

Arming the Melanoma Sentinel Lymph Node through Local Administration of CpG-B and GM-CSF: Recruitment and Activation of BDCA3/CD141⁺ Dendritic Cells and Enhanced Cross-Presentation

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Abstract

Melanoma-induced suppression of dendritic cells (DC) in the sentinel lymph node (SLN) interferes with the generation of protective antitumor immunity. In an effort to strengthen immune defense against metastatic spread, we performed a three-arm phase II study comprising 28 patients with stage I–II melanoma randomized to receive intradermal injections around the primary tumor excision site of saline or low-dose CpG-B, alone or combined with GM-CSF, before excision of the SLNs. After pathologic examination, 5 patients were diagnosed with stage III melanoma based on the presence of tumor cells in the SLNs. Combined CpG/GM-CSF administration resulted in enhanced maturation of all identifiable conventional (cDC) and plasmacytoid (pDC) DC subsets and selectively induced increased frequencies of SLN-resident BDCA3/CD141⁺ cDC

subsets that also expressed the C-type lectin receptor CLEC9A. Correlative *in vivo* analyses and *in vitro* studies provided evidence that these subsets were derived from BDCA3⁺ cDC precursors in the blood that were recruited to the SLNs in a type I IFN-dependent manner and subsequently matured under the combined influence of CpG and GM-CSF. In line with their reported functional abilities, frequencies of *in vivo* CpG/GM-CSF-induced BDCA3/CD141⁺ DCs correlated with increased *ex vivo* cross-presenting capacity of SLN suspensions. Combined local CpG/GM-CSF delivery thus supports protective anti-melanoma immunity through concerted activation of pDC and cDC subsets and recruitment of BDCA3⁺ cDC subsets with T cell-stimulatory and cross-priming abilities. *Cancer Immunol Res*; 3(5); 495–505. ©2015 AACR.

Introduction

The first melanoma-draining lymph node, also known as the sentinel lymph node (SLN), has been identified as an important site of tumor-induced immune suppression. As professional antigen-presenting cells (APC) and principal orchestrators of the innate and adaptive immune response, dendritic cells (DC) are prime targets for tumor-induced immune suppression as a means of immune escape: DCs in SLNs express lower levels of activation

markers and display a less activated morphology compared with DCs in second-line melanoma-draining lymph nodes (LN; 1). As a result, the most probable site of initial micrometastasis, i.e., the SLN, is severely hampered in its ability to generate protective T cell-mediated immunity. We and others have shown previously that local administration of DC-stimulatory agents, such as GM-CSF, CpG oligodeoxynucleotides (ODN), and IFN α , can lead to increased activation of DCs in the SLNs and tip the local cytokine balance in favor of cell-mediated immunity (2–6). Such DC-targeted immunopotentiating strategies may be generally applied and afford a measure of protection against early metastatic spread (7, 8).

Two major types of DCs can be discerned on the basis of their phenotype and morphology: conventional DCs (cDC) and plasmacytoid DCs (pDC). Both have been reported to directly activate CD4⁺ T-helper (Th) cells and cross-prime CD8⁺ cytotoxic T lymphocytes (CTL), and both have been implicated in the generation of effective antimelanoma immunity (9, 10). Upon Toll-like receptor (TLR) ligation, pDCs rapidly release large amounts of IFN α (11), which activate effector CTLs and natural killer (NK) cells as well as promoting the differentiation and maturation of cDCs (12–15). We previously reported on the respective cDC- and pDC-activating effects in the SLNs of

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early-stage melanoma patients of locally administered GM-CSF or the CpG type B (CpG-B) ODNs and TLR9 ligand PF-3512676 (3, 16). As both compounds act through activation of different DC subsets, we hypothesized that their combined administration should most effectively activate a cell-mediated immune response. In keeping with this notion, CpG ODN combined with GM-CSF proved to facilitate vaccine-induced tumor rejection more effectively *in vivo* than GM-CSF alone (17) and also proved to be clinically safe (18).

We and others recently characterized different cDC subsets in SLN suspensions in relation to their ability to stimulate T cells (19, 20). On the basis of their phenotype, we identified both CD1a⁺ Langerhans cells (LC) and dermal DCs (DDC) in SLN suspensions. Both subsets were previously reported to be able to migrate to draining LNs and to prime specific Th cells or CTLs (21, 22). Besides these two migratory subsets, we also identified two CD1a⁻ LN-resident cDC subsets (CD14⁻ or CD14⁺; ref. 19). In a comparative analysis, the LN-resident cDC subsets proved to be more powerful T-cell stimulators in terms of allogeneic T-cell priming and IFN γ induction, despite their lower phenotypic maturation level, than the skin-derived subsets. By studying the effects of immune modulators on these cDC subsets in SLNs in relation to DC precursor rates in peripheral blood, we may learn the relationship between these cDCs and their putative blood-borne precursors and how best to recruit them for therapeutic purposes.

Herein, we present results from a three-arm clinical trial, demonstrating that combined local administration of GM-CSF and CpG-B at the primary melanoma excision site leads to full-range activation of cDC and pDC subsets, both in SLNs and in blood. Moreover, we describe a type I IFN-related recruitment of LN-residing BDCA3/CD141⁺ cDC subsets (19) from BDCA3/CD141⁺ blood precursors and their CpG/GM-CSF-related activation. In keeping with the reported cross-priming ability of BDCA3/CD141-expressing cDC subsets (23), we further report their CpG/GM-CSF-mediated mobilization *in vivo* to be associated with increased *ex vivo* cross-presenting capacity of SLN cells, suggesting that their recruitment may contribute to protection against metastatic spread through increased CTL induction against melanoma-derived antigens.

Materials and Methods

Patients

From April 2006 to June 2007, 28 patients were included in this single-blinded phase II study and randomly assigned to receive preoperative local administration of either GM-CSF (Leukine; Berlex Laboratories Inc.) and synthetic CpG-B (PF-3512676; Coley Pharmaceutical Group), CpG-B alone, or saline (NaCl 0.9%). All patients were diagnosed with clinically stage I/II melanoma, according to criteria of the American Joint Committee on Cancer, and were scheduled to undergo a sentinel node procedure (SNP). Inclusion criteria were as described previously (16). The medical ethics committee of the VU University Medical Center (Amsterdam, the Netherlands) approved the study and written informed consent was obtained from each patient before treatment. After pathologic examination, 5 patients were diagnosed with stage III melanoma based on the presence of tumor cells in the SLNs (1/9 patients in the GM-CSF/PF-3512676 group, 0/10 patients in the PF-3512676 group, and 4/9 patients in the saline group). One of these patients from the saline group was diagnosed with micrometastasis (<0.1 mm) and did not undergo additional lymph node dissection; all additional nodes from the other 4 patients were negative. Patient characteristics are listed in Table 1A.

