Diagnosing Melanoma and Immunomodulation of the Melanoma Sentinel Lymph Node
The publication of this thesis was financially supported by:
Mw. M.J.H. Aben
Pfizer B.V.
Novartis Pharma B.V.
Janssen-Cilag B.V.
Erbe Benelux B.V.
BD Biosciences
Greiner Bio-One B.V.
Siemens Medical Solutions

Diagnosing Melanoma and Immunomodulation of the Melanoma Sentinel Lymph Node,
VU University Medical Center, Amsterdam, the Netherlands

The work described in this thesis was performed within the framework of the Graduate School Oncology Amsterdam at the departments of Surgery (Head Prof.dr. J.A. Rauwerda) and Pathology (Head Prof.dr. C.J.L.M. Meijer), VU University Medical Center, Amsterdam, the Netherlands.

Copyright © Barbara Molenkamp, Amsterdam, 2007
No part of this thesis may be reproduced, stored or transmitted, in any form or by any means, without prior permission of the author.

Cover Art: Courtesy of the artist Jeroen Krabbé, Champagne Beach, Espírito Santo, Vanuatu, 1989
Lay-out: Chris Bor
Printed by: Buijten en Schipperheijn, Amsterdam
Diagnosing Melanoma and Immunomodulation of the Melanoma Sentinel Lymph Node

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op vrijdag 11 januari 2008 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Barbara Gerarda Molenkamp

tegenover te Hoogeveen-Sappemeer
promotoren:  prof.dr. P.A.M. van Leeuwen  
prof.dr. R.J. Scheper  
prof.dr. S. Meijer  
copromotor:  dr. T.D. de Gruijl
### CONTENTS

**Introduction**

<table>
<thead>
<tr>
<th>Chapter 1a</th>
<th>Immunomodulation of the melanoma sentinel lymph node: a novel adjuvant therapeutic option</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Immunobiology</em> 2006;211:651-61</td>
<td></td>
</tr>
</tbody>
</table>

| Chapter 1b | Outline of this thesis | 23 |

**Diagnosing melanoma**

| Chapter 2 | Non-radical diagnostic biopsies do not negatively influence melanoma patient survival | 27 |
|  | *Annals of Surgical Oncology* 2007;14:1424-30 |  |

**Immunomodulation of the melanoma sentinel lymph node: GM-CSF**

| Chapter 3 | Local administration of granulocyte/macrophage colony-stimulating factor increases the number and activation state of dendritic cells in the sentinel lymph node of early-stage melanoma | 41 |
|  | *Cancer Research* 2004;64:8456-60 |  |

| Chapter 4 | Tumor-specific CD8+ T cell reactivity in the sentinel lymph node of GM-CSF-treated stage I melanoma patients is associated with high myeloid dendritic cell content | 55 |
|  | *Clinical Cancer Research* 2006;12:2826-33 |  |

| Chapter 5 | Matched skin and sentinel lymph node samples of melanoma patients reveal exclusive migration of mature dendritic cells | 75 |

**Immunomodulation of the melanoma sentinel lymph node: PF-3512676**

| Chapter 6 | Intradermal CpG-B activates both plasmacytoid and myeloid dendritic cells in the sentinel lymph node of melanoma patients | 91 |
|  | *Clinical Cancer Research* 2007;13:2961-9 |  |

| Chapter 7 | Local administration of PF-3512676 instigates tumor-specific CD8+ T cell reactivity in melanoma patients | 109 |
|  | *Submitted Clinical Cancer Research* |  |

| Chapter 8 | Summary, discussion and future prospects | 127 |

**Publications**

<table>
<thead>
<tr>
<th></th>
<th>139</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nederlandse samenvatting</strong></td>
<td>143</td>
</tr>
<tr>
<td><strong>Dankwoord</strong></td>
<td>149</td>
</tr>
<tr>
<td><strong>Curriculum vitae</strong></td>
<td>153</td>
</tr>
</tbody>
</table>
chapter 1

Immunomodulation of the melanoma sentinel lymph node: a novel adjuvant therapeutic option

Immunobiology 2006;211:651-61

BG Molenkamp
PAM van Leeuwen
AJM van den Eertwegh
BJR Sluijter
RJ Scheper
S Meijer
TD de Gruijl
ABSTRACT

Cutaneous melanoma is the most aggressive type of skin cancer. Paradoxically, melanoma is also the most immunogenic tumour identified to date: tumour-reactive T cells are detectable both in the blood and in tumour draining lymph nodes (TDLN) of melanoma patients and their frequency can be increased by specific vaccination. However, early melanoma development is accompanied by impaired immune effector functions in the initial tumour-draining lymph node, the sentinel lymph node (SLN). Most notably, a reduced frequency and activation state of dendritic cells (DC) interferes with the uptake and presentation of tumour-associated antigens (TAA) to specific anti-tumour cytotoxic T-lymphocytes (CTL) and T helper cells (Th). These impaired immune effector functions may contribute to the early metastatic events that are associated with this tumour type. Since complete surgical excision at an early stage remains the only curative treatment option (adjuvant therapy options are limited and show no survival benefits), immunopotentiation of the SLN to jump-start or boost tumour specific immunity in early stage melanoma may be a valuable adjuvant treatment option that can be generally applied with minimal discomfort to the patient. Early clinical studies indicate that local Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) or Cytosine-phosphate-Guanine (CpG) administration leads to activation of different DC subsets and conditions the SLN microenvironment to be more conducive to the generation of T cell-mediated anti-tumour immunity.
The Sentinel Lymph Node concept in the staging and treatment of melanoma

Cutaneous melanoma is the most aggressive type of skin cancer and in fair-skinned Caucasian populations is an important and growing public health problem. Both its incidence and mortality rates have been increasing in Europe over the past decades. For instance, in The Netherlands it accounts for 3% of all new cancer cases per year with an incidence of 14 per 100,000, which is currently increasing at 5% per year. The absolute total number of new cases of melanoma in The Netherlands is expected to be more than 4800 in 2015, as compared to around 2400 in 2000. Complete surgical excision at an early stage remains the only curative treatment option for cutaneous melanoma. Adjuvant therapy options are limited and show low survival benefits.

The first proximal lymph node (LN) to directly drain a primary tumour, the so-called sentinel lymph node (SLN), is the preferential site of early metastasis. Lymphatic mapping and selective SLN excision is a minimally invasive procedure, which allows for the identification of patients at risk for LN metastasis who should undergo a full therapeutic LN dissection. Since the introduction of the SLN procedure in the early 1990s, this procedure is widely used in the management of patients with cutaneous melanoma without clinical evidence of nodal metastases. The SLN concept is based on the theory of an orderly progression of initial tumour cell metastasis within the lymphatic system. It assumes that early lymphatic metastases, if present, are always found first within the most proximal tumour draining lymph node (TDLN), the SLN. A tumour negative SLN thus predicts the absence of metastatic disease in the rest of the TDLN basin and negates the necessity of an elective lymph node dissection (ELND), which, unlike SLN excision, is associated with considerable morbidity. Several studies have validated this assumption. So far, the SLN procedure has been used mainly for staging of melanoma patients and to select patients for experimental adjuvant therapies at an early stage. The American Joint Committee on Cancer (AJCC) has declared the SLN procedure standard of care for melanoma patients. 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.

Immune control of melanoma

Melanoma is the most immunogenic tumour identified to date. T cells reactive to tumour-associated antigens (TAA) are already detectable at early stages of tumour development, both in peripheral blood and in TDLN and their frequency can be increased by specific vaccination strategies. This intrinsic immunogenicity makes melanoma particularly amenable to adjuvant immunotherapy. For this, sufficient numbers of properly activated...
Chapter 1a

Dendritic cells (DC) in the lymphatic tumour-drainage catchment area are essential. Myeloid DC (MDC) are antigen-presenting cells that are critical to the initiation of T cell-mediated immunity and offer the basis for promising new treatment modalities in cancer. MDC have an important sentinel function; excessive tissue damage or signs of microbial invasion can prompt maturation and migration of MDC to LN where they alert the immune system. In cutaneous melanoma, skin-resident MDC (i.e. Langerhans Cells [LC]) take up and transport TAA to TDLN. In order to subsequently activate melanoma-specific T cells, the migrated LC need to become activated, i.e. express high levels of the maturation marker CD83 and of co-stimulatory molecules (e.g. CD40, CD86), as well as appropriate chemokine receptors to migrate to the paracortical T cell areas of the TDLN (e.g. CCR7). There, LC interact with recirculating naive and memory T-helper (Th) cells and cytotoxic T lymphocytes (CTL). Upon TAA-specific recognition, Th cells further activate LC through CD40L-CD40 interactions and enable upregulation of CD86 and the production of IL-12, both of which are essential for efficient CTL priming. Activated effector CTL leave the LN via efferent vessels and home to and infiltrate tumour sites in order to eradicate cancer cells. Unfortunately, under tumour conditions DC functions may be disturbed, leading to immune tolerance rather than immune activation.

**Melanoma-associated immune suppression**

Immune suppression in TDLN has been reported at the level of different immune effector cells. Below an overview is given of tumour-associated suppressive effects on myeloid and plasmacytoid DC and on T cells.

