Phase I clinical trial of Mixed Bacterial Vaccine (Coley’s Toxins) in patients with NY-ESO-1 expressing cancers: Immunological effects and clinical activity

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Running title: Effects of Mixed Bacterial Vaccine

Key words: Coley’s toxins, fever, innate immune system, immunomodulation, cancer vaccines, NY-ESO-1

Grant support: This study was supported by the Cancer Vaccine Collaborative of the Cancer Research Institute, the Ludwig Institute for Cancer Research and the Krebsforschung Rhein-Main e.V.

Conflict of interest: No potential conflict of interest was disclosed by the authors.

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Translational Relevance

There is increasing evidence that non-specific activation of the innate immune system may critically support the initiation of a functional specific immune response against cancer. Based on anecdotal reports on the therapeutic effects of Coley’s toxins, we developed a new, well-defined and cGMP compliant bacterial preparation (Mixed Bacterial Vaccine, MBV) that led to the production of immuno-regulatory cytokines at fever inducing dose levels in the treatment of 12 patients. The study also showed that MBV-induced fever and elevated levels of various cytokines may have mediated the tumor regression in a metastatic bladder cancer patient. One patient showed a partial tumor response, six patients showed prolonged overall survival. The novelty of our study is that the immuno-stimulatory effects of mixed bacterial vaccine were investigated systematically based on a defined endotoxin quantity of the preparation. The immuno-modulatory effects render MBV as a potent adjuvant that needs to be evaluated in combination with different strategies of cancer vaccines including prophylactic therapy for the establishment of a protective immune response.
Abstract

**Purpose:** Mixed Bacterial Vaccine (MBV, Coley’s Toxins) is a historical, vaguely defined preparation of heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens* used as non-specific immunotherapy in the treatment of cancer. The mechanism of action is suspected to have an immunological basis, yet it is poorly defined up to now. We developed a new, biochemically well-defined and cGMP-compliant MBV preparation, which has been investigated in patients with NY-ESO-1 expressing cancers.

**Experimental Design:** Patients received MBV subcutaneously at a starting dose of 250 EU (endotoxin units) twice weekly. The MBV dose was escalated in each patient until a body temperature of 38°-39.5°C was induced or up to the maximum dose of 547,000 EU. Changes in serum cytokine levels were determined and immune responses to NY-ESO-1 were evaluated. Tumor response was assessed according to RECIST.

**Results:** Twelve patients were enrolled and 11 of them developed fever after the administration of MBV. 10/12 patients showed a consistent increase in serum IL-6 levels with the highest levels coinciding with the highest body temperature. A subgroup of patients showed increasing levels of TNF-α, IFN-γ and IL1-β. A patient with metastatic bladder cancer showed a partial tumor response strongly correlated with MBV-induced fever and highly elevated levels of several cytokines.

**Conclusions:** MBV at fever-inducing dose levels can lead to a massive induction of immunoregulatory cytokines that may be involved in inducing tumor regressions. We propose to further explore the role of MBV as a potent immune modulator at higher dose levels and in conjunction with antigen-specific cancer vaccines.
Introduction