GM-CSF and PF-3512676 administration, triple-technique SNP and cell sampling

All patients received 4-mL intradermal (i.d.) injections directly into the scar of the primary melanoma excision 7 and 2 days before SNP. Patients received either a combination of 1 mg PF-3512676 and 100 μ g of GM-CSF, or 1 mg PF-3512676, or 4 mL of plain saline. Heparinized blood was drawn before the first injection and on the day of the SNP. Viable peripheral blood mononuclear cells (PBMC) were isolated and cryopreserved for further analysis as previously described (4). One week after the first injection, all patients underwent SNP and reexcision of the primary melanoma site as described previously (24). We and others have previously described a detailed method to collect viable cells from SLNs without interference in standard diagnostic procedures (25, 26). In short, the SLNs were collected in sterile ice-cold complete medium (CM), consisting of IMDM supplemented with 25 mmol/L HEPES buffer (BioWhittaker) with 10% FCS, 50

Table 1. Patient and SLN characteristics (A) and inflammatory cytokine concentrations in supernatants from 24-hour cultures of SLN cells (B)

	Saline	GM-CSF + PF3512676	PF3512676	P ^b
A. Patient and SLN characteristics				
Sex (male:female)	4:5	4:5	6:4	0.755
Age (mean \pm SD)	47 \pm 12	50 \pm 12	58 \pm 12	0.183
Breslow thickness (mm; mean \pm SD)	1.67 \pm 0.55	2.19 \pm 1.37	1.67 \pm 0.55	0.493
Volume SLN (mm ³ ; mean \pm SD) ^a	1.22 \pm 0.71	^c 2.94 \pm 1.78	2.04 \pm 1.30	0.031
Weight SLN (g; mean \pm SD)	0.95 \pm 0.35	1.36 \pm 1.19	1.62 \pm 0.80	0.294
Yield scraping ($\times 10^6$; mean \pm SD)	29 \pm 25	77 \pm 61	70 \pm 55	0.125
SLN-containing tumor cells	4	1	0	0.031
B. Cytokines released by SLN				
IL12p70	12 \pm 4	11 \pm 4	12 \pm 3	0.732
TNF α	30 \pm 34	34 \pm 31	38 \pm 40	0.910
IL10	44 \pm 73	77 \pm 55	83 \pm 68	0.454
IL1 β	23 \pm 23	42 \pm 31	29 \pm 13	0.243
IL6	198 \pm 174	^c 1,217 \pm 1,385	361 \pm 293	0.026
IL8	2,872 \pm 2,047	10,140 \pm 12,350	4,167 \pm 2,030	0.108

NOTE: All concentrations are in pg/mL (mean \pm SD).

^aVolume: height \times width \times length.

^bStatistical significance among the three patient groups by one-way ANOVA.

^cStatistically significant difference from that of saline control group by the Tukey *post hoc* analysis ($P < 0.05$).

IU/mL penicillin–streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L β -mercaptoethanol (i.e., CM), after which they were bisected in a sterile environment and viable cells were scraped from the cutting surface. The SLN cells were washed twice in CM, counted, and further processed.

Cytokine profiling

Freshly isolated SLN cells were cultured overnight at 37°C (1×10^5 per 100 μ L) in CM. The supernatants were harvested and stored at –20°C until detection of cytokine levels by BD-cytometric bead array (CBA; BD) following the manufacturer's instructions.

Flow cytometry

Freshly isolated SLN cells or thawed PBMCs were directly stained with antibodies labeled with either FITC, PE, PE-CY5.5, PerCP-CY5.5, or APC and analyzed by flow cytometry at 100,000 or 200,000 events per measurement, as previously described (25, 27). Monoclonal antibodies against CD1a, CD3, CD11c, CD14, CD16, CD19, CD25, CD40, CD56, CD80, CD86, CD123, CCR7, HLA-DR, CLEC9A, IgM, rIgG2a (BD), CD11c, CD40, CD83 (Immunotech), BDCA-1/CD1c, BDCA-2/CD303, BDCA-3/CD141 (Miltenyi Biotec), MDC-8 (kindly provided by Dr. E.P. Rieber, Institute for Immunology, Technical University of Dresden, Germany), and mIgM (Southern Biotechnology) with matching isotype control antibodies were used.

Quantitative real-time polymerase chain reaction

RNA (0.25 μ g) was isolated from PBMCs and reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas) according to the manufacturers' instructions and as described previously (28). Quantitative real-time PCR (qRT-PCR) was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems) using SybrGreen (Applied Biosystems). Primers were designed using Primer Express software and guidelines (Applied Biosystems): *MxA* (*Genes Genbank accession no.* NM 002462): sense: TTCAGCACCTGATGGCTATC, antisense: GTACGTCTGGAGCATGAAGAACTG; *GAPDH*: sense: GCCAGCCGAGCCACATC, antisense: TGACCAGGCGCCCAATAC. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies, a standard curve was constructed. Expression levels of *MxA* were expressed relative to the housekeeping gene *GAPDH*.

PBMC cultures

PBMCs from 4 healthy donors were cultured for 2 days at 5×10^6 cells/mL in CM at 37°C, without additives (neg. control), with 5 μ g/mL CpG-A (ODN 2216), or with 5 μ g/mL CpG-B (ODN 7909, PF-3512676; both from Coley Pharmaceutical Group), the latter either with or without 1,000 IU/mL GM-CSF (Leukine; Berlex Laboratories Inc.), at 1 mL/well/condition in a 48-well tissue culture plate. After 2 days, cells were harvested and analyzed by flow cytometry.

Cross-presentation assay

Cryostored SLN single-cell suspensions from HLA-A2⁺ patients were thawed, washed in serum-free medium, and 1×10^5 SLN cells/50 μ L/well were plated in 96-well round-bottom wells; triplicate wells were used for all below listed test conditions. To the wells, either no additives or a long (MART-1 aa16-40L)

synthetic peptide (concentration 100 μ g/mL) were added and the cells were left at 37°C for 2 hours, after which 50 μ L of 20% FCS CM was added to each well and the cells were cultured overnight at 37°C in a humidified 5% CO₂ atmosphere. The following day, the cells were washed, and to the no-additive wells, either a short MART-1 aa26-35L or a short HIV RT aa476-484 (irrelevant) synthetic peptide were added at a concentration of 1 μ g/mL in serum-free medium together with 3 μ g/mL β 2-microglobulin. After 2 hours of incubation at 37°C, all cells were washed and resuspended in 100 μ L/well CM. Subsequently, 50,000 cells of a >90% pure MART-1 aa26-35L-specific CD8⁺ T-cell bulk culture (determined by tetramer staining) were added in 100- μ L CM per well, together with 0.5 μ L of Golgi Plug (BD Biosciences), and incubated at 37°C in a humidified 5% CO₂ atmosphere for 5 hours. As a positive control, HLA-A2⁺ JY stimulator cells loaded with MART-1 aa26-35L were also cocultured with the MART-specific HLA-A2–matched CD8⁺ effector T cells. Subsequently, all cells were harvested, washed, stained for MART-1 aa26-35L HLA-A2-tetramer binding and intracellular (i.c.) IFN γ , and analyzed by flow cytometry as previously described (29).

