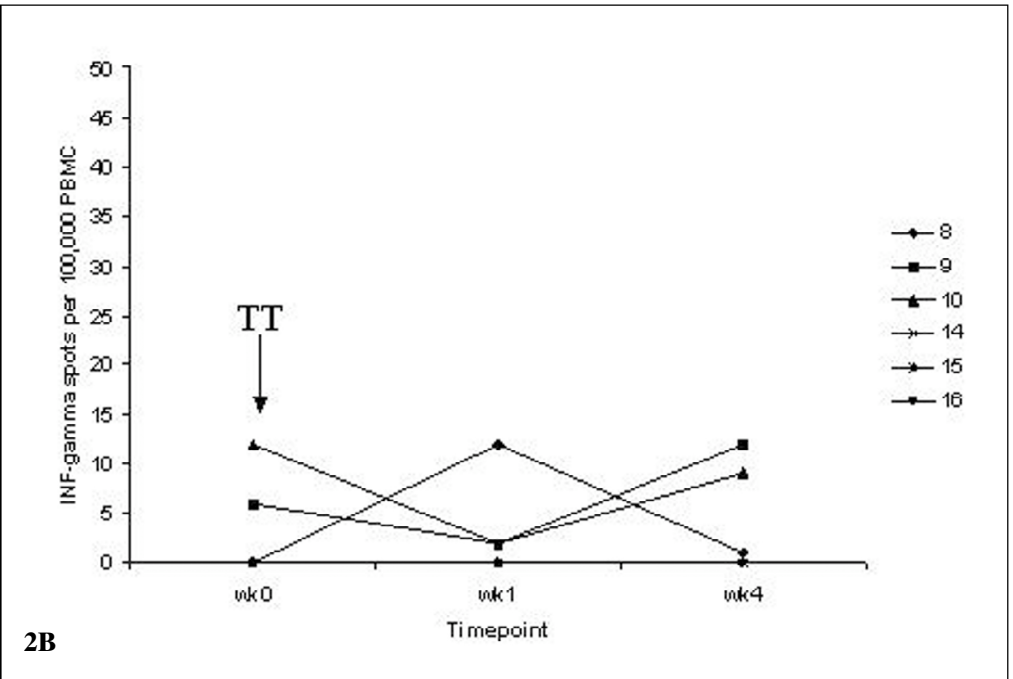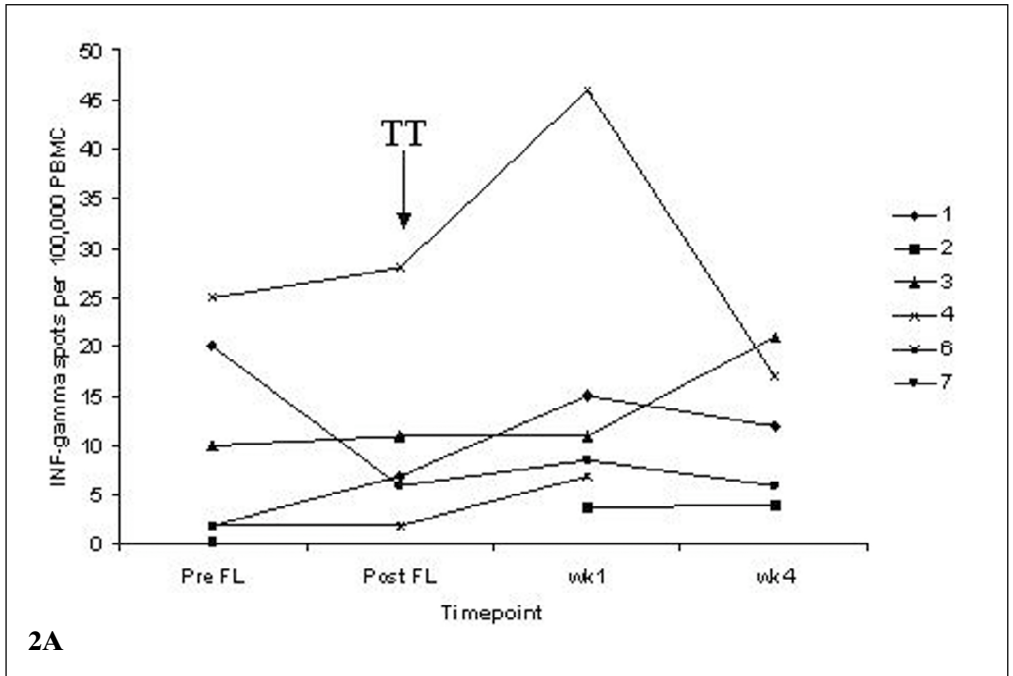
2A