Nonspecific immunotherapy’s with bacterial products have been used in the treatment of cancer for more than a century and have shown beneficial effects in multiple clinical settings. The prototype composition for this approach is Coley’s toxins, a mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* named after William B. Coley, who developed the mixture in the late 19th century as a treatment for cancer. This bacterial mixture was used in a large number of patients by Coley and others (1-6). Coley observed that a strong fever reaction was the key indicative aspect of a successful intervention leading to tumor regression. However, a systematic investigation of the mechanisms involved in mediating tumor regression was not possible at the time. In a review of 186 cases of soft tissue sarcomas treated with Coley’s toxins, Helen Coley Nauts concluded that the toxins were most effective when given every 24 or 48 hours initially followed by less frequent further injections, when they caused fever of 38.9 - 40°C, and if the toxins were injected into or in close vicinity to the tumor. In addition, beneficial effects were observed when the toxins were given over an extended period of 6 to 12 months (7-8). In the 1970’s, Coley’s toxins were investigated again. The mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* used in these more recent clinical trials was called Mixed Bacterial Vaccine (MBV) that was administered against different types of cancer with varying results (9-13). Although single factors of the bacterial product were identified as active components, e.g. lipopolysaccharide (LPS), a component in the cell wall of gram-negative bacteria (14), the detailed mechanisms of action has not been identified yet. It is suggested, however, that MBV triggered the release of a broad range of fever-inducing substances like interleukins, tumor necrosis factor (TNF) and interferons. The recent renewed interest in MBV is prompted by two new basic developments. First, humoral and cellular immunity to cancer antigens, among them the so-called CT-antigens, expressed by many solid tumors, can now be measured in greater detail. NY-ESO-1 is one of these antigens. It is highly immunogenic in cancer patients and has the capacity for inducing spontaneous antibody responses in as many as 50% of patients with NY-ESO-1–expressing tumors (15). Spontaneous antibody responses are frequently associated with concurrent NY-ESO-1-specific CD4 and CD8 T cells in cancer patients (16). Immunity to NY-ESO-1 has been recently been described as a good prognostic biomarker to predict clinical responders to anti-CTLA4 immunotherapy (17). For this reason, we have selected anti- NY-ESO-1 directed immune responses as a surrogate marker to gauge for tumor antigen-specific immune responses. Second, the discovery of the human Toll-like receptor (TLR) gene family in the 1990s as receptors for pathogen-associated molecular
patterns led to a better understanding of downstream cytokines that contribute to immunological cancer recognition and rejection (18-19). Unmethylated CpG, bacterial DNA and other TLR agonist are likely to stimulate the innate immune system to initiate the production of a complex cascade of cytokines (20-22). These include IL-1β, TNF-α and IL-6, which act as endogenous pyrogens that elevate the body temperature. TNF-α regulates the inflammatory response, induces apoptosis and disrupts neovascularization of solid tumors. TNF-α also amplifies the innate immune response by inducing IL-12 production that provides a critical link between innate and adaptive immunity by driving type-1 responses through the induction of IFN-γ production by NK cells, type-1 helper cells and cytotoxic T-cells. IFN-γ plays potentially several important roles in the immune response to tumors including tumor immunosurveillance and direct anti-tumor activity. Thus, IL-6, TNF-α, IL-12, and IFN-γ, collectively represent cytokines that are critical at the various stages of the host response to bacterial products. Over the years, numerous versions of Coley’s toxins have been produced under different names (e.g. Vaccineurin, Novopyrexal, MBVax) but each of these products had a different specific formulation and a standard dosage or standardized route or time frames of administration have never been explored systematically. To address these issues we have developed a new, well defined and biochemically well characterized cGMP-compliant preparation of MBV and have initiated a dose escalation phase I clinical study to determine the fever-inducing dose of MBV and to explore the immunological and clinical effects following treatment with MBV. We focused on NY-ESO-1 specific immune responses and on serum cytokine induction for TNF-α, IFN-γ, IL-1β, and IL-6. We found that increased levels of cytokines strongly correlated with the increase of MBV-related body temperature and, in one patient, with favorable clinical outcome.