Statistical analysis

Overall differences in patient or SLN characteristics and immune parameters were analyzed using the one-way ANOVA test. The *post hoc* multiple comparison Tukey test was used to analyze differences between two patient study groups. The paired samples *t* test was used to calculate differences in immune parameters between the PBMCs from the first and second time point. Correlations were determined using the Pearson *r* test. Differences were considered statistically significant when $P \leq 0.05$.

Results

Clinical observations

No statistically significant differences were found among the three patient groups receiving combined GM-CSF and PF-3512676 (hereafter referred to as CpG), CpG alone, or saline, in terms of age, gender, or Breslow Thickness (see Table 1A). Injections with CpG and/or GM-CSF were tolerated well. Common side effects were mild flu-like symptoms and induration at the injection site, all of which were transient and easily controlled by paracetamol. According to common toxicity criteria (NCI CTC Toxicity scale Version 2.0), 70% of patients receiving CpG and 56% of patients receiving GM-CSF/CpG had grade 1 fatigue and fever and/or grade 1 myalgia. Grade 2 injection site reactions were observed in 89% of patients receiving GM-CSF/CpG and in 50% of patients receiving CpG; grade 1 reactions were observed in 22% of patients receiving GM-CSF/CpG and in 30% of patients receiving CpG. Induration of the injection site remained present for 2 to 7 days after injection but was considered manageable by most patients. One patient's concern about the injection site induration led to the decision not to administer the second dose of GM-CSF and CpG but did not result in exclusion from the trial as the measured immune parameters in this patient did not deviate from others in the test group. No toxicity was observed in patients receiving saline. After pathologic examination, 5 SLNs were found to contain tumor cells, i.e., disease stage III. Four of these stage III patients had received saline, resulting in an uneven distribution over the three groups ($P = 0.031$, see Table 1A). The dissected SLNs of patients receiving GM-CSF/CpG were found larger in

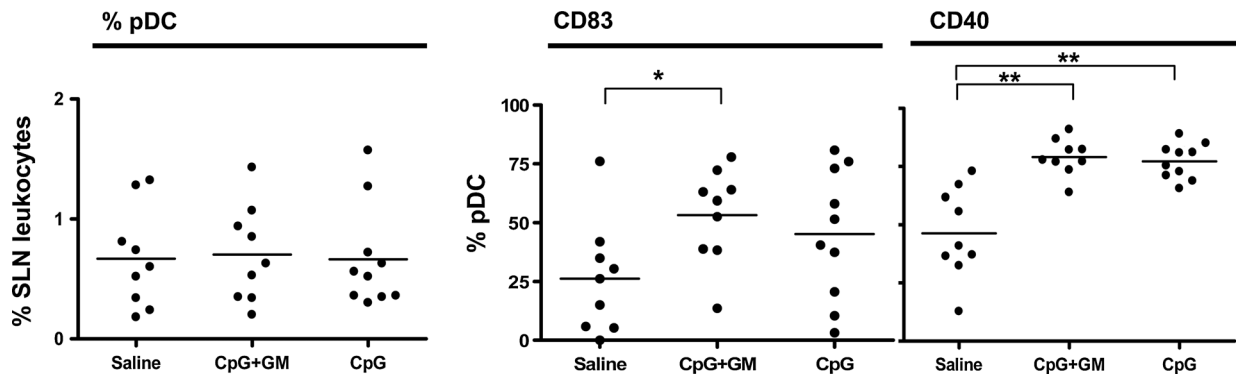


Figure 1. Frequency and phenotype of pDCs in melanoma SLN. Shown are pDC frequencies (as percentage of SLN cells), CD83 and CD40 expression by percentage positivity of pDCs (scatter plots, means indicated), divided by patient group receiving either saline placebo, or 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM), or 1 mg CpG-B alone (CpG). Statistical significance: *, $P < 0.05$; **, $P < 0.01$.

comparison with the SLNs of the other groups ($P = 0.031$; see Table 1A). Also, cell yields were higher, but this did not reach statistical significance.

SLN cytokine release profiles

As an indication of innate immune activation, spontaneous cytokine release by SLN leukocytes was determined after 24-hour culture. As shown in Table 1B, compared with those of saline controls, the combined i.d. administration of GM-CSF and CpG resulted in the release of higher levels of the proinflammatory cytokines IL1 β , IL6, and IL8; only the difference in the levels of IL6

reached statistical significance. This result is in keeping with reported CpG-induced IL6 release by pDCs (9) and consistent with an induced inflammatory environment in the SLN, which was enhanced by the addition of GM-CSF as evidenced by further elevated levels of IL1 β , IL6, and IL8. Of note, we were unable to detect IFN α at this time point in any of the supernatants.

Effects on SLN DC subset composition

SLN cells, isolated on the day of operation, were analyzed by flow cytometry for the frequency and activation state of lymphocytic and DC subsets. No major shifts in overall lymphocyte subset

