Neoadjuvant GM-CSF and Modulation of the Immune Cell Profile of the SLN in Primary Cutaneous Stage I-III Melanoma

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Neoadjuvant GM-CSF and Modulation of the Immune Cell Profile

of the SLN in Primary Cutaneous Stage I-III Melanoma

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CONTENTS

PROTOCOL SYNOPSIS

Protocol Title	Neoadjuvant GM-CSF treatment and modulation of the immune cell profile of the SLN in melanoma (as above)
# Site and Names:	One Site: Mayo Clinic Rochester
Study Schema:	Twenty patients with a primary melanoma 1-4mm Breslow depth will be randomized to either undergo standard care (10 patients), or be treated with 14 days of GM-CSF administered subcutaneously daily in a dose of 125 $\mu g/m^2$ preoperatively (10 patients), prior to sentinel lymph node biopsy. The regional lymph nodes will undergo detailed analysis defining their immune features
Trial Objective:	To characterize and compare the regional nodal immune features of the GM-CSF treated patients, including the Th1/Th2 environment, with the regional immune environment of the control cohort
Study Design:	Open-label, single-center, randomized controlled trial
Accrual Goal:	20 patients
Accrual Rate:	1-2 patients per month

Followup:	30 days postoperatively
Inclusion Criteria:	• Histologically confirmed, 1-4mm Breslow depth melanoma • Clinical stage I or II disease • Undergoing sentinel lymph node biopsy
Exclusion Criteria	• Clinical stage III or IV disease Immunosuppressive therapy in the past 6 weeks (e.g. high-dose steroids, methotrexate, anti-TNF, IL-1Ra antagonist) • Pregnant or nursing women • Patients under 18
Analysis	Immunologic features of the sentinel lymph node will be evaluated by FACS and RT-PCR. Patient safety of GM-CSF will be evaluated using the NCI CTCAE criteria, version 4.0. Patients will be encouraged to keep a diary of events during GM-CSF administration and will be encouraged to report any symptoms or adverse events to the study team.
Statistics	The study will be a double-arm open label randomized study including 20 evaluable subjects. A sample size of 10 in each group will have 80% power to detect an effect size of 1.325 on mRNA expression and cell type prevalence in the sentinel node using a two group t-test with a 0.050 two-sided significance level. The effect size is the expected difference in the means divided by the pooled standard deviation. It is estimated that accrual will be completed in 18 months.

INTRODUCTION

Background

The prognosis for patients with melanoma and lymph node involvement is highly variable, with the 5-year survival ranging from 40-78% (Nading et al., 2010). A proportion of this variability is thought to be attributable to the host immune response to the disease process, with high T cell infiltration correlating with improved survival (Cochran et al., 2006). The sentinel lymph node (SLN) is in direct communication with the primary tumor via the afferent lymphatics (Cochran et al., 2006). This site of direct downstream drainage of tumor associated antigens should allow for a robust host immune response; however, we have demonstrated evidence of Th2 polarization and early signs of immunodysfunction within the SLN of patients with melanoma (Grotz et al., 2012; Mansfield et al., 2011). This proposal builds off of our ongoing research and is the next logical step in not only defining but reversing this immune perturbation. This study aims to reverse this early immune dysfunction and preserve the immune regulation in melanoma, potentially leading to better clinical outcomes.

We have previously demonstrated that SLNs in melanoma are fundamentally immunologically distinct from normal lymph nodes; the SLNs consistently have less expression of Th1 transcription factor T-bet and almost complete absence of cytotoxic T cells and mature antigen presenting cells ($CD86^{+}$) (Grotz et al., 2012; Mansfield et al., 2011). Th2 cytokines depress macrophage and T cell activation and function, and as such are independent predictors of poor prognosis in melanoma (Cochran et al., 2006). We have shown this regional nodal immune dysfunction occurs in cases with and without evidence of lymph node metastases (Mansfield et al., 2011). In particular, immunohistochemical analysis indicated that cytotoxic T cell accumulation within the SLN declines early in melanoma and that the degree of CD8 depletion corresponds to the risk of disease recurrence (unpublished data). Furthermore, there is a Th2 skewed cytokine expression within the SLNs of patients with melanoma compared to control lymph nodes. These changes precede SLN metastasis and we theorize are necessary for regional spread of disease. Other studies have confirmed that exposure of peripheral

blood monocytes to melanoma produces a Th2-type cytokine profile. Cytokine analysis of SLN from melanoma demonstrates an initial increase in GM-CSF observed in SLNs could lead to the attraction of a high number of dendritic cells (DC to SLNs). However, the presence of immunosuppressive molecules, such as IL-10 and COX-2, could block their maturation and prohibit their ability to become efficient antigen presenters.

Restoring the immune profile of the SLN to a normal state preoperatively may allow a robust host response and offer improved regional control, disease free survival and potentially overall survival to patients with melanoma. In this study we plan to build off of our prior work and improve the immunologic state of the SLN in patients undergoing definitive surgery for primary melanoma by administering neoadjuvant therapy with granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF has been shown to be safe for human administration and has been previously postulated to exert an anti-tumor effect by activating macrophages, T cells and dendritic cells (Pan et al., 2004). Analysis within animal models has demonstrated GM-CSF can prompt reversal of the early melanoma SLN immunomodulation in mouse B16 melanoma models (Pan et al., 2004). Furthermore, Vuylsteke et al. found that preoperative intratumoral injections of GM-CSF resulted in larger T-cell areas within the SLNs resulting in upregulation of expression of dendritic cell markers and improved outcomes (Vuylsteke et al., 2004), while Vaquerano showed regression of in-transit melanoma metastases with intralesional GM-CSF (Vaquerano et al., 1999). Most recently, our group has demonstrated improved overall survival in patients with resected advanced stage III disease who received adjuvant GM-CSF compared with a control population who underwent similar surgical management but were observed (Grotz et al., 2013).

