Intradermal CpG-B Activates Both Plasmacytoid and Myeloid Dendritic Cells in the Sentinel Lymph Node of Melanoma Patients

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Abstract

Purpose: A decrease in the frequency and activation state of dendritic cells in the sentinel lymph node (SLN) has been observed in early stages of melanoma development. This may hinder the generation of effective antitumor T-cell responses and increase the likelihood of metastatic spread. Immunopotentiation of the melanoma SLN may therefore be a valuable adjuvant treatment option. One way to achieve this is through the use of bacterially derived unmethylated cytosine-phosphate-guanine (CpG) DNA sequences that bind Toll-like receptor 9 and activate plasmacytoid dendritic cells (PDC). CpG-activated PDC, in turn, release IFNα and may thus boost T-cell and natural killer cell responses as well as activate conventional myeloid dendritic cells (MDC).

Experimental Design: We studied the effects of preoperative local administration of the CpG B-type oligodeoxynucleotide (ODN) PF-3512676 (formerly known as CPG 7909) on dendritic cell and T-cell subsets in the SLN of 23 stage I to III melanoma patients, randomized to receive intradermal injections of either PF-3512676 or saline (NaCl 0.9%).

Results: PF-3512676 administration resulted in bulkier SLN, higher yields of isolated SLN leukocytes, and activation of BDCA-2$^+$CD123$^+$ PDC as well as of CD1a$^+$ MDC. In addition, PF-3512676 administration was associated with the presence of a newly identified CD11c^{hi} CD123$^+$CD83$^+$TRAIL$^+$ mature SLN-MDC subset, an increased release of a variety of inflammatory cytokines, and lower frequencies of CD4$^+$CD25^{hi}CTLA-4$^+$FoxP3$^+$ regulatory T cells in the SLN.

Conclusions: These findings point to the possible utility of the conditioning of SLN by PF-3512676 as an adjuvant immunotherapeutic modality for early-stage melanoma.

Early melanoma development is accompanied by impaired immune effector functions in the initial tumor-draining lymph node, the so-called sentinel lymph node (SLN; refs. 1–4). Most notably, the observed reduced maturation state of professional antigen-presenting cells, the dendritic cells, may interfere with the presentation of tumor-associated antigens to specific antitumor CTLs and T-helper cells in the SLN (5). This immune evasion may facilitate the early metastatic events that characterize this tumor type. Immunopotentiation of the SLN microenvironment in early stages of melanoma development may therefore be a valuable treatment option.

We previously described the effects of local granulocyte/macrophage-colony stimulating factor administration on the number and activation state of myeloid dendritic cell (MDC) and melanoma-specific CTL reactivity in the SLN of early-stage melanoma patients, showing the clinical feasibility and immunopotentiating effects of this approach, specifically targeting MDC subsets (6, 7). Plasmacytoid dendritic cells (PDC) constitute another important dendritic cell subset in lymph nodes with potential antigen-presenting and T-cell activating capabilities, which may present an additional immunotherapeutic target.

PDC were first identified as a dendritic cell subset distinct from conventional MDC in peripheral blood and secondary lymphoid organs and were found to be responsible for most of the type I IFN that is produced in response to viral infection (8). PDC have a CD11c$^-$CD123^{hi} phenotype and differentiate from CD34$^+$ precursors under the influence of interleukin (IL)-3 (8). In blood and lymph node, immature PDC are characterized by their expression of the C-type lectin BDCA-2 (9, 10). Maturation induction *in vitro* results in an up-regulation of costimulatory molecules and the maturation marker CD83 and a complete down-regulation of BDCA-2 (10). L-Selectin– and CXC chemokine receptor 3–mediated homing and migration allow PDC to travel from the blood to lymph nodes (8). PDC bind microbial products through specific receptors such as Toll-like receptor-9 (TLR9). TLR9 expression in human immune effector cells seems to be restricted to PDC and B cells (11, 12). Bacterially derived

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unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODN) directly stimulate PDC through TLR9 triggering. TLR- and/or CD40L-activated PDC preferentially release large amounts of IFNα (13–15), which may facilitate direct activation of $CD8^+$ T cells and natural killer cells as well as promote the differentiation and maturation of neighboring MDC (precursors) and, thus, also indirectly stimulate T-cell activation (9, 16–18), all of which are effects with potential antitumor benefits.

In studies of murine tumor models, CpG ODN administration was found to increase the size of vaccine-draining lymph nodes, elevate their type 1 T-cell content, and enhance tumor rejection (17, 19, 20). More recently, peritumoral administration of CpG in cutaneous melanoma was shown to result in a T-cell–mediated reduction of the primary tumor mass and to afford protection against the outgrowth of lung metastases, whereas immunization against a tumor-associated epitope in the presence of CpG-ODN resulted in a strong $CD8^+$ T-cell response and an increased survival (21, 22). The relevance of these findings to humans is disputable due to incongruous TLR9 expression profiles in mouse and man, with TLR9 being expressed on MDC as well as on PDC in the mouse. It is therefore vitally important to study the immune effects of CpG ODN in humans.

The clinically most extensively studied CpG ODN, PF-3512676 (formerly known as CPG 7909), belongs to the B-class of CpG (CpG-B) ODN, which have a strong PDC maturation–inducing capacity but are relatively poor inducers of IFNα release (23). In a recent trial, PF-3512676 was administered in combination with incomplete Freund's adjuvant to vaccinate melanoma patients against a MART-1 peptide and shown to rapidly induce strong and specific $CD8^+$ T-cell responses (24). However, *in vitro* data also point to less desirable effects such as an increased generation of regulatory T cells (T_{reg}) by PF-3512676–activated PDC (25).