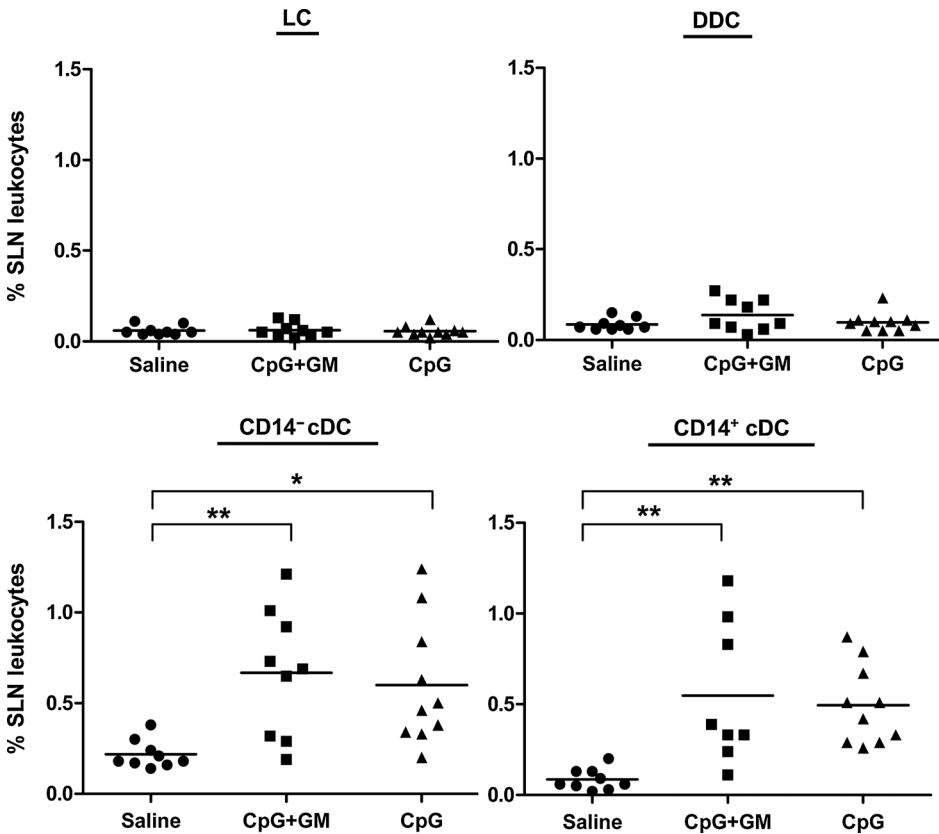


Figure 2. Frequencies and phenotype of cDC subsets in melanoma SLN. Frequencies are shown as percentage of SLN cells, divided by patient group receiving either saline placebo, or 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM), or 1 mg CpG-B alone (CpG). The cDC subsets include LC, CD1a⁺ DDCs, and CD1a⁻ CD14⁻ and CD14⁺ LN-resident cDC subsets. Statistical significance: *, $P < 0.05$; **, $P < 0.01$.

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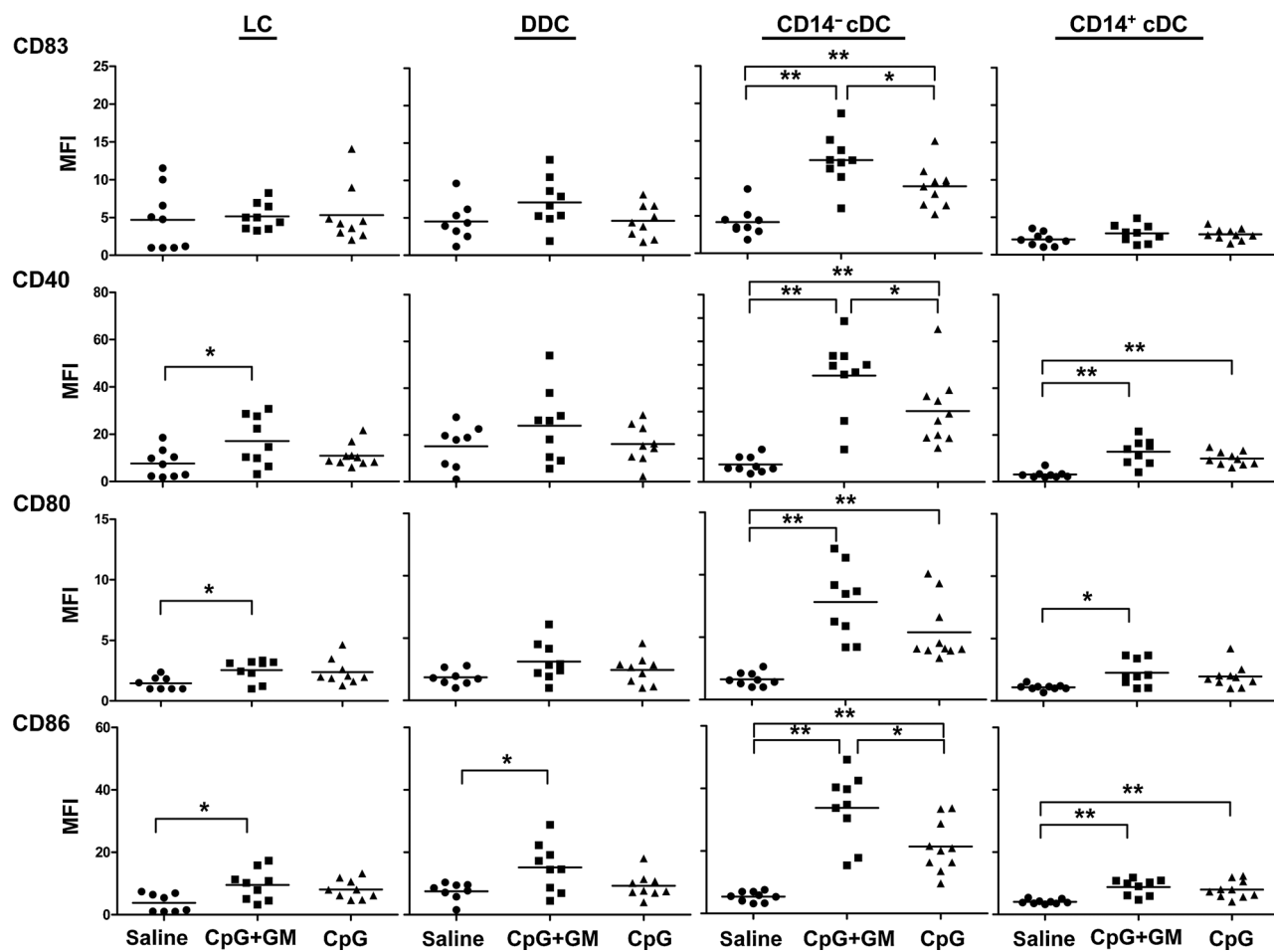


Figure 3. Phenotype of cDC subsets in melanoma SLN. Expression levels of CD83 and costimulatory molecules of LC, CD1a⁺ DDC, CD1a⁻ CD14⁻ and CD14⁺ cDC subsets by mean fluorescence index (MFI) are shown. Means are indicated; patient groups received saline placebo, 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM), or 1 mg CpG-B alone (CpG). Statistical significance: *, $P < 0.05$; **, $P < 0.01$.

composition were observed between the test groups (data not shown). Although pDC rates were not elevated by administration of either GM-CSF/CpG or CpG alone, their activation state was, as evidenced by increased percentages of pDCs expressing the activation markers CD83 and CD40 (Fig. 1).