Patients, Material and Methods

Investigational Agent: Mixed Bacterial Vaccine was manufactured by Cobra Biomanufacturing Plc, Oxford, UK and consists of 250,000 Endotoxin Units (EU)/mL of heat killed gram positive Streptococcus pyogenes and gram negative Serratia marcescens in phosphate buffered saline (PBS) containing 2% glycerol. Both strains were developed from non-passaged, freshly isolated clinical virulent bacterial isolates from patients with a sepsis syndrome. The identity of the strains was confirmed by standard microbiology technologies and by 16sRNA sequencing. All manufacturing, formulation and packaging of the investigational agent were done in accordance with applicable current Good Manufacturing Practices and met the applicability criteria for use in humans.
Patients and Treatment Plan: This was a phase 1, open-label, multiple dosing, single arm study according to study protocol LUD2005-003, which was approved by the Ludwig Institute for Cancer Research as well as by the medical and ethical committees of Hessen/Germany. Twelve patients with NY-ESO-1 expressing tumors or NY-ESO-1 serum antibodies were enrolled to receive MBV subcutaneously at a starting dose of 250 EU twice weekly. In the absence of a dose-limiting toxicity, the MBV dose was escalated in each patient to the MBV dose level that elicits a body temperature of 38°C - 39.5°C or up to the maximum dose level 8 (547.000 EU). If a fever of 39.5°C - 40°C was observed, the patient continued to receive MBV at the previous dose level for four additional doses. For patients who had not achieved the desired pyrogenic effect at dose level 8, no additional MBV was administered. The trial design schema is illustrated in SI Figure 1. Based on MBV pyrogenicity in rabbits and the assumption that endotoxins are equipotent in rabbits and in man, the initial MBV injection dose of 250 EU was considered acceptable for use in patients without undue risk of overwhelming endotoxin reactions. Toxicity was evaluated according to the National Cancer Institute CTCAE Scale (Version 3.0). Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Body Temperature: Body temperature measurement was performed prior to each MBV vaccination and every hour for 6 hours after each MBV injection using a tympanic thermometer. The patient’s body temperature was monitored using a tympanic thermometer hourly on the day of vaccination up to six hours as an outpatient. Results were documented in a body temperature log.

NY-ESO-1 serum antibody: NY-ESO-1 specific antibodies were measured in the serum by ELISA analysis at baseline and every 4 weeks during MBV treatment as described (23).

Analysis of NY-ESO-1 specific T cells: For the analysis of CD8+ and CD4+ T cells in enzyme-linked immunospot (ELISPOT), purified CD8+ and CD4+ T cells were presensitized with NY-ESO-1 peptide - pulsed irradiated autologous peripheral blood mononuclear cells (PBMC) depleted of CD4 and CD8 T-cells. Presensitized CD4+ and CD8+ effector cells were tested on day 10-14 by IFN-γ ELISPOT against NY-ESO-1 peptide-pulsed autologous antigen-presenting cells (EBV-transformed B-cells, T-APC or dendritic cells) as described (23). A response was considered positive if the number of spots in the peptide-exposed well was two-fold or more higher than the number of spots in the unstimulated well, and if there
was a minimum of ten (after subtraction of background spots) peptide-specific spots/25,000 T-cells or fewer cells if T-cell clones were used.

**Analysis of cytokines:** Serum samples were obtained 6 hours after each MBV injection, processed immediately and stored at -80°C for subsequent cytokine measurement by ELISA assays using multiplex technology from Meso Scale Discovery as described by the manufacturer.

**Anti-MBV antibodies:** MBV specific antibodies were measured in the serum by standard ELISA analysis using 1250 EU/well MBV lysate as antigen coating.

## Results

### Patients

Twelve patients with NY-ESO-1 expressing tumors were entered into the clinical trial at Krankenhaus Nordwest, Frankfurt, Germany: 6 patients with melanoma; 2 with sarcoma; 2 with prostate; 1 with HNC and 1 patient with bladder cancer. Two patients had resected disease at study entry. The patients presented with tumor stages III and IV. Seven patients (2, 3, 4, 5, 7, 9 and 12) had received NY-ESO-1 specific immunotherapy before entering this study. All patients were clinically followed until progression or death (Table 1).

### Toxicity

Treatment with MBV was considered safe. In all patients, MBV-related local and systemic reactions were mild to moderate. Three patients had no reactions at all. Five patients experienced local reactions such as reddening, swelling, pain or pruritus at the injection site. Four patients had systemic and local reactions, which were likely to be related to pyrogenicity and associated inflammation. Any side effects resolved within one to five days. No dose limiting toxicity and no treatment-related serious adverse events occurred. Inflammatory local reactions were seen predominantly after injection of dose level 6 and higher (Table 1).

