Local Administration of PF-3512676 CpG-B Instigates Tumor-Specific CD8+ T-Cell Reactivity in Melanoma Patients

Barbara G. Molenkamp,1 Berbel J.R. Sluijter,1 Paul A.M. van Leeuwen,1 Saskia J.A.M. Santegoets,2 Sybren Meijer,1 Pepijn G.J.T.B. Wijnands,3 John B.A.G. Haanen,4 Alfons J.M. van den Eertwegh,2 Rik J. Scheper,3 and Tanja D. de Gruijl2

Abstract

Purpose: Impaired immune effector functions in the melanoma sentinel lymph node (SLN) may allow for early metastatic events. Local administration of PF-3512676 (formerly known as CpG 7909) has shown immunostimulatory effects of both dendritic cell and T-cell subsets in the melanoma SLN. Here, we set out to ascertain whether these PF-3512676-induced immunostimulatory effects translate into higher frequencies of melanoma-specific CD8+ Tcells.

Experimental Design: Twenty-four stage I to III melanoma patients were randomized to preoperative local administration of either PF-3512676 or saline. CD8+ T cells from SLN and peripheral blood were tested for reactivity by IFN-γ ELISPOT assay against several HLA-A1/A2/A3-restricted epitopes derived from various melanoma-associated antigens (MAA) in 21 of 24 enrolled patients. Frequencies of natural killer (NK) cells and frequencies and maturation state of dendritic cell subsets in the SLN were determined by flow cytometry.

Results: Melanoma-specific CD8+ T-cell response rates against >1 MAA epitope in the SLN were 0 of 11 for the saline group versus 5 of 10 for the PF-3512676-administered group (P = 0.012). Of these 5 responding patients, 4 also had a measurable response to >1 MAA epitope in the blood. Increased frequencies in the SLN of both MAA-specific CD8+ T cells and NK cells correlated to CpG-induced plasmacytoid dendritic cell maturation.

Conclusions: These data show an increase in melanoma-specific CD8+ T-cell frequencies as well as an increased effector NK cell rate after a single dose of PF-3512676 and thus support the utility of local PF-3512676 administration as adjuvant treatment in early-stage melanoma to try and halt metastatic spread.
precursors and thus also indirectly stimulate T-cell activation (15–19).

NK cells are powerful innate effectors of the immune system with an ability to limit tumor burden before the onset of adaptive T-cell immunity (20). They are defined by the expression of CD56 and different NK cell subgroups can be distinguished by the surface density of CD56. The CD56lo subset has been shown to express perforin as well as the killer cell immunoglobulin-like receptors, whereas the CD56hi subset does not carry perforin and killer cell immunoglobulin-like receptors but seems to exhibit immunoregulatory functions through the secretion of various cytokines [IFN-γ, tumor necrosis factor-α, or interleukin (IL)-10; refs. 20, 21].

We recently reported on the immunostimulatory effects of intradermal (i.d.) injections of the CpG-B oligodeoxynucleotide PF-3512676 (formerly known as CpG 7909) around the excision site of stage I to III melanoma tumors, resulting in increased PDC and MDC activation, increased proinflammatory type 1 T-cell cytokine profile, and reduction in immunosuppressive regulatory T-cell (Treg) frequencies (22). We hypothesized that these combined PF-3512676-induced immunostimulatory effects would translate into higher frequencies of melanoma-specific CD8+ T cells. Indeed, this hypothesis is supported by findings presented here, showing increased local and systemic CD8+ T-cell responsiveness to melanoma associated epitopes, together with an increased CD56lo effector NK cell rate, on i.d. administration of PF-3512676.

Materials and Methods

Patients and PF-3512676 administration. In this single-blinded phase II study, 24 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 8 mg PF-3512676 (Coley Pharmaceutical Group) dissolved in 1.6 mL saline or 1.6 mL plain saline (0.9% NaCl). i.d. injections were given directly adjacent to the scar of the primary melanoma excision 1 week before surgery. 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. As reported previously, PF-3512676 injections were tolerated well by all patients with transient and mild flu-like symptoms and induration at the injection site (22).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>PF-3512676</th>
<th>Saline</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>6/5</td>
<td>9/4</td>
<td>0.469*</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>51 ± 13</td>
<td>55 ± 15</td>
<td>0.622*</td>
</tr>
<tr>
<td>Breslow thickness (mm; mean ± SD)</td>
<td>1.51 ± 0.88</td>
<td>1.92 ± 1.44</td>
<td>0.534*</td>
</tr>
<tr>
<td>Tumor cells in the SLN</td>
<td>2/11</td>
<td>4/13</td>
<td>0.649†</td>
</tr>
<tr>
<td>Additional lymph node dissection</td>
<td>2/11</td>
<td>2/13</td>
<td>1.000†</td>
</tr>
<tr>
<td>HLA-A1</td>
<td>2/11</td>
<td>5/13</td>
<td>0.386†</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>6/11</td>
<td>6/13</td>
<td>1.000†</td>
</tr>
<tr>
<td>HLA-A3</td>
<td>4/11</td>
<td>3/13</td>
<td>0.659†</td>
</tr>
<tr>
<td>Time from primary excision to SLN procedure (d; mean ± SD)</td>
<td>52 ± 16</td>
<td>45 ± 18</td>
<td>0.338*</td>
</tr>
</tbody>
</table>