Therapies that reverse peripheral tolerization, eradicate suppressive regulatory cells, re-establish a Th1 cytokine milieu and/or prevent lymphangiogenesis are likely to make a profound impact on the outcomes of patients with melanoma. Immune modulation of the SLNs and regional lymphatics offers a promising treatment option for patients. The

use of these agents before surgical resection and SLN biopsy presents a unique translational setting to study the immune enhancing effects of these therapies in vivo.

Hypothesis

We hypothesize that the regional immune cell profile of the SLN will be modified preoperatively by neoadjuvant use of GM-CSF, and that this preoperative modulation will restore the host regional immune surveillance towards a healthy Th1 dominant state.

Aims

- To characterize the immune cell environment of the SLN in patients with melanoma following the preoperative administration of GM-CSF
- To determine if the SLN Th1/Th2 ratio is restored in patients with clinically node negative melanoma receiving a short course of neoadjuvant GM-CSF compared with the immune environment of the SLN in a control untreated population.

GM-CSF INTERVENTION

GM-CSF Immunologic Effects

GM-CSF (sargramostim, Leukine, Genzyme Corporation, Cambridge, MA) is a hematopoietic growth factor which stimulates proliferation and differentiation of hematopoietic progenitor cells and is approved for this purpose (Leukine, Bayer 2007). In addition, GM-CSF has immunologic activities that play a vital role in various diverse functions of the immune system. These include its ability to activate macrophages, which distinguish tumor cells from normal cells and kill only the tumor cells (Fidler et al., 1984), stimulation of peripheral blood monocytes in vitro to become cytotoxic for human melanoma cells (Grabstein et al., 1986; Thomasson et al., 1989), production of monocyte activation and tumoricidal activity following in vivo administration (Chachoua et al., 1994; Demir et al., 2003) and stimulation of production of an angiogenesis inhibitor by macrophages (Dong et al., 1997). GM-CSF also serves as the principal

mediator of proliferation, maturation and migration of dendritic cells (Young et al., 1995; Szabolcs et al., 1995; Szabolcs et al. 1996). Dendritic cells are antigen presenting cells that play a major role in the induction of primary and secondary T-cell immune responses.

The recommended dose of GM-CSF for the FDA-approved use of neutrophil recovery after chemotherapy in acute myelogenous leukemia is 250µg/m2/day administered intravenously or subcutaneously (Leukine, Bayer 2008). However, the dose required to achieve the goal of activation of macrophages and monocytes is lower (Demir et al., 2003; Grabstein et al., 1986). GM-CSF has been used in several clinical trials of adjuvant therapy of melanoma and in almost all of these trials the dose has been 125 µg/m2/day for 14 consecutive days followed by 14 days off therapy for a period of a year (Daud et al., 2008; Elias et al., 2005; Lutzky et al., 2009; Spitler et al., 2000). In one study, the GM-CSF was given in the same dosing schedule for 3 years (Spitler et al, 2009). A cooperative group trial (ECOG 4697), in which 800 patients were randomized, has just been completed and used the 125ug/m2/day dose of GM-CSF for a period of 1 year (Lawson et al., 2006).

We propose to use the dose of GM-CSF that has been used in the clinical trials of adjuvant therapy of melanoma, 125 µg/m2/day for 14 consecutive days before patients undergo their definitive surgery (wide local excision and sentinel lymph node biopsy).

GM-CSF Pharmacology/pharmacokinetics/Toxicology - see appendix

GM-CSF Clinical Safety

GM-CSF has been under clinical investigation since 1987 in many countries and has been marketed in the United States under the trade name Leukine® since 1991. Thus, there has been extensive experience with the use of this product and it has generally been regarded as safe. The side effects described in the Package Insert are for doses of GM-CSF higher than those proposed for this trial. Moreover, the Package Insert describes

side effects for administration of GM-CSF by the intravenous route. In the current trial, a lower dose of GM-CSF will be used and it will be administered subcutaneously, not intravenously. Accordingly, the side effects described in the Package Insert aren't those expected in this trial.

Ravaud et. al. administered a slightly higher dose of GM-CSF (5 μg/kg) to patients with metastatic melanoma in a similar 21-day cycle of 14 days on followed by 7 days rest (Ravaud et al., 2001). This was a randomized Phase II trial in which administration of GM-CSF monotherapy was compared to administration of combination therapy of DTIC with GM-CSF. Toxicity was manageable and there were no Grade 4 adverse events. Additionally, this study was conducted in France with the E.Coli-derived version of GM-CSF (molgramostim), which has been reported to be more toxic than the yeast-derived version (sargramostim) to be used in our study. In studies in patients with hormone refractory prostate cancer, Small et. al. used a dose of GM-CSF of 250 μg/m2 for 14 days and reported that the data suggest the GM-CSF may have antitumor activity in advanced prostate cancer. This is twice the dose that will be used in our study and there were no untoward side effects (Small et al., 1999). In a follow-on study, Fong et. al. treated 24 patients with metastatic, castration-resistant prostate cancer in a phase I trial where sequential cohorts were treated on a 28-day cycle with increasing doses of ipilimumab and subcutaneous injections of GM-CSF at a dose of 250 μg/m2 for the first 14 days of the 28-day cycle (Fong et al., 2009). Toxicity was manageable, even in combination with ipilimumab and a GM-CSF substantially higher than our trial.