2B

Figure 2. Interferon-gamma ELISPOT response of normal donor peripheral blood mononuclear cells to TT prior to and following tetanus vaccination. Donors either received FL (2A) or did not receive FL (2B) before tetanus vaccination (denoted by the arrow). PBMC were incubated with TT for 20-24 hours prior to development of the ELISPOT and automated image analysis on a Zeiss Axiomat ELISPOT reader. Responses are reported as the mean number of spots from 6 replicate wells each containing 100,000 PBMC.

Table 1. Area of Skin Reactivity (mm2)*

	TT-E	TT-I	Mumps-E	Mumps-I	Candida-E	Candida-I
Pre-FL	579±610	364±484	1059±504	426±676	1045±518	323±225
Post FL	1205± 820 *P*=0.11	460±572 *P*=0.28	1668± 2126 *P*=0.24	754±936 *P*=0.18	1919± 1555 *P*=0.13	748±689 *P*=0.08
Post TT	1927± 2006 *P*=0.09	1823± 2040 *P*=0.08	669±579 *P*=0.07	405±348 *P*=0.48	1831± 1132 *P*=0.10	494±392 *P*= 0.19
Pre TT Post TT	335±255 255±225 *P*=0.35	220±351 129±157 *P*=0.31	348±328 336±299 *P*=0.46	247±337 543±752 *P*=0.21	842±504 1410± 1803 *P*=0.19	660±726 404±405 *P*=0.22

*Mean cross-product of the perpendicular diameters of the skin reactivity to each antigen.
P values are for comparison of Post FL and pre FL, Post TT and pre FL, and Post TT with Pre TT.

FL Has Inconsistent Effects on Activation of TT-Specific T Cells by ELISPOT

To complement the lymphocyte proliferation assays, and in order to enumerate the expansion of TT-specific T cells, we also performed an IFN-γ ELISPOT assay directly from freshly isolated PBMC samples (Figures 2A and B). Amongst the subjects who received FL (Figure 2A), there was a detectable ELISPOT response (at least 5 spots/100,000 PBMC) in 3 out of 6 subjects at baseline. The FL administration alone did not have a consistent effect on the ELISPOT response with one subject experiencing an increase, and one a decrease, with the remainder unchanged. At week 1, following the TT immunization, the ELISPOT response increased above baseline in 4 of 6 subjects, and by week 4, the ELISPOT response was above baseline in 3 of 5 evaluable subjects. In comparison, amongst those who did not receive FL (Figure 2B), there was a detectable ELISPOT response in 2 of 6 subjects prior to TT injection, and in 1 of 6 and 2 of 6 subjects at weeks 1 and 4 following the TT injection respectively. These data suggest that FL has an inconsistent effect on TT-specific responses in vitro.