*Two-sample Mann-Whitney U test.
†Two-tailed Fisher’s exact test.

Triple-technique SLN procedure and isolation of viable SLN cells. To identify and retrieve the SLN, the triple technique was used as described previously (23–25). 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 injected i.d. next to the original site of the primary melanoma. During surgery, guided by a hand-held gamma probe and the blue staining of the draining tissues, the SLN was removed. Immediately after removal, SLN were collected in sterile ice-cold complete medium, comprising IMDM supplemented with 25 mmol/L HEPES buffer (BioWhittaker) with 10% FCS (HyClone), 50 U/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 (26). 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 (Swann Morton, size no. 22). SLN cells were rinsed from the blade with medium supplemented with 0.2 mg/mL DNase I, 1 mg/mL collagenase A (Boehringer), and 5% FCS and incubated for 45 min at 37°C. Finally, the SLN cells were washed twice in complete medium, counted, and further processed. After isolation of viable SLN cells, the SLN was handed over and examined meticulously by the pathologist (27).

Isolation of peripheral blood mononuclear cells and flow cytometry. From each patient, blood (40-50 mL) was taken before, 1 week after, and 3 weeks after PF-3512676/saline administration. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Nycomed Pharma). Cells were washed twice with sterile PBS with 0.1% bovine serum albumin after which they were counted, frozen, and stored in liquid nitrogen for functional analysis at a later date. To determine the patients’ HLA restriction for the planned functional CD8+ T-cell studies, freshly isolated SLN cells were directly stained with monoclonal antibodies (mAb) 8.1L.104 (HLA-A1; US Biological), B7.2, MA 2.1 (HLA-A2), and GAP A3 (HLA-A3: American Tissue Culture Collection). Freshly isolated SLN cells were also directly stained with mAbs against CD3, CD4, CD8, CD25, CD56, CD69, CD123, HLA-DR (BD), CD1a, CD40, CD86, CD123, CTLA-4 (PharMingen), CD40, CD83 (Immunootech), BDCA-2 (Miltenyi Biotec), and matching isotype control antibodies, labeled with either FITC, PE, PE-CY5-5, PerCP-CY5-5, or APC, and analyzed by flow cytometry at 100,000 events per measurement as described previously (26).

T-cell expansion. To obtain sufficient numbers of T cells from the SLN for functional analysis, T cells from all SLN were expanded as described previously (26). Briefly, cells were incubated for 1 h on ice with 2 μg anti-CD3 and 0.4 μg anti-CD28 per 1 × 10⁶ cells (kindly
provided by Dr. René van Lier, CLB) in 100 to 200 μL complete medium with 5% FCS. After incubation and washing, cells were placed on 24-well plates (Greiner Bio-One) and coated with affinity-purified goat anti-mouse immunoglobulin (1:100; DAKO) in complete medium with 10% FCS at a concentration of 1 × 10^6/mL/well for 1 h at 4°C. The cells were cultured for 48 h in a humidified 5% CO2 incubator at 37°C. After 48 h, the cells were resuspended and the contents of each well were divided over four new uncoated wells at a volume of 250 μL/well. To each new well, 750 μL complete medium supplemented with 14 IU/mL recombinant human IL-2 (CLB) was added, resulting in a final concentration of 10 IU/mL rHL-2. The cells were cultured for another 5 days, after which they were harvested and stored in liquid nitrogen for functional analysis at a later date.