In a clinical trial including 98 patients with melanoma at high-risk of recurrence, adjuvant GM-CSF therapy was well tolerated administered for 14 days on followed by a 14 day break, for 3 consecutive years (Spitler et al., 2009). Eighty patients (82%) had at least one Grade 1 (74%) or Grade 2 (8%) treatment-related adverse event. The most common side effects were mild injection site reactions (68%), erythema at the injection

site (57%), and flu-like symptoms (54%). Six patients experienced Grade 3 or 4 adverse events but these were all thought to be unrelated to the study drug.

Leukocytosis is expected in patients receiving GM-CSF, even in short courses such as our trial, but, to our knowledge, has not been reported to be associated with adverse clinical side effects, other than sternal pain associated with bone marrow stimulation. We are not aware of thrombocytosis as a result of GM-CSF therapy and have not seen that in our experience. Institutionally we have administered GM-CSF at this dose off protocol for patients with resected stage III and IV melanoma and published our results in over 150 patients (Grotz et al., 2013). Our standard is for patients to receive an education on self-administration by an oncology RN and proceed home with the drug and self-administer. Off protocol these patients are typically on the drug for months to years and we are not aware of any acute toxicity within the first 2 weeks, aside from minor irritation at the injection site.

Taken together, these reports indicate that the dose of 125 µg/m2 of GM-CSF for 14 days is very safe and tolerable.

GM-CSF - Overall Risk/Benefit Assessment

GM-CSF has been marketed since 1991 and has been used in thousands of patients. It has an excellent safety record. It is not thought to provide clinical benefit as monotherapy in patients with metastatic melanoma that cannot be surgically excised. However, it may provide benefit as monotherapy in the adjuvant setting for patients with melanoma after all gross disease is resected (Spitler et al., 2000) and also may provide benefit in patients with metastatic melanoma when used in combination with other agents (O'Day et al., 2002; Weber et al., 2005).

STUDY OBJECTIVES

Primary objectives:

- To characterize and compare the regional nodal immune features of the GM-CSF treated patients, including the Th1/Th2 environment, with the regional immune environment of the control cohort

STUDY DESIGN

Twenty adult (>18 years old) patients undergoing lymphatic mapping and sentinel lymph node biopsy for cutaneous melanoma, Breslow depth 1.00 – 4.00 mm of the trunk or extremity, with clinically negative regional lymph nodes will be enrolled. Ten patients will be randomized to an intervention arm. These 10 patients will be treated with 14 days of GM-CSF administered subcutaneously in a dose of 125 $\mu g/m^2$.The timing of starting the drug will be coordinated with an ending date within 1-5 days of their surgical date.

Eligible patients will not have a primary or secondary immunodeficiency. Patients who are taking immunosuppressive drugs including high-dose steroids, TNF inhibitors, methotrexate or IL-1Ra antagonists within the previous six weeks will be excluded. Patients who have had prior GM-CSF therapy or chemotherapy within the previous 3 months will be excluded. Patients with evidence of metastatic disease will be excluded.

All inclusion criteria must be met for a subject to be eligible for participation in this study. Patients will be thoroughly informed about all aspects of the study including administration of the drug and all regulatory requirements before informed consent is sought.

Inclusion Criteria

To be eligible for the study, patients must satisfy the following criteria:

1) Histologically confirmed primary cutaneous malignant melanoma
2) 1-4mm Breslow depth

3) Scheduled for sentinel lymph node biopsy as part of their standard surgical management
4) Man or woman, age >/= 18 years
5) Women of childbearing potential (WOCBP) must be using an adequate method of contraception to avoid pregnancy throughout the study and for up to 2 weeks after the study in such a manner that the risk of pregnancy is minimized. WOCBP include any female who has experienced menarche and who has not undergone successful surgical sterilization (hysterectomy or bilateral oophorectomy) or is not postmenopausal. Sexually active WOCBP must use an effective method of birth control during the course of the study, in a manner such that risk of failure is minimized. All WOCBP must have a negative pregnancy test prior to first receiving GM-CSF.
6) Men must agree to use and utilize an adequate method of contraception throughout treatment and for at least 2 weeks after study drug is stopped
7) Patients on the following immunosuppressive medications can be considered for accrual (but use of these medications will be documented by the study coordinator if used within 6 weeks of surgery)
    a. Aspirin (daily)
    b. Ibuprofen
    c. NSAIDs
    d. Low dose steroids (≤5mg/day)
    e.
8) All patients must be willing and able to give written informed consent.