**Myeloid DC**

Hampered MDC differentiation and activation has been reported in many tumours and decreased tumour infiltration by MDC is a poor prognostic factor. Immature MDC with ready access to TAA from the tumour may induce specific tolerance through inappropriate or abortive T cell activation, as was indeed shown for MDC isolated from metastatic melanoma lesions. MDC in TDLN were similarly reported to display immature characteristics. MDC development and activation can both be frustrated by inhibitory factors commonly associated with melanoma, such as IL-10 or gangliosides. The degree of immunosuppression in TDLN is directly related to their distance to the primary tumour, indicating the causative agents to be tumour-derived. Thus, the SLN, being most proximal to the tumour, shows the most pronounced immunosuppression, as early as Stage-I of melanoma development. Paracortical SLN-MDC density is reduced and most SLN-MDC lack dendritic morphology and display lower expression levels
Immunomodulation of the melanoma sentinel lymph node

of costimulatory molecules as compared to non-SLN-MDC. Lee et al. reported significantly higher levels of the immunosuppressive cytokine IL-10 in tumour positive SLN, which may further exacerbate this MDC suppression. IL-10-conditioned DC were previously shown to be resistant to maturation and to display deregulated chemokine receptor patterns with low CCR7 levels. The upshot of these characteristics is the induction of Ag-specific T cell anergy. Indeed, IL-10-conditioned MDC and MDC isolated from metastatic melanoma lesions, which generally contain high levels of IL-10, were found to be responsible for the induction of melanoma-specific T cell anergy and possibly tumour progression. These studies indicate that crippled MDC functions in the first line of immunological defence will frustrate specific T cell activation and likely increase the chance of tumour immune escape and metastatic spread.

Recent studies have shown that these tumour-induced inhibitory effects in MDC are often accompanied by an aberrant constitutive phosphorylation state of STAT3, a member of the signal transducer and activation of transcription (STAT) proteins, which interferes with proper MDC maturation. STAT3 appears to play an important role in melanoma cell immune evasion and blocking STAT3 signaling is therefore a promising novel approach to melanoma therapy.

Plasmacytoid DC

Plasmacytoid DC (PDC) constitute another important DC subset with potential antigen presenting and T cell activating capability. PDC were first identified as a separate DC subset in peripheral blood and secondary lymphoid organs with a CD123\textsuperscript{hi}CD11c\textsuperscript{-} phenotype and shown to differentiate from lymphoid precursors under the influence of IL-3. In normal blood and LN PDC reside in an immature state and are characterized by expression of the C-type Lectin BDCA-2. Maturation induction \textit{in vitro} results in an upregulation of the costimulatory molecules CD40, CD80 and CD86 as well as the activation marker CD83 and a complete down-regulation of BDCA-2. L-Selectin- and CXCR3-mediated homing and migration allow PDC to travel between the blood and LN. PDC reside in the LN and are able to bind microbial products through specialised receptors such as toll-like receptor (TLR)-9. TLR9 expression in man appears to be restricted to B cells and PDC. Unmethylated Cytosine-phosphate-Guanine oligonucleotide-containing motifs (CpG-ODN), derived from bacterial DNA directly stimulate PDC through TLR9 triggering. Activated PDC rapidly release large amounts of IFN\textalpha which may facilitate direct activation of CD8+ T cells and NK cells as well as promote the differentiation and maturation of neighbouring MDC (precursors) and thus also indirectly stimulate T cell activation. These studies clearly point to a possible role of PDC in (melanoma-specific) CTL activation. However, evidence is accumulating that tumour-associated PDC may induce immunosuppression rather than
activation. Tumour-infiltrating PDC can be found in melanomas and are likely recruited by stromal cell-derived factor-1 (SDF-1), which is secreted by melanoma cells.\textsuperscript{49,52,53} Although \textit{in vitro} generated PDC can prime melanoma-specific CTL,\textsuperscript{49} tumour-associated PDC have been shown to prime immunosuppressive IL-10-producing T cells.\textsuperscript{53} Moreover, PDC infiltrating head and neck squamous cell carcinomas show low TLR9 expression and a diminished capacity for IFN\(\alpha\) production.\textsuperscript{54}

\textbf{T cells}

Spontaneous tumour-specific CD8+ T cell responses are detectable in primary melanoma, in TDLN, and in the blood of melanoma patients.\textsuperscript{16,55-58} In primary melanoma and TDLN tumour specific T cell infiltration is found to correlate with improved patient survival, in contrast to melanoma-specific CD8+ T cells recirculating in the blood.\textsuperscript{55,58,59} High frequencies of melanoma-specific CD8+ T cells are able to reach the tumour site but unfortunately most metastatic melanoma patients undergo disease progression despite a detectable T cell response, suggesting tumour escape from T cell effector functions.\textsuperscript{60} The anti-tumour T cell response thus appears to be ineffective, either because the tumour cells have become insensitive to the effector T cells or because the effector T cells themselves have become unable to be stimulated by the antigen or to exert their function, a state referred to as anergy.\textsuperscript{61} Tumour cell variants that no longer present the TAA that are the targets of the detected effector T cells have been described\textsuperscript{62} but do not seem to be the major cause of tumour immune escape.\textsuperscript{61} To establish the extent to which systemic T cell anergy contributes to immune escape further investigations are required, as circulating tumour specific T cells in melanoma patients have been found to be unresponsive by some investigators and fully functional by others.\textsuperscript{56,63} In contrast, the evidence for local T cell anergy is more convincing.\textsuperscript{63-65} Classic anergy induction is effected by T cell receptor (TCR) ligation by MHC/peptide complexes in the absence of sufficient co-stimulation. However, tumour cells and tumour-conditioned stroma have developed additional ways to directly or indirectly anergise effector T cells. For example, in tumour-conditioned environments CD4+CD25\textsuperscript{hi} regulatory T cells (T\textsubscript{reg}) are often found. The exact mechanism behind T\textsubscript{reg}-mediated immune suppression is currently unknown, but elevated numbers have been found in several human cancers, including lung, breast and ovarian tumours,\textsuperscript{66-68} as well as in human metastatic melanoma lymph nodes.\textsuperscript{69} Three observations collectively provide strong evidence that T\textsubscript{reg} do in fact interfere with protective immunity against a wide variety of human tumours: 1) the presence of increased frequencies of T\textsubscript{reg} in the peripheral blood and/or SLN of cancer patients, 2) the identification of T\textsubscript{reg} with specificity for antigens expressed by human tumours, and 3) the finding that an accumulation of T\textsubscript{reg} in tumours is predictive of poor prognosis and survival.\textsuperscript{70} Other mechanisms resulting in T cell hyporesponsiveness include tryptophan
Immunomodulation of the melanoma sentinel lymph node

depletion via indoleamine-2,3-dioxygenase (IDO) secreted by tumour cells,\textsuperscript{71} arginine depletion via high activity of Arginase I,\textsuperscript{72,73} and engagement of the inhibitory receptor PD-1 on T cells by PD-L1.\textsuperscript{74,75} Furthermore, human melanoma cells secrete galectin-1, which inhibits T cells,\textsuperscript{76} and also produce IL-10, which can inhibit the production of pro-inflammatory cytokines such as TNF\(\alpha\), IFN\(\gamma\) and IL-2.\textsuperscript{77} This state of functional T cell tolerance is reversible \textit{in vitro} and adoptive transfer of anti-tumour T cells restimulated and amplified \textit{in vitro} has shown clinical efficacy.\textsuperscript{78,79} Interestingly, T cell anergy may also be reversible \textit{in vivo}, as recent studies demonstrated: upon melanoma-specific vaccination, reinvigorated pre-existent anti-tumour T cells, rather than newly primed anti-vaccine T cells, were reported to be responsible for subsequent anti-tumour effects.\textsuperscript{57,60,61} It was suggested that a temporary break of tumour-induced immunosuppression by the vaccination (possibly due to pro-inflammatory agents derived from the newly primed anti-vaccine CTL) might have reactivated previously primed anti-tumour CTL and might thus have set in motion an effective anti-tumour response.

\textbf{Boosting anti-tumour immunity through DC: systemic versus local SLN-targeted approaches}

Cytokines and microbial pro-inflammatory factors may be applied to overcome tumour-induced immunosuppression of the above listed immune effector cell subsets. In this regard, both systemic and local immunotherapies of melanoma are explored for their efficacy in clinical settings.

\textbf{Systemic cytokine administration}

Currently, the only available adjuvant therapy for melanoma approved by the US Food and Drug Administration (FDA) is the systemic administration of high-dose IFN\(\alpha\)-2b. But the role of IFN\(\alpha\)-2b as an adjuvant therapy in melanoma patients is under debate since the improved overall survival originally found was no longer evident after a prolonged follow up.\textsuperscript{80-83} The long-term complete remissions in metastatic cancer patients seen with high-dose IL-2 suggest that this agent may have activity in the adjuvant setting, but this has not yet been tested in a randomised fashion in melanoma patients.\textsuperscript{84} In stage III/IV melanoma patients systemic Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) has been used as an adjuvant therapy following surgery and led to an increased median overall survival in the treated patients as compared to the historical control group.\textsuperscript{85} The effect of long-term administration of GM-CSF on MDC activation and disease-free survival was also reported in patients with locally advanced breast cancer (LABC).\textsuperscript{86} A significantly higher mean percentage of MDC in TDLN of the GM-CSF treated patients was found as compared to control groups. Moreover, intrapatient
comparison before and after treatment showed the percentage of MDC to significantly increase over the course of GM-CSF treatment. In a univariate survival analysis with a median follow up of 5 years, relatively high percentages of MDC were associated with longer disease-free survival. In patients with high tumour load, where immunosuppressed conditions generally prevail, long-term administration of GM-CSF may thus contribute to survival through enhanced MDC activation and consequently improved chances of efficacious anti-tumour immunity.

Local cytokine administration

Whereas systemic treatment is indicated in advanced melanoma stages, local immunotherapeutic approaches aimed at the control of early metastasis may present novel and valuable adjuvant treatment options. In this regard TDLN, and SLN in particular, are obvious targets for local immunotherapy. To study the effect of local immunotherapy on immune effector cells in SLN a special technique was developed to investigate DC and T cell functions without interfering with routinely performed diagnostic procedures. SLN were bisected cross-wise and the cutting surface scraped with a surgical blade to obtain viable cells for phenotypic and functional testing.