Peptides and peptide loading of T2 stimulator cells. A panel of HLA-A1, HLA-A2, or HLA-A3 binding peptides (Peptide Synthesis Facility, IHB-LUMC), derived from various melanoma-associated tumor antigens and containing previously described CD8+ T-cell epitopes, was used for CD8+ T-cell reactivity testing in the IFN-γ ELISPOT assay. HLA-A1: MAGE-A161-169 (EADPTGHSY), MAGE-A168-176 (EVDPIGHLY), TYR164-172 (KDICTDEY), TYR243-251 (KCDICTDEY), TYR454-463 (DSDPDSFQDY), INF A NP44-52 (FLU) (CTELKLSDY), and hTERT1036-1044 (ISDTASLLY); HLA-A2: gp100154-162 (KTWGQYWQV), MAGE-A3 (FLWGPRALV), MART126-35 (EAAGIGILTV), NY-ESO157-165 (SLLMWITQ), TYR369-377 (YMDGTMSQV), INF A MP58-66 (FLU) (GILGFVFTL), and HPV-16 E711-20 (YMLDLQPETT); and HLA-A3: gp10017-25 (ALLAVGATK), MAGE-A3 (FLWGPRALV), MART126-35 (EAAGIGILTV), NY-ESO157-165 (SLLMWITQ), TYR369-377 (YMDGTMSQV), INF A MP58-66 (FLU) (GILGFVFTL), and HPV-16 E711-20 (YMLDLQPETT) were dissolved in complete medium without FCS at 5 mg/mL and stored at -20°C until use. Nontransfected T2 cells (in case of A2 binding peptides) or HLA-A1 or HLA-A3 transfected T2 cells (in case of A1 or A3 binding peptides; kindly provided by Dr. E.M. Jaffee, Johns Hopkins...
University School of Medicine) were loaded overnight in serum-free medium with h2-microglobulin (50 μg/mL; Sigma) and melanoma-associated or control peptides (50 μg/mL) at 37°C in a humidified 5% CO2 incubator. After overnight pulsing, stimulator cells were washed, counted, and used for CD8+ T-cell activation testing.

Melanoma-specific ex vivo CD8+ T-cell activation and IFN-γ ELISPOT analysis. To test the CD8+ T cells for functional melanoma-specific reactivity, an IFN-γ ELISPOT assay was done (28, 29). As effector cells, CD8+ T cells were isolated from expanded T cells from the SLN and from PBMC using the untouched CD8+ mini MACS selection kit according to the manufacturer’s instructions (Miltenyi Biotec). After isolation, CD8+ T cells were washed and resuspended in complete medium with 10% FCS and added directly to the peptide-pulsed T2 cells in anti-IFN-γ precoated Multiscreen 96-well filtration plates (Millipore). Plates were seeded with 10,000 stimulator cells (peptide-pulsed T2 cells) per well and 100,000 or 50,000 effector cells (CD8+ T cells), resulting in effector/stimulator ratios of 10:1 and 5:1, respectively. Assays were tested in quadruplicate where possible, but at least in triplicate at each of the effector/stimulator ratios. After overnight (18 h) incubation, the cells were flicked off and an ELISPOT assay was done as described previously (30) using a commercially available anti-IFN-γ mAb pair (Mabtech). After development of the plates, spots were counted by an automated ELISPOT reader (AID Diagnostika). CD8+ T-cell ELISPOT activity was expressed either as the mean number of spots per well or as the number of specific effector CD8+ T cells per 1 x 10^6 CD8+ T cells (obtained by subtracting the mean frequency of spot-forming CD8+ T cells in control conditions from the mean spot-forming CD8+ T-cell frequencies in the test conditions based on results from the effector/stimulator ratio of 5:1). ELISPOT CD8+ T-cell responses were considered positive when (a) the number of spots in the test condition was significantly higher than the number of spots in the control condition in an unpaired two-sided Student's t test (P < 0.05), (b) the mean number of spots of the test condition exceeded the mean number of spots for the corresponding effector/stimulator ratio of the control condition by at least 2-fold, and (c) the absolute difference in number of spots between the test and control condition was at least 5 (31).