### Pyrogenicity of MBV

The pyrogenic dose levels varied among the patients between dose level 2 and dose level 8. Eleven of 12 patients developed fever $\geq 38 ^\circ$C, eight of them at dose level 5 or higher. There were three patients where fever was observed at dose level 8, two patients at dose level 7, two
patients at dose level 6, and one patient at dose level 5. Three patients developed fever at dose level 3 and lower. Of those, patient #2 possibly suffered from a latent infection that might have caused fever unrelated to MBV. Patient #9 did not experience any fever though the dose was escalated up to level 8. Fever was symptomatically managed with the antipyretic drug paracetamol when measured 38°C or higher. Therefore fever of 39°C or higher was seen just once in a single patient. Although the treatment was continued at the pyrogenic dose level for 4 times, five patients (#1, 4, 6, 7 and 12) did not respond again with fever to the subsequent MBV administrations. Two patients (#10 and 11) had one more and three patients (#2, 3 and 5) had two more fever reactions at the pyrogenic dose level. One patient (#8) responded with fever after every subsequent MBV administration (Figure 1).

**In-vitro immunostimulatory activity of MBV**

To evaluate the immunostimulatory activity of our vaccine preparation in-vitro, human peripheral blood mononuclear cells were stimulated with various concentration of MBV and cytokine levels in supernatant including TNF-α, IFN-γ, IL-6, IL-12, IL-10 were measured by ELISA. As shown in SI Figure 2, MBV induced all 5 cytokines in a dose-dependent manner at a level comparable to or higher than that induced by PMA or LPS. We further tested activation of human monocyte-derived dendritic cells (mo-DC) by MBV. After stimulation with MBV, mo-DC up-regulated surface MHCs (HLA-DR and HLA-ABC), co-stimulatory molecules (CD80 and CD86) and the DC-maturation marker CD83 (SI Figure 3A-B). In addition, MBV stimulation of mo-DC induced cytokine production including immunopotentiating IL-12, inflammatory TNF-α and immunosuppressive IL-10 (SI Figure 3C).

**In-vivo cytokine activation by MBV treatment and correlation with body temperature**

In addition to TNF-α, IFN-γ, IL-1β, and IL-6 that served as immunological readouts in the study objectives we examined a panel of 13 different cytokines in the serum from each patient in order to compare cytokine levels with increasing MBV dose levels and elevated body temperature. Changes in serum cytokine concentrations were observed for TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10 and IL-12. TNF-α levels rose in 5 patients (#1, 3, 4, 9, 11) and dropped again in 3 of these patients (#3, 4, 11). IFN-γ levels rose in 4 patients to a moderate level (#1, 2, 4, 10) and in one patient to a high level (#11). Levels for IL-1β were generally low but significantly rose in 2 patients (#9 and #11). Levels for IL-2 rose in 3 patients (#1, 4, 11). Levels for IL-5 were low in all but 1 patient (#7) and did not increase during MBV
treatment. Levels of IL-6 increased in 11 of 12 patients (#1, 2, 3, 4, 5, 6, 8, 9, 10, 11 and 12) and dropped again in 8 patients. Two of these patients (#2 and #4) showed baseline cytokine levels of IL-6 significantly higher as compared to normal endogenous levels. Levels for IL-8 and IL-10 rose in two patients (#4 and #11). IL-12 levels rose in 1 patient (#11). IL-13 levels decreased in 1 patient (#7), whereas all other patients did not respond with changes of IL-13 levels. Levels of GM-CSF, IL-4 and IFN-α were undetectable in all patients throughout the study. In summary, all but one patient (#8) responded to MBV with increasing levels of at least two cytokines. For IL-6, maximum increase and duration of the peak showed a direct correlation to the fever-inducing MBV dose level except for patient #9 who showed an increase of IL-6 at maximum MBV dose level 8 but did not develop fever of 38°C. Cytokine results of all patients are presented in Figure 2. The most impressive changes in cytokine levels were observed in patient #11 who developed high serum levels of TNF-α, IFN-γ, IL-2, IL-6, IL-8 and IL-10 simultaneously after the injection of MBV at the pyrogenic dose level 8. Maximum cytokine values were 10-100 fold higher as compared with baseline values or individual and median normal ranges. Serum levels further increased with the subsequent administration and then rapidly decreased to baseline values during the following MBV administrations. Levels of cytokines IL-1β, IL-5 and IL-12 clearly became detectable with the increase of body temperature but values did not reach very high levels. These results indicate, that MBV treatment at a fever-inducing dose level can lead to a massive induction of immuno-regulatory cytokines.