Exclusion Criteria

Subjects meeting any of the following criteria are ineligible for study entry

1) Clinical stage III or IV disease
2) Autoimmune disease: Patients with a history of inflammatory bowel disease are excluded from this study as are patients with a history of immunologic disease (e.g. rheumatoid arthritis, scleroderma, systemic lupus erythematosus, autoimmune vasculitis, motor neuropathy considered of autoimmune origin)
3) Any underlying medical conditions which, in the opinion of the investigator, will make the administration of GM-CSF hazardous or obscure the interpretation of

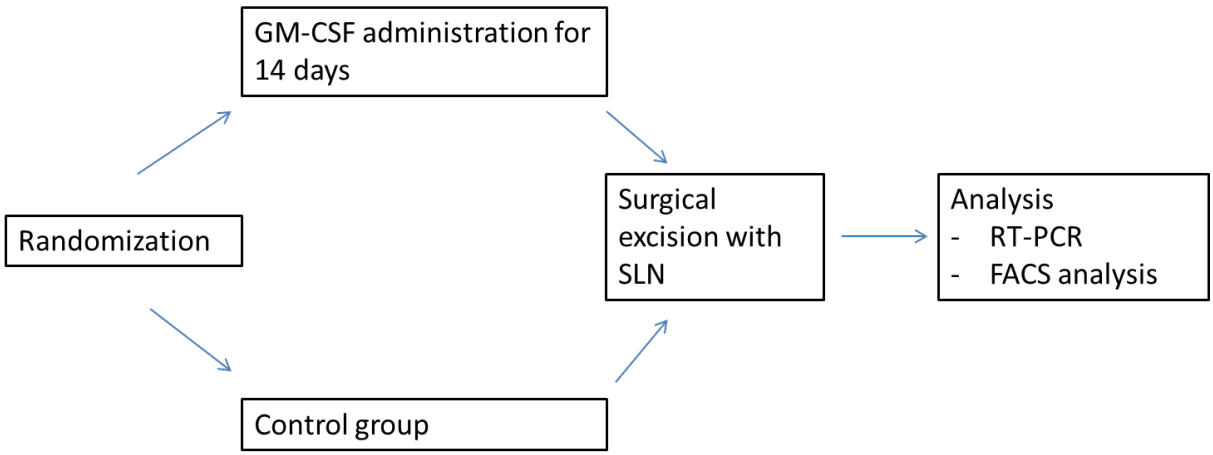
adverse events; such as, psychological, familial, sociological or geographical conditions potentially hampering compliance with the study protocol and followup schedule

4) Any vaccination therapy within 4 weeks prior to GM-CSF administration
5) Concomitant therapy with any of the following within the past 3 months: GM-CSF, interferon, other non-study immunotherapy regimes; cytotoxic chemotherapy

- Immunosuppressive mediations within the past 6 weeks including:
  - High dose steroids (>5mg/day)
  - TNF-inhibitors
  - Methotrexate
  - IL-1Ra antagonist

6) Active or chronic infection with HIV, hepatitis B or hepatitis C
7) WOCBP who are unwilling or unable to use an acceptable method to avoid pregnancy for the entire study period and 2 weeks after cessation of the study drug.
8) Prisoners or subjects who are compulsorily detained

Study Scheme



Patients will be randomized to control or intervention group. Patients will be treated with 14 days of GM-CSF self-administered subcutaneously daily for 14 days in a dose of 125 μg/m2. Patients will be instructed in the self-administration of GM-CSF and after they have demonstrated competency with the procedure, they will self-administer the treatment at home. Patients will undergo surgery within 1 day to 5 days after cessation of the GM-CSF therapy.

Surgical intervention will follow our institutional standard of care, including wide local excision of the primary melanoma according to National Comprehensive Cancer Center (NCCN) guidelines and SLN biopsy (7).

DETAILS OF PROCEDURES

This study is divided into five phases (Recruitment, Preoperative treatment, Surgery, Laboratory analysis, Followup) with associated procedures that will be performed at specific time points as described within the following sections.

Recruitment

As soon as a patient is considered for this study and prior to any study procedures, the patient will have the nature of the study explained to him or her, and will be asked to give written informed consent and Health Insurance Portability and Accountability (HIPAA) authorization. Informed consent must be obtained prior to any procedures that do not constitute part of the patient's normal care.

Patients will be introduced to the project at their meeting with their surgeon. (S)he will outline the project to them and answer any questions. The study coordinator will meet with the patient to discuss the trial further and formal consent obtained if the patient wishes to be part of the study. Following informed consent and after the patient decides to participate in the study, the patient will be randomized into either the intervention or control arm.

The following checks will be completed by the surgeon and by the study coordinator.

- Eligibility confirmation
- Histologic diagnosis of malignant melanoma (based on original biopsy, no new biopsies required)
- Medical history
- Physical examination
- Concomitant medication review

A surgical date will be set and based on this, patients enrolled in the GM-CSF arm will be given written directive on the start and stop date of the GM-CSF.

We will prospectively record the demographic, clinical and pathological data of all patients including age, sex, primary location, lymph node basin, if the SLN and non SLN analyzed were positive or negative for metastatic disease, Breslow depth, Clark's level, mitotic rate, ulceration, presence of regression, and tumor infiltrating lymphocytes, if any residual disease is present at the primary site at the time of WLE and time period between original biopsy confirming melanoma and the sentinel lymph node procedure. The 10 second radioactive count of the SLNs and if the SLN is blue or not will be captured. If the SLN is positive the size of the metastatic focus will be recorded. All SLNs will be sent to pathology as is our standard. Only tissue from SLN#1 and if applicable SLN#2 will be submitted for research purposes. All data will be stored in a secure file on the Mayo intranet.

Preoperative treatment

Patients who have been randomized to undergo GM-CSF treatment preoperatively will be educated in administration of subcutaneous GM-CSF.