Tetramer binding and Cytokine Bead Array analysis. PE-labeled HLA-A2 tetramers (Tm) presenting melanoma-associated HLA-A2 epitopes were obtained from The Netherlands Cancer Institute or from Sanquin (both in Amsterdam, The Netherlands) and were prepared and used as described previously (31). HLA-A2 restriction of melanoma-specific CD8+ T-cell reactivity in SLN and PBMC was determined by flow cytometric Cytokine Bead Array (CBA) analysis. To this end, 500,000 MACS-isolated CD8+ T cells were stimulated with pools of peptide-loaded T2 stimulator cells (10:1 ratio per peptide-loaded T2 cells) for 18 h at 37°C. Target cells used were T2 cells loaded with negative control peptide HIV-1 RT476-484 (ILKEPVHGV), a pool of T2 cells individually loaded with the positive control peptides INF-A MP95-96 (HLA-A2) and EBV 280-288 (GLCTLVAML) or pools of T2 cells individually loaded with the five HLA-A2 binding melanoma peptides listed above. To confirm that recognition was HLA-A2 restricted, the melanoma-peptide loaded T2 pools were incubated with a neutralizing anti-HLA-A2 antibody (BB7.2) or control IgG1 antibody for 1 h at 4°C before addition of the CD8+ T cells. Following stimulation, supernatants were harvested and the cytokine levels were determined by CBA (human Th1/Th2 cytokine kit; BD Biosciences) according to manufacturers’ protocols.

Statistical analysis. Differences between patient study groups were analyzed with the two-sample Mann-Whitney U test or the Fisher’s exact test (all two-tailed). Correlations were calculated using the Spearman’s ρ test. Differences and correlations were considered significant when P < 0.05.

Results

Patients. There were no significant differences in patient characteristics between the saline-administered control group and the PF-3512676-administered test group (Table 1). I.d.
administration of PF-3512676 (hereafter referred to as CpG) was tolerated well with transient flu-like symptoms (22). Pathologic examination revealed 6 patients with stage III melanoma based on the presence of tumor cells in the SLN (2 of 11 patients in the CpG-administered group and 4 of 13 patients in the saline-administered group). In 4 of 6 of these patients (n = 2 in the saline group and n = 2 in the CpG 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. Based on flow cytometric positivity for HLA-A1, HLA-A2, or HLA-A3, 11 patients in the saline and 10 patients in the CpG group could be tested for CD8+ T-cell reactivity against a panel of melanoma-associated epitopes. There was no significant difference in time elapsed from primary excision to SLN procedure between both study groups (Table 1).

CD8+ T-cell reactivity in the SLN and peripheral blood. Before expansion, CD4/CD8 ratios were determined among the T cells from the freshly sampled SLN (7 days after saline or CpG administration) and found to be higher in the saline control group than in the CpG-modulated SLN samples (Fig. 1A; median for saline and CpG, 7.74 and 4.95, respectively). Although this difference did not reach significance, it is suggestive of preferential CD8+ T-cell expansion in the SLN on CpG administration. As a measure of T-cell activation, flow cytometric analysis of T-cell activation markers on CD4+ and CD8+ T cells in the SLN was also done before T-cell expansion (Fig. 1B). No difference was observed in expression of the T-cell

### Table 2. Melanoma-specific CD8+ T-cell responses

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
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<th>P</th>
</tr>
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<tbody>
<tr>
<td>FLU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC pre</td>
<td>1/11</td>
<td>4/10</td>
<td>0.149</td>
</tr>
<tr>
<td>PBMC post</td>
<td>2/11</td>
<td>7/10</td>
<td>0.030</td>
</tr>
<tr>
<td>SLN</td>
<td>2/11</td>
<td>5/10</td>
<td>0.183</td>
</tr>
<tr>
<td>MAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC pre</td>
<td>0/11</td>
<td>2/10</td>
<td>0.214</td>
</tr>
<tr>
<td>PBMC post</td>
<td>0/11</td>
<td>7/10</td>
<td>0.001</td>
</tr>
<tr>
<td>SLN</td>
<td>0/11</td>
<td>5/10</td>
<td>0.012</td>
</tr>
<tr>
<td>&gt;1 MAA</td>
<td></td>
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<tr>
<td>PBMC pre</td>
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<td>0/10</td>
<td>1</td>
</tr>
<tr>
<td>PBMC post</td>
<td>0/11</td>
<td>5/10</td>
<td>0.012</td>
</tr>
<tr>
<td>SLN</td>
<td>0/11</td>
<td>5/10</td>
<td>0.012</td>
</tr>
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NOTE: P values determined by Fisher’s exact test.

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Fig. 2. HLA-A2 Tm binding and CBA analysis confirm the presence of MAA-specific and HLA-A2-restricted CD8+ T cells in SLN and peripheral blood of CpG-treated patients. A, MAA-specific Tm binding on CD3+CD8+ T cells from post-treatment SLN and blood samples (d7 and d21; epitopes presented by the employed Tm were derived from the indicated MAA). Top right, number of Tm+ cells per 10⁵ CD8+ T cells; bottom right, frequencies from corresponding IFN-γ ELISPOT tests (number of spots per 10⁵ CD8+ T cells; Tm frequencies over the detection threshold (0.02%) and positive ELISPOT reactivity are indicated in bold).
activation markers CD69 or HLA-DR on either CD4⁺ or CD8⁺ T cells. Interestingly, on both CD8⁺ and CD4⁺ T cells, the levels of CD25 and CTLA-4 (both T-cell activation markers associated with a regulatory phenotype) were consistently low in the CpG-modulated SLN in contrast to the higher but more heterogeneous expression levels observed on T cells from the saline controls (Fig. 1B).