Using this technique a small-scale phase II study was performed in which Stage-I melanoma patients were randomized to receive intracutaneous injections, either with GM-CSF or plain saline around the scar of the primary excision, preceding re-excision and SLN dissection. Analysis of the CD1a+ MDC phenotype and frequency (through immunocytochemistry and flowcytometry) was performed and flowcytometric analysis showed a significant increase in the number and maturation of the SLN-MDC to be associated with GM-CSF treatment. These results were confirmed by cytospin staining. The injection site was also studied for the effects of GM-CSF on local epidermal and dermal DC and the frequency of CD1a+CD83+ DC in the SLN correlated perfectly with the number of CD1a+CD83+ DC in the superficial dermis for all tested patients.

Further cytochemical analysis of the SLN demonstrated a significant increase in the number of T cells bound to the SLN-MDC upon GM-CSF administration, possibly reflecting protracted periods of binding between DC and T cells upon antigen recognition. Melanoma-specific CD8+ T cell reactivity was determined by IFNγ ELISPOT assay and by HLA-tetramer binding analysis against a range of HLA-A1, -A2, and -A3-binding epitopes derived from melanoma TAA. Results showed a more robust melanoma-specific CD8+ T cell response in the SLN as compared to the peripheral blood, consistent with past local priming of tumour-specific CTL in the tumour-draining SLN. These SLN-resident melanoma-reactive T cells may well have derived from reactivated local (central) memory T cells, which were previously reported to persist in LN for over a year subsequent to local priming and to rapidly home to the target
organ upon amplification in the LN.\textsuperscript{93} A significant association between high SLN-MDC frequencies and the presence of melanoma-reactive CD8\textsuperscript{+} T cells further provided evidence for the CTL-stimulatory effects of local GM-CSF administration. In aggregate this pilot study clearly showed powerful effects of local GM-CSF administration on DC number and activation state and melanoma-specific CTL reactivity in the SLN, demonstrating the clinical feasibility and immunopotentiating effects of this approach. In keeping with these findings local administration of GM-CSF was recently reported to also result in a type-1 cytokine profile skewing consistent with enhanced cell-mediated immunity.\textsuperscript{31}

**Local TLR ligand administration**

Besides DC-activating cytokines, microbial TLR ligands with specificity for different DC subsets are also promising compounds for DC activation and subsequent T cell stimulation. Another pilot study was therefore initiated to investigate the influence of local administration of the TLR9 ligand PF-3512676 (formerly CPG 7909) around the primary tumour excision scar, on the SLN of clinically Stage-I/II melanoma patients (Molenkamp \textit{et al.}, submitted). The obtained results certainly confirm the potential of PF-3512676 as an adjuvant immunotherapeutic modality for early-stage melanoma. Both PDC and MDC subsets in SLN showed signs of increased phenotypic activation, while cytokine and T cell subset profiles further evidenced improved conditions for the generation of cell-mediated anti-tumour immunity in patients receiving PF-3512676. In conclusion, intradermal injections of both GM-CSF and PF-3512676 are tolerated well and have shown to exert immunostimulatory effects on different SLN-DC and T cell subsets in early stage melanoma patients. It will be of particular interest to establish the effects of a combination of GM-CSF and PF-3512676 on the studied immune effector functions.

**CONCLUSIONS**

Melanoma patients who are not eligible for adjuvant therapy but who are at risk for occult nodal metastasis (Breslow thickness of 1-4mm)\textsuperscript{12} might particularly benefit from immunopotentiation of the SLN. Local administration of immunostimulatory agents like GM-CSF and PF-3512676 will counteract the prevailing tumour-associated immunosuppression observed in SLN and may thus help prevent further metastatic spread (Fig. 1). Immunomodulation of the melanoma SLN offers a promising adjuvant treatment option and the SLN procedure in early stage melanoma patients presents a unique translational setting to study these adjuvant therapies \textit{in vivo}. 
Figure 1. Immune status of the melanoma sentinel lymph nodes (SLN) and possible consequences for metastatic spread: comparison between non-intervention and immune intervention strategies. 1. Quiescent Langerhans cells (LC) in the epidermis of the skin may take up cellular fragments from the primary melanoma (in grey), but will also be conditioned by tumour-derived suppressive factors to remain in a suboptimally activated state, prohibiting efficient migration to the SLN. 2a. Upon excision of the primary tumour, but without additional intervention, low-rate steady state migration of mature LC in a low activation state will take place. 2b. In the case of immune intervention through intradermal injection of cytokines and/or TLR ligands (TLR-L) around the excision scar of the primary tumour, optimal activation and migration of the LC through the dermis to the SLN is induced (e.g. by GM-CSF). 3a. Most likely leading to 1) suboptimal or even a complete lack of tumour-specific cytotoxic T lymphocyte (CTL) activation by the migrated LC, 2) a lack of plasmacytoid dendritic cell activation (PDC) and 3) possible outgrowth and further spread of micrometastases through the efferent lymphatics. 3b. In the case of immune intervention through intradermal injection of cytokines and/or TLR ligands (TLR-L) around the excision scar of the primary tumour, optimal activation and migration of the LC through the dermis to the SLN is induced (e.g. by GM-CSF).
ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the Fritz Ahlqvist Foundation and the Netherlands Organization for Scientific research (NWO VIDI grant 917-56-32).

REFERENCES


Outline of this thesis
Diagnostic studies
With adjuvant therapies still under investigation, early detection is the only way to improve melanoma patient survival. The influence of incisional biopsies on melanoma patient survival has been discussed for many years. **Chapter 2** investigates both the influence of diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen on disease free and overall survival.

**Immunomodulation of the melanoma sentinel lymph node: GM-CSF**
Early melanoma development is accompanied by impaired immune effector functions in the initial tumor-draining lymph node, the sentinel lymph node (SLN), and may facilitate early metastatic events that characterize this tumor type. Most notably, a reduced frequency and activation state of professional antigen-presenting cells, the so-called dendritic cells (DC), interferes with uptake and presentation of tumor-associated antigens to tumor-specific CD8+ T cells and T helper cells. Immunopotentation in these early stages may therefore be a valuable adjuvant treatment option. Granulocyte/macrophage colony-stimulating factor (GM-CSF) is known to stimulate myeloid DC (MDC) at all stages of development, by mobilizing DC precursors to the blood and differentiated DC to the site of administration, by activating them, stimulating their migration to lymph nodes and by endowing them with an increased resistance to apoptosis. **Chapter 3** investigates the effect of intradermal administration of GM-CSF around the excision site of primary melanoma tumors on the number and activation state of SLN MDC and finds both to be significantly increased. In view of the critical role of MDC in the initiation of T cell-mediated immunity, **Chapter 4** investigates the influence of potentiated MDC functions in the GM-CSF-administered group on numbers of tumor-specific CD8+ T cells in the SLN and blood of melanoma patients. In secondary lymphoid tissues mature and immature DC are thought to differentially modulate T cell responses. While under pro-inflammatory conditions mature DC are believed to induce T cell activation, under steady state conditions immature DC are believed to maintain a state of T cell tolerance. Yet, little is known about the actual activation state of human DC under these different conditions. In **Chapter 5** the frequency and activation state of human DC between matched skin and SLN samples, following intradermal administration of either GM-CSF or saline, are compared.

**Immunomodulation of the melanoma sentinel lymph node: PF-3512676**
**Chapter 3 and 4** report the effect of GM-CSF administration on the number and activation state of MDC and the frequency of melanoma-specific CD8+ T cells in the SLN of early-
stage melanoma patients, demonstrating the clinical feasibility and immunopotentiating effects of this approach, specifically targeting MDC subsets. However, plasmacytoid DC (PDC) constitute another important DC subset in lymph nodes with potential antigen presenting and T cell activating capabilities. Bacterially derived unmethylated Cytosine-phosphate-Guanine oligodeoxynucleotides (CpG ODN) directly stimulate PDC through Toll-like receptor 9 triggering. CpG-activated PDC in turn release interferon-α and may thus boost T- and natural killer cell responses as well as activate MDC. Chapter 6 delineates the effect of intradermal administration of immunostimulatory agent CpG-B ODN PF-3512676 (formerly known as CpG 7909) around the excision site of primary melanoma tumors on both DC and T cell subsets in the melanoma SLN. Local administration of PF-3512676, as described in Chapter 6, resulted in increased PDC and MDC activation status, the induction of a novel TRAIL⁺ MDC subset, a pro-inflammatory type-1 T cell cytokine profile, and reduced regulatory T cell frequencies. Encouraged by these findings Chapter 7 investigates whether, like with GM-CSF, these PF-3512676-induced immunostimulatory effects on both DC and T cell subsets in the SLN translate into higher melanoma-specific CD8⁺ T cell frequencies in both the SLN and blood of melanoma patients.

In Chapter 8 a summary and implications of the findings presented in this thesis are discussed and directions for future research are outlined.
Non-radical diagnostic biopsies do not negatively influence melanoma patient survival

Annals of Surgical Oncology Oncology 2007;14:1424-30

BG Molenkamp
BJR Sluijter
B Oosterhof
S Meijer
PAM van Leeuwen
ABSTRACT

Background: In fair-skinned Caucasian populations both the incidence and mortality rates of cutaneous melanoma have been increasing over the past decades. With adjuvant therapies still being under investigation, early detection is the only way to improve melanoma patient survival. The influence of incisional biopsies on melanoma patient survival has been discussed for many years. This study investigates both the influence of diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen on disease free and overall survival.

Methods: After (partial) removal of a pigmented skin lesion 471 patients were diagnosed with stage I/II melanoma and underwent re-excision and a sentinel node biopsy. All patients were followed prospectively, mean follow up >5 years. Patients were divided according to their diagnostic biopsy type (wide excision biopsy, narrow excision biopsy, excision biopsy with positive margins and incisional biopsy) and the presence of residual tumor cells in their re-excision specimen. Survival analysis was done using Cox’s proportional hazard model adjusted for eight important confounders of melanoma patient survival.