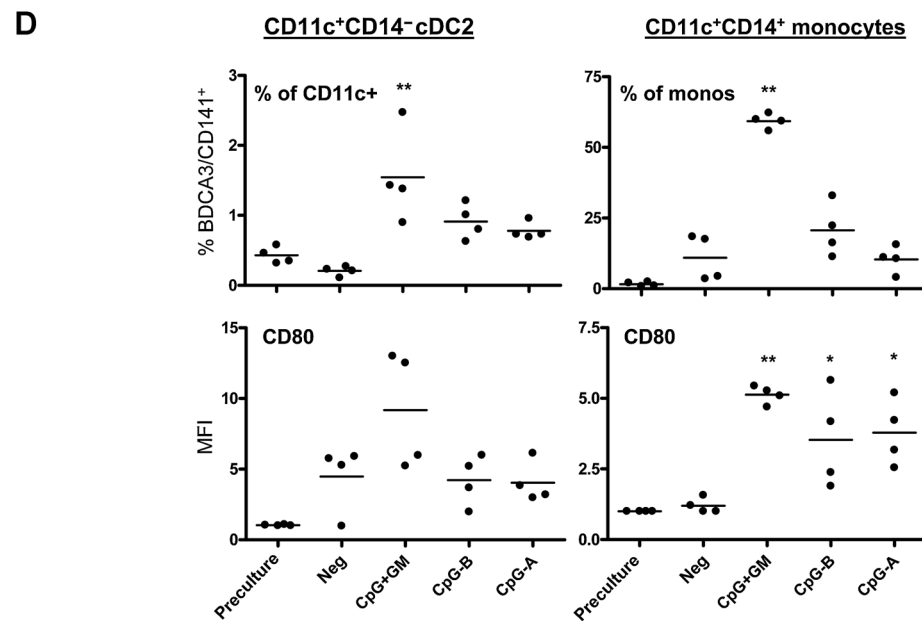
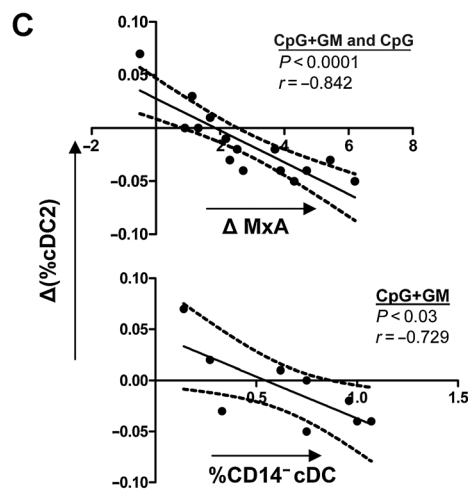
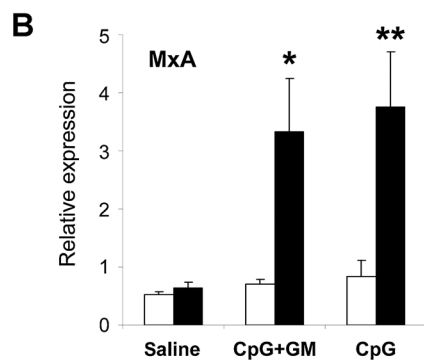
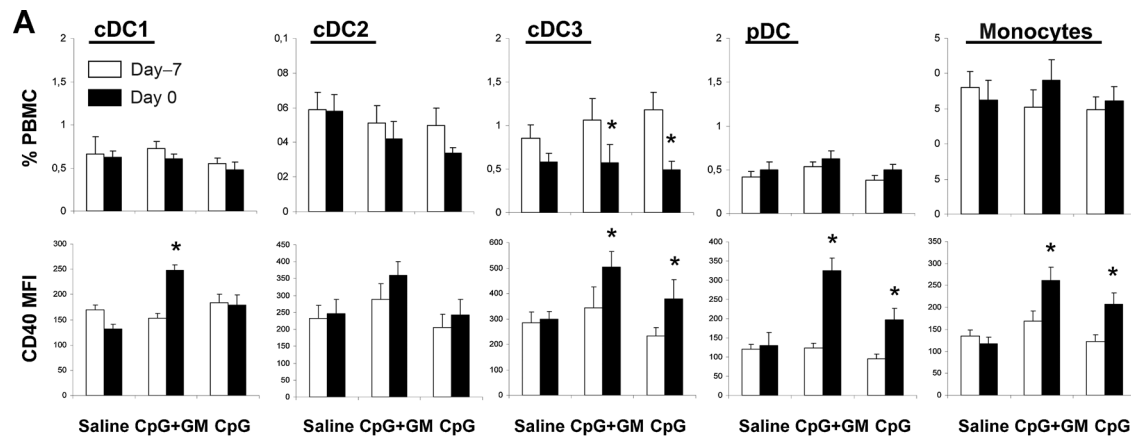
On the basis of CD1a, CD11c, and CD14 expression, four cDC subsets were discerned and phenotypically analyzed: (i) LC, (ii) DDC, (iii) CD14⁻ cDC, and (iv) CD14⁺ cDC, with the first two corresponding to skin-derived DCs (19, 30), and the latter two more likely representing LN-resident, blood-mobilized DC subsets (19). For gating strategies, we refer to Supplementary Fig. S1. Only the frequencies of both LN-resident cDC subsets were significantly upregulated in equal measure by either combined GM-CSF/CpG or CpG single administration (Fig. 2). These LN-resident subsets were further characterized in the steady state by relatively high expression levels of BDCA3 and, in particular for the CD14⁺ cDCs, by CLEC9A (Supplementary Fig. S2), suggestive of their ability to take up necrotic cells and cross-present antigens to CTLs (23, 31, 32).

To assess cDC subset activation, expression levels of the maturation/costimulatory markers CD83, CD40, CD80, and

CD86 were determined. As shown in Fig. 3, both CD1a⁻ LN-resident cDC subsets, but the CD14⁻ LN-resident subset in particular, displayed a stronger increase in activation state upon GM-CSF and/or CpG administration than the CD1a⁺ skin-derived subsets. Whereas the CD14⁺ cDCs were equally activated by combined GM-CSF/CpG or CpG alone, the CD14⁻ subset was more activated by the combined regimen, with all activation markers consistently elevated to higher levels (Fig. 3).

Effects on DC (precursor) rates in peripheral blood: evidence for CpG + GM-CSF-induced activation and recruitment of BDCA3/CD141⁺ cDC subsets

To ascertain systemic effects of low doses of locally administered CpG and GM-CSF, and to identify putative circulating precursors to the cDC subsets recruited to the SLN, frequencies and activation state of DC and monocytic subsets in PBMCs (27) were analyzed on the day of the first injection (day -7) and on the day of the SNP (day 0). Three cDC subsets were discerned in blood: cDC1 (BDCA1/CD1c⁺), cDC2 (BDCA3/CD141⁺), and cDC3 (M-DC8⁺, also known as CD16⁺CD14^{dim} or 6-Sulfo



LacNac⁺ (SLAN) inflammatory DCs (33). pDCs were defined as CD123⁺BDCA2/CD303⁺ and monocytes as CD11c^{hi}CD14⁺. cDC2 and cDC3 frequencies were decreased upon either combined GM-CSF/CpG or single CpG administration, reaching statistical significance for cDC3 only (Fig. 4A). On the basis of CD40, CD86, and HLA-DR expression levels, combined i.d. GM-CSF/CpG delivery most profoundly affected myeloid maturation, with all APC subsets showing a measure of activation (shown for CD40 with statistical significance levels indicated in Fig. 4A). Of note, none of these subsets were activated by i.d. saline administration before SLN excision.