CD8⁺ T-cell reactivity in response to a range of melanoma-derived epitopes was measured at three different time points (before, 1 week after, and 3 weeks after CpG or saline administration) by an IFN-γ ELISPOT readout (as shown in Fig. 1C). Beside MAA-specific CD8⁺ T-cell reactivity, reactivity against influenza epitopes (FLU) was determined as a measure of general immunocompetence (see Fig. 1C). ELISPOT CD8⁺ T-cell responses were considered positive only after meeting a strict definition of positivity as described previously by Vuylsteke et al. (31). Complying with these criteria, five patients showed specific post-treatment reactivity to more than one MAA-derived epitope either in blood-derived CD8⁺ T cells at 1 or 3 weeks after CpG administration or in SLN-derived CD8⁺ T cells 1 week after CpG administration [all shown for the tested MAA and FLU epitopes in Fig. 1D and E [HLA-A3⁺ or HLA-A2⁺ patients, respectively; positive responses marked by an asterisk]. Importantly, none of these or any other of the tested patients showed MAA-specific reactivity in the blood before treatment (Table 2). Post-treatment CD8⁺ T-cell response rates against at least one of the tested MAA epitopes were 0 of 11 for the saline control group in both blood and SLN and 7 of 10 and 5 of 10 for the CpG-administered group in blood and SLN, respectively (P = 0.001 and 0.012; Table 2). Response rates against more than one MAA epitope were 5 of 10 in the post-treatment blood and 5 of 10 in the SLN (Table 2). Four of these responding patients had measurable responses to more than one MAA epitope in both the post-treatment blood and the SLN. In total, 6 of 10 patients in the CpG-administered group and 0 of 11 patients in the saline-control group had a positive response to more than one MAA epitope (P = 0.004).

Of note, increased FLU response rates in the blood on CpG administration [saline versus CpG: before treatment, 1 of 11 versus 4 of 10 (P = 0.149); after treatment, 2 of 11 versus 7 of 10 (P = 0.030); Table 2] are indicative of a generalized increase in CD8⁺ T-cell activation state resulting from CpG injection.

To verify that the MAA-specific CD8⁺ T-cell responses measured by IFN-γ ELISPOT assay were not a mere reflection of a generally heightened (nonspecific) T-cell activation state, we did HLA-A2 Tm binding analyses of T cells from stored SLN and PBMC samples that were still available from four positive ELISPOT responders. MAA-Tm results from one representative patient (resp.4 in Fig. 1E) are shown in Fig. 2A as number

![Fig. 2](Continued. B, modest but specific IL-2, IL-10, and IFN-γ release was measurable by CBA on MAA peptide stimulation of CD8⁺ T cells from post-treatment peripheral blood (day 7) of a CpG-administered patient (positive ELISPOT responder: resp.5) and was blocked by addition of the HLA-A2-neutralizing mAb BB7.2. C, similarly, CD8⁺ T cells from the SLN of the same patient released IL-2, IL-10, and IFN-γ in response to MAA, which was blocked by anti-HLA-A2. In contrast, no MAA-specific cytokine release was observed by SLN CD8⁺ T cells from a CpG-administered patient who also failed to respond in the IFN-γ ELISPOT assay (non-resp.).)
of Tm+ cells per 1 x 10^5 CD8+ T cells; also listed are the corresponding number of spots (per 1 x 10^5 CD8+ T cells) measured in the IFN-γ ELISPOT assay (Tm frequencies over the detection threshold and positive ELISPOT reactivity indicated in bold). Between the four tested HLA-A2+ patients, Tm+ T cells were measurable for a total of 10 of 14 tested epitopes to which also a positive ELISPOT reactivity was found. Despite a lack of quantitative correlation between the frequencies measured by ELISPOT or by Tm analysis (illustrated by the data shown in Fig. 2A), overall concordance between the two tests in the presence or absence of detectable T cells to the tested epitopes was quite high at 74% (based on a total of 23 Tm analyses and assuming a detection threshold of 0.02% for the Tm stainings and adhering to the definition of positive ELISPOT reactivity as described in Materials and Methods). This is not too dissimilar to the 88% concordance rate reported previously by us for MAA-specific CD8+ T-cell frequencies similarly measured by IFN-γ-ELISPOT and Tm analysis in melanoma SLN after local administration of granulocyte macrophage colony-stimulating factor (31).