Humoral and cellular immunity to NY-ESO-1

NY-ESO-1 antibodies: Nine out of 12 patients (#1, 3, 5, 6, 7, 9, 10, 11 and 12) were baseline seropositive and remained seropositive during the course of MBV treatment. Changes in antibody titers were observed in 2 patients; patient #7 increased from baseline titer 1:12,800 to 1:100,000 at the end of study and patient #10 decreased from baseline titer 1:100,000 to 1:25,000 at the end of study. One out of three baseline antibody negative patients (#8) seroconverted late at the end of the study to a low titer of 1:400. NY-ESO-1 antibody results are shown in Table 1 and Figure 3A. In patients #5, 7, 9, 10 and 11, follow up sera were obtained 24, 9, 21, 5 and 12 months after the end of study, respectively. We observed an increase of NY-ESO-1 antibody titer in patient #9 from 1:1600 to 1:200,000 twenty-one months after the end of study. The other follow up serum samples did not show any changes in NY-ESO-1 specific antibody titer. In addition to NY-ESO-1, we tested serological responses against other cancer antigens such as MAGE-A1, -A3, -A4, -A10, SSX1, SSX2,
SSX3, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1 and CT47. We observed a number of antibody responses in individual patients against several proteins including MAGE and CT47. Almost all responses were already present at baseline and no significant changes in titers were observed during MBV treatment. Patient #10 (melanoma) developed a clear de novo antibody response to the melanoma antigen Melan-A during MBV treatment (Data not shown).

**NY-ESO-1 specific T-cell responses:** Pre-existing CD4+ and CD8+ T-cells were detected in 5/12 and 7/12 patients, respectively. The epitopes recognized by CD4 and CD8 T-cells were clustered in the known immunodominant NY-ESO-1 regions p81-110, p119-143 and p151-170. Following MBV treatment changes in NY-ESO-1 specific epitope recognition were observed in two patients. Patient #5 developed a de novo CD8 T-cell response against p119-143 and patient #3 developed a de novo CD4 T-cell response against p151-170. Patients who had no baseline NY-ESO-1 specific T-cell reactivity remained negative during the study. A summary of CD4 and CD8 responses pre and post MBV treatment is presented in Table 1. Immunodominant NY-ESO-1 epitopes recognized by CD4+ and CD8+ T-cells are shown in **Figure 3B**. ELISPOT data of the entire patient cohort and all time points including background spots are presented in the **Supplemental Table 1**. These results indicate that treatment with MBV did not appear to impact on NY-ESO-1 specific immune responses during the course of MBV treatment.

**MBV specific antibodies**

Since the pyrogenic effect of MBV at a certain dose level and the accompanying elevated cytokine levels were only transient and additional administrations at the same dose level did not further influence the body temperature, we analyzed patients sera for antibody responses against MBV. Anti-MBV serum IgG antibodies developed in nearly all patients to varying levels. Strong MBV specific antibody responses were found in patients #1, 5, 10 and 11. These patients received MBV at the maximum dose level of 547,000 EU (**Figure 4**). MBV antibodies decreased to baseline values after completion of MBV treatment.

**Tumor Responses**

Two patients (#9 and #10) had no evidence of disease at study entry. Patient #9 relapsed two months after study termination. Patient #10 entered the study in a status of resected lymph node metastases associated to an unknown primary melanoma and has remained free of disease until today. Ten of twelve patients had measurable disease at study entry and eight of
them progressed under MBV treatment. Six patients (#5, 7, 9, 10, 11 and 12) have over-average survival times and four of these patients (#5, 10, 11 and 12) are still alive with survival times of 45+, 36+, 34+, and 25+ months calculated from study entry date until today. A partial tumor response defined according RECIST criteria was seen in patient #11, who entered the study in a status of progressing metastases to abdominal lymph nodes and abdominal soft tissue as target lesions. For examples, a mediastinal lymph node metastasis decreased from 20 mm to 15 mm, an abdominal paraortic lymph node metastasis diminished from 19 mm to 8 mm. In this patient the most significant changes in cytokine levels were observed (Figure 5A/B). The decreasing tumor marker CEA from 16.4 μg/l to 10.0 μg/l correlated with these findings in this patient.