A likely mechanism of CpG-induced cDC recruitment to the SLN would be through IFN α -mediated activation of circulating cDC precursors, followed by their extravasation at inflammatory sites (e.g., CpG-conditioned skin or SLN). Although we were unable to detect IFN α either in supernatants of *ex vivo* cultured SLN suspensions or in plasma samples (data not shown), we did detect an increase in mRNA levels of the type I IFN-responsive gene product MxA by qRT-PCR in PBMC samples of the CpG-treated patient groups (Fig. 4B), clearly demonstrating a systemic impact of type I IFN release induced by locally administered CpG-B (most likely pDC-derived IFN α). Interestingly, CpG-related decreases of BDCA3/CD141⁺ cDC2 frequencies in the blood directly and strongly correlated to Δ MxA transcript levels (Fig. 4C, top). Moreover, correlating frequencies of the four separate cDC subsets in SLNs and posttreatment changes in frequencies of the different cDC subsets and monocytes in peripheral blood, the only significant correlation found was between decreased cDC2 subset frequencies in the blood and higher BDCA3/CD141⁺CD14⁻ cDC rates in the SLNs of patients that received the combined administration of GM-CSF/CpG (Fig. 4C, bottom), but *not* of patients receiving CpG only (data not shown). This potential relationship was confirmed by the results of 2-day *in vitro* PBMC cultures, showing a significant increase and enhanced activation state (exemplified by CD80 expression) of BDCA3⁺cDC2 (and note: also of *de novo* BDCA3-expressing monocytes), upon culture with combined CpG-B and GM-CSF as compared with either CpG-B or CpG-A alone (Fig. 4D). CpG-A was taken along in these cultures as this class of ODN is known to induce higher release levels of type I IFN. Indeed, concentrations of IFN α in CpG-B-stimulated cultures averaged 27.6 pg/mL, while those in CpG-A-stimulated cultures exceeded 500 pg/mL. Nevertheless, the combined CpG-B/GM-CSF culture induced superior activation of both cDC2 and monocytes. These data are consistent with a CpG/type I IFN-related recruitment of BDCA3⁺ cDC2 to the

SLN, followed by their CpG/GM-CSF-induced maturation to BDCA3/CD141⁺CD14⁻ LN-resident cDCs.

Effects on cross-presentation in SLNs

As the observed expression of both BDCA3 and CLEC9A on the LN-resident cDC subsets in the steady state was suggestive of cross-presentation capacity, and as they were preferentially induced by CpG + GM-CSF, we compared the *ex vivo* (cross-) presentation ability of SLN suspensions among patients from all three treatment groups (Fig. 5A–C). Cryostored SLN suspensions of 3 HLA-A2⁺ patients from each of the three treatment groups were available for this analysis. In line with the CpG + GM-CSF (CpG+GM)-induced activation and recruitment of putative cross-presenting BDCA3/CD141⁺ cDC subsets to the SLN, it seemed that SLN cells from patients that received the combined administration of CpG+GM most efficiently cross-presented the immunodominant aa26-35L HLA-A2-binding epitope of MART-1 to a specific CD8⁺ T-cell line after uptake and processing of a long 25-mer MART-1 (aa16-40L) peptide, whereas direct presentation of the aa25-35L short peptide was comparable among all three groups (Fig. 5B and C). Cross-presentation efficiency was determined by i.c. IFN γ expression in the MART-1 aa26-35L-specific T cells relative to the i.c. IFN γ level detected after presentation of the short peptide, to standardize between donors, samples, and assays. Unfortunately, statistical significance was not reached because of the low number of cryostored SLN samples available for this analysis (Fig. 5C). Of note, the observed increased cross-presentation capacity corresponded to predominant frequencies of CD1a⁻ LN-resident subsets (with relatively high expression levels of BDCA3 and CLEC9A) and, accordingly, the observed cross-presentation efficiencies correlated with the sum-total rate of LN-resident CD1a⁻ cDC subsets in the used SLN suspensions rather than with that of either CD1a⁺ skin-migrated cDCs or pDCs (Fig. 5D).

Discussion

The results presented in this article are the first to show the effects of combined GM-CSF and CpG-B administration on human DC subsets in melanoma SLNs and the consequent outcome in terms of antigen cross-presentation. We previously showed that four daily i.d. injections of GM-CSF (at 3 μ g/kg) increased the number and activation state of CD1a⁺ cDCs in the SLNs (16) and presented evidence to suggest that these cells were contiguous with dermal CD1a⁺CD83⁺ DCs and had migrated

Figure 4.

Effects of CpG \pm GM-CSF on the frequency and activation state of APC subsets in peripheral blood. A, frequency of various DC subsets and monocytes and expression levels of CD40 by mean fluorescence index (MFI). Patient groups are indicated: saline placebo, 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM), or 1 mg CpG-B alone (CpG). Open bars, day -7 (basal levels); closed bars, day 0, time of SLN excision. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; mean \pm SEM are shown. B, relative expression of transcripts from the type I IFN-responsive *mxa* gene, determined by reverse transcriptase quantitative PCR on PBMCs. Patient groups are indicated in the bar graphs: saline placebo, 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM), or 1 mg CpG-B alone (CpG). Open bars, day -7 (basal levels); closed bars, day 0, time of SLN excision. Statistical significance: *, $P < 0.05$ and **, $P < 0.01$. C, correlation between changes in BDCA3/CD141⁺ peripheral blood cDC (cDC2) frequencies (between day -7 and 0) and corresponding MxA transcript levels in peripheral blood, and cDC2 frequencies and CD14⁻ LN-resident cDC rates in the SLNs of patients who were administered 1 mg CpG-B and 100 μ g GM-CSF (CpG+GM) at the primary melanoma excision site. Pearson r^2 and P values are listed and 95% confidentiality intervals indicated by dotted lines. D, frequencies of BDCA3/CD141⁺ cDC2 (as % of gated CD11c⁺ cells) and of BDCA3/CD141⁺ monocytes (as % of CD14⁺ monocytes) in PBMCs, before (preculture) and after 2 days of culture without additions (neg) or with added CpG-B and GM-CSF (CpG+GM), CpG-B, or CpG-A. Also shown are CD80 expression levels (in MFI) on the CC14⁻BDCA3/CD141⁺ cDC2 or monocytes. Asterisks denote statistically significant differences versus negative control cultures (neg): **, $P < 0.01$; *, $P < 0.05$. Note that MFI CD80 on CD11c⁺CC14⁻BDCA3/CD141⁺ cDC2 were also significantly different between preculture and CpG+GM conditions ($P < 0.01$).

from the dermis, where GM-CSF was delivered at the primary melanoma excision site (30). In contrast, a single high dose of CpG-B (8 mg) did not result in increased rates of either the pDC or CD1a⁺ cDC subsets, but increased the frequency of a CD1a⁻CD11c⁺CD83⁺ cDC subset (3). In the current trial, patients received two i.d. injections of lower doses of CpG-B alone (1 mg), or in combination with GM-CSF (100 µg). These doses were based on studies in which these compounds were added as adjuvants to i.d. applied vaccines (34, 35) and were chosen to minimize the chances of any side effects from their combined administration in early-stage melanoma patients participating in this trial.