Finally, HLA-A2 restriction of the measured MAA-specific CD8+ T-cell responses was verified in another positive responder (res.p5 in Fig. 1E). CD8+ T cells from post-treatment PBMC and SLN samples were stimulated for 24 h by a pool of T2 cells, individually loaded with MAA peptides (to which this donor responded previously in the IFN-γ ELISPOT assay), in the presence of either an isotype control mAb (IgG1) or a HLA-A2 neutralizing mAb (BB7.2). Supernatants were harvested and tested for the presence of six T-cell-secreted cytokines by CBA analysis. Results for the PBMC sample are shown in Fig. 2B: on MAA stimulation, specific release of IFN-γ, IL-2, and IL-10 was detected, which was prevented by blocking of HLA-A2 during T-cell stimulation. Equivalent results were obtained with CD8+ T cells from a SLN sample of the same donor, whereas no specific cytokine release was observed in a parallel test of SLN-derived CD8+ T cells from a CpG-treated patient who failed previously to respond to MAA in the IFN-γ ELISPOT assay (Fig. 2C). Immune competence for both the MAA responder and nonresponder was shown by specific IFN-γ release to influenza and EBV epitopes (at 29.2 and 15.4 pg/mL in the SLN samples, respectively).

CD8+ T-cell reactivity to >1 MAA in the SLN and blood correlates to activation of BDCA-2+CD123+ PDC. CpG-activated PDC rapidly release large amounts of IFN-α (12–14), which may facilitate direct activation of CD8+ T cells as well as promote the differentiation and maturation of neighboring MDC and thus also indirectly stimulate T-cell activation (15–19). Interestingly, a significant association was found between high expression levels of the costimulatory and activation markers CD86 (P = 0.003) and CD40 (P = 0.002) on BDCA-2+CD123+ PDC and the presence of CD8+ T-cell reactivity to >1 MAA in the SLN (Fig. 3A), but no such association was found for the expression of CD86 and CD40 on CD1a+ MDC (Fig. 3A). These data suggest a direct causal relationship between PDC and CD8+ T-cell activation rather than an involvement of bystander MDC activation.

Similar results were found when the expression of activation markers CD86 and CD40 on BDCA-2+CD123+ PDC and CD1a+ MDC were correlated to qualitative (+ or -) CD8+ T-cell reactivity to >1 MAA in the post-treatment blood samples. Figure 3B clearly shows the relationship among CpG administration, MAA-specific CD8+ T-cell reactivity, and PDC activation state. As with SLN, a significant association was also found between the presence of CD8+ T-cell reactivity to >1 MAA in the postinjection blood (all on CpG administration) and the level of expression of the activation markers CD86 (P = 0.001) and CD40 (P = 0.003) on BDCA-2+CD123+ PDC (Fig. 3B). Again, neither the expression of activation markers CD86 and CD40 on CD1a+ MDC nor the expression of maturation marker CD83 on either BDCA-2+CD123+ PDC or CD1a+ MDC correlated significantly to melanoma-specific CD8+ T-cell reactivity to >1 MAA in the post-treatment blood (data not shown).

To make a more quantitative correlation between SLN-PDC activation state and treatment-induced MAA-specific CD8+ T-cell reactivity, we carried out the following analysis: for all HLA-A2+ patients [saline (n = 6) and CpG (n = 6); all tested for the same five MAA epitopes derived from five different MAA; see Materials and Methods], an aggregate increase in MAA-specific frequencies was determined by adding up the specific increases in measured spots for all tested MAA epitopes (per 10^5 CD8+ T cells) between pretreatment and post-treatment PBMC samples. The results presented in Fig. 3C reveal a quantitative correlation between high aggregate MAA-specific CD8+ T-cell frequency increases in blood (observed only in the CpG-administered patients) and CD86 and CD40 expression levels on SLN-PDC. A significant correlation in this analysis was only observed for CD40 levels on the PDC (P = 0.005; Fig. 3C).

From these analyses, we conclude that local CpG-induced PDC activation in the SLN may afford systemic T-cell-mediated protection against tumor spread in addition to local tumor control in the SLN.