Here, we set out to delineate the *in vivo* effects of PF-3512676, intradermally administered at the excision site of the primary tumor, on dendritic cell and T-cell subsets in the melanoma SLN. The observed favorable changes in the activation state of both PDC and MDC subsets, T_{reg} frequencies, and cytokine release profiles are clear indicators of the usefulness of PF-3512676 in an immunomodulatory adjuvant treatment scheme to ultimately minimize the risk of (micro) metastatic spread in early-stage melanoma patients.

Materials and Methods

Patients. In this single-blinded phase II study, 23 patients with clinically stage I/II melanoma, according to criteria of the American Joint Committee on Cancer, who were scheduled to undergo a SLN procedure, were assigned randomly to preoperative local administration of either synthetic CpG-B PF-3512676 (Coley Pharmaceutical Group) or saline (NaCl 0.9%). Pathologic examination revealed six patients with stage III melanoma based on the presence of tumor cells in the SLN (2 of 11 patients in the PF-3512676 administered group and 4 of 12 patients in the saline-administered group). In four of six of these patients ($n = 2$ in the saline group and $n = 2$ in the PF-3512676 group), the metastases were deemed to be of sufficient size to warrant an additional lymph node dissection and all lymph nodes in the additional lymph node dissection were found to be tumor negative. Patients who had undergone previous immunotherapy or chemotherapy were excluded as well as patients receiving immunosuppressive medication or suffering from any autoimmune disorder. The study was approved by the medical ethical committee of the VU University Medical Center and written informed consent was obtained from each patient before treatment. Patient characteristics are listed in Table 1. PF-3512676 injections were tolerated well by all patients with transient and mild flu-like symptoms and induration at the injection site. According to common toxicity criteria (National Cancer Institute Common Toxicity Criteria scale, version 2.0), ~80% of the PF-3512676–administered patients had mild constitutional symptoms (grade 1 fatigue, fever, and rigors) and grade 1 myalgia. Injection site reaction was present in all PF-3512676–administered patients with ~85% grade 1 and 15% grade 2 reactions. No toxicity was observed in the saline-administered patient population.

PF-3512676 administration and triple-technique SLN procedure. Both patient groups received an intradermal injection directly adjacent to the scar of the primary melanoma excision a week before operation with either 8-mg PF-3512676 dissolved in 1.6-mL saline or 1.6-mL plain saline. All patients were treated according to the same protocol and underwent a SLN biopsy as well as a re-excision of the primary melanoma site (26). To identify and retrieve the SLN, the triple technique was used as previously described (27). In short, the day before surgery, patients underwent a dynamic and static lymphoscintigraphy to determine the lymphatic drainage pattern. Just before surgery, Patent Blue V (Laboratoire Guerbet) was intradermally injected next to the excision scar of the primary melanoma. During surgery, guided by a handheld gamma probe and the blue staining of the draining tissues, the SLN was removed and, after isolation of viable SLN cells, examined meticulously by the pathologist (28).

Isolation of viable SLN cells. Immediately after removal, SLN were collected in sterile ice-cold complete medium, composed of Iscove's modified Dulbecco's medium supplemented with 25 mmol/L HEPES

Table 1. Patient and SLN characteristics in the PF-3512676 test and saline control groups

	PF-3512676	Control	*P*
Sex (male/female)	6:5	8:4	0.561*
Age (y)	51 ± 13	55 ± 13	0.424*
Breslow thickness (mm)	1.51 ± 0.88	1.68 ± 1.20	0.758*
Volume SLN (mm^3) †	1,805.3 ± 1,519.6	727.1 ± 492.0	0.052*
Weight SLN (g)	1.1 ± 0.8	0.49 ± 0.2	0.038*
Yield scraping ($\times 10^6$)	36.0 ± 30.6	15.6 ± 6.1	0.023*
Tumor cells in the SLN	2	4	0.640 ‡
Additional lymph node dissection	2	2	1.000 ‡

NOTE: Values in table expressed as *n* or mean ± S.D.
* From Mann-Whitney *U* test.
† Volume: height × width × length.
‡ By Fisher's exact test.

buffer (BioWhittaker) with 10% FCS, 50 IU/mL penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L β-mercaptoethanol. Before routine histopathologic examination of the SLN, viable cells were isolated using a previously described scraping method (29). In short, after measuring the size and weight of the SLN, it was bisected crosswise with a surgical scalpel and the cutting surface of the SLN was scraped 10 times with a surgical blade (size no. 22, Swann Morton Ltd.). SLN cells were rinsed from the blade with medium containing 0.1% DNase I, 0.14% collagenase A (Boehringer), and 5% FCS, then incubated for 45 min at 37°C and subsequently in PBS with 5 mmol/L EDTA for 10 min on ice. Finally, the SLN cells were washed twice in complete medium, counted, and further processed.

Flow cytometry. Freshly isolated SLN cells were directly stained with antibodies labeled with FITC, phycoerythrin, phycoerythrin-Cy5.5, peridinin chlorophyll protein-Cy5.5, or allophycocyanin and analyzed by flow cytometry at 100,000 events per measurement, as previously described (29). Monoclonal antibodies against CD3, CD4, CD8, CD11c, CD14, CD25, CD123, CCR7 (Becton Dickinson), CD1a, CD14, CD40, CD80, CD86, CD123, tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), CTLA 4 (PharMingen), CD11c, CD40, CD83 (Immunotech), BDCA-2 (Miltenyi Biotec) and FabαIgM (Southern Biotechnology) and matching isotype control antibodies were used. Intracellular FoxP3 staining was done using the eBioscience PE antihuman FoxP3 Staining Set following the manufacturer's instructions (eBioscience).