Discussion

In addition to modern antigen-specific antibody- and vaccine-based immunological cancer therapies, “nonspecific” immunotherapies with bacterial products have also shown anti-tumor effects (24-26). Over the past two decades, immunological research has broadened our understanding of how these bacterial products may stimulate the immune system and how endogenous mediators, induced by non-specific immune stimulation influence the immune defense (22, 27-28). In previous studies with MBV, also known as Coley’s toxins, fever induction has been considered to be the key indicative aspect of successful treatment (29). However, there has been no systematic investigation of the mechanism by which MBV induced fever would contribute to mediate tumor regression. Therefore, a primary objective of our study was to identify the dose of standardized MBV that increased the body temperature to 38°-39.5°C. Eleven of 12 patients developed fever of ≥38°C at a given MBV dose level. The pyrogenic dose level varied among patients. Considering the fact that the pyrogenic dose level distribution in most of the patients (7 of 11) was within a 3 levels span (3, 2 and 2 patients at dose levels 8, 7 and 6, respectively), a starting dose of MBV level 6, i.e., 60,000 EU, may be determined for future trials using this approach. Due to the intervention with anti-pyrogenic drugs, fever was well managed and did generally not increase over 39°C. However, antipyretic therapy in this immunological approach may be critical, since these preparations include e.g. inhibitors of the prostaglandin synthesis and glucocorticosteroids that might interfere with the efficacy of the treatment approach at the immunological level (30, 31). Also, the short pulse of relatively low fever in
the patients may have had an adverse impact on cytokine levels and clinical outcome. Repeated induction of fever over several days or at frequent intervals may be required to optimize the production and maintenance of effective cytokine levels.

As a second objective, the study evaluated the effects of MBV on changes in serum cytokine levels. As expected, we observed a wide inter- and intra-patient variability of cytokine levels as to their baseline detectability and to their changes during MBV treatment. Notably, the evaluation of cytokine levels in cancer patients is complicated because of concomitant conditions and of disease-related situations. Also, a typical pretreatment cytokine profile has not yet been identified in cancer patients and data regarding the behaviour of cytokines during different treatment approaches are very few (32). In our study, the most remarkable effect of MBV on changes in cytokine levels was observed for IL-6. Eleven of 12 patients showed a consistent increase in serum levels of IL-6 with the highest levels coinciding with the MBV dose that elevated body temperature. These results clearly demonstrated the efficacy of MBV to induce fever and cytokines in a dose-dependent manner. In a proportion of patients we found significant increases in serum levels of TNF-α (5/12), IFN-γ (4/12), IL-1β (2/12) and IL-2 (5/12). Highest levels of these cytokines mostly appeared unrelated to the body temperature. Some patients showed baseline serum cytokine levels significantly higher compared to normal endogenous levels. In particular, patient #1 had very high baseline levels of IL-8, patients #2 and #4 had elevated levels of IL-6, patient #7 had very high baseline levels of IL-5, IL-10, IL-12 and IL-13 and patient #11 had elevated levels of IL-13. During the course of MBV treatment significant changes of baseline elevated cytokine levels were observed in patient #7 who responded with a consistent decrease of cytokines IL-5, IL-10 and IL-13. The impact of high baseline serum levels of the anti-inflammatory cytokines IL-10 and IL-13 was significant, since this patient was the only one who did not respond to MBV with the production of IL-6, although fever was developed. These findings suggest that the pattern of cytokines at baseline and during MBV treatment may be related to significant biological differences most likely associated with the tumor disease and the ongoing immune responses in individual patients. In conclusion, the most remarkable cytokine response during MBV treatment was the increase of IL-6 in 11 of 12 patients. IL-6 is a multipotent cytokine engaged in numerous biological activities (22) and it is also a potent mediator of fever and the acute-phase immune response. Along with TNF-α, these inflammatory signals are essential for the recruitment of tumor-infiltrating lymphocytes, as well as natural killer cells. Although the treatment was continued at the pyrogenic dose level for 4 times, most of the patients had only one more fever reaction and did not respond again with fever and cytokine
production to subsequent MBV administrations. This could be due to the well known property of endotoxin to induce tolerance to its own biological effects (33-36) as well as to the release of anti-inflammatory cytokines e.g. IL-10 in patients #3, 4, 11 and 12, that inhibit TNF-α and IL-1β production in macrophages and thus contribute to the control of the magnitude of pro-inflammatory responses. IL-10 also enhances B-cell proliferation and, along with other factors, may support the immune activation against the bacterial product itself leading to a pathogen-specific antibody response. In fact, several patients developed anti-MBV antibodies during treatment with MBV that decreased to baseline levels after MBV treatment was completed (Figure 4). The MBV-directed antibodies might have had a neutralizing effect on further MBV administrations and may explain, among other aspects like endotoxin tolerance, the only transient effect of fever and cytokine induction. To overcome tolerance to the bacterial vaccine, increasing doses of MBV or variations of the bacterial species for MBV may be effective to repeatedly induce fever and cytokines at significant levels. In addition, since endotoxin tolerance has been shown to be reversible, patients who failed to respond to further MBV administrations may have a rest and then start again with less frequent further injections. In fact, preliminary results of patient #11 who received MBV treatment on the basis of a compassionate single-patient protocol (SPP) after a 3-months break for 16 additional injections at dose level 8, responded again with fever and cytokine production (SI Figure 4).