FL Effect on Th1 or Th2 Responses to TT As Measured by Intracellular Cytokine Assay

Because proliferation and bulk ELISPOT analyses evaluate aggregate CD4 and CD8 T cell responses, we sought assays that would allow simultaneous determination of the phenotype and frequency of TT-specific T cells following immunization. A novel assay employed to monitor the T-cell response to the vaccine is intracellular cytokine detection in CD4+ and CD8+ T cells. Amongst evaluable 6 subjects who received FL, none had a detectable percentage of CD4+ or CD8+ T cells that secreted IFN-gamma in response to TT at baseline, but at week 1, two of 5 had a CD4+ T cell response and 3 of 6 subjects had a CD8+ response. In contrast, amongst those who did not receive FL, only 1 of 6 subjects had a CD4+ response and CD8+ response following the TT injection.

FL Does Not Affect the Antibody Response to TT

The anti-TT antibody titer was measured by a hemagglutinin assay (Table 2). Amongst those who received FL, 3 of 6 subjects had an increase in their anti-TT titer following the FL. In these three,

Table 2. Anti-tetanus Toxoid Titers*

FL group	Pre-FL	Post FL	Week 1	Impact of FL	Impact of TT
Ft 1	729	19683	19683	+	NC
Ft 2	6561	6561	19683	NC	+
Ft 3	19683	59049	19683	+	NC
Ft 4	6561	531441	6561	+	NC
Ft 6	2187	2187	531441	NC	+
Ft 7	59049	59049	19683	NC	+
No FL group					
Ft 10	6561	NA	6561	NA	
Ft 12	6561	NA	6561	NA	+
Ft 13	531441	NA	531441	NA	NC
Ft 14	729	NA	177147	NA	+

*Results depicted are the reciprocal of the serum anti-TT titer+, increase in titer; NC, no change; NA, not applicable. Note: Two in the No FL group did not have baseline titers available, so they are not included.

there was no further increase in anti-TT titer after TT immunization. In the 3 of 6 subjects, who had no augmentation with the FL alone, there was an increase in anti-TT titer following the tetanus injection. In comparison, amongst those who received no FL, 3 of 4 subjects had an increase in their anti-TT titer. The magnitude of the anti-TT titer was similar in both groups. These data do not indicate an affect on anti-TT antibody responses by FL.

DISCUSSION

The goal of this study was to determine if systemic mobilization of DC by FL could augment an antigen-specific immune response under ideal circumstances in healthy individuals using an immunogenic recall antigen. We chose to study FL because it increases the number of DC in peripheral blood and tissues, and we hypothesized that DC mobilization would result in improved antigen uptake and presentation. We observed that FL administration prior to TT immunization was safe with only transitory grade 1 and 2 systemic and local injection site toxicities observed. FL tended to amplify DTH responses to TT immunization, but the in vitro assays yielded inconsistent results. Several subjects demonstrated an increase in TT-specific responses by ELISPOT and

cytokine flow cytometry following FL treatment, but similar albeit less frequent responses were observed in the control group who did not receive FL. The proliferative response was not augmented by FL and in fact was lower than for those who did not receive FL.

There are several possible explanations for our results. First, prior studies have shown that FL treatment generally leads to the mobilization of immature DC. There are some suggestions that immature DC may possess immunomodulatory characteristics, leading to antigen tolerance. Lack of augmentation of immune response in some individuals could have been due to development of tolerance, but this was not observed overall since some individuals had augmentation of TT-specific immune responses. Second, a non-specific activation of T cells could have left the cells unable to proliferate in response to the specific antigen. Mosley et al[26] reported that in vivo administration of FL increased the frequency and absolute number of effector/memory T cells, particularly Th1 cells, as well as their proliferation in response to Concanavalin A. Third, the lack of proliferative response could reflect the methodology of the assay. In our proliferative assay, we use the same number of PBMC in each well of the plate and add graded amounts of

antigen. The PBMC consists of predominantly monocytes, lymphocytes, and a few dendritic cells. Although it is usually assumed that the number of responders (lymphocytes) and stimulators (monocytes and dendritic cells) is similar in all wells, in fact, the percentage of each of these cell types is dramatically different in FL mobilized PBMC. Prior to FL, monocytes represented 7.5% of the total WBC and lymphocytes 28%. Following FL, monocytes represented 30%, but lymphocytes were only 16% of the total WBC. Therefore, it is possible that a decrease in proliferative response could be due to fewer responders being present in the wells of the assay plate.