Cytokine profiling. Fresh and viable SLN leukocytes were incubated overnight (1×10^5/100 μL) in 10% FCS–containing complete culture medium. The supernatants were harvested and stored at -20°C and analyzed by BD cytometric bead array (Becton Dickinson) for sensitive fluorescence-activated cell sorting detection of the levels of the inflammatory cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor α (TNFα). Supernatants were also taken from overnight anti-CD3/anti-CD28–stimulated SLN T-cell cultures (before addition of 10 IU/mL IL-2) to check type 1/type 2 cytokine profiles. IL-4, IL-10, and IFNγ were determined by ELISA (Pelikine compact ELISA, Sanquin) following the manufacturer's instructions. IL-5 was captured in a sandwich ELISA by using purified rat anti-human IL-5 monoclonal antibody, which was paired with the biotinylated JES1-5A10 antibody as the detection antibody (both antibodies from Becton Dickinson).

Immunohistochemistry. Paraffin sections were mounted on Superfrost Plus glass slides and dried overnight at 37°C. After deparaffinization, the tissue sections were hydrated through decreasing (v/v) percentages of ethanol and endogenous peroxidase was blocked with 0.1% hydrogen peroxide in methanol. Tissue sections were pretreated with 10 mmol/L citrate (pH 6) in an autoclave for 21 min at 121°C [for CD83 (1:25); Novocastra] or in a microwave at 100°C for 10 min [for CD68 (1:400); DAKO]. The CD83 antibody was applied and incubated at room temperature for 1 h. Detection and visualization were done with the DAKO Chemmate Envision detection kit (DAKO) according to the manufacturer's instructions. For the CD68 antibody, an automated immunostainer (Ventana) was used for all incubation, detection, and visualization steps according to the manufacturer's instructions. Sections were counterstained with hematoxylin, dehydrated, and mounted.

Statistical analysis. Differences in patient characteristics, SLN characteristics, and measured immune parameters between patient study groups were analyzed using the two-sample Mann-Whitney *U* test and considered significant when $P < 0.05$.

Results

Clinical observations. Twenty-three patients with clinical stage I/II melanoma, who were scheduled to undergo a SLN procedure, were assigned randomly to preoperative local administration of either recombinant human PF-3512676 or saline. No significant differences in patient characteristics were observed between the two study groups (Table 1). The PF-3512676 injections were well tolerated by all patients with transient and mild flu-like symptoms and mild to moderate fevers between 8 and 24 h after PF-3512676 administration, all easily controlled by paracetamol administration. Induration at the injection site remained palpable for 3 to 5 days after injection. Metastatic melanoma cells were detected in the SLN of 2 of 11 patients in the PF-3512676–administered group and in 4 of 12 patients in the saline-administered group. On average, SLN from PF-3512676–administered patients were larger in volume ($P = 0.052$) and weighed more ($P = 0.038$) than SLN from patients receiving saline injections (Fig. 1A and B; Table 1). In addition, significantly higher SLN cell yields were obtained after scraping the cutting surface of the SLN from PF-3512676–administered patients, as compared with saline-administered patients ($P = 0.023$). Histologic architecture of the SLN was conserved on conditioning by CpG without an obvious increase in the number of follicles (Fig. 1C and D). This was also supported by the finding of similar T-cell (70-80%) and B-cell (20-30%) frequencies among the harvested SLN lymphocytes in both patient groups (data not shown). In addition, a normal distribution was observed for $CD68^+$ macrophages [preferentially in the (marginal) sinuses; Fig. 1C and D] and for $CD83^+$ dendritic cells (in the paracortical T cells areas; Fig. 1E and F).

Activation of both PDC and MDC on PF-3512676 administration. SLN-derived PDC (SLN-PDC; defined as $CD123^{hi}$ and $BDCA\text{-}2^+$) were analyzed by flow cytometry for the expression of dendritic cell maturation (CD83) and activation markers (CD86 and CD40; Fig. 2A). Although expression of BDCA-2 and CD83 on *in vitro* mature PDC was previously reported to be mutually exclusive (10), our analyses clearly showed *de novo* expression of the maturation marker CD83 on the otherwise immature $BDCA\text{-}2^+$ SLN-PDC to be associated with intradermal administration of PF-3512676 ($P = 0.007$). PF-3512676–induced maturation of the SLN-PDC was also evidenced by significantly increased expression levels of the costimulatory markers CD86 ($P = 0.006$) and CD40 ($P < 0.001$; Fig. 2B). Furthermore, a moderate but not significant increase in the mean frequency of SLN-PDC was observed in the PF-3512676–administered group (0.48 ± 0.30%) as compared with the saline-administered group (0.34 ± 0.25%).

SLN-MDC (most likely skin derived and identified by expression of the Langerhans cell–associated marker CD1a, as previously described; ref. 6; Fig. 2C) were similarly analyzed for the expression of CD83, CD86, and CD40 (Fig. 2D); PF-3512676 administration was associated with significantly higher expression levels of CD83 ($P = 0.036$) and CD86 ($P < 0.001$) but did not result in increased $CD1a^+$ SLN-MDC frequencies (overall mean, 0.32 ± 0.29%).

The expression of activation markers on both the PDC and $CD1a^+$ MDC was also compared between the SLN tumor–positive and SLN tumor–negative patients within both treatment groups, but no significant differences were found (data not shown). This may be due to the low numbers of SLN-positive patients that were enrolled in this study ($n = 2$ in the saline control group and $n = 4$ in the PF-3512676 test group).

Identification of a PF-3512676–induced $CD11c^+CD83^+$-$CD1a^-$ mature and TRAIL-expressing MDC subset. Evidence for a novel PF-3512676–induced $CD1a^-$ SLN-MDC subset emerged when, after subtraction of the measured $CD1a^+$ MDC

and $CD14^+$ monocyte/macrophage frequencies, we found a remaining population of $CD11c^{hi}CD123^+$ myeloid cells (phenotypically distinct from $CD11c^-CD123^{hi}$ PDC) in the SLN of the PF-3512676–administered patients (at a mean frequency of 1.23 ± 1.34%) but not of the saline-administered control group (-0.14 ± 0.35%; $P < 0.001$).