NK cell frequency in relation to PF-3512676 administration and melanoma-specific CD8+ T-cell reactivity. IFN-α released by activated PDC potentially boosts not only CD8+ T-cell responses but also NK cell responses (12–14). From 4 saline-administered patients and 10 CpG-administered patients, frozen stored samples of the original SLN single-cell suspensions were available to establish the frequency of NK cells. A clear correlation was found between CD56+ NK cell frequency in the SLN and PDC activation state by CD86 expression (Fig. 4A). Increases in the frequency of CD56lo (“cytotoxic effector”) and CD56hi (“regulatory”) NK cells were found in the CpG test group compared with the saline control group (both nonsignificant; see Fig. 4B). In addition, the calculation of NK subset frequencies in the SLN of CpG-administered patients according to the presence (n = 5) or absence (n = 5) of CD8+ T-cell reactivity to >1 MAA in the SLN (Fig. 4C) revealed a significant association between MAA CD8+ T-cell reactivity and high frequencies of CD56lo cytotoxic effector NK cells. Altogether, these data clearly show a simultaneous increase in melanoma-specific CD8+ effector T cells and innate NK effector cells, resulting from i.d. PF-3512676 CpG-B administration around the primary tumor excision site and subsequent PDC activation in the draining SLN.

Discussion

More and more, the SLN procedure is becoming standard of care for melanoma patients. Not only does it provide
important prognostic information, it also identifies patients with nodal metastases whose survival may be prolonged by lymphadenectomy (32). In addition, routine application of this procedure in early-stage melanoma patients presents a unique translational setting to study adjuvant therapies in vivo, aimed at immunopotentiation of the SLN (33). We recently reported immunostimulatory effects of local administration of the CpG-B oligodeoxynucleotide PF-3512676 on the SLN of stage I/III melanoma patients, leading to an increase in the activation state, but not the frequency, of PDC and MDC, the induction of a novel TRAIL+ MDC subset, a proinflammatory type 1 T-cell cytokine profile, and reduced Treg frequencies (22).

Our hypothesis that these CpG-induced immunostimulatory effects on both dendritic cell and T-cell subsets in the SLN would translate into higher frequencies of melanoma-specific CD8+ T cells has now been confirmed by data presented in this article. Even in this is relatively small study of CD8+ T cells obtained from blood or by scraping the cutting surface of bisected SLN (26, 34), we found convincing evidence that melanoma-specific CD8+ T-cell responses (detected by specific IFN-γ release) were enhanced by i.d. injection of PF-3512676 around the excision site of the primary melanoma. Thus, as reported previously for granulocyte macrophage colony-stimulating factor (30), PF-3512676 is able to (re-)activate

Fig. 3. Activation of PDC is associated with melanoma-specific CD8+ T-cell reactivity. A, presence of melanoma-specific CD8+ T-cell reactivity to >1 MAA in the SLN (open columns, negative; filled columns, positive reactivity) in relation to the activation status of BDCA-2+CD123+ PDC and CD1a+ MDC (by the expression levels of activation markers CD86 and CD40) for all patients. P values were determined by Mann Whitney U test. B, activation of PDC (defined by the expression of activation markers CD86 and CD40) in the SLN correlates to PF-3512676 administration and melanoma-specific CD8+ T-cell reactivity in the blood. CD8+ T-cell reactivity (+, positive ELISPOT response to >1 MAA in the postinjection blood) in PF-3512676-administered (+) and NaCl 0.9% saline-administered (−) patients are listed in relation to the activation of BDCA-2+CD123+ PDC (ordered by percentages of CD86 and CD40 expression). C, aggregate increase in the IFN-γ ELISPOT for all tested epitopes (n = 5) was determined for all 12 HLA-A2+ patients (+ aggregate s/spots per 10^5 CD8+ Tcells) and correlated to percentage positivity of CD86 and CD40 on the SLN-PDC. Open triangles, saline-administered patients; filled triangles, CpG-administered patients. ρ and P values from Spearman ρ correlation tests.
tumor-reactive CD8+ T cells that appear to be already recruited to the SLN in early stages of melanoma development but remain functionally “dormant.” Furthermore, a clear correlation was found between CD56+ NK cell frequency and PDC activation, indicating a simultaneous increase in specific and innate effector cells on PF-3512676 treatment.