The intervention group will be issued a 14 day supply of GM-CSF to self-administer daily. They will be provided phone numbers and details of who to contact with any concerns for adverse events, as well as a diary in which to record time of administration, whether entire vial was injected, and any adverse events. Patients will be provided a list of adverse events and to call immediately for any grade 3 or high adverse event. Grade I-II AEs will be logged into their diary. The diary will be collected on the day of surgery.

Patients in the control arm, who have been randomized to not undergo preoperative GM-CSF, will not receive GM-CSF in the preoperative phase.

Surgery

Patients will undergo formal consent for their operative procedure which will be separate from the consent for trial enrollment. Patients will undergo sentinel lymph node biopsy and the sentinel lymph nodes will be sent to pathology. The lymph node (LN) will undergo gross sectioning along the long axis at 1-2mm intervals until the LN is exhausted. The sections are then placed on a cryotome where they freeze from the bottom up. Prior to freezing, the top of one of these 2mm sections will be divided in half. One half will be reviewed by pathologists, the other half will be submitted for research. The remaining sections of the lymph node will be examined by standard pathological analysis. Sentinel lymph node number one will be the preferred SLN from which tissue will be collected, unless it is < 7mm in greatest dimension, in which cases sequential SLNs will be evaluated for gross size and ½ of a top will be taken from the next SLN with a gross size ≥7mm as described above. If all SLNs are <7mm tissue will not be collected for research. In addition, if there is any fresh or frozen tissue in excess of diagnosis and not critical to the pathologic interpretation this should be submitted for research purposes as well. If there is no remaining fresh or frozen tissue then paraffin fixed tissue will be collected after pathological diagnosis for this study. Only tissue from SLN #1 and if applicable SLN #2 will be submitted for research purposes. The tissue specimen will be submitted fresh for research purposes as described below and as is our practice with TRAG approval for our currently active study (IRB 10-000806).

Laboratory analysis

Lymph node tissue analysis

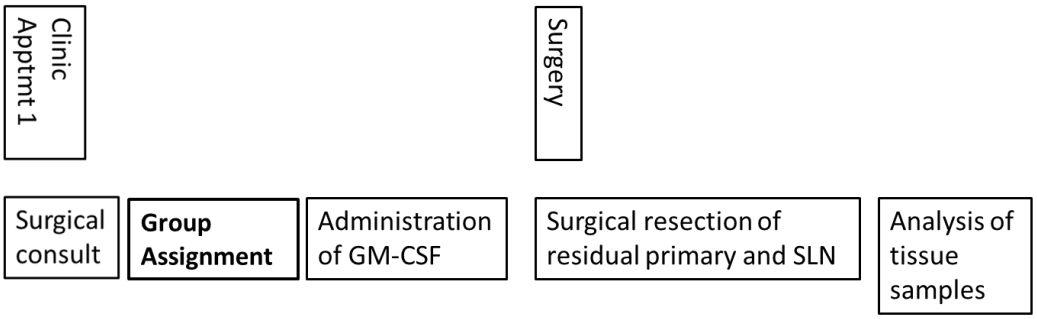
The SLN(s) will be dissected free of surrounding fat and then undergo gross sectioning along the long axis at 1-2mm intervals. The sections are then placed on a cryotome where they freeze from the bottom up. Prior to freezing, one of these 2mm sections will be divided in half and submitted for research. The remainder of the lymph node will be processed, examined and stored per the institutional standard. The fresh tissue obtained for research purposes will be collected in a sterile fashion and resuspended in RPMI buffer that will be provided by the co-investigator. The lymph node samples will be processed into a single cell suspension using a Milltenyi gentleMACS dissociator (Milltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's directions. Immune cell profiling and activation statuses will be performed utilizing flow cytometry and potentially other phenotyping methods. One approach to assess Th1/Th2 biasing in T cells of the LN cells, will include lymphocyte suspensions which will be stimulated with PMA (5ng/ml) and Ionomycin (0.1 μg/ml) ex vivo and incubated for 4 h at 37^{o}C with 5% CO_2. After stimulation, the cells will be positively selected for CD4 T cells using Miltenyi magnetic beads as prescribed by the manufacturer (Miltenyi Bergish Gladbach, Germany). The purity of the T cell population will be validated using flow cytometry. Total RNA will be isolated from the isolated cell populations using the Qiagen RNA extraction kit (Qiagen, Germantown, MD). The quality of the RNA will be evaluated by obtaining electropherograms on Agilent 2100 Bioanalyzer and RNA integrity number using 2100 Expert software (Agilent Technologies, Santa Clara, CA). cDNA will be reverse transcribed from a total of 0.3 μg of RNA using RT^2 First strand Kit (Qiagen) per manufacturer's instructions. To make the PCR cocktail the RT^2 SYBR Green ROX qPCR Mastermix (Qiagen) will be used. The Th1/Th2/Th3 RT-qPCR arrays (SABioscience, Valencia CA) will be used to quantify RNA expression of Th1 and Th2 mRNAs as well as housekeeping controls.