Two other human studies have reported conflicting results regarding the effect of FL on antigen-specific immune responses following immunization. FL administered with tumor antigen peptides to melanoma patients did not enhance immune responses to melanoma antigens (using an ELISA for interferon-gamma release) and did not lead to clinical responses.[6] In contrast, Disis et al[20] studied 10 patients with HER-2/neu-overexpressing cancer who received 20 µg/kg FL per day subcutaneously for 14 days. Five patients received the HER2/neu peptide-based vaccine alone on day 7 of the 14-day cycle, and 5 patients received the vaccine admixed with 150 µg GM-CSF on day 7 of the FL cycle. Similar to our experience, they did not observe an increase in the proliferative response to the HER2/neu intracellular domain protein, but did observe a boosting of the precursor frequency of interferon-gamma-secreting HER-2/neu-specific T cells. Due to small numbers, they could not compare the effect of GM-CSF. Autoimmune phenomena was observed in this study of HER2/neu + FL vaccination, whereas we did not detect any evidence of autoimmunity in our study.

Analogous to these early human trials, murine studies investigating the impact of FL administration on immune responses have also come to conflicting conclusions. Parajuli et al[13] observed that FL administered for 10 days prior to immunization with a prime-boost strategy of adenovirus/plasmid DNA vectors expressing human wild-type p53 stimulated a type 1 T-cell response and induced protection against challenge from a metastatic tumor that expressed mutated murine p53. Spleen cells from mice immunized with p53 and FL exhibited a higher Ag-specific proliferative response than mice immunized with p53 alone. Furthermore, by ELISpot analysis, the frequency of IFN-gamma-secreting cells was significantly higher in mice immunized with p53 and FL than in mice receiving FL, or p53 alone. Fong et al[10] demonstrated that naked DNA encoding the extracellular domain (secreted form) of human FL given via the tail vein or into the spleen and followed by immunization intrasplenically with an immunogenic muc-1 peptide resulted in potent muc-1-specific CTL responses. In contrast, Baca-Estrada et al[19] reported lower antigen-specific cellular and humoral immune responses, when mice were immunized mice with hen egg lysozyme along with systemically administered FL, compared with mice that did not receive FL. It is possible that differing results in these studies could be due to differences in the state of the DC maturation differed. When Baca-Estrada administered the lysozyme along with cholera toxin, FL-treated mice developed significantly higher cellular and humoral immune responses. It is possible the cholera toxin caused maturation of DC. Indeed, other DC maturational agents such as immunostimulatory DNA have augmented the anti-tumor antigen response to immunizations with FL plus tumor antigen.[12]

Other cytokines may be required to

realize the full activity of FL on induction of antigen-specific immune responses by increasing DC recruitment to the injection site, modulating the maturity of DC, or promoting proliferation of T cells activated by the DC. Mwangi et al,[8] using plasmid DNA encoding GM-CSF and FL, given prior to immunization with Anaplasma marginale major surface protein 1a, observed enhanced antigen-specific CD4+ T cell proliferation and IFN-gamma secretion as compared with immunizations without FL and GM-CSF. Pisarev et al[15] demonstrated that only when FL was given with a bioactive fragment of IL-1beta conjugated to an antigenic epitope of HIV gag were there significant antigen-specific ELISPOT responses. Other cytokines may be important as well. Zeis et al[17] using a murine model of myeloma, observed that only the addition of IL-2 resulted in increased survival of mice immunized with myeloma-specific immunoglobulin concurrent with systemic FL. We have recently reported that human immature DC mobilized by FL, required additional cytokines to undergo maturation.[27] Therefore, differences in previous reports may be related to the presence or absence of the complete set of cytokines required for maturation of FL mobilized DC. We did not administer other cytokines or immunostimulary molecules in our study and thus cannot make a direct comparison to these data. Interestingly, we observed the highest DTH, proliferation assay, and ELISPOT results in the same individual who also experienced generalized fatigue and it is possible other inflammatory cytokines contributed to the immunologic activity observed in this individual.