Although the MBV effects on fever and increased synthesis of several cytokines were transient, the short pulse of high systemic cytokine levels may have influenced the induction of an individual tumor response considerably. This might have been the case in patients #10 who has been experiencing a disease free survival of 36.4+ months and patient #11 who responded to MBV treatment with a strong increase of a number of cytokines followed by regression of multiple tumor manifestations. The tumor response in this patient may be related to the MBV induced production of cytokines such as TNF-α, IFN-γ, IL-6, IL-2 and IL-12 (Figure 5A/B).

Regarding NY-ESO-1 specific immunity, we did not observe significant changes in humoral and cellular immune responses mediated by MBV treatment. Antibody responses against other cancer antigens such as MAGE, SSX, CT7, CT10 or CT45-47 were observed in individual patients. Again, all responses were already present at prestudy and no significant changes in titers were observed during or after MBV treatment. However, follow-up blood samples need to be tested, since changes in humoral and cellular immune responses to tumor antigens that might have been initiated during the phase of massive cytokine production may
became detectable at a later time after successful translation of the innate response into the adaptive immune response (37). Patient #11 who had preexisting NY-ESO-1 specific antibody and T-cell response showed a partial tumor response after MBV treatment suggesting that MBV possibly broke tolerance and stimulated functional effector cells (38). It is of interest to note that this was the only patient who showed elevated levels of IL-12, a key cytokine augmenting the function of pre-existing tumor-specific T-cells capable of inducing tumor rejection (39).

In conclusion, this study has shown that MBV treatment at fever inducing dose levels can lead to a massive induction of immuno-regulatory cytokines that may have supported tumor regression mediated by immunological mechanisms in a patient with advanced bladder cancer. In six of 12 patients a prolonged overall survival was observed without any other treatment correlation. However, because of the small number of patients, the preliminary data on clinical outcome have to be taken with caution and warrant confirmation in a larger phase II clinical study. The individual immune response to MBV seems to be the result of dose, timing, potential additive or synergistic effects and genetically determined responsiveness. Our results indicate that depending on individual conditions of cancer patients, the restoration of basic regulatory mechanisms through MBV treatment may be important to introduce an effective anti-tumor response. In this context, intratumoral MBV administration may ‘induce a danger signal’ and produce a more pro-inflammatory environment balancing immuno-suppressive signals including regulatory T-cells (Tregs). Further studies will have to show whether MBV treatment given at higher doses and over extended periods of time will correlate to anti-tumor immune responses in larger patient populations. The immuno-modulatory effects render MBV as a potent adjuvant that may benefit from the combination with different strategies to treat cancer, including cancer vaccines.