Furthermore, we will utilize flow cytometry to further investigate the immune cell phenotype using the following extracellular, anti-human monoclonal antibodies to important immune cell subsets including: anti-CD3 PE-Cy 7, anti-CD8 PE-Cy 5, anti-CD4 FITC, anti-CD62L PE-Cy 5, anti-CD69 FITC, anti-CD152 (CTLA-4) PE, anti-CD11c PE-Cy 5, anti-CD86 PE-Cy 7, anti-HLA-DR PE, anti-CD3 FITC, anti-CD14 FITC, anti-CD16 FITC, anti-CD19 FITC, anti-CD123 PE-Cy 7, anti-CD141 PE, anti-CD68 PE-Cy 5, anti-CD20 PE, anti-CD56 PE, anti-CD279 (PD-1) APC, anti-CD11b PE, anti-CD64 FITC (BD Pharmingen, San Jose, CA). The human monoclonal antibodies, anti-CD4 PE-Cy 5 and anti-CD25 PE, will be purchased from Biolegend (Sand Diego, CA) and used in conjunction with intracellular staining with anti-FoxP3 FITC for the quantification of regulatory T cells. Cell death will be determined by propidium iodine antibody (eBioscience, San Diego, CA). Four-color flow cytometry will be performed on a Guava 8HT flow cytometer (EMD Millipore, Billerica, MA) capturing 25,000 events for all samples. Results will be analyzed using Guava Soft Incyte (EMD Millipore, Billerica, MA).

Followup

All patients will be offered a postoperative visit as is standard and all patients will receive a 30 day follow-up phone call from the surgical RN as is our standard.

Timeline



GM-CSF ADMINISTRATION

Storage of GM-CSF

GM-CSF will be stored in a secure area in the pharmacy. Pharmacy will dispense the GM-CSF and necessary supplies for self-administration (syringes, needles, alcohol wipes, and sterile water for injection) only after the patient has been consented and randomized to the treatment arm. Vials of GM-CSF will be stored refrigerated at 2-8C. Lyophilized drug is stable for 36 months at 2-8C.

Handling of GM-CSF

Patients will be educated on the proper handling and preparing of GM-CSF. It should be prepared using standard precautions for the safe handling of agents applying aseptic technique.

Recombinant human GM-CSF should be reconstituted in water for injection USP containing 0.9% benzyl alcohol and stored at 2-8C prior to administration. Aseptically inject 1.0ml water for injection USP into the vial to dissolve the lyophilized power. The diluent should be directed against the side of the vial and the contents gently swirled to avoid foaming during dissolution. The reconstituted solution is stable for 14 days at 2-8C. Recombinant GM-CSF may be administered immediately or held for a period of no more than 14 days in the original vial. Do not freeze after reconstitution. If the GM-CSF

concentrate or solution comes into contact with skin or mucosa, immediately and thoroughly wash with soap or water.

Administration of GM-CSF

Patients will be educated on handling and administration of GM-CSF. The patients will be encouraged to call with any questions during the 14 day course of self-administration. The patients in the intervention arm will maintain a log, documenting their daily injection, time of injection and if the total dose was injected. If they miss a dose they are not to double up the next day. They will bring the administration log with them the day of surgery.

Disposal of GM-CSF

After final drug reconciliation, unused GM-CSF should be disposed at the site following procedures for disposal of anticancer drugs. The investigator will ensure that the arrangements have been made for disposal and that procedure for proper disposal are present according to the applicable regulations, guidelines and institutional procedures.

TRIAL SAFETY

Adverse Event Reporting

We project minimal toxicity from GM-CSF administration, but we will grade for and measure as per the National Cancer Institute Common Terminology Criteria of Adverse Events, version 4.0 (http://ctep.cancer.gov). An adverse event is defined as any new untoward medical occurrence or worsening of a pre-existing medical condition in a patient or clinical investigation subject administered an investigational product. Patients will be provided phone numbers and details of who to contact with any concerns for adverse events, as well as a diary in which to record time of administration, whether entire vial was injected, and any adverse events. Patients will be provided a list of adverse events and to call immediately for any grade 3 or high adverse event. Grade I-II AEs will be logged into their diary. The diary will be collected on the day of surgery.

Once the 14 day course is stopped no AE's from the GM-CSF are expected. All patients will be offered a postoperative visit as is standard and all patients will receive a 30 day follow-up phone call from the surgical RN as is our standard.

All adverse events will be graded according to the NCI CTCAE version 4.0. The following categories and definitions of casual relationship to investigational product as determined by a physician should be used for adverse events:

RELATIONSHIP	ATTRIBUTION	DESCRIPTION
Unrelated to investigational agent/intervention	Unrelated	The AE is clearly **NOT** related to the intervention
	Unlikely	The AE is **doubtfully related** to the intervention
Related to investigational agent/intervention	Possible	The AE **may be related** to the intervention
	Probable	The AE **is likely related** to the intervention
	Definite	The AE **is clearly related** to the intervention

Adverse events can be spontaneously reported or elicited during open-ended questioning. The following information will be captured for all AEs: onset, duration, intensity, seriousness, relationship to investigational product, action taken and treatment required. The investigator will supply the DSMB with any additional information.

The following hospitalizations are not considered SAEs in this study

- Elective surgery planned before signing the consent
- Admissions per the protocol for the planned medical or surgical procedure
- Routine health assessment requirement and admission for baseline/trending of health status

All AEs will be reported to the department of surgery DSMB at Mayo Clinic, Rochester, MN. Any morbidity caused by GM-CSF administration within this cohort will be captured and reported from the day of administration through 30 days postoperatively. Specific

risks are detailed below that have been experienced in patients taking long term GM-CSF use. It is not anticipated patients will experience side effects beyond local injection site irritation and 'first treatment effect' as described below, during this 14 day administration.