From 14 patients (4 saline control and 10 PF-3512676 administered), frozen stored samples of the original SLN single-cell suspensions were available to establish the actual percentage of $CD11c^{hi}CD123^+CD1a^-CD14^-$ cells through four-parameter flow cytometry (Fig. 3A). We did find detectable numbers of these cells in the saline-administered patient population but at far lower frequencies (Fig. 3B; $P = 0.013$) and seemingly expressing lower levels of dendritic cell activation markers (Fig. 3C) as compared with the PF-3512676–administered patient population. In forward scatter analyses, these $CD11c^{hi}CD123^+CD1a^-CD14^-$ cells were found to be of intermediate size between monocytes and $CD1a^+$ MDC and, in terms of side scatter levels, showed a granular/dendritic morphology (Fig. 3C). Phenotypic analysis of this novel MDC population, gated as $CD11c^{hi}CD1a^-CD14^-$, showed it to be DC-SIGN negative but positive for CD83, CD40, CD86, CD80, and CCR7 (all indicative of a mature dendritic cell phenotype), as well as for TRAIL and CD123 (Fig. 3C). Of note, this phenotype is compatible with previously described *in vitro* IFNα-induced monocyte-derived dendritic cells (30, 31).

PF-3512676–induced inflammatory cytokine release. BD cytometric bead array fluorescence-activated cell sorting analysis for the detection of cytokines was done with supernatants from 24-h cultures of the total SLN leukocyte population. Significantly increased levels of IL-6, IL-8, IL-10, and TNFα were found in the supernatants of the PF-3512676–administered patient population as compared with the supernatants from the saline-administered patient population, whereas there was no significant difference in the concentration of IL-1β and IL-12p70 between the two patient groups (Table 2A). IFNα was below detection level by ELISA in both PF-3512676– and saline-administered patients (data not shown). This was not unexpected because any PF-3512676–induced IFNα release by PDC that might be expected is an acute/immediate event whereas both PF-3512676 and saline were administered 1 week before the SLN procedure and subsequent supernatant collection.

T-cell cytokine skewing and T_{reg} frequencies. Because of the significant increase in the release of IL-10 (but not of IL-12p70) by SLN leukocytes from the PF-3512676–administered patients, we set out to determine if this translated into skewing toward a more suppressive T-cell profile, either by increased type 2 cytokine release or by elevated T_{reg} frequencies.

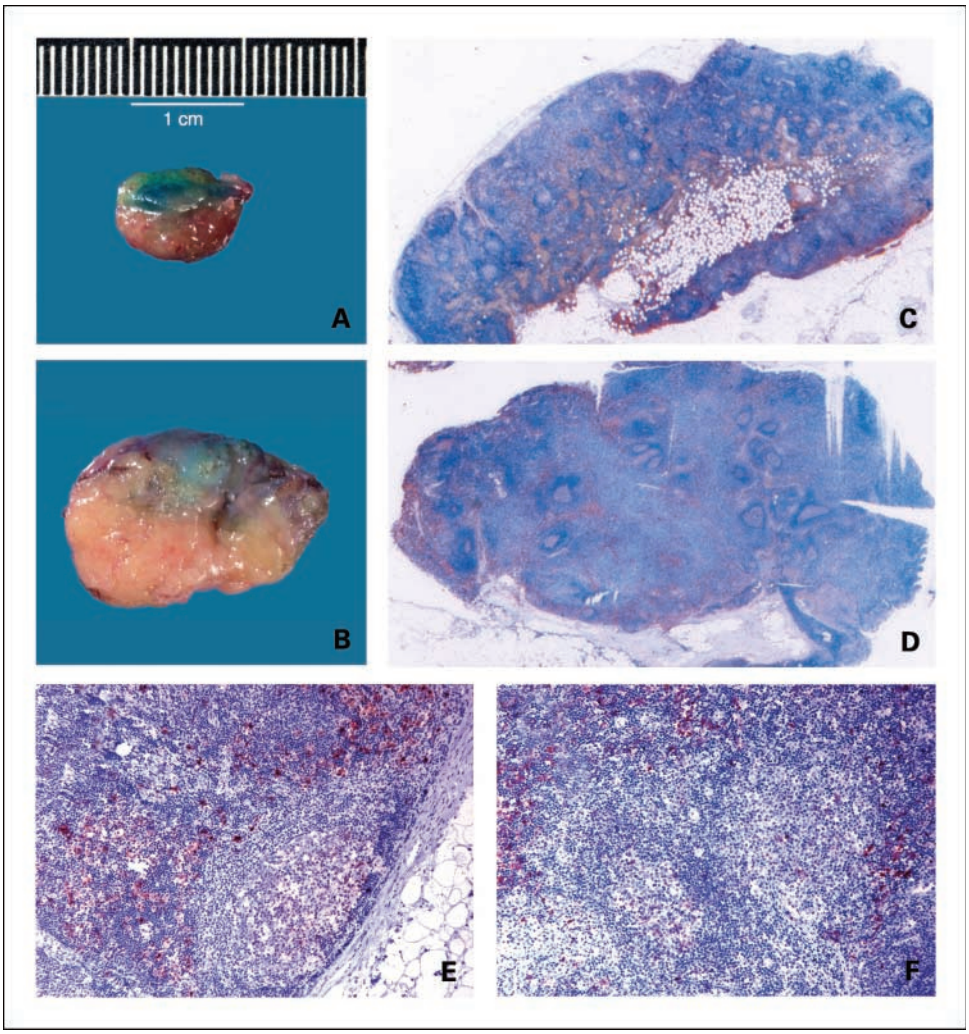

Fig. 1. SLN from PF-3512676 administered patients were increased in volume and weight as compared with SLN of saline-administered patients but maintained normal histology. Representative SLN are shown of a saline-administered patient (*A*) and of a PF-3512676–administered patient (*B*). Histologic examination shows normal architecture on saline (*C*) and PF-3512676 (*D*) administration; follicles are clearly discernable and sinuses are distinguishable by the red-brown CD68 staining (fragments of representative SLN are shown at a 6-fold magnification). Similar distribution of $CD83^+$ dendritic cells in the paracortical areas was observed on saline (*E*) or PF-3512676 (*F*) administration (magnification, ×100).