Results: The diagnostic biopsy was wide in 279 patients, narrow in 109 patients, 52 patients underwent an excision biopsy with positive margins and 31 patients an incisional biopsy. In 41 patients residual tumor cells were present in the re-excision specimen. Both the diagnostic biopsy type and the presence of tumor cells in the re-excision specimen did not influence disease free and overall survival of melanoma patients.

Conclusions: Non-radical diagnostic biopsies do not negatively influence melanoma patient survival.
INTRODUCTION

In fair-skinned Caucasian populations cutaneous melanoma is an important growing public health problem, causing a heavy burden on healthcare services. Both its incidence and mortality rates have been increasing in Europe over the past decades.\(^1\) The absolute total number of new cases of melanoma in the Netherlands is expected to be more than 4800 in 2015, compared with around 2400 in 2000.\(^2\) The Netherlands, as many other countries, has a two-tiered medical care system in which patients need to seek a medical opinion initially from a general practitioner (GP) before referral if necessary, to a specialist.

The mean number needed to treat (NNT), defined as the mean number of pigmented lesions needed to be excised to identify one melanoma, among 468 GPs in Perth, Australia, was 29 and ranged from 83 in the youngest patients (≤19 years) to 11 in the oldest patients (≥70 years).\(^3\) Assuming that for each new case of melanoma another 20-50 patients with pigmented skin lesions will visit the GP, the demand for detection will increase quite markedly. With adjuvant therapies still being under investigation, early detection is the only way to improve melanoma patient survival.\(^4\) Dutch guidelines recommend pigmented skin lesions suspect for melanoma to be removed through a diagnostic excision biopsy with a minimal lateral clearance of 2 mm.\(^5\) This recommendation is inline with the advice of the National Institutes of Health (NIH) to remove any suspicious lesion through excisional biopsy with a narrow margin of normal-appearing skin.\(^6\) The influence of an incisional biopsy on melanoma patient survival has been discussed for many years and different investigators have found contradicting results.\(^7\)\(^-\)\(^15\) Two recent publications concluding incisional biopsies not to interfere with melanoma patient survival were not able to end this discussion since patient groups were not fully comparable\(^16\) or follow up (FU) was short.\(^17\)

This study investigated both the influence of diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen on melanoma patient disease free survival (DFS) and overall survival (OS), in 471 patients with a mean FU of more than 5 years. Survival analysis was done using Cox’s proportional hazard model adjusted for; gender, age, site of primary melanoma, Breslow thickness, type of melanoma, ulceration, lymphatic invasion and sentinel node (SN) status. Both the diagnostic biopsy type and the presence of tumor cells in the re-excision specimen were found not to influence melanoma patient survival.
METHODS

Patients
Between August 1993 and September 2004, 551 patients were diagnosed with clinical stage I/II cutaneous melanoma according to criteria of the American Joint Committee on Cancer (AJCC) and underwent re-excision of the primary melanoma site and a SN biopsy. If patients were referred to us from another institution the pathologic characteristics of the primary melanoma were reviewed in our hospital before the SN procedure. All patients were treated according to the same protocol; re-excision margins were 1 cm for melanomas with a Breslow thickness of ≤2 mm and 2 cm for melanomas with a Breslow thickness of >2 mm. To identify and retrieve the SN, the triple technique was used as described previously. In short, the day before surgery patients underwent a dynamic and static lymphoscintigraphy to determine the lymphatic drainage pattern. Just prior to surgery, Patent Blue V (Laboratoire Guerbet, Aulnay-sous-Bois, France) was injected intradermally next to the initial site of the melanoma. During surgery, guided by a hand held gamma probe and the blue staining of the draining tissues, the SN was removed. To investigate the influence on survival, patients were divided both according to their diagnostic biopsy type; wide excisional biopsy (lateral clearance ≥2 mm), narrow excisional biopsy (lateral clearance <2 mm), excisional biopsy with positive margins and incisional biopsy (includes punch) and the presence of residual tumor cells in their re-excision specimen.

Statistical analysis
Data were processed with the Statistical Package for the Social Sciences software for Windows 2000 (SPSS 11.5, Chicago, IL). Cox’s proportional hazard model was used for survival analysis. P values <0.05 were considered significant.

RESULTS

Patient population
Between August 1993 and September 2004, 551 patients were diagnosed with clinical stage I/II cutaneous melanoma, 257 male (46.6%) and 294 female (53.4%) with a mean age of 49.9 years (Table 1). Most primary melanomas were located on the trunk (43.7%) or on the lower extremities (36.7%). Breslow thickness was categorized into four groups (≤1.00 mm; 1.01-2.00 mm; 2.01-4.00 mm; >4.01 mm), but due to spontaneous regression of the primary lesion remained unknown in 38 patients. The majority of patients had a
Diagnostic biopsy and melanoma patient survival

Superficial spreading melanoma (65.0%) or a nodular melanoma (26.7%). In 46 patients the type of melanoma was different or remained unknown (8.3%). Ulceration, defined as the absence of intact epidermis overlying the major portion of primary melanoma, was diagnosed in 80 patients (14.5%), unknown in 1 patient (0.2%) and absent in 470 patients (85.3%). Lymphatic invasion was present in 25 patients (4.5%), absent in 521 patients (94.6%) and remained unknown in 5 patients (0.9%). The SN was negative in 446 patients (80.9%) and positive in 94 patients (17.1%). In 11 patients the SN was not removed and the SN status remained unknown (2.0%). In total, there were 101 missing variables in 80 patients; all were excluded from the study.

### SN identification

In 11 of the 551 patients the SN status remained unknown (2.0%), in 5 of these patients the SN was located in the deep lobe of the parotid gland and in one patient the SN was located high in the left axilla, in all cases the decision was made not to remove the SN to avoid potential morbidity associated with the intervention. The SN was not identified in 3 cases due to non-visualization by preoperative lymphoscintigraphy. In one patient the SN was located in the right axilla and could not be removed because the patient was suffering from frozen shoulder syndrome, the physical condition of another patient did not allow further treatment. Therefore, the success rate of SN identification was 98% (540 of 551 patients). Two of the patients with the SN located in the deep lobe of the parotid gland experienced metastasis of the parotid gland, one patient is still alive with disease and one patient is dead of disease. The patient whose physical condition did not

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
<th>Patients (n=551)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristics</strong></td>
<td><strong>Follow up (years)</strong></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>5.1 (2.8)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Male 257 (46.6%)</td>
</tr>
<tr>
<td></td>
<td>Female 294 (53.4%)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean (SD) 49.9 (15.3)</td>
</tr>
<tr>
<td><strong>Site of primary melanoma</strong></td>
<td>Lower extremity 202 (36.7%)</td>
</tr>
<tr>
<td></td>
<td>Upper extremity 63 (11.4%)</td>
</tr>
<tr>
<td></td>
<td>Head/Neck 45 (8.2%)</td>
</tr>
<tr>
<td></td>
<td>Trunk 241 (43.7%)</td>
</tr>
<tr>
<td><strong>Breslow thickness (mm)</strong></td>
<td>0 ≤ x ≤ 1 153 (27.8%)</td>
</tr>
<tr>
<td></td>
<td>1 ≤ x ≤ 2 207 (37.6%)</td>
</tr>
<tr>
<td></td>
<td>2 ≤ x ≤ 4 114 (20.7%)</td>
</tr>
<tr>
<td></td>
<td>&gt; 4 39 (7.1%)</td>
</tr>
<tr>
<td></td>
<td>Unknown (regression) 38 (6.9%)</td>
</tr>
<tr>
<td><strong>Type of melanoma</strong></td>
<td>Superficial spreading 358 (65.0%)</td>
</tr>
<tr>
<td></td>
<td>Nodular 147 (26.7%)</td>
</tr>
<tr>
<td></td>
<td>Other/Unknown 46 (8.3%)</td>
</tr>
<tr>
<td><strong>Ulceration</strong></td>
<td>No 470 (85.3%)</td>
</tr>
<tr>
<td></td>
<td>Yes 80 (14.5%)</td>
</tr>
<tr>
<td></td>
<td>Unknown 1 (0.2%)</td>
</tr>
<tr>
<td><strong>Lymphatic invasion</strong></td>
<td>No 521 (94.6%)</td>
</tr>
<tr>
<td></td>
<td>Yes 25 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>Unknown 5 (0.9%)</td>
</tr>
<tr>
<td><strong>Sentinel node status</strong></td>
<td>Negative 446 (80.9%)</td>
</tr>
<tr>
<td></td>
<td>Positive 94 (17.1%)</td>
</tr>
<tr>
<td></td>
<td>Unknown 11 (2.0%)</td>
</tr>
</tbody>
</table>
allow further treatment, passed away soon after re-excision of the primary melanoma site, from massive hematogenic and lymphogenic metastasis. The 8 remaining patients have shown no evidence of disease.

### Diagnostic biopsy type and survival

The influence of diagnostic biopsy type on DFS and OS was tested in 471 patients with a mean FU of more than 5 years; 279 patients (59.3%) underwent a wide excision biopsy, 109 patients (23.1%) a narrow excision biopsy, 52 patients (11.0%) an excision biopsy with positive margins and 31 patients (6.6%) an incision biopsy (Table 2A).

In 91/471 patients (19.3%) the SN was positive, 58/279 patients (20.8%) after a wide excision biopsy, 14/109 patients (12.8%) after a narrow excision biopsy, 15/52 patients (28.8%) after an excision biopsy with positive margins and 4/31 patients (12.9%) after an incisional biopsy.