Adverse effects from surgery

This study involves removing 1 half of one (1-2mm) tissue section from the center of the lymph node prior to pathological analysis. Harvesting alternating 1-2 mm sections of sentinel lymph nodes for H&E, IHC and research studies is something that has been done in numerous clinical studies and as is part of some institutional standard practices. It is accepted that only a small percentage of a lymph node is ever pathologically examined. Much more gets wasted down the cryotome or not seen histologically because of sampling issues or stored in blocks then would be submitted in this study for research purposes. The research tissue would not be evaluated by the pathologist, therefore creating a small risk (1-2%) of understaging patients. A micrometastatic foci, while unlikely, could be missed in these cases if the metastasis is less than 1mm in size. However, the SLN tissue being reviewed is removed from the patient, therefore there is not an increased risk of leaving disease behind as the micrometastatic foci would still be removed as part of the surgical procedure. We do not see this risk as any different than if the patients were not on study and these shaves were not placed on a slide for pathologic review.

Data Safety Monitoring Board

The Mayo Clinic Department of Surgery data safety monitoring board (DSMB) will meet and review the study every six months to assure the safety of the procedure. Specifically, the DSMB will evaluate the progress of the trial, including an interim assessment of participant recruitment and accrual. We will also report to the DSMB the feasibility of the trial, in terms of how many consented patients received the drug from pharmacy, were educated on the self-administration technique, how many doses they administered per their self-assessment log and how many withdrew. The demographics

of the participants are expected to match the demographics of our melanoma population.

Safety assessments

Safety will be evaluated for all treated patients using the NCI CTCAE version 4.0. Safety assessments will be based on medical review of AE reports. Safety will be reviewed by the Department of Surgery DSMB with reference to stopping rules for the study as below.

Stopping rules for patients

For GM-CSF, toxicity is assessed according to the NCI CTCAE, Version 4. The major non-hematologic toxicities expected for GM-CSF are injection site reactions and a flu-like syndrome. GM-CSF must be discontinued if the patient has an allergic reaction which, in the opinion of the investigator would pose an unacceptable hazard to the patient. GM-CSF will also be discontinued for any grade 3 or greater AE or any unacceptable toxicity attributed to GM-CSF at the request of the patient.

Subjects must be discontinued from any further study therapy for the following reasons:

- Any clinically significant adverse event (grade 3 or grade 4)
- Any clinical adverse event which in the opinion of the investigator indicates that continued treatment is not in the best interest of the patient
- Intercurrent illness which would, in the judgement of the investigator require discontinuation of the drug
- Noncompliance with respect to taking the drug

Patients who have a drug related Grade 3 or greater adverse event at the time of discontinuation from study treatment will continue to be followed until resolution of the event or until the event is considered irreversible.

Subjects must be discontinued from the study therapy AND withdrawn from the study for the following reasons:

- Withdrawal of informed consent for any reason
- Patient request to withdraw from the study
- Pregnancy
- Termination of the study
- Imprisonment or the compulsory detention of the patient.

Stopping rules for the study

The trial will be stopped if 3 consecutive patients or at any time after 6 patients have been treated, if 30% of patients, have been required to be removed from the study due to drug-related toxicities, accrual will be halted and the study reviewed by the DSMB.

Prohibited therapies

Patients may not use any of the following during the study:

- Immunosuppressive agents, except if needed to treat AEs or provided during anesthesia administration
- Chronic systemic corticosteroids
- Any non-oncology vaccine therapies used for the prevention of infectious diseases
- Other investigational agents

STATISTICAL CONSIDERATIONS

Randomization: Patients will be randomized in a 1:1 fashion using a varying permuted block randomization (block sizes of 2 and 4).

Primary endpoint: The primary endpoint is the Th1/Th2 ratio. Th1/Th2 ratio will be compared between the two groups. The trial will be considered a success if the Th1/Th2 ratio is statistically significantly higher in the GM-CSF treated group.

Sample Size and power: There will be 10 patients in each group. It is expected that this treatment will have a considerable effect on the Th1/Th2 ratio. If it does not, it will not be of further interest. A sample size of 10 in each group will have 80% power to detect an effect size of 1.325 using a two group t-test with a 0.050 two-sided significance level. The effect size is the expected difference in the means divided by the pooled standard deviation.

Analysis plan: The primary endpoint will be compared between the two groups using a two-sample t-test. If the distribution of the Th1/Th2 ratios is considerably skewed, the analysis will be done on the log transformed values. If the log transformed distribution is also skewed, then a rank-sum test will be used to compare the Th1/Th2 values between the two groups. Given the small sample size, all other analyses will be exploratory. Continuous measurements will be summarized with means and standard deviations and compared between groups using a two-sample t-test (on log transformed data if necessary). Categorical variables will be summarized as frequencies and relative frequencies and will be compared between groups with a Fisher's exact test. We will also summarize disease-free survival with Kaplan-Meier curves. The adverse event data will be reported as frequencies and relative frequencies. The number/proportion of patients with Grade 3+ AEs will be compared between the two groups using Fisher's exact test. All tests will be two-sided and a p-value of 0.05 will be considered statistically significant.

PROJECTED RESULTS

We will compare demographic, clinical and pathological variables between the two cohorts. Given the unselected nature of this study we anticipate that the two groups will be comparable.