Murine research has not shown DTH reactivity to antigen immunizations to be greater with FL treatment. The DTH response to p53 was increased similar whether mice were mice immunized with p53 alone or p53 and FL.[13] In Mwangi's study,[8] the combination of FL and GM did not increase the DTH reactivity above that of FL alone.

There are also conflicting reports of the effect of FL on antibody production following immunization. Pulandren et al[17] observed that FL-treated mice injected with soluble ovalbumin display increased in antigen-specific antibody titers, IgG2a more than IgG1. In contrast, Kwon et al[12] reported that intramuscular co-injection of FL with a DNA vaccine encoding hepatitis B core antigen resulted in significantly suppressed hepatitis B-specific antibody responses in a murine model. These results suggest that FL may inhibit humoral response induced by DNA-type vaccination. Similar to our experience, Evans et al[19] did not observe an increase in hepatitis B antibody titers when FL administration was followed by a series of three hepatitis B protein vaccines.

In summary, in the optimized setting of vaccination with an immunogenic recall antigen in normal volunteers with healthy immune systems, we observed a trend towards a higher in vivo DTH response, but an inconsistent or negative effect on in vitro TT-specific T cell responses. There may be patients who will have a significant augmentation of response, but it is not possible from our study to identify them a priori. If FL is to have a value in augmenting the immune response to vaccine therapies, modifications of this approach such as the additional administration of other cytokines or molecules that cause DC maturation will need to be studied. One approach is to mobilize DC, collect them by leukapheresis, load them with antigen, and administer the cell product as a cancer vaccine.

DISCLOSURES

REFERENCES

1. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell.* 2001;106:255-8.

2. Berger TG, Feuerstein B, Strasser E, et al. Large-scale generation of mature monocyte-derived dendritic cells for clinical application in cell factories. *J Immunol Methods.* 2002;268:131-40.

3. Lyman SD, Jacobsen SEW. C-kit and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood.* 1998;91:1101.

4. Maraskovsky E, Daro E, Roux E, et al. In vivo generation of human dendritic cell subsets by Flt3 ligand. *Blood.* 2000;96:878-84.

5. Morse MA, Nair S, Fernandez-Casal M, et al. Preoperative mobilization of circulating dendritic cells by Flt3 ligand administration to patients with metastatic colon cancer. *J Clin Oncol.* 2000;18:3883-93.

6. Marroquin CE, Westwood JA, Lapointe R, et al. Mobilization of dendritic cell precursors in patients with cancer by flt3 ligand allows the generation of higher yields of cultured dendritic cells. *J Immunother.* 2002;25:278-88.

7. Gasparetto C, Gasparetto M, Morse M, et al. Mobilization of dendritic cells from patients with breast cancer into peripheral blood stem cell leukapheresis samples using Flt-3-Ligand and G-CSF or GM-CSF. *Cytokine.* 2002;18:8-19.

8. Mwangi W, Brown WC, Lewin HA, et al. DNA-encoded fetal liver tyrosine kinase 3 ligand and granulocyte macrophage-colony-stimulating factor increase dendritic cell recruitment to the inoculation site and enhance antigen-specific CD4+ T cell responses induced by DNA vaccination of outbred animals. *J Immunol.* 2002;169:3837-46.

9. Baca-Estrada ME, Ewen C, Mahony D, Babiuk LA, Wilkie D, Foldvari M. The haemopoietic growth factor, Flt3L, alters the immune response induced by transcutaneous immunization. *Immunology.* 2002;107:69-76.