In 79/471 patients (16.8%) a recurrence was found during FU, 45/279 patients (16.1%) after a wide excision biopsy (21 locoregional skin, 8 SN basin and 16 systemic), 17/109 patients (15.5%) after a narrow excision biopsy (7 locoregional skin, 2 SN basin and 8 systemic), 10/52 patients (19.2%) after an excision biopsy with positive margins (5 locoregional skin, 2 SN basin and 3 systemic) and 7/31 patients (22.6%) after an incisional biopsy (5 locoregional skin, and 2 systemic). But confounding factors were not equally distributed between the diagnostic biopsy groups. Therefore, survival analysis was done using Cox's proportional hazard model adjusted for; gender, age, site of primary melanoma, Breslow thickness, type of melanoma, ulceration, lymphatic invasion and sentinel node (SN) status.

In univariate analysis; gender, age, Breslow thickness, type of melanoma, ulceration, lymphatic invasion and SN status were all significantly related to both DFS and OS.

### Table 2. Patient distribution according to A) diagnostic biopsy type and B) the presence of residual tumor cells in the re-excision specimen

<table>
<thead>
<tr>
<th>A</th>
<th>Patients (n=471)</th>
<th>Follow up (years;mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnostic biopsy type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wide excision biopsy (≥2 mm)</td>
<td>279 (59.3%)</td>
<td>5.0±3.0</td>
</tr>
<tr>
<td>Narrow excision biopsy (0&lt;x&lt;2 mm)</td>
<td>109 (23.1%)</td>
<td>6.0±2.7</td>
</tr>
<tr>
<td>Excision biopsy with positive margins</td>
<td>52 (11.0%)</td>
<td>5.8±2.9</td>
</tr>
<tr>
<td>Incision biopsy</td>
<td>31 (6.6%)</td>
<td>5.4±2.4</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>Patients (n=441)</td>
<td>Follow up (years;mean±SD)</td>
</tr>
<tr>
<td><strong>Re-excision specimen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No residual tumor cells</td>
<td>400 (90.7%)</td>
<td>5.1±2.9</td>
</tr>
<tr>
<td>Residual tumor cells</td>
<td>41 (9.3%)</td>
<td>5.8±2.6</td>
</tr>
</tbody>
</table>
Diagnostic biopsy and melanoma patient survival

Diagnostic biopsy and melanoma patient survival (Table 3). Site of primary melanoma on the head/neck was in univariate analysis only significantly related to DFS (Table 3). Diagnostic biopsy type did not have a significant relation with either DFS or OS (Table 3).

Even though, diagnostic biopsy type did not have a significant relation with either DFS or OS in univariate Cox regression analysis it was also tested in multivariate Cox regression analysis together with all significant variables from univariate analysis, to investigate its influence on DFS and OS after correction for the confounding factors. In multivariate analysis, the only significant and independent predictors of both DFS and OS were; age, Breslow thickness, lymphatic invasion and SN status (Table 3). Site of primary melanoma on the lower extremities or head/neck and ulceration were in multivariate analysis only significant and independent predictors of DFS (Table 3). Also in multivariate analysis, diagnostic biopsy type did not have a significant relation with either DFS or OS (Fig. 1A, B).

Table 3. Univariate and multivariate Cox regression analysis of disease-free survival and overall survival according to diagnostic biopsy type

<table>
<thead>
<tr>
<th>Tested variables</th>
<th>Disease Free Survival</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>HR</td>
</tr>
<tr>
<td>Diagnostic biopsy type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wide excision biopsy</td>
<td>0.691</td>
<td>1.00</td>
</tr>
<tr>
<td>Narrow excision biopsy</td>
<td>0.360</td>
<td>0.71</td>
</tr>
<tr>
<td>Excision biopsy with positive margins</td>
<td>0.870</td>
<td>0.59</td>
</tr>
<tr>
<td>Incision biopsy</td>
<td>0.581</td>
<td>0.47</td>
</tr>
<tr>
<td>Gender</td>
<td>0.018</td>
<td>1.15</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.004</td>
<td>1.02</td>
</tr>
<tr>
<td>Site of primary melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower extremity</td>
<td>0.091</td>
<td>1.00</td>
</tr>
<tr>
<td>Upper extremity</td>
<td>0.825</td>
<td>0.91</td>
</tr>
<tr>
<td>Head/Neck</td>
<td>0.014</td>
<td>3.40</td>
</tr>
<tr>
<td>Trunk</td>
<td>0.731</td>
<td>0.93</td>
</tr>
<tr>
<td>Breslow thickness (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 &lt; x ≤ 1</td>
<td>&lt;0.001</td>
<td>1.00</td>
</tr>
<tr>
<td>1 &lt; x ≤ 2</td>
<td>0.003</td>
<td>13.19</td>
</tr>
<tr>
<td>2 &lt; x ≤ 4</td>
<td>&lt;0.001</td>
<td>34.11</td>
</tr>
<tr>
<td>&gt; 4</td>
<td>&lt;0.001</td>
<td>15.73</td>
</tr>
<tr>
<td>Type of melanoma</td>
<td>&lt;0.001</td>
<td>1.58</td>
</tr>
<tr>
<td>Ulceration</td>
<td>&lt;0.001</td>
<td>1.83</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>&lt;0.001</td>
<td>3.98</td>
</tr>
<tr>
<td>Sentinel node status</td>
<td>&lt;0.001</td>
<td>3.87</td>
</tr>
</tbody>
</table>
Figure 1. A) Disease free survival rates and B) overall survival rates according to the initial biopsy type.

Figure 2. A) Disease free survival rates and B) overall survival rates according to the presence of residual tumor cells in the re-excision specimen.
The same analysis was done after combining the groups; the wide excision biopsy group was joined with the narrow excision biopsy group and compared to the excision biopsy group with positive margins joined with the incision biopsy group. Still, diagnostic biopsy type did not have a significant influence on either DFS or OS (data not shown).

Residual tumor cells in the re-excision specimen and survival

441/471 Patients (93.6%) underwent re-excision of the primary melanoma site and in 30 patients (6.4%) the diagnostic biopsy was found to be sufficient. In 41/441 patients (9.3%) residual tumor cells were found in the re-excision specimen (Table 2B). All 41 patients with residual tumor cells in the re-excision specimen underwent either an excision biopsy with positive margins or an incision biopsy. In none of the patients with a wide or narrow excision biopsy residual tumor cells were found.

In univariate analysis, gender, age, Breslow thickness, type of melanoma, ulceration, lymphatic invasion and SN status were all significantly related to both DFS and OS (Table 4). Site of primary melanoma on the lower extremity or head/neck was in univariate analysis significantly related to DFS and site of primary melanoma on the head/neck or trunk was significantly related to OS (Table 4). Residual tumor cells in the re-excision specimen did not have a significant relation with either DFS or OS (Table 4).

<table>
<thead>
<tr>
<th>Tested variables</th>
<th>Disease Free Survival</th>
<th>Overall Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>Residual tumor cells</td>
<td>0.094</td>
<td>0.79</td>
</tr>
<tr>
<td>Gender</td>
<td>0.025</td>
<td>1.14</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.007</td>
<td>1.02</td>
</tr>
<tr>
<td>Site of primary melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower extremity</td>
<td>0.032</td>
<td>1.00</td>
</tr>
<tr>
<td>Upper extremity</td>
<td>0.830</td>
<td>0.95</td>
</tr>
<tr>
<td>Head/Neck</td>
<td>0.004</td>
<td>3.22</td>
</tr>
<tr>
<td>Trunk</td>
<td>0.647</td>
<td>0.96</td>
</tr>
<tr>
<td>Breslow thickness (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 &lt; x ≤ 1</td>
<td>&lt;0.001</td>
<td>1.00</td>
</tr>
<tr>
<td>1 &lt; x ≤ 2</td>
<td>0.005</td>
<td>12.82</td>
</tr>
<tr>
<td>2 &lt; x ≤ 4</td>
<td>&lt;0.001</td>
<td>30.16</td>
</tr>
<tr>
<td>&gt; 4</td>
<td>&lt;0.001</td>
<td>15.91</td>
</tr>
<tr>
<td>Type of melanoma</td>
<td>&lt;0.001</td>
<td>1.61</td>
</tr>
<tr>
<td>Ulceration</td>
<td>&lt;0.001</td>
<td>1.55</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>&lt;0.001</td>
<td>4.16</td>
</tr>
<tr>
<td>Sentinel node status</td>
<td>&lt;0.001</td>
<td>3.30</td>
</tr>
</tbody>
</table>
Even though, residual tumor cells in the re-excision specimen did not have a significant relation with either DFS or OS in univariate Cox regression analysis it was also tested in multivariate Cox regression analysis together with all significant variables from univariate analysis, to investigate its influence on DFS and OS after correction for the confounding factors. In multivariate analysis, the only significant and independent predictors of both DFS and OS were; age, site of primary melanoma on the head/neck, Breslow thickness, lymphatic invasion and SN status (Table 4). Also in multivariate analysis, residual tumor cells in the re-excision specimen did not have a significant relation with either DFS or OS (Fig. 2A, B).

**Consistent confounders of melanoma patient survival**

Age, Breslow thickness, lymphatic invasion and SN status were the most consistent, independent and significant confounders of melanoma patient DFS and OS (Table 3, 4). Site of the primary melanoma was not always an independent and significant confounder of melanoma patient survival, but location on the head/neck region consistently carried the highest hazard ratio (HR) (Table 3, 4). Surprisingly, ulceration was not an independent and significant confounder of OS (Table 3, 4).