We anticipate that the gene expression phenotype (as analyzed by RT-PCR) and the immune milieu (as analyzed by flow cytometry) of the SLN in patients who received preoperative GM-CSF will have maintained a more Th1 biased immune profile. We

anticipate that the improved Th1:Th2 profile will not only apply to the SLN but to neighboring lymph nodes as well.

Data Management Plan

All data will be stored confidentially on the Mayo Clinic server and access will be provided on a password protected basis. Data will be processed as stated in protocol section titled Data Analysis, Calculations, and Statistical Power.

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Appendix

GM-CSF Pharmacology

GM-CSF is a human granulocyte-macrophage colony stimulating factor (rhu GM-CSF) produced by recombinant DNA technology in a yeast (S. cerevisiae) expression system. It is a glycoprotein of 127 amino acids characterized by three primary molecular species having molecular masses of 19,500, 16,800 and 15,500 daltons. The amino acid sequence of GM-CSF differs from the natural human GM-CSF by a substitution of leucine at position 23, and the carbohydrate moiety may be different from the native protein. GM-CSF belongs to a group of growth factors termed colony stimulating factors which support survival, clonal expansion, and differentiation of hematopoietic progenitor cells (Metcalf et al., 1986). GM-CSF induces partially committed progenitor cells to divide and differentiate in the granulocyte-macrophage pathways which include neutrophils, monocytes/macrophages and myeloid-derived dendritic cells. GM-CSF is also capable of activating mature granulocytes and macrophages. GM-CSF is a multilineage factor and, in addition to dose dependent effects on the myelomonocytic lineage, can promote the proliferation of megakaryocytic and erythroid progenitors. However, other factors are required to induce complete maturation in these two lineages. The various cellular responses (i.e., division, maturation, activation) are induced through GM-CSF binding to specific receptors expressed on the cell surface of target cells (Park et al., 1986).

GM-CSF Pre-Clinical Toxicology

The biological activity of GM-CSF is species-specific. Consequently, in vitro studies have been performed on human cells to characterize the pharmacological activity of GM-CSF. In vitro exposure of human bone marrow cells to GM-CSF results in the proliferation of hematopoietic progenitors and in the formation of pure granulocyte, pure macrophage and mixed granulocyte-macrophage colonies. Chemotactic, anti-fungal and anti-parasitic activities of granulocytes and monocytes are increased by exposure to GM-CSF in vitro (Reed et al., 1987). GM-CSF increases the cytotoxicity of monocytes toward certain neoplastic cell lines (Grabstein et al., 1986) and activates polymorphonuclear neutrophils to inhibit the growth of tumor cells. Pharmacology/toxicology studies of GM-CSF were performed in cynomolgus monkeys. An acute toxicity study revealed an absence of treatment-related toxicity following a single IV bolus injection at a dose of 300 mcg/kg. Two subacute studies were performed using IV injection (maximum dose 200 mcg/kg/day × 14 days) and subcutaneous injection (SC) (maximum dose 200 mcg/kg/day × 28 days). No major visceral organ toxicity was documented. Notable histopathology findings included increased cellularity in hematologic organs and heart and lung tissues. A dose-dependent increase in leukocyte count, which consisted primarily of segmented neutrophils, occurred during the dosing period; increases in monocytes, basophils, eosinophils and lymphocytes were also noted. Leukocyte counts decreased to pretreatment values over a 12 week recovery period.

GM-CSF - Pharmacokinetics in Normal Subjects

Pharmacokinetic profiles have been analyzed in controlled studies of 24 normal male volunteers (Spitler et al., 2012). Liquid and lyophilized GM-CSF, at a dose of 250 mcg/m2, has been determined to be bioequivalent based on the statistical evaluation of AUC. When GMCSF (either liquid or lyophilized) was administered IV over two hours to normal volunteers, the mean beta half-life was approximately 60 minutes. Peak concentrations of GM-CSF were observed in blood samples obtained during or

immediately after completion of GM-CSF infusion. For liquid GM-CSF, the mean maximum concentration (Cmax) was 5.0 ng/mL, the mean clearance rate was approximately 420 mL/min/m2 and the mean AUC (0inf) was 640 ng/mL•min. Corresponding results for lyophilized GMCSF in the same subjects were mean Cmax of 5.4 ng/mL, mean clearance rate of 431 mL/min/m 2, and mean AUC (0inf) of 677 ng/mL•min. GM-CSF was last detected in blood samples obtained at three or six hours. When GM-CSF (either liquid or lyophilized) was administered SC to normal volunteers, GM-CSF was detected in the serum at 15 minutes, the first sample point. The mean beta half-life was approximately 162 minutes. Peak levels occurred at one to three hours post injection, and GM-CSF remained detectable for up to six hours after injection. The mean Cmax was 1.5 ng/mL. For liquid GM-CSF, the mean clearance was 549 mL/min/m2 and the mean AUC (0inf) was 549 ng/mL•min. For lyophilized GM-CSF, the mean clearance was 529 mL/min/m 2 and the mean AUC (0inf) was 501 ng/mL•min. The pharmacokinetic profile of the EDTA-containing GM-CSF formulation is not the same as that of reconstituted lyophilized and previous liquid formulations. These differences include an earlier Tmax, a decrease in clearance and two peaks of absorption as compared to a single peak of the other formulations. The magnitude of these differences is small and unlikely to result in meaningful pharmacodynamic changes. This difference is attributed to the presence of EDTA in GM-CSF.