10. Fong CL, Hui KM. Generation of potent and specific cellular immune responses via in vivo stimulation of dendritic cells by pNGVL3-hFLex plasmid DNA and immunogenic peptides. *Gene Ther.* 2002;9:1127-38.

11. Kwon TK, Park JW. Intramuscular co-injection of naked DNA encoding HBV core antigen and Flt3 ligand suppresses anti-HBc antibody response. *Immunol Lett.* 2002 May 1;81(3):229-34.

12. Merad M, Sugie T, Engleman EG, Fong L. In vivo manipulation of dendritic cells to induce therapeutic immunity. *Blood.* 2002;99:1676-82.

13. Parajuli P, Pisarev V, Sublet J, et al. Immunization with wild-type p53 gene sequences coadministered with Flt3 ligand induces an antigen-specific type 1 T-cell response. *Cancer Res.* 2001;61:8227-34.

14. Smorfesi A, Shiota F, Disis ML. Flt3-ligand (Flt3L) as a vaccine adjuvant: results in the generation of antigen-specific IFN-g producing T cells after immunization. *Proc Am Assn Cancer Res.* 1999;40:313.

15. Pisarev VM, Parajuli P, Mosley RL, et al. Flt3 ligand and conjugation to IL-1beta peptide as adjuvants for a type 1, T-cell response to an HIV p17 gag vaccine. *Vaccine.* 2002;20:2358-68.

16. Pulendran B, Smith JL, Caspary G, et al. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci USA.* 1999;96:1036-1041.

17. Zeis M, Zunkel T, Steinmann J, Schmitz N, Uharek L. Enhanced antitumoral effectiveness of idiotype vaccination induced by the administration of Flt3 ligand combined with interleukin 2 against a murine myeloma. *Br J Haematol.* 2002 Apr;117(1):93-102.

18. Marroquin CE, Westwood JA, Lapointe R, et al. Mobilization of dendritic cell precursors in patients with cancer by flt3 ligand allows the generation of higher yields of cultured dendritic cells. *J Immunother.* 2002;25:278-88.

19. Evans TG, Hasan M, Galibert L, Caron D. The use of Flt3 ligand as an adjuvant for hepatitis B vaccination of healthy adults. *Vaccine.* 2002;21:322-329.

20. Disis ML, Rinn K, Knutson KL, et al. Flt3 ligand as a vaccine adjuvant in association with HER-2/neu peptide-based vaccines in patients with HER-2/neu-overexpressing cancers. *Blood.* 2002;99:2845-50.

21. Armitage KB, Duffy EG, Mincek MA, et al. Transient normalization of lymphocyte blastogenic and specific antibody responses following boosting of healthy elderly subjects with tetanus toxoid. *J Gerontol.* 1993;48:M19-25.

22. Shohat T, Marva E, Sivan Y, Lerman I, Mates A, Cohen A. Immunologic response to a single dose of tetanus toxoid in older people. *J Am Geriatr Soc.* 2000;48:949-51.

23. Peel MM, Edsall G, White WG, Barnes GM. Relationship between lymphocyte response to tetanus toxoid and age of lymphocyte donor. *J Hyg (Lond).* 1978;80:259-65.

24. Suni MA, Picker LJ, Maino VC. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods.* 1998;212:89-98.

25. Boyden SV. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J Med.* 1951;93:107.

26. Mosley RL, Parajuli P, Pisarev V, et al. Flt3 ligand augmentation of T cell mitogenesis and expansion of type 1 effector/memory T cells. *Int Immunopharmacol.* 2002;2:925-40.

27. Mosca PJ, Hobeika AC, Colling K, Clay TM, Thomas EK, Caron D, Lyerly HK, Morse MA. Multiple signals are required for maturation of human dendritic cells mobilized in vivo with Flt3 ligand. *J Leukoc Biol.* 2002;72:546-53.