**DISCUSSION**

Numerous investigators have studied the influence of incisional biopsy on melanoma patient survival and found contradicting results. Fitzpatrick et al found a five-year OS rate of 30% in the incisional biopsy group as compared to 48% in the excisional biopsy group but the different biopsy groups were not matched for important prognostic factors.\(^8\) Epstein et al found a more favorable ten-year OS in the biopsy group (65%) as compared to the primary wide excision group (56%) but biopsy was loosely defined as less than optimal or complete surgical excision.\(^9\) Ironside et al found a five-year OS rate of 66% in the excision- and 43% in the incision biopsy group but failed to describe the distribution of prognostic factors between both groups.\(^10\) Rampen et al also found a worse prognosis for patients after an incision biopsy (14 patients) but the study was small and retrospective.\(^11\) Griffiths et al found no difference in survival but 7/19 incisional biopsy patients were excluded because of missing histopathological data.\(^12\) Survival rates were not significantly different between the incision- and excision biopsy groups of Lederman et al, but patient groups were matched for Breslow thickness only.\(^13\) Lees et al also indicated no significant averse effect of incisional biopsy in 96 patients, but 40% of the histopathological data was not assessable.\(^14\) Austin et al did find a significantly reduced survival in the incisional biopsy group, but patients in the incisional biopsy group were also significantly older.\(^15\) The two most recent publications found no negative influence...
of incisional biopsies on melanoma patient survival but in Bong et al patients groups were not fully matched and in Martin et al median FU was only 28 months.\textsuperscript{16,17}

This study not only investigated the influence of diagnostic biopsy type but also the presence of residual tumor cells in the re-excision specimen on melanoma patient survival in patient groups adjusted for 8 important confounders of survival with a mean FU > 5 years. Excision biopsies with positive margins and incisional biopsies were found not to influence melanoma patient survival. Interestingly, DFS and OS even seemed slightly better in the non-radical biopsy groups (Fig. 1A, B). In line with this, patients with residual tumor cells in their re-excision specimen also had a slightly better survival as compared to patients without residual tumor cells in their re-excision specimen (Fig. 2A, B). Melanoma is the most immunogenic tumor identified to date; melanoma specific T cells are detectable both in the blood and in tumor draining lymph nodes from melanoma patients and their frequency can be increased by specific vaccination.\textsuperscript{21-23}

This intrinsic immunogenicity makes melanoma lesions particularly amenable to therapeutic approaches aimed at strengthening tumor immune surveillance. Did residual tumor cells combined with biopsy induced wound healing trigger the melanoma patient’s immunosystem? In non-melanoma skin cancer (NMSC) biopsy induced tumor regression has previously been described, Swetter et al reported that 24% of NMSCs transected on the initial biopsy showed no residual tumor in the excision specimens and suggested biopsy induced wound healing to play an important role.\textsuperscript{24}

Even though non-radical diagnostic biopsies and residual tumor cells in the re-excision specimen do not have a negative influence on melanoma patient survival, the routine use of incisional biopsies is not recommended. Incisional biopsies often consist only of a small percentage of the surface area of the pigmented skin lesion making it difficult to sample a representative area within the tumor. Somach et al found that 40% of the histopathological features were more pronounced in the re-excision specimen as compared to the incision biopsy and in 20% areas of invasive melanoma not detected in the incisional biopsy were observed in the re-excision specimen.\textsuperscript{25} Further more when melanoma is diagnosed, attempting to evaluate the depth of invasion in an incisional biopsy is treacherous and may lead to over- or underestimation of the invasion.\textsuperscript{26,27} Of course these problems are less prominent in excision biopsies with positive margins, here the majority of the lesion has been removed and only the outer borders are compromised making a sampling error highly unlikely.

In conclusion; age, Breslow thickness, lymphatic invasion and SN status were the most consistent, independent and significant confounders of melanoma patient DFS and OS. The site of primary melanoma and ulceration were also important confounders of survival. Both the diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen did not have a negative influence on melanoma patient DFS and
OS. With melanoma incidence rates rising and early detection of melanoma still being the only way to improve melanoma patient survival, it is important for all physicians to feel confident about removing a pigmented skin lesion suspect for melanoma. Incisional biopsies are not recommended but there is no cause for concern when an excision biopsy turns out to have positive margins.

REFERENCES


Local administration of granulocyte/macrophage colony-stimulating factor increases the number and activation state of dendritic cells in the sentinel lymph node of early stage melanoma

Cancer Research 2004;64:8456-60

RJCLM Vuylsteke
BG Molenkamp
HA Gietema
PAM van Leeuwen
PGJTB Wijnands
W Vos
PJ van Diest
RJ Scheper
S Meijer
TD de Gruijl
ABSTRACT

The initial tumor-draining lymph node, the sentinel lymph node, not only constitutes the first expected site of micrometastasis, but also the first point of contact between tumor-associated antigens and the adaptive immune system. A tumor-induced decrease in the frequency and activation state of sentinel lymph node dendritic cells will impair the generation of effective antitumor T cell responses and increase the likelihood of metastatic spread. Here, we demonstrate that intradermal administration of granulocyte macrophage-colony stimulating factor around the excision site of stage I primary melanoma tumors increases the number and activation state of dendritic cells in the paracortical areas of the sentinel lymph node and enhances their binding to T cells. We conclude that local treatment of melanoma patients with granulocyte macrophage-colony stimulating factor, before surgery, conditions the sentinel lymph node microenvironment to enhance mature dendritic cell recruitment and hypothesize that this may be more conducive to the generation of T cell-mediated antitumor immunity.
INTRODUCTION

Dendritic cells are bone marrow-derived antigen-presenting cells that are critical to the initiation of T cell-mediated immunity. In melanoma, skin-resident myeloid dendritic cells (i.e. the Langerhans Cells) take up and transport tumor-associated antigens to tumor-draining lymph nodes. In order to subsequently activate melanoma-specific T cells, the migrated Langerhans cells need to become activated (i.e. mature) and express high levels of co-stimulatory molecules, as well as appropriate chemokine receptors for their migration to the paracortical T cell areas of the tumor-draining lymph node. Melanoma is the most immunogenic tumor identified to date; tumor-associated antigen-reactive T cells are detectable both in the blood and in tumor-draining lymph nodes from melanoma patients, and their frequency can be increased by specific vaccination. This intrinsic immunogenicity may make melanoma lesions particularly amenable to therapeutic approaches aimed at strengthening tumor immune surveillance. High numbers of sufficiently activated dendritic cells in the lymphatic tumor-drainage catchment area are very important in this regard. Dendritic cell development and activation can both be frustrated by inhibitory factors commonly associated with melanoma. The degree of such immunosuppression in tumor-draining lymph nodes is directly related to their distance to the primary tumor. Indeed, the first lymph node to directly drain the primary tumor, the so-called sentinel lymph node, is the preferential site of early metastasis and shows the most pronounced immunological downregulation. The frequency of paracortical sentinel lymph node-dendritic cell is reduced, and most sentinel lymph node-dendritic cell lack dendritic morphology and display lower expression levels of costimulatory molecules as compared to non-sentinel lymph node-dendritic cell. Such a paralysis of dendritic cells in the first line of immunological defense may well facilitate the metastatic spread of melanoma cells to more distal tumor-draining lymph nodes. Local release of granulocyte/macrophage colony-stimulating factor (GM-CSF) has previously been reported to result in enhanced recruitment of activated dendritic cells to draining lymph nodes in a murine model. Here, we demonstrate that intradermal injection of GM-CSF around the excision site of primary melanoma tumors increases the number and activation state of dendritic cells in the paracortical areas of the sentinel lymph node. A concomitant increase in dendritic cell–T cell clustering is indicative of intensified immune surveillance. These observations lend support to peri-operative local administration of GM-CSF as a valuable immunoadjuvant option in the treatment of early-stage melanoma.
Chapter 3

METHODS

Patients
In this single-blinded Phase II study, twelve patients with stage I melanoma according to criteria of the American Joint Committee on Cancer (all patients with Breslow thickness $\leq 1.5$ mm, aged 18-70 years), who were scheduled to undergo a sentinel lymph node procedure, were assigned randomly to preoperative local administration of either recombinant human GM-CSF or saline. 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.

Triple-technique Sentinel Lymph Node procedure and GM-CSF administration
On day 0, patients underwent a triple-technique sentinel lymph node procedure as described previously.\textsuperscript{11,16} In short, a lymphoscintigraphy was performed at least four hours prior to surgery to determine the lymphatic drainage pattern in a dynamic fashion. Just prior to surgery a blue inert dye was injected adjacent to the site of the primary melanoma excision. During surgery, guided by the blue staining of the draining lymphoid tissues and a hand-held gamma probe, the sentinel lymph node was positively identified, removed and, after isolation of viable sentinel lymph node cells, examined meticulously by the pathologist.\textsuperscript{17}
Both patient groups received four daily intradermal injections directly adjacent to the scar of the primary melanoma excision, from day –3 until day 0 just prior to surgery, with either 3 $\mu$g per kg body-weight recombinant human GM-CSF (Leucomax®; Schering Plough, Maarssen, the Netherlands), dissolved in 1.0 mL saline, or 1.0 mL plain saline.

Isolation of viable Sentinel Lymph Node cells
Immediately after removal, sentinel lymph nodes were collected in sterile ice-cold complete medium, comprising Iscove’s modified Dulbecco’s medium supplemented with 25 mmol/L HEPES buffer (BioWhittaker/CAMBREX, Verviers, Belgium) with 10% FCS, 50 IU/mL penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L $\beta$-mercaptoethanol. Before routine histopathological examination of the sentinel lymph node, viable cells were isolated using a previously described cytological scraping method.\textsuperscript{18} In short, after measuring the size of the sentinel lymph node, it was bisected...
crosswise with a surgical scalpel and the cutting surface of the sentinel lymph node was scraped 10 times with a surgical blade (size no.22; Swann Morton Ltd., Sheffield, United Kingdom). Sentinel lymph node cells were rinsed from the blade with medium containing 0.1% DNAse I, 0.14% Collagenase A (Boehringer Mannheim, Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently placed in PBS with 5 mmol/L EDTA for 10 minutes on ice. Finally, the sentinel lymph node cells were washed twice in complete medium, counted, and further processed.

**Flow cytometry**

Freshly isolated sentinel lymph node cells were directly stained with antibodies labeled with either phycoerythrin or fluorescein isothiocyanate, and analyzed by flow cytometry at 100,000 events per measurement, as previously described. Monoclonal antibodies against CD1a, CD14, CD86 (Pharmingen, San Diego, CA), CD40, and CD83 (Immunotech, Marseille, France) were used.