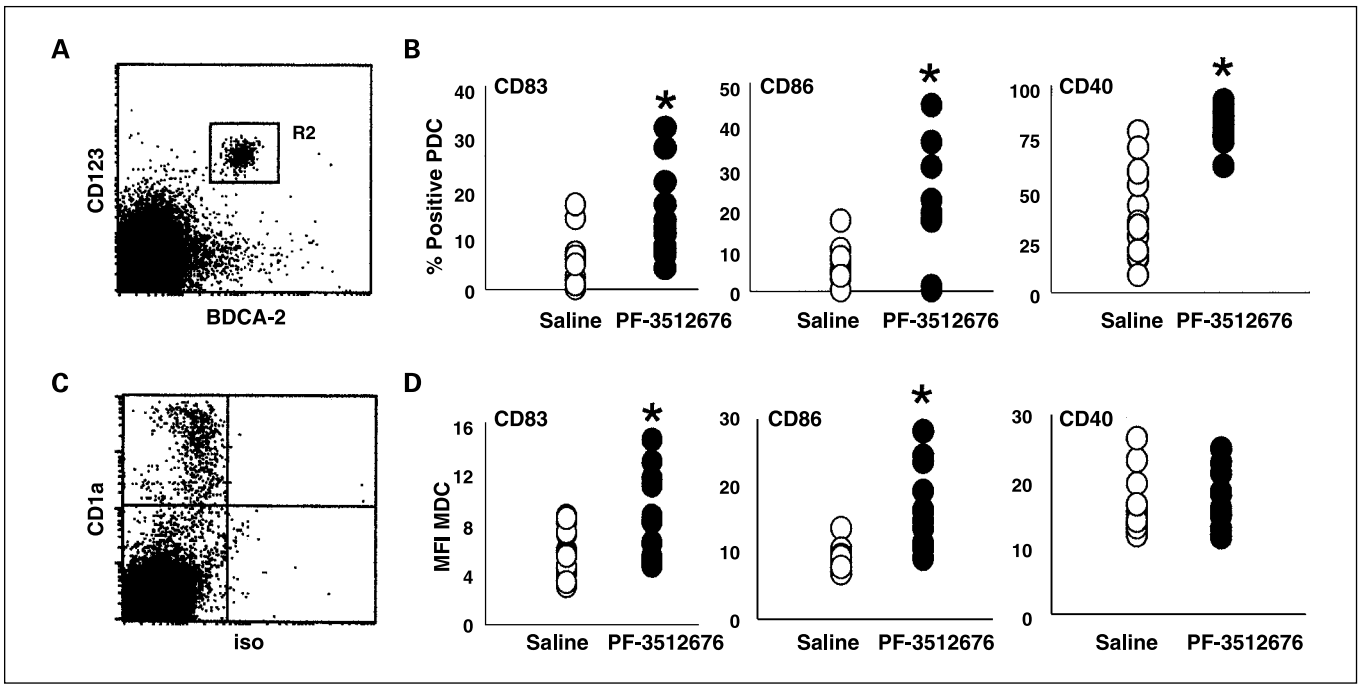

Fig. 2. Administration of PF-3512676 leads to the activation of SLN PDC and MDC. *A,* PDC were defined and gated as $CD123^{hi}$ and $BDCA\text{-}2^{+}$. *B,* flow cytometric analyses revealed significantly higher expression (indicated as percentage positive cells) of the maturation marker CD83 ($P = 0.007$) and the costimulatory markers CD86 ($P = 0.006$) and CD40 ($P < 0.001$) on the SLN-PDC of PF-3512676–administered patients (●) as compared with saline-administered patients (○). *C,* SLN-MDC were defined and gated by their CD1a expression (*left top quadrant*). *D,* flow cytometric analyses revealed significantly higher expression levels [in mean fluorescence indices (*MFI*)] of the maturation marker CD83 ($P = 0.036$) and the costimulatory marker CD86 ($P < 0.001$) in the PF-3512676–administered patient population (●) as compared with the saline-administered patient population (○).

In the supernatants from overnight anti-CD3/anti-CD28–stimulated fresh SLN T-cell cultures, increased concentrations of IFNγ, accompanied by decreased IL-4 and IL-5 levels, were detectable in the PF-3512676–administered patient population as compared with the saline-administered patient population, whereas IL-10 levels did not differ between the two groups (Table 2B). Resulting IFNγ/IL-4 and IFNγ/IL-5 ratios, indicative of type 1/type 2 immune skewing, were higher for the PF-3512676–administered patient population (significantly so in the case of the IFNγ/IL-5 ratio; $P = 0.002$; Table 2B). Thus, despite a considerable increase in IL-10 release by the total SLN leukocyte population, a slight bias toward a type 1 T-cell response rather than a type 2 response was found to be associated with PF-3512676 administration.

From frozen stored SLN samples of 14 patients (4 saline control and 10 PF-3512676–administered), T_{reg} frequencies (defined as $CD3^{+}CD4^{+}CD25^{hi}$, and found to coexpress high levels of both CTLA4 and FoxP3; Fig. 4A) were determined. Significantly lower SLN T_{reg} frequencies were found in the PF-3512676–administered patients ($P = 0.004$; Fig. 4B), again arguing against a PF-3512676/IL-10–induced immunosuppressive microenvironment.