Whereas in trials in which patients underwent multiple rounds of vaccination, suppressive effects were ascribed to the use of GM-CSF as vaccine adjuvant (34), limited low dosing as local immune stimulant was shown by us to lead to cDC maturation and concomitant antitumor T-cell activation (5, 16, 30). As anticipated, combined CpG/GM-CSF administration led to a more wide-ranging pDC and cDC subset activation than CpG alone, but it also led to the preferential recruitment of BDCA3/CD141⁺ blood-derived cDC subsets, corresponding to the CD1a⁻CD11c⁺CD83⁺ cDC population identified after CpG-B treatment in our previously reported trial (3), and which, in turn, we found to correlate with an increased functional *ex vivo* cross-presentation capacity of cells derived from the *in vivo* conditioned SLNs.

We previously identified two major BDCA3/CD141⁺ LN-resident cDC subsets, i.e., CD1a⁻CD14⁻ and CD1a⁻CD14⁺ cDCs (19). In the steady state (e.g., in saline-administered patients), CD1a⁻CD14⁻ cDCs express CD83 and costimulatory molecules at lower levels than the CD1a⁺ skin-migratory cDC subsets (19). However, CpG and/or GM-CSF administration enhanced expression of these markers to levels exceeding those of CD1a⁺ cDCs, demonstrating the great potential of this subset for T-cell activation under inflammatory conditions. Indeed, this is in keeping with our own previous findings showing that upon isolation from SLNs, this subset proved to be the most powerful T cell-stimulatory cDC subset, based on proliferation and induction of cytokine release (19). We found the frequencies of CD1a⁻CD14⁻ cDCs to be elevated in patients receiving CpG-B and to even higher levels in patients receiving combined CpG-B/GM-CSF. GM-CSF coadministration also further enhanced the CpG-induced maturation of this subset. Although CpG-B ODNs are generally poor IFNα inducers (11), increased MxA transcript levels in peripheral blood nevertheless demonstrated a systemic impact of the CpG-B-induced type I IFN release. Indeed, the CD14⁻ cDC subset may be the *in vivo* equivalent of IFN-DCs generated *in vitro* in the presence of GM-CSF and IFNα (36). Like IFN-DCs, they have a dendritic morphology (19), are mature (CD83⁺), and display high levels of costimulatory molecules; they express CCR7 (3), lack CD1a, but also express CD123 (19, 37). IFN-DCs express higher levels of MHC class I and associated antigen-processing elements than classical IL4-DCs (38, 39) and were shown to be superior Th1 and CTL inducers with low-level coactivation of Tregs (38–40). Moreover, they were reported to be optimally equipped for high-efficiency CTL cross-priming (41, 42). These phenotypic and functional characteristics strongly favor antitumor immunity.

Although CD1a⁻CD14⁺ cDCs morphologically resemble monocytic or macrophage-like cells, their CD83 expression (rang-

ing from 12% to 95%) suggests a semimature DC phenotype (19). The observed increase in their frequencies upon local CpG-B administration is suggestive of their recruitment from blood-derived monocytes, most likely dependent on CpG-induced type I IFN. Monocytes express extremely low levels of TLR9 transcripts, and their *in vitro* activation by CpG requires the presence of pDCs, a rich source of IFNα (43). IFNα-activated monocytes acquire a CD14⁺CD123⁺CD83⁺ phenotype but maintain a monocytic appearance, reminiscent of the CD14⁺ cDC subset in SLNs (19), and have been shown to stimulate memory T cells (44). This may be relevant to their activity in SLNs, where previously primed antimelanoma T cells are present and "poised" for reactivation (4, 5).

Locally administered CpG and GM-CSF also affected the *in vivo* activation state of DC subsets in peripheral blood. The exact relationship between DC subsets and SLN subsets may not be simply deduced by phenotypic similarities and correlation between frequencies in blood and SLNs, because of their phenotypic plasticity and possible simultaneous recruitment to the periphery and mobilization from the bone marrow. It nevertheless seems plausible that the activated M-DC8⁺ SLAN-cDC subset (significantly reduced in posttreatment blood) was recruited to the site of CpG administration, because this subset has been associated with rapid migration to sites of inflammation (33). Most notably, a significant correlation between decreased BDCA3/CD141⁺ cDC2 rates in peripheral blood and increased CD1a⁻CD14⁻ cDC frequencies in the SLNs of patients receiving combined CpG/GM-CSF, suggests cDC2 to be the precursors of the BDCA3⁺ CD1a⁻CD14⁻ LN-resident cDC subset. Indeed, this was suggested by our *in vitro* observation that cDC2 frequencies and their maturation state both increased significantly upon culture in the presence of CpG combined with GM-CSF. Results from the same experiments also identified monocytes as possible precursors of the BDCA3/CD141⁺ CD14⁺ cDC subset, as they acquired both BDCA3/CD141 and CD80 expression upon 48 hours exposure to CpG ± GM-CSF.

We showed both CD1a⁻ LN-resident cDC subsets to express BDCA3/CD141 (also known as thrombomodulin) and CLEC9A in the steady state (i.e., in untreated SLN samples). Intriguingly, genome-wide transcriptional profiling pointed to BDCA3⁺ DCs as the human equivalent of the CD8α⁺ DC subset in the murine spleen, which is known to be the subset with powerful CTL cross-priming ability (45), an important feature for the generation of antitumor immunity. *In vitro* studies demonstrated the ability of human BDCA3⁺ DCs to cross-prime CTLs and thus confirmed this hypothesis (23, 31, 32). CLEC9A, a C-type Lectin receptor was recently shown to be preferentially expressed on BDCA3⁺ cDCs (32) in the blood and to bind extracellularly exposed actin, thus facilitating cross-priming from necrotic cell-derived proteins (46). We found CLEC9A expressed to varying extent on all cDC subsets in the SLNs but in particularly high levels on the CD14⁺ cDC subset that also expressed relatively high levels of BDCA3. Our finding of selective *in vivo* recruitment of BDCA3⁺ cDC subsets to the SLNs that can also express CLEC9A, a corresponding enhanced *ex vivo* cross-presenting ability of *in vivo* CpG+GM-CSF-exposed SLN cells, and the *in vitro* maturation and induction of BDCA3 expression of both cDC2 and monocytes by CpG-B and GM-CSF, thus attests to the utility of this combination as an adjuvant for protein or long peptide vaccines that require cross-priming. This was confirmed