**Immunocytochemistry**

Cytospin preparations were acetone-fixed and stained immunocytochemically as described previously. Monoclonal antibodies against CD1a, CD3, CD14, CD86 (Becton Dickinson, San Jose, CA), CD40, CD83 (Immunotech, Marseille, France) and S100 (DAKO A/S, Glostrup, Denmark) were used.

The number of positively stained dendritic cells and the number of T cells clustered per dendritic cell were determined with an interactive video morphometry system (Q-PRODIT®, Leica, Cambridge, United Kingdom). The outer border of each cytospot was demarcated at a 100-fold magnification, and 40 fields of vision were randomly selected in an automated manner for subsequent evaluation. The total number of CD3+ T cells was counted in these 40 fields of vision and used to correct for cell density of the cytospots of each patient. In each field of vision, the number of dendritic cells was counted on the basis of positive staining of specific markers and dendritic cell morphology. Furthermore, numbers of T cells directly clustered to the dendritic cells were counted. Results are listed as total number of dendritic cells, normalized per 600 CD3+ T cells (i.e., the mean number of T cells detected per 40 fields of vision), and a mean number of T cells clustered per dendritic cell.

**Immunohistochemistry**

Paraffin sections were mounted on Superfrost Plus glass slides and dried overnight at 37°C. After deparaffination, the tissue sections were hydrated through decreasing (v/v) percentages of etomidade, and endogenous peroxidase was blocked with 0,1% hydrogen
peroxide in methanol. Tissue sections were pre-treated with 10 mmol/L citrate (pH 6) in an autoclave for 21 minutes at 121°C [for CD14 (1:25), CD83 (1:25) and an isotype-matched control antibody] or in a microwave at 100°C for 10 minutes [CD1a (1:25) and CD68 (1:400; DAKO A/S)]. All antibodies (except CD68) were applied and incubated at room temperature for one hour. Detection and visualization were performed with the DAKO Chemmate™ Envision™ detection kit (DAKO A/S) according to manufacturers’ instructions. For the CD68 antibody, an automated immunostainer (Ventana, Tuscon, AZ) was used for all incubation, detection and visualization steps according to the manufacturers’ instructions. Sections were counterstained with haematoxylin, dehydrated, and mounted. Tonsillar tissue sections were used as positive control samples.

Statistical analysis

Differences between patient study groups were analyzed using the two-sample Mann-Whitney U test and considered significant when $P<0.05$.

RESULTS

Clinical observations

No significant differences in patient characteristics were observed between the two study groups (Table 1). Administration of recombinant human GM-CSF was well tolerated by all patients. Apart from minor musculoskeletal pain, mild fever, and general tiredness after the first injection in some patients, no significant side effects or skin abnormalities were observed. The sentinel lymph node contained no metastatic tumor cell deposits in any of the patients after routine pathological examination. The sentinel lymph nodes in the GM-CSF group were significantly larger in volume as compared to the control group (Table 1). Also, higher sentinel lymph node cell yields were obtained after scraping the cutting surface of the sentinel lymph node from GM-CSF-administered patients, although this did not reach significance in comparison to the control group.

Table 1. Patient and SLN characteristics in the GM-CSF and saline control groups

<table>
<thead>
<tr>
<th></th>
<th>GM-CSF</th>
<th>Control</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>4:2</td>
<td>3:3</td>
<td>NS</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td>56±11</td>
<td>57±15</td>
<td>NS</td>
</tr>
<tr>
<td>Breslow thickness (mm; mean±SD)</td>
<td>1.04±0.33</td>
<td>0.84±0.27</td>
<td>NS</td>
</tr>
<tr>
<td>Volume* SN (mm$^3$; mean±SD)</td>
<td>1668±296</td>
<td>759±146</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Yield scraping (x10$^6$; mean±SD)</td>
<td>69.6±23.8</td>
<td>20.4±5.69</td>
<td>NS</td>
</tr>
<tr>
<td>Positive SLN</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Volume: height x width x length; Abbreviations: SLN, sentinel lymph node; NS, not significant
Distribution and morphology of Sentinel Lymph Node-Dendritic Cells in situ

Immunohistochemical staining for the dendritic cell markers CD1a and CD83 revealed colocalization of both markers in the paracortical regions (i.e., T cell areas) of the sentinel lymph node (Fig. 1A). CD1a+ and CD83+ sentinel lymph node cells had distinct irregular dendritic cell morphology (Fig. 1A). In contrast to CD1a+ and CD83+ dendritic cells, cells positive for the monocyte/macrophage markers CD14 and CD68 are preferentially colocalized in the marginal sinuses of the sentinel lymph node and displayed a more rounded macrophage-like morphology (Fig. 1B). In none of the GM-CSF-administered patients, hyperplasia of lymphoid follicles or paracortical areas was observed, nor did the distribution patterns of the CD1a+/CD83+ dendritic cells or the CD14+/CD68+ macrophages inside the sentinel lymph node differ between the GM-CSF and the control group (data not shown).

Increased frequency and activation state of Sentinel Lymph Node-Dendritic Cells upon intradermal administration of GM-CSF

Flowcytometric analysis showed a significantly increased frequency of CD1a+ sentinel lymph node-dendritic cells to be associated with GM-CSF administration, while no such association was apparent for the frequency of CD14+ monocytes/macrophages (Fig. 2A). Mean percentages of CD1a+ sentinel lymph node-dendritic cells were 0.68% (range 0.31 to 1.03%) and 0.15% (range 0.02 to 0.35%) for the GM-CSF and the control group, respectively (P<0.05). Of note, no CD1a/CD14 double-positive cells were detected, indicating that these markers defined two distinct myeloid populations in the sentinel lymph node, i.e., CD1a+ dendritic cells and CD14+ macrophages. These data clearly demonstrate a specific stimulatory effect of local GM-CSF on the migration and recruitment of dendritic cells to the draining lymph nodes. The CD1a+ sentinel lymph node-dendritic cells were selectively gated and analyzed for expression levels of dendritic cell activation markers (Fig. 2B). Expression levels of the maturation marker CD83 and of the costimulatory markers CD86 and CD40 were all significantly increased in the GM-CSF group as compared to the control group (Fig. 2C), demonstrating an enhanced activation state of the sentinel lymph node-dendritic cell. Of note, neither in the GM-CSF-administered patients nor in the patients receiving saline injections, could any CD1a+/CD83- sentinel lymph node-dendritic cell be detected, indicating the absence of immature Langerhans cells in the sentinel lymph node. Immunocytochemical analysis was in agreement with the flowcytometric data, showing significant and comparable increases in the frequency of CD1a+ sentinel lymph node-dendritic cells after intradermal recombinant human GM-CSF administration: from on average one dendritic cell per 600 CD3+ T cells in the control group (equaling about 0.15%) to 6 to 10 DC per 600 T
Figure 1. Colocalization of CD1a and CD83 positive dendritic cells (DC) and of CD68 and CD14 positive macrophages in sentinel lymph nodes (SLN). Positive cells in SLN paraffin sections are stained brown. A) CD1a and CD83 positive cells are colocalized in the paracortical areas (top panels, magnification 25x) and have an irregular DC-like morphology (bottom panels, magnification 400x). Representative data are shown from a patient who had received intradermal saline placebo injections. B) CD68 and CD14 positive cells are both found in the (subcapsular) sinuses (top panels, magnification 25x) and display a rounded macrophage-like morphology (bottom panels, magnification 400x). Representative data are shown from a patient who had received intradermal GM-CSF injections.
Local GM-CSF administration activates SLN-DC

Figure 2. Flowcytometric analysis reveals increased frequencies and activation state of CD1a+ dendritic cells (DC) in melanoma sentinel lymph nodes (SLN) upon local GM-CSF administration. A) Percentages of CD1a+ and CD14+ cells in SLN single-cell suspensions in patients who received four consecutive daily intradermal injections of GM-CSF or of placebo (saline control). Horizontal bars represent mean percentages. B) CD1a+ SLN-DC were gated (see dot plot scatter diagrams [CD1a/iso]) and analyzed for the expression of maturation markers (see the corresponding histograms for isotype control, CD83, CD86, and CD40). Expression of the indicated markers is listed as mean fluorescence (MFL). Data are shown from two representative patients, one receiving placebo injections (saline control) and one receiving GM-CSF. C) Mean fluorescence intensities obtained with isotype control mAbs or with mAbs against CD83, CD86, or CD40, on CD1a+ SLN-DC in patients receiving injections with placebo (saline control, n=6) or with GM-CSF (GM-CSF, n=6). Error bars represent standard error of the mean (SEM). All significant differences (P<0.05) are indicated by asterisks.
cells (1-1.5%) in the GM-CSF group ($P<0.05$, Fig. 3A). Similar and significant increases were observed for the dendritic cell activation markers CD83, CD86, CD40, and S100 (all at $P<0.05$), but not for the macrophage/monocyte marker CD14 (Fig. 3A). Equal counts for CD1a and the dendritic cell activation markers reflect the mature state of the CD1a+ sentinel lymph node-dendritic cells. On the sentinel lymph node cytospots clear sentinel lymph node-dendritic cell/T cell clusters (determined on the basis of CD3 positivity; data not shown) were observed in the samples from the patients that had received intradermal recombinant human GM-CSF (Fig. 3B). Quantitation showed significantly higher numbers (four on average) of T cells in direct contact to sentinel lymph node-dendritic cells in GM-CSF-administered patients than in patients that had

**Figure 3.** Increased frequencies of mature dendritic cells (DC) in melanoma sentinel lymph nodes (SLN) and increased T cell/SLN-DC clustering upon local GM-CSF administration. A) Immunocytochemical analysis revealed higher numbers of mature SLN-DC in patients receiving GM-CSF (n=6) than in patients receiving saline (i.e. control, n=6), as judged by the number of DC positive for CD1a and positive for the maturation markers CD83, CD86, CD40, and S100. CD14+ macrophages were not significantly increased. Counts are based on the evaluation of 40 (100x) fields of vision (FOV) and corrected for the cell density of the cytospot by normalization in relation to the number of T cells in 40 FOV (quantified by CD3 staining); expressed as number of DC per 600 T cells. B) Clustering of T cells to S100+ SLN-DC (magnification 400x). Results are shown for a representative control and GM-CSF-treated patient. C) Mean number of T cells clustered per DC or macrophage- by staining of CD1a and the activation markers CD83, CD86, CD40, and S100, or the monocyte/macrophage marker CD14 and based on the evaluation of 40 (100x) FOV. Error bars represent standard error of the mean (SEM). Asterisks denote significant differences between the saline control the GM-CSF treated patient groups ($P<0.05$).
received intradermal saline injections (one T cell per sentinel lymph node-dendritic cell on average; Fig. 3C). Again, this T cell clustering effect was found to be dendritic cell specific because the numbers of T cells in direct contact to CD14+ macrophages was not increased by GM-CSF administration (Fig. 3C).