Discussion

The first lymph node to directly drain the primary tumor, the SLN, is the preferential site of early metastasis and shows the most pronounced suppression of dendritic cell phenotype and numbers, as early as stage I of melanoma development (2–4). Dendritic cells represent a heterogeneous population of bone marrow–derived cells that are both powerful initiators and modulators of immune responses (32). Besides the conventional MDC, lymph nodes contain $CD11c^{-}$ PDC. The primary task of PDC seems to be the production of large amounts of type 1 IFN, most notably IFNα, on activation by specific TLR and/or CD40L, to stimulate components of both the innate (e.g., natural killer cells, MDC) and the adaptive immune system (e.g., CTL; refs. 9, 16–18). However, evidence is accumulating that tumor-associated PDC may induce immunosuppression rather than activation. Tumor-associated PDC have been shown to prime immunosuppressive IL-10–producing T cells (33). Moreover, PDC infiltrating head and neck squamous cell carcinomas show low TLR9 expression and a diminished capacity for IFNα production (34). It may well be that activation of these PDC by appropriate CpG ODN will abrogate their suppressive traits and lead to enhanced antitumor CTL activation. Target cells for CpG ODN in the human immune system are restricted to TLR9-expressing B cells and PDC. However, indirect bystander activation of other immune effector cells may be expected through the induction of local cytokine release. To show the clinical applicability of the CpG-B ODN PF-3512676 in immunotherapeutic strategies aimed at the immunopotentiation of the melanoma SLN, it is therefore important to study the effects of PF-3512676 in a complex *in vivo* setting with all possible interplays between the different SLN cell populations in place.

In this randomized clinical trial, intradermally injected PF-3512676 has been shown to exert *in vivo* immunostimulatory effects on both PDC and MDC subsets in the SLN of early-stage melanoma patients. A significant *de novo* expression of the

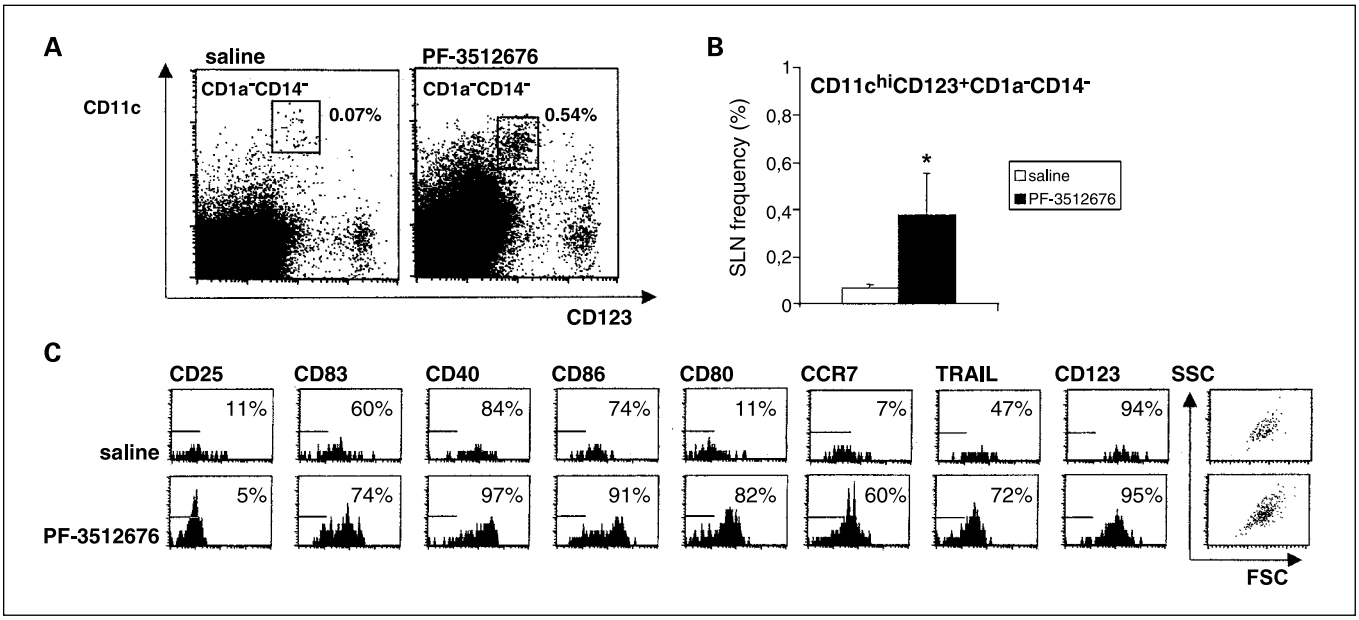

Fig. 3. Identification of a novel PF-3512676–induced CD1a$^-$TRAIL$^+$ MDC subset in the SLN of melanoma patients. *A,* using four-parameter flow cytometry, we were able to gate and quantify the novel MDC subset as CD11c^{hi}CD123$^+$CD1a$^-$CD14$^-$ cells, shown for a PF-3512676–administered patient and a saline-administered patient. *B,* in PF-3512676–administered patients (n = 10), there was a significantly higher frequency of CD11c^{hi}CD123$^+$CD1a$^-$CD14$^-$ SLN-MDC as compared with the saline-administered control group (n = 4, P = 0.013). *C,* phenotypic analysis of the PF-3512676–induced SLN-MDC subset, gated on its CD11c^{hi}CD1a$^-$CD14$^-$ phenotype, showed it to be CD25 negative but positive for CD83, CD40, CD86, CD80, CCR7, TRAIL, and CD123 (shown for a saline- and PF-3512676–administered patient). Forward (*FSC*) and side scatter (*SSC*) plots are also shown for the gated MDC.