These effects of CpG administration on T-cell and NK cell activation are in keeping with previously reported in vivo effects of CpG oligodeoxynucleotides. It is known that PF-3512676 activates PDC through Toll-like receptor 9 triggering and that activated PDC release IFN-α (12–14, 35). The rapid release of large amounts of IFN-α may enable PDC to boost CD8+ T-cell (36–38) and NK cell (13, 18) responses as well as promote the differentiation and maturation of neighboring MDC (precursors; ref. 19) and thus also indirectly stimulate T-cell activation. However, the relatively poor induction of IFN-α release by CpG-B oligodeoxynucleotides like PF-3512676 (11, 34), coupled to the observed correlation between CpG-induced phenotypic PDC activation and increased frequencies of functional CD8+ effector T cells and, importantly, the absence of such a correlation with increased activation of CD1a+ MDC (or with a recently described in vivo CpG/IFN-α-induced CD83+TRAIL+ MDC subset; ref. 21; data not shown), is suggestive of direct activation of specific CD8+ T cells through PDC-mediated antigen presentation as reported previously (16). Indeed, expression levels on SLN-resident PDC of the costimulatory molecules CD86 and CD40, both essential for effective T-cell stimulation, correlated directly with robust CD8+ T-cell reactivity against more than one MAA epitope in both the SLN and peripheral blood. A generalized induction of preferential CD8+ T-cell proliferation was further suggested by overall lower CD4/CD8 ratios observed in CpG-modulated SLN compared with saline controls. The relative contribution of either CpG-induced contact-dependent stimulation or cytokine release by PDC to the observed increase in effector frequencies of CD8+ T cells and NK cells remains to be clarified.

Tumor environmental factors interfere with dendritic cell maturation and/or differentiation, resulting in immature and/or partially differentiated dendritic cells (39, 40). Tumor-associated PDC have been reported to induce CD8+ Treg (41–43) and tumor-associated immature MDC are capable of promoting Treg proliferation (44–47). We showed previously that a single dose of PF-3512676 was sufficient to both increase the PDC and MDC activation state and decrease the Treg frequencies in the melanoma SLN (22). In this study, consistently lower CD25 and CTLA-4 expression levels were observed on both CD8+ and CD4+ T cells in CpG-modulated SLN than in control SLN. Expression of these markers signals immunosuppressive, regulatory functions that may be induced in memory T cells by tumor-modulated PDC or MDC (8, 40–42). Due to low patient numbers and highly variable expression levels in the control SLN, the observed differences between the study groups were not significant. Nevertheless, these observations

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**Fig. 4.** Activation of PDC and melanoma-specific CD8+ T-cell reactivity are associated with increased frequencies of NK cells in the SLN. **A.** Positive and significant correlation between PDC activation (by percentage CD86 positivity) and frequencies of CD56+CD3- NK cells in the SLN (expressed as percentage of total SLN leucocytes). ρ and P values from Spearman ρ correlation tests. Frequencies of CD56+ cytotoxic effector NK cells and CD56+ regulatory NK cells in relation to (B) PF-3512676 (filled columns) or saline administration (open columns) and (C) melanoma-specific CD8+ T-cell reactivity (open columns, negative; filled columns, positive) in the SLN of PF-3512676-administered patients. P values were determined by Mann Whitney U test.
suggest that, beside the down-regulation of natural Treg (22), CpG may also contribute to increased antimelanoma T-cell reactivity through down-modulation of negative feedback loops in activated effector T cells maintained by inhibitory molecules such as CTLA-4/B7 interactions. In this regard, it is tempting to speculate that CpG may also be able to break the vicious circle of ongoing back-and-forth stimulation through CTLA-4/B7 interactions in inactivated effector T cells maintained by inhibitory molecules. Reactivity through down-modulation of negative feedback loops. Finally, Ghiringhelli et al. found an inverse correlation between NK cell activation and Treg frequency in tumor-bearing patients, indicating a role for Treg frequencies in blunting the NK cell arm of the innate immune system (50). Therefore, PF-3512676 might boost the innate immune system not only by triggering PDC to release large amounts of IFN-α, IL-10 was released. This observation points to the need for a broader assessment of T-cell effector and regulatory functions in follow-up studies.

In conclusion, whether effected through direct immune activation or through interference with tumor-related immune regulatory mechanisms, the demonstrated ability of locally administered PF-3512676 to harness both adaptive and innate effector arms of the immune system (through local and systemic MAA-specific CD8+ T-cell reactivity and NK effector cell mobilization) and thus contribute to protection against melanoma spread clearly indicates its utility as a possible adjuvant therapy option for early-stage melanoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Local Administration of PF-3512676 CpG-B Instigates Tumor-Specific CD8 + T-Cell Reactivity in Melanoma Patients

Barbara G. Molenkamp, Berbel J.R. Sluijter, Paul A.M. van Leeuwen, et al.


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