


In the mix: the potential benefits of adding GM-CSF to CpG-B in the local treatment of patients with early-stage melanoma

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ABSTRACT

Whereas TLR9 agonists are recognized as powerful stimulators of antitumor immunity, GM-CSF has had mixed reviews. In previously reported randomized trials we assessed the effects of local immune modulation in early-stage melanoma with CpG-B alone or with GM-CSF. Here we discuss the added value of GM-CSF and show sex-related differences.

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Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an important hematopoietic growth factor that plays multiple roles in the development and differentiation of progenitor cells into granulocytes, macrophages and dendritic cells (DC). Results from clinical trials that evaluated GM-CSF for the treatment of advanced melanoma, extensively reviewed by Hoeller *et al.*, have been ambiguous.¹ GM-CSF can enhance anti-tumor responses by stimulating DC but it can also promote myeloid-derived suppressor cell (MDSC) expansion and act as a chemo-attractant for neutrophils which can dampen the immune response and even promote tumor growth and disease progression.¹ Much is influenced by the context in which GM-CSF is administered² and more research is warranted in order to find optimal dosing, combinations, and administration routes for GM-CSF as a potential treatment for melanoma. For instance, in combination with ipilimumab, an overall survival advantage has been reported, which, beside immune activation, may have been related to lower toxicity rates.^{3,4} There has also been the further suggestion that clinical benefit may be associated with lower doses, leading to DC activation, rather than with higher doses, which may result in detrimental MDSC mobilization.¹ In line with this, localized expression of GM-CSF, encoded by the intratumorally delivered oncolytic virus Talimogene laherparepvec (T-VEC), led to an actual decrease in intratumoral MDSC and regulatory T cell (Treg) rates and increased T cell infiltration.⁵ In patients with early metastatic melanoma (IIIB/C-IVM1a) impressive clinical outcomes were observed after T-VEC treatment with a best overall response rate and complete response rate of 88.5% and 61.5% respectively.⁶

In localized melanoma, disease recurrence after resection of the primary tumor is dependent on disease stage, which includes risk factors such as Breslow thickness, tumor ulceration and whether or not the tumor has metastasized to the regional lymphatics.⁷ As a prognostic measure, a sentinel node biopsy (SNB) is performed to assess lymphogenic metastasis, although so far the treatment implications are very limited after identifying a tumor positive (sentinel) lymph node (stage III disease). A complete lymph node dissection is no longer indicated as this does not improve overall survival⁸ and thus far only high-risk stage III patients have an indication for treatment with checkpoint inhibitors. For patients without lymphatic spread or with stage IIIA disease, the only available treatment is surgery followed by a wait-and-see policy, even though the chances of recurrence can be considerable. Our clinical work has clearly demonstrated the potential clinical benefits of local immune modulation prior to SNB.

In three previously reported randomized controlled phase II trials, we intradermally injected GM-CSF alone,⁹ the TLR9 agonist CpG-B/CPG7909 alone,^{10,11} or CpG-B/CPG7909 and GM-CSF combined¹⁰ directly adjacent to the excision scar of the primary tumor in early-stage (i.e. localized) melanoma in the week leading up to the SNB. In the first trial, patients received four consecutive daily doses of 3 µg/kg body weight GM-CSF (n = 6) or placebo (n = 6), immediately preceding SNB. In the second trial patients received one dose of 8 mg CpG-B (n = 11) or placebo (n = 13) one week prior to SNB, and in the third trial, patients received 1 mg CpG-B (n = 10), 1 mg CPG-B with 100 µg GM-CSF (n = 9) or placebo (n = 9), 7 and 2 days prior to SNB. We previously reported potential down staging and an improved recurrence-free survival (RFS) in patients who

were treated with CpG-B in the second and third trial.¹² Of note, 30% of the patients in the intervention group (9/30) of this meta-analysis actually received combinational treatment with CpG-B and GM-CSF. Here, we elaborate on our previous findings from immune monitoring and clinical follow-up studies,¹⁰ and, based on previously published data as well as data presented here for the first time, demonstrate and discuss the added benefits of including GM-CSF in CpG-based immune potentiation of the melanoma sentinel lymph node (SLN).