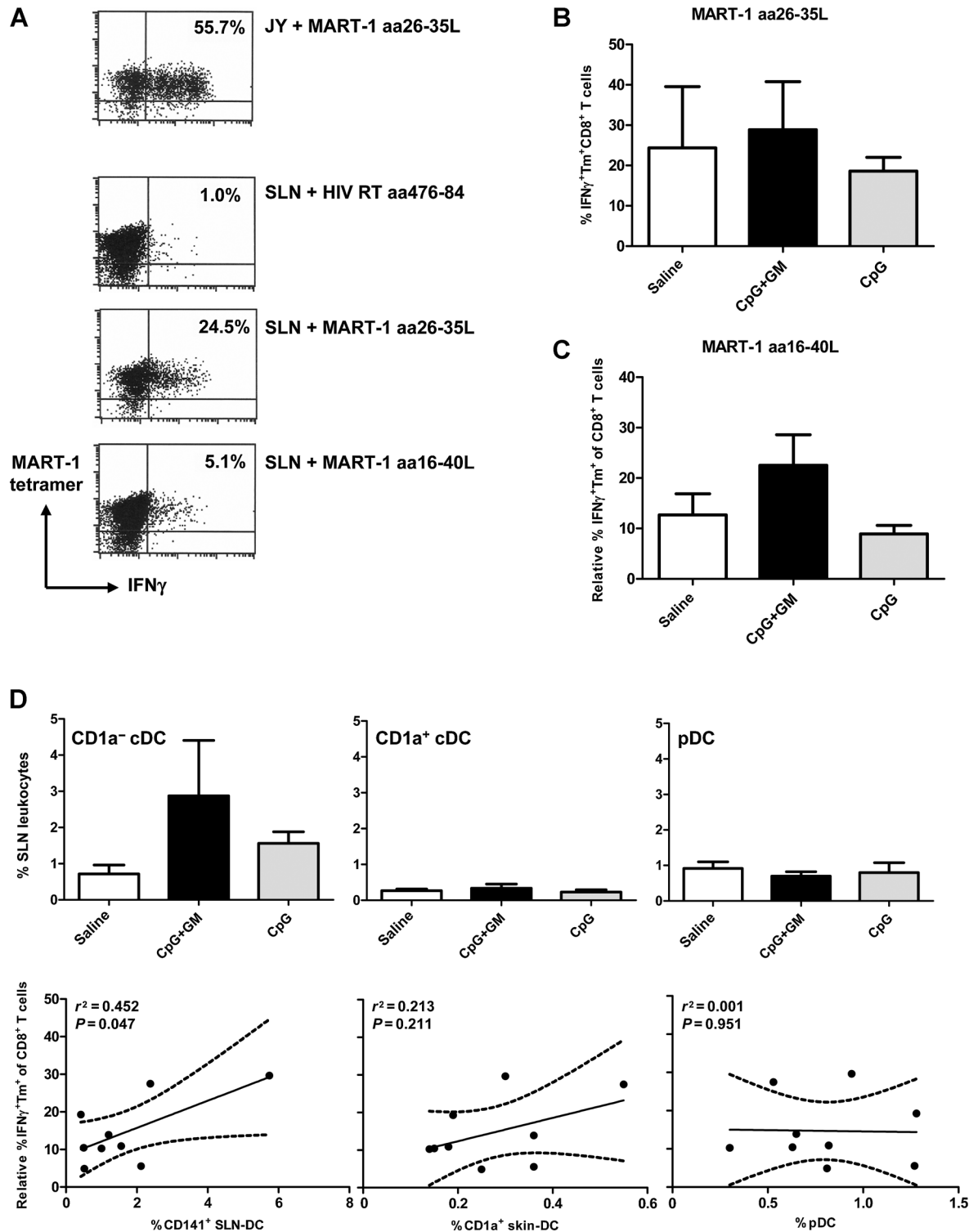


Figure 5. Increased cross-presenting capacity of SLN single-cell suspensions from patients who received local CpG-B and GM-CSF. A, representative plots of intracellular (i.c.) IFN γ production by >90% pure HLA-A2-matched MART-1 aa26-35L-specific CD8⁺ bulk T cells after incubation with respectively (from top to bottom): MART-1 aa26-35L (short peptide)-loaded JY cells, HIV RT aa476-484 (short-peptide)-loaded SLN cells, MART-1 aa26-35L (short peptide)-loaded SLN cells and MART-1 aa16-40L (long peptide)-loaded SLN cells. SLN single-cell suspension was from a CpG+GM-treated patient. B, average (\pm SEM) i.c. IFN γ -positive MART-1 aa26-35L-specific CD8⁺ T cells after incubation with MART-1 aa26-35L (short peptide)-loaded and HLA-A2-matched SLN cells; Tm, tetramer. C, average (\pm SEM) i.c. IFN γ -positive MART-1 aa26-35L-specific CD8⁺ T cells after incubation with MART-1 aa16-40L (long peptide)-loaded SLN cells (relative to i.c. IFN γ expression after incubation with the short peptide). Results shown are from SLN single-cell suspensions derived from HLA-A2⁺ patients from the three indicated groups ($n = 3$ per group). D, percentages of CD1a⁻ skin-resident cDC subsets, CD1a⁺ skin-migrated cDC subsets, and pDCs in each of the three indicated patient groups tested for SLN cross-presenting ability (top; $n = 3$ per group) and correlation between the measured MART-1 aa16-40L cross-presenting efficiencies and frequencies of the respective CD1a⁻ or CD1a⁺ cDC subsets and the pDC subset (bottom). Pearson r^2 and P values are listed and 95% confidentiality intervals indicated by dotted lines.

in vivo, showing superior cross-priming by splenic CD8 α after CpG stimulation, leading to tumor rejection (15, 47); remarkably, GM-CSF synergized with CpG to optimize this effect (47). Recently, CD1a⁺/CD1c⁺ as well as BDCA3/CD141⁺ cDC subsets from LNs and tonsils were shown to have cross-presenting abilities (20, 48). Although the small numbers of viable SLN cells that we obtained from our treated patients prohibited DC subset sorting, we found the *ex vivo* cross-presenting ability of SLN cells of a MART-1–derived synthetic long peptide to correlate with frequencies of CD1a⁺BDCA3/CD141⁺ cDCs rather than of CD1a⁺ skin-derived cDCs or pDCs, indicating that the CD1a⁺CD141⁺/BDCA3⁺ LN-resident cDC subsets in particular may contribute to CTL-mediated immunity against melanoma antigens afforded by combined local administration of CpG-B and GM-CSF.

In conclusion, cross-talk between pDCs and cDCs has been shown to enhance their mutual activation and to benefit the generation of cell-mediated immunity (10, 15, 49, 50). As such, concerted activation and recruitment of pDC and BDCA3/CD141⁺ cDC subsets, achieved by local delivery of combined CpG-B and GM-CSF, may greatly enhance antitumor immunity and support immunotherapy or other conventional therapies that induce the release of (necrotic cell-associated) tumor antigens and thus facilitate cross-priming of antitumor CTLs. The resulting immunity may afford a level of protection against (distant) recurrences. Indeed, preliminary follow-up analyses of patients participating in this study point to prolonged recurrence-free survival of the intervention groups (B.D. Koster and colleagues, manuscript in preparation). If this observation holds up in more extensive analyses and larger follow-up trials, this simple and generally applicable approach of local immune potentiation may provide an effective adjuvant therapy option for patients with early-stage melanoma.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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