DISCUSSION

In this randomized trial, we have shown that short-term local administration of GM-CSF significantly increases the number and activation state of dendritic cells in the sentinel lymph nodes of stage I cutaneous melanomas. Although the study of non-sentinel lymph nodes in these patients would have provided valuable information on the specificity of the observed effects for sentinel lymph nodes, we were bound by restrictions from the local ethical committee and could not perform these comparative studies. Previous reports, however, clearly demonstrated a preferential decrease in sentinel lymph node-dendritic cell frequency and maturation state as early as stage I of melanoma development.\textsuperscript{12,13} The fact that these inhibitory effects on dendritic cell activation and migration to the tumor-draining lymph node are directly related to the proximity of the primary tumor indicates the causative agents to be tumor-derived. Data from earlier studies suggest that IL-10 and gangliosides may be good candidates in this regard.\textsuperscript{7,8} Clearly, the crippling of dendritic cell functions in the principal nodes involved in immune surveillance will frustrate specific T cell activation and likely increase the chance of tumor immune escape and metastasis.

GM-CSF has powerful stimulatory \textit{in vivo} effects on dendritic cell recruitment, activation, and survival.\textsuperscript{20} This makes it a prime candidate to test its modulatory effects on sentinel lymph node-dendritic cell numbers and on their activation state in a clinical setting. Evidence that systemic and long-term GM-CSF administration can increase tumor-draining lymph node-dendritic cell frequencies and improve clinical outcome was recently reported by us in patients with locally advanced breast cancer.\textsuperscript{21} Here, we demonstrate that in a relatively short period (four injections in four days) and in a localized fashion (intradermal injections around the tumor excision scar), the frequency of sentinel lymph node-dendritic cells can be quadrupled and their phenotypic maturation state increased (as determined by a quantitative flow cytometric method). This indicates the applicability of GM-CSF administration in the treatment of early-stage melanoma. Previous reports of suppressed sentinel lymph node-dendritic cells in stage I melanoma\textsuperscript{12,13} suggest that this GM-CSF-induced upregulation of both sentinel lymph node-dendritic cell frequency and maturation state signifies a restoration of dendritic cell functions to more normal levels. Increased numbers of CD83+ dendritic cells infiltrating the paracortical areas of
the sentinel lymph node upon GM-CSF administration indicate an enhanced migration of mature dendritic cells to the sentinel lymph node T cell zones, which has previously been reported to be CCR7 mediated.22,23 Interestingly, no CD1a+/CD83- immature dendritic cells were present in the single-cell suspensions of either the GM-CSF- or the saline-treated sentinel lymph node. In keeping with this, similar in situ densities and anatomic distributions were observed for both CD1a+ and CD83+ sentinel lymph node-dendritic cells by immunohistochemistry in both study groups. These findings seem to argue against the migration of immature CD83- dendritic cells to lymph nodes where they might effect T cell tolerance under steady-state and tumor conditions.24 Nevertheless, the GM-CSF-induced upregulation of the costimulatory molecules CD40 and CD86 described here is consistent with suboptimal sentinel lymph node-dendritic cell maturation in stage I melanoma patients.12,13

A previous study of dendritic cell-T cell interactions in intact lymph nodes has revealed protracted periods of binding between dendritic cells and T cells upon antigen recognition.25 Our finding of a significant increase in the numbers of T cells bound to the sentinel lymph node-dendritic cells upon GM-CSF administration may well be a reflection of this phenomenon, as protracted binding of T cells to dendritic cells will increase the likelihood of finding T cell-dendritic cell clusters at a given time. Thus, local peritumoral GM-CSF administration might lead to enhanced tumor-associated antigen presentation and T cell recruitment and activation in the sentinel lymph node. Indeed, our observation of GM-CSF-induced increases in sentinel lymph node size and cell numbers may also result from this enhanced T cell attraction and retention by activated sentinel lymph node-dendritic cells. In keeping with this, we have found evidence for specific recruitment of tumor-specific cytotoxic T lymphocytes (CTLs) to sentinel lymph nodes (Molenkamp et al., manuscript in preparation). Peritumoral treatment with GM-CSF may be expected to facilitate the activation of tumor specific CTLs, which was recently suggested to offer protection from the outgrowth of micrometastases.22 Nevertheless, the finding of GM-CSF-induced increased numbers of tumor specific-CTLs and a possible diminished outgrowth of micrometastases in the sentinel lymph node has yet to confirm this hypothesis. To this end, we are planning a larger prospective study, which should include patients with melanomas between 1 and 4 mm of Breslow thickness, who are not eligible for adjuvant therapy but who are at risk for occult nodal metastasis.16 Particularly these patients may benefit from local GM-CSF treatment because nodal metastases, despite offering a ready source of tumor-associated antigens, may be expected to interfere with dendritic cell maturation and migration to the sentinel lymph node26 and to thus interfere with proper anti-tumor CTL activation. GM-CSF may counteract these detrimental effects and prevent further metastatic spread and outgrowth.
ACKNOWLEDGEMENTS

The authors would like to thank Ingeborg A. de Jonge for her efforts during this study. She passed away shortly after we finished the first draft of this manuscript. Our thoughts are with her family and friends. Furthermore, the authors wish to thank Schering Plough, Maarssen, the Netherlands, for their kind provision of recombinant human GM-CSF and Dr. Elisabeth Bloemena and Dr. Paula Pohlmann for helpful suggestions and fruitful discussions.

REFERENCES

Tumor-specific CD8+ T cell reactivity in the sentinel lymph node of granulocyte/macrophage-colony stimulating factor-treated stage I melanoma patients is associated with high myeloid dendritic cell content

Clinical Cancer Research 2006;12:2826-33

BG Molenkamp
RJCLM Vuylsteke
PAM van Leeuwen
S Meijer
PGJTB Wijnands
JBAG Haanen
RJ Scheper
TD de Gruijl
ABSTRACT

Purpose: Impaired immune functions in the sentinel lymph node (SLN) may facilitate early metastatic events during melanoma development. Local potentation of tumor-specific T cell reactivity may be a valuable adjuvant treatment option.

Experimental Design: We examined the effect of locally administered Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF) on the frequency of tumor-specific CD8+ T cells in the SLN and blood of Stage I melanoma patients. Twelve patients were randomly assigned to preoperative local administration of either recombinant human GM-CSF or NaCl 0.9%. CD8+ T cells from SLN and peripheral blood were tested for reactivity in an IFNγ ELISPOT-assay against the full-length MART-1 antigen and a number of HLA-A1/-A2/-A3-restricted epitopes derived from a range of melanoma-associated antigens.

Results: Melanoma-specific CD8+ T cell response rates in the SLN were 1/6 for the control group and 4/6 for the GM-CSF-administered group. Only one patient had detectable tumor-specific CD8+ T cells in the blood, but at lower frequencies than in the SLN. All patients with detectable tumor-specific CD8+ T cells had a percentage of CD1a+ SLN-dendritic cells (DC) above median (i.e. 0.33%). This association between above-median CD1a+ SLN-DC frequencies and tumor-antigen-specific CD8+ T cell reactivity was significant in a two-sided Fisher’s Exact Test (p=0.015).

Conclusions: Locally primed anti-tumor T cell responses in the SLN are detectable as early as Stage I of melanoma development and may be enhanced by GM-CSF-induced increases in SLN-DC frequencies.
INTRODUCTION

Melanoma is the most immunogenic tumor identified to date and as such a prime candidate for the implementation of novel immunotherapeutic approaches. In vivo primed T cells reactive to a wide range of melanoma-specific tumor-associated antigens (TAA) are detectable in tumors, in tumor-draining lymph nodes (TDLN), and in the blood of melanoma patients and, most importantly, their frequency can be increased by TAA-specific vaccination.\textsuperscript{1-8} T cell infiltration of melanoma tumor fields has been identified as an important prognostic factor.\textsuperscript{9} Indeed, we recently found that in patients with late stage melanoma tumor-specific tumor-infiltrating CD8\textsuperscript{+} T cells may afford a survival advantage whereas circulating tumor-specific CD8\textsuperscript{+} T cells do not.\textsuperscript{7,10} These findings offer a clear indication of the ability of effector T cells to control melanoma development locally.

For the generation of an effective anti-melanoma T cell response high numbers of sufficiently activated myeloid dendritic cells (MDC) are essential.\textsuperscript{11} MDC take up antigens from the tumor tissue environment and upon sufficient activation transport these to draining lymph nodes for presentation and activation of specific T cells. Unfortunately, immune effector functions in tumor-conditioned microenvironments are often disturbed, resulting in tolerance rather than immune activation. Specifically, MDC differentiation and activation can both be