Results and discussion

Clinical findings

All patients who had been enrolled in one of the two previous randomized trials which included treatment arms with GM-CSF pre-SNB,^{9,10} alone ($n = 5$) or combined with CpG-B ($n = 9$), were combined into one treatment arm. Their recurrence-free survival (RFS) was compared to that of a corresponding combined control group of patients receiving a saline placebo from the same two clinical trials ($n = 15$). Remarkably, we did not find any loco-regional or distant disease recurrences in the treatment arm after a median follow up of 90 months (Figure 1a). In comparison, we found five recurrences in the combined control groups ($n = 15$) from the same trials ($p = .002$). Although we did not find any recurrences in the GM-CSF group, one patient had never attended any follow-up visits and further data could not be retrieved (this patient was therefore not included in the analyses) and one patient died from an unrelated cause at 19 months after treatment (this patient was censored at 19 months). Although these patient groups are too small to draw any firm conclusions, locally administered GM-CSF, either alone or combined with CpG-B, certainly appeared to improve clinical outcome in terms of RFS of patients with early-stage melanoma.

Myeloid subset modulation and sex disparities

We have previously identified two skin-derived migratory conventional DC subsets (cDC), i.e. Langerhans cells (LC) and dermal dendritic cells (DDC), and two lymph node resident cDC subsets (LNR-cDC) in melanoma SLN.¹³ The LNR-cDC subsets differ in their expression of CD14, with the CD14⁻ LNR-cDC phenotypically resembling CD141⁺ cDC1 with particular cross-presenting abilities. We found locally administered GM-CSF to activate and induce the migration of skin-derived cDC subsets to the SLN.¹⁴ The combination of GM-CSF with CpG-B also resulted in superior activation of skin derived cDC.¹⁰ These findings are in keeping with our previous observation of increased activation of skin explant-emigrated cDC subsequent to intradermal GM-CSF injection.¹⁵ In contrast, no such effect was observed for CpG-B, but rather cDC1 and pDC were mobilized to the deep dermis upon intradermal delivery of CpG-B (Koster *et al.*, manuscript in preparation). We have also reported that the addition of GM-CSF to locally administered CpG-B prior to SNB, resulted in more profound activation of the LNR-cDC subsets, and indeed increased the cross-presentation capacity of SLN-derived leukocyte suspensions.¹⁰ We also observed that frequencies of the CD14⁻ LNR-cDC in SLN correlated with the decrease in cDC1

frequencies in PBMC of patients who had received the combination of CpG-B and GM-CSF (Figure 1b, top panel previously published¹⁰). We now report that this correlation was absent in patients who received CpG-B only (Figure 1b, lower panel). These data indicate that the addition of GM-CSF to CpG-B enhances the mobilization of cDC1 to the SLN as well as their activation. Remarkably, when we subdivided patients by sex, disparities in terms of DC activation became apparent (see Figure 1c). Whereas CpG-B monotherapy in men induced significant activation of both CD14⁻ LNR-cDC and pDC in the SLN (by CD83 expression), and in equal measure as when combined with GM-CSF, in women an equivalent activation induction in these subsets required combined CpG-B and GM-CSF activation. Of note, similar observations were made for the CD14⁺ LNR-cDC subset (data not shown). The role of sex specific hormones and genetics on the immune system^{16,17} and cancer progression^{18,19} has been well documented, but this has not yet led to cancer treatment implications based on the patient's sex. Our data suggest that optimal activation of DC and, as a consequence, subsequent priming of antitumor effector T cells in women with early-stage melanoma may require combined administration of GM-CSF and CpG-B. This may be related to sex-dependent differences in TLR9 expression levels as previously observed in a mouse study, wherein increased TLR9 expression levels in males led to improved clearance of a viral infection.²⁰ Moreover, a sex-dependent inflammatory cytokine pattern during melanoma development was also described before in an experimental mouse model.²¹ Improved pDC activation and IFN α release by GM-CSF has also been previously reported,²² but, as far as we know, has never previously been linked to sex-dependent mechanisms. It is important to note that despite these remarkable differences in DC subset activation, we did not find any differences in RFS between women receiving CpG-B only or CpG-B combined with GM-CSF. However, again, caution is warranted due to the small sample size.