Acknowledgment: We wish to thank Drs. Linda Pan, Eric Hoffman and Ralph Venhaus for clinical trial management.
Table 1: Patient characteristics, highest MBV dose level and summary of vaccine-related toxicity and NY-ESO-1 specific humoral and cellular immune responses.

**Figure legends**

**Figure 1:** Pyrogenicity of MBV. For each patient, MBV dose levels 1-8 are shown on the left x-axis in blue. Highest body temperatures measured 4-12 hours after MBV treatment are shown on the 2nd x-axis in red. Dot line indicates fever level above 38°C. MBV dose levels 1-8 correspond to 250, 750, 2205, 6750, 20300, 60800, 182000 and 547000 Endotoxin Units (EU), respectively. MBV dose levels that increased body temperature above 38°C varied among patients.

**Figure 2:** Serum cytokine levels for TNF-α, IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12 and IL-13 measured at baseline (b), six hours after each MBV administration and 4 weeks after the last MBV administration (post = p) in correlation with the increase of body temperature. Cytokine levels are shown on the left x-axis (blue bars) in pg/ml. Body temperatures are shown on the right x-axis (red lines) in °Celsius. Related MBV dose levels are indicated on the y-axis. Cytokine levels under the detection limit are recorded as 0. Normal range in pg/ml; TNF-α: 1-8, IFN-γ: 0-30, IL-1β: 0.2-7, IL-2: 0.7-90, IL-5: 0.1-2, IL-6: 0.8-10, IL-8: 3-300, IL-10: 0.7-20, IL-12: 1-80 and IL-13: 1-45

**Figure 3 A/B:** NY-ESO-1 specific antibody (A) and T-cell response (B) in all patients. NY-ESO-1 antibody titers did not change during MBV treatment, except in patient #7 whose titer increased from pre-study and #8 who seroconverted late to a reciprocal titer of 1: 400. MAGE-A3 was used as an internal assay control. No antibody reactivity against MAGE-A3 protein was observed. NY-ESO-1 specific CD4 and CD8 T-cell responses generally remained unchanged during the study as shown by the recognition of NY-ESO-1 overlapping 20mer peptides at baseline (○) and after MBV treatment (●).
Figure 4: Development of anti-MBV specific antibodies throughout MBV treatment. MBV was used as antigen and coated at 1250 EU lysate/well. A pool of serum samples obtained from 16 individual healthy donors (HD - pool) served as background control.

Figure 5 A/B: Cytokine and tumor response of patient #11 following MBV treatment
A: Except for IL-13, all other cytokines increased after administration of the fever-inducing MBV dose level. Bars represent baseline (dose level 1) and highest cytokine values (dose level 8) during MBV treatment for each individual cytokine. B: Representative computer tomography images pre and post MBV treatment demonstrate partial remission of mediastinal and abdominal lymph node metastases (marked with arrows). Post CT-scans were taken 5 weeks after the last MBV administration.
References


# Table 1

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* L: local injection reaction including Reddening, Swelling, Pain, Pruritus
S: systemic toxicity including Flu like-syndrome, Sweating, Weakness, CRP Elevation
§ relapse 2 months after study termination
¶ Antibody reciprocal serum titer:  +  < 25,000,  ++  50-100,000,  +++ > 100,000
Figure 2
Figure 3

3a: NY-ESO-1 antibody

3b: NY-ESO-1 T-cells
Fig. 5A

Fig. 5B

Pre-MBV 2/09

Post-MBV 5/09
Phase I clinical trial of Mixed Bacterial Vaccine (Coley's Toxins) in patients with NY-ESO-1 expressing cancers: Immunological effects and clinical activity


Clin Cancer Res  Published OnlineFirst July 30, 2012.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-1116

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