dendritic cell maturation marker CD83 and of the costimulatory marker CD86, as well as a significant up-regulation of CD40 levels, was induced on PDC on PF-3512676 administration. In contrast to Dzionek et al. (10), we did not find maturation of PDC to result in BDCA-2 down-regulation, as we did not find any evidence of a CD11c$^-$BDCA-2$^-$CD123^{hi}CD83$^+$ cell population. However, our study design was not entirely comparable with theirs: Dzionek et al. studied human blood PDC after IL-3–mediated maturation in culture (10), whereas we studied human SLN-PDC after PF-3512676–mediated maturation *in vivo*. Probably due to CpG-induced PDC-derived cytokines (IFNα being the most obvious candidate), we also found maturation of neighboring MDC in the PF-3512676–administered patient population, revealed by significant up-regulation of CD83 and CD86 on CD1a$^+$ MDC (17, 18). Unlike the observed PDC activation, this finding is more remarkable because PF-3512676, a CpG-B ODN, is a more powerful inducer of phenotypic PDC activation than of IFNα release. Nevertheless, the *in vivo* induced cytokine release, either directly by PDC or in second instance by other immune effector cells, seems to be sufficient for subsequent MDC activation.

IFNα released by CpG-activated PDC is also most likely responsible for the recruitment of the CD1a$^-$CD83$^+$TRAIL$^+$

Table 2. PF-3512676–induced cytokine release measured by cytometric bead array in supernatants from 24 h-cultures of the total SLN leukocyte population (A) and supernatants from overnight anti-CD3/anti-CD28–stimulated SLN T-cell cultures (B)

(A) Total SLN leukocyte population

	IL-1β	IL-6	IL-8	IL-10	IL-12p70	TNFα
Saline*	41 ± 33	49 ± 28	908 ± 398	9 ± 4	8 ± 6	12 ± 5
PF-3512676 †	47 ± 31	172 ± 103	1,988 ± 1,105	22 ± 16	9 ± 4	19 ± 6
P ‡	0.602	0.002	0.022	0.001	0.310	0.009

(B) SLN T cells

	IFNγ	IL-4	IL-5	IL-10	IFNγ/IL-4	IFNγ/IL-5
Saline*	1,682 ± 972	61 ± 82	84 ± 81	5,528 ± 5,169	45 ± 29	33 ± 41
PF-3512676 †	2,597 ± 1,983	44 ± 40	47 ± 47	5,817 ± 5,171	74 ± 43	111 ± 119
P ‡	0.284	0.884	0.191	0.839	0.099	0.002

* Mean ± SD (pg/mL) in the saline-administered patient population.
† Mean ± SD (pg/mL) in the PF-3512676–administered patient population.
‡ By Mann-Whitney U test.

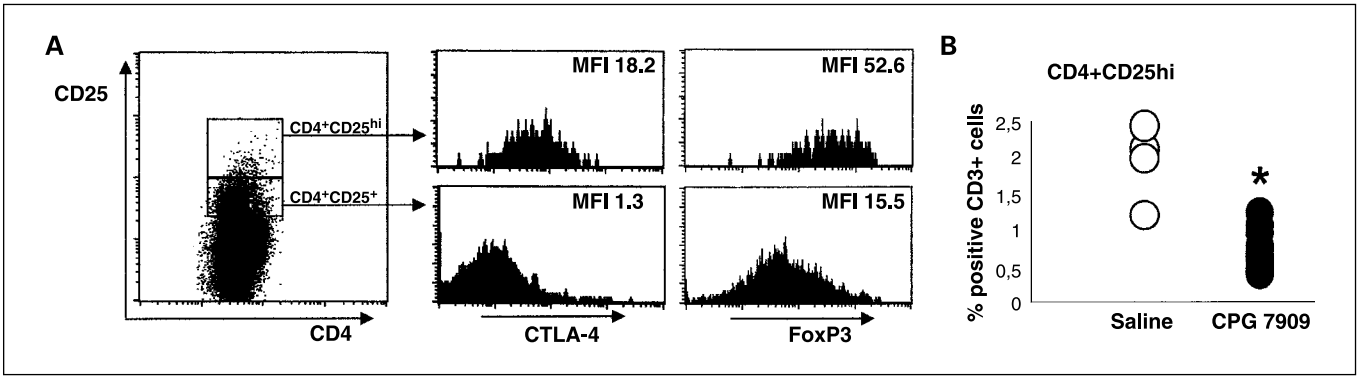

Fig. 4. T_{reg} frequencies in the SLN of PF-3512676–administered patients ($n = 10$) compared with saline-administered patients ($n = 4$). *A,* T_{reg} were defined and gated as $CD3^+CD4^+CD25^{hi}$ (the events shown in the dot plot were pregated on CD3 positivity) and express characteristically high levels of intracellular CTLA4 and FoxP3, as compared with levels of these markers on $CD4^+CD25^+$ non-T_{reg} cells, also shown. Mean fluorescence indices, determined by dividing the mean fluorescence intensity by that of the corresponding isotype control, are listed in the histograms. *B,* T_{reg} frequencies (expressed as percentage of the total $CD3^+CD4^+$ T-cell population) were significantly decreased in the PF-3512676–administered patients as compared with the saline-administered patients ($P = 0.004$).

MDC found in the SLN of PF-3512676–administered patients. This previously unidentified *in vivo* MDC subset bears a strong phenotypic resemblance to *in vitro* IFNα-induced monocyte-derived dendritic cells. Parlato et al. (30) found blood-derived monocytes, when cultured with IFNα for 3 days, to exhibit a wide spectrum of phenotypic and functional features typical of mature dendritic cells. They described IFN-induced monocyte-derived dendritic cells as negative for CD14 and positive for CD80, CD83, CD86, CD123, and CCR7 (30). Santini et al. (31) found IFN-induced monocyte-derived dendritic cells to also express TRAIL on their membranes. In keeping with this reported phenotype, our population of PF-3512676–induced MDC was positive for CD11c, CD80, CD83, CD86, CD123, and CCR7, as well as for TRAIL. These phenotypic similarities strongly suggest that the PF-3512676–associated dendritic cell subset identified by us actually represents IFNα-induced monocyte-derived dendritic cells. In support of this, we found the PF-3512676 ODN used in our study to induce TRAIL expression on blood-derived monocytes *in vitro* (data not shown), most likely mediated through PDC-derived IFNα as previously reported by Kemp et al. (35). The origin of the monocytes that might have differentiated into the novel $TRAIL^+$ MDC subset in the SLN remains unknown. PF-3512676 was administered locally but at high doses so that any systemic effects cannot be excluded. Monocytes in the skin as well as in peripheral blood or in the SLN may have differentiated into the observed $TRAIL^+$ MDC under the influence of IFNα produced by PDC at all three locations. Indeed, the induction of functional TRAIL expression seems to be generally associated with CpG stimulation and has been reported for PDC and B cells as well as for monocytes (35–37). Functional testing of the PF-3512676–induced $CD1a^-TRAIL^+$ MDC was not possible due to their low frequency and the overall low cell yields from the SLN scrapes. However, the observed CCR7 expression indicates an ability to migrate to the paracortical areas of the SLN, and TRAIL-expressing monocytes and dendritic cells have previously been shown able to kill TRAIL-sensitive tumor cells (35, 38, 39). These phenotypic traits thus suggest an ability of this novel inducible MDC subset to (*a*) home to the paracortical T-cell areas of the SLN, possibly to activate (tumor-) specific T cells (in keeping with their mature T cell-stimulatory phenotype), and (*b*) to directly lyse sensitive tumor cells in a TRAIL-dependent fashion. Both these characteristics remain to be confirmed in functional read-outs.

PDC release IFNα within 12 h of *in vitro* CpG-mediated activation (40). It is therefore not surprising that we were unable to detect any spontaneous IFNα release in 24 h supernatants of freshly isolated SLN cells that were collected 7 days after the *in vivo* administration of PF-3512676 (we opted for this supposed optimal time point for the monitoring of CpG-associated effects at the T-cell level, based on previous murine experiments; ref. 20). In addition, as previously mentioned, CpG-B ODN like PF-3512676 are relatively poor IFNα release inducers, in contrast to CpG-A and CpG-C ODN. However, in the same supernatants from PF-3512676–administered patients, we did detect significantly increased levels of IL-6, IL-8, IL-10, and TNFα. Of these, IL-6, IL-8, and TNFα were previously reported to be produced by (CpG-) activated PDC (41). Both IL-6 and TNFα may have contributed to the observed MDC maturation induction, alongside any IFNα-mediated effects in the PF-3512676–administered patient population. Although *in vitro* CpG-induced IL-10 production by PDC was not reported, the elevated IL-10 release detected by us may have resulted either from CpG-stimulated B cells (40, 41) or from secondarily activated immune effector cells *in vivo*, possibly providing a negative feedback loop for the proinflammatory effects of the administered CpG ODN (42). There was no difference in IL-1β and IL-12p70 concentrations between the two patient groups, which, in the case of IL-12p70, is in keeping with previous reports of a lack of IL-12 production on CpG-mediated activation of human PDC *in vitro* (15). Despite this lack of detectable IL-12p70, we found no indication of any type 2 cytokine skewing in *ex vivo* stimulated SLN T cells, but rather of the opposite, which is in keeping with findings in murine models (20).

Finally, we studied the influence of a PF-3512676–conditioned SLN environment on T_{reg} frequencies. Whether human $CD4^+CD25^{hi}$ T_{reg} (also strongly positive for CTLA-4 and the T_{reg}-associated transcription factor FoxP3; see Fig. 4A) are a separate lineage of cells or arise from a highly specialized process of differentiation remains to be determined, but elevated numbers have been found in several human cancers including lung, breast, and ovarian tumors (43–45), as well as

in human metastatic melanoma lymph nodes (46), and are predictive for a poor prognosis (47). T_{reg} may directly inhibit T-cell priming, inducing tolerance under tumor conditions in a contact-dependent way, but may also indirectly inhibit T-cell priming by influencing dendritic cell differentiation and maturation. T_{reg}-mediated immune suppression may be overcome by decreasing the number of T_{reg} or by blocking/reversing their influence on dendritic cells and/or T cells. In the PF-3512676–administered patients, a significant decrease in $CD4^+CD25^{hi}$ T_{reg} frequencies was found as compared with the saline control group. Both immature PDC and immature or IL-10–modulated MDC are known to induce T_{reg} (48, 49). In view of this, the reduced T_{reg} frequencies observed in the SLN of the PF-3512676–administered patients may have resulted either directly from the induced maturation of PDC or indirectly from MDC maturation induction. However, the latter option seems to be more likely in view of a report by Moseman et al. (25) who showed that CpG-activated PDC actually induce $CD4^+CD25^+$ T_{reg} when cultured *in vitro* with naïve $CD4^+CD25^-$ T cells. Thus, indirect MDC-mediated effects may account for the opposite effect of CpG on the T_{reg} frequencies that we observed *in vivo*.

In conclusion, PF-3512676 certainly seems to be promising as an adjuvant immunotherapeutic modality for early-stage melanoma based on the following observations in the SLN: (*a*) increased PDC and MDC activation status; (*b*) induction of a novel $TRAIL^+$ MDC subset with a mature T-cell stimulatory phenotype; (*c*) an increased proinflammatory and type 1 cytokine profile; and (*d*) reduction in immunosuppressive T_{reg} frequencies. However, final judgment should be reserved, pending the outcome of investigations that are ongoing to ascertain if these PF-3512676–induced immunostimulatory effects on dendritic cells and T_{reg} in the SLN translate into higher frequencies of melanoma-specific $CD8^+$ effector T cells, which would further confirm the clinical usefulness of CpG-ODN in the immunotherapy of melanoma. Altogether, these immune effects should then contribute to an increased protection against early metastatic spread.

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