Whereas CpG-B monotherapy led to a significant reduction in circulating monocytic MDSC (mMDSC) rates (defined as CD14^{hi}HLA-DR⁻) at day 7 after the first injection (i.e. day of SNB, see Figure 1d), this reduction was abrogated by the addition of GM-CSF. This is consistent with the reported systemic mobilization of MDSC by GM-CSF,¹ apparently counteracting the mMDSC-reducing effect of CpG-B. Whereas high doses of CpG and chronic type-I IFN exposure may lead to increased MDSC levels,^{23,24} local CpG may actually attenuate MDSC development and their suppressive activity by inducing their maturation.²⁵ The latter is consistent with our observation.

IFN response gene expression

Type-I IFN responses have been identified as crucial to the generation of an effective antitumor T cell response,²⁶ inducing the maturation of cDC1 and enhancing their cross-presenting ability, while simultaneously boosting the effector functions of cytotoxic T cells and NK cells alike. We analyzed transcript levels of IFN Response Genes (IRGs) in PBMC before treatment ($t = 0$), one week after treatment ($t = 7$) and three weeks after treatment ($t = 21$). Cluster analysis revealed concerted up-regulation of a group of 33 IRGs at $t = 7$ in the CpG-treated patient groups (shown in

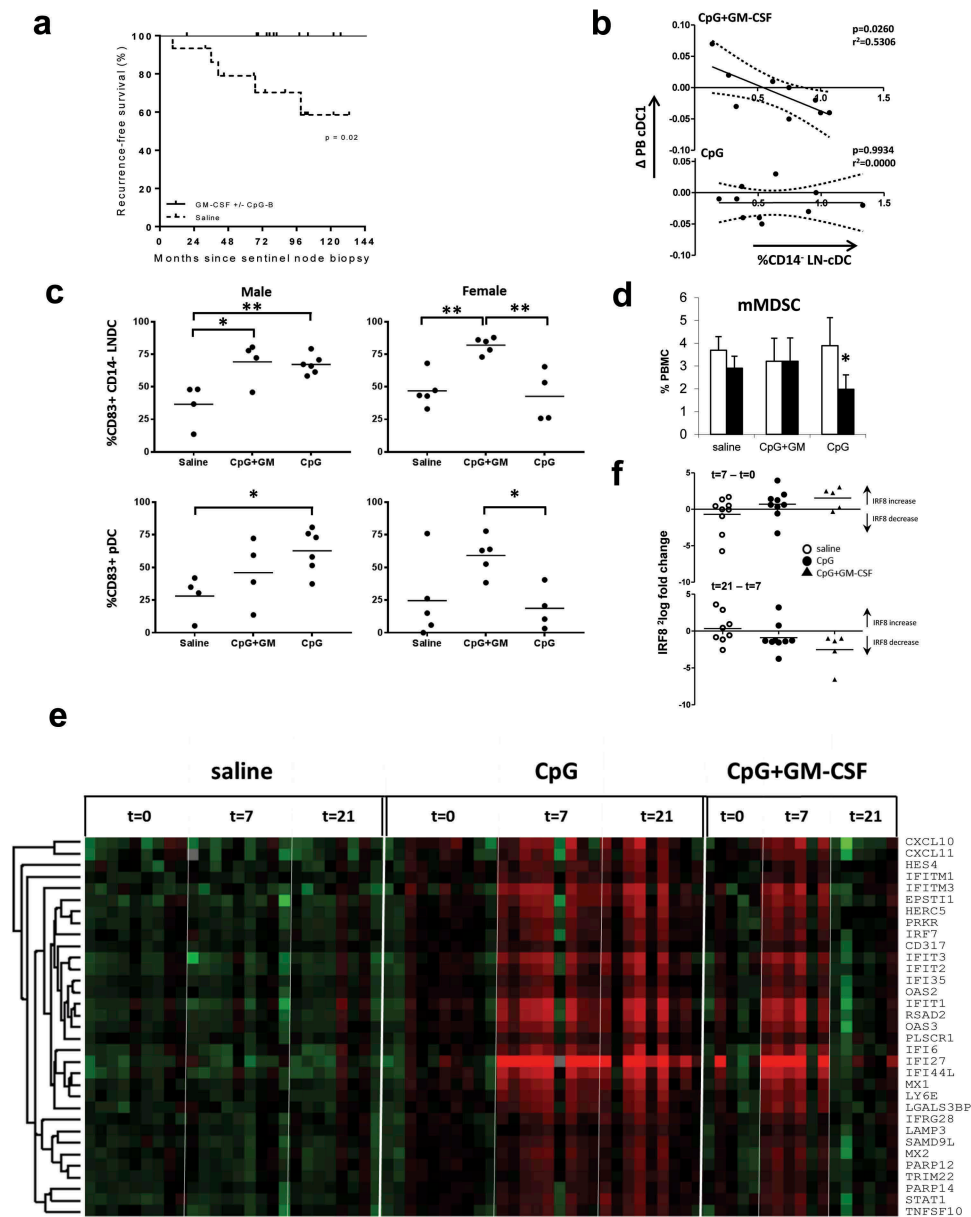


Figure 1

Figure 1. Added local and systemic effects of GM-CSF, co-delivered locally with CpG-B, in early-stage melanoma. Results shown are from patients receiving either a saline placebo (saline), or two administrations of CpG-B (CpG, 1 mg) or CpG combined with GM-CSF (CpG+GM, 1 mg + 100 μ g) at day -7 and day -2 before sentinel lymph node biopsy (SNB), or GM-CSF alone (GM-CSF), 4 doses of 3 μ g/kg divided over the four days leading up to SNB. All were administered at the excision site of the primary tumor. (a) Recurrence-free survival of patients who were treated with GM-CSF with or without CpG-B ($n = 14$) versus patients that received saline ($n = 15$, p value is listed). (b) Correlation between changes in BDC3/CD141⁺ peripheral blood cDC (cDC1) frequencies (between day -7 and 0) and CD14⁻CD11c^{hi}CD14⁻ cDC (CD14-LNDC) rates in the SLN of patients who received CpG+GM or CpG. (c) CD14⁻ LNDC and pDC activation (by CD83) in men and women after local treatment with CpG or CpG+GM. (d) Pre- (day -7, open bars) and post-treatment (day 0, closed bars) frequencies of monocytic myeloid derived suppressor cells (mMDC) in peripheral blood of patients receiving either saline, CpG, or CpG+GM-CSF. (e) Transcriptional profiling reveals post-treatment induction of a type-I Interferon (IFN) response signature in peripheral blood mononuclear cells in patients receiving CpG or CpG+GM. (f) Changes in IRF8 transcript levels (relative to GAPDH) between $t = 7$ and $t = 0$ and $t = 21$ and $t = 7$ for saline ($n = 9$); CpG ($n = 9$); CpG+GM-CSF ($n = 5$). Statistical significance: * $P < .05$; ** $P < .01$; either by One-way ANOVA with Tukey post-hoc test or by paired two-sided student's T test.

Figure 1e). Of the in total 47 tested IRGs, 33 IRGs were significantly upregulated at $t = 7$ as compared to matching baseline values in the CpG-treated patient groups (p values, corrected for multiple testing, ranging from 0.019 to 7.4x10e-6), but none in the saline placebo group. Of these 33 significantly up-regulated genes, 31 were also part of the identified co-regulated cluster of 33 genes shown in **Figure 1e**. Interestingly, whereas the IRG expression levels

remained high on $t = 21$ in the CpG-B only group, their levels had gone down by then in the combined CpG-B and GM-CSF group (see **Figure 1e**). This would fit with reports that GM-CSF may initially enhance pDC-derived type-I IFN responses through up-regulation of IRF8, but at later time points would lead to down-regulation of IRF8 and so attenuate IFN responses.^{27,28} Analysis of changes in IRF8 transcript levels from $t = 0$ to $t = 7$ versus from $t = 7$ to $t = 21$

between the treatment groups would indeed seem to support this hypothesis (figure 1f). Interestingly, these differential expression levels of IRF8 transcripts between CpG-B only and combined GM-CSF/CpG-B may also explain the observed effects on mMDSC levels (Figure 1d), as GM-CSF-induced down-regulation of IRF8 has been implicated in increased MDSC development.²⁹ Of note, although type-I IFN responses favor antitumor immunity and facilitate immune checkpoint blockade, chronic type-I IFN exposure can also have detrimental effects, up-regulating MDSC rates and PD-L1 expression and actually mediating resistance to certain cancer therapies, including immune checkpoint blockade.^{26,30} Thus, timely attenuation of IRGs by GM-CSF may actually be beneficial.

Conclusion

Although GM-CSF can have both adverse and favorable effects on parameters contributing to antitumor immunity, its clearly positive effects on LNR-cDC recruitment and activation and its apparent ability to secure LNR-cDC and pDC activation in women, warrants its further clinical exploration in combination with next-generation CpG oligodeoxynucleotides for the local treatment and conditioning of melanoma SLN in patients with early-stage melanoma. Certainly, the resulting significantly improved clinical outcome, with none of the fourteen GM-CSF-treated patients experiencing recurrences, seems to underline this notion.

Patients and methods

For patient characteristics, we refer to Vuylsteke *et al.*⁹ and Sluijter *et al.*¹⁰ Importantly, for both trials, the control groups and treatment groups were balanced in terms of sex, age, ulceration, Breslow thickness, and disease staging. For methods and statistics employed in the flow cytometry analyses, we refer to Sluijter *et al.*¹⁰ and for methods and statistics employed for clinical follow-up analysis, we refer to Koster *et al.*¹² For methods and statistics employed in the flow cytometry analyses, we refer to Sluijter *et al.*¹⁰ and for methods and statistics employed for clinical follow-up analysis, we refer to Koster *et al.*¹² The studies were approved by the Institutional Review Board of the VU University Medical Center, and written informed consent was obtained from each patient before treatment in accordance with the Declaration of Helsinki.

Type-I interferon (IFN) response analysis

PBMC were isolated and total RNA isolated and reverse transcribed as previously described.¹⁰ Forty-seven type I IRGs were selected based on significant upregulation in more than 3 experiments published on the Interferome database³¹ (<http://www.interferome.org/>). Custom-designed TaqMan[®] assays for each gene were supplied by Applied Biosystems. Quantitative PCR (qPCR) analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark[™] Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, USA), according to the manufacturer's instructions. Thermal cycling

and real-time imaging of the BioMark array was done on the BioMark instrument, and cycle threshold (C_T) values were extracted using the BioMark Real-Time PCR analysis software. Relative quantities were calculated using the standard curve method, using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. Expression levels were ²log-transformed. Cluster analysis was used for categorization of IRGs with respect to their relative expression between treatment arms.³² TreeView was used to visualize the clustering of genes (Eisen Lab, Berkeley, CA, USA). Comparison of IRG expression between time points was assessed using paired *t* tests. The Benjamini-Hochberg procedure was applied to correct for multiple testing. Corrected *P* values of <0.05 were considered significant.

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Disclosure of Potential Conflicts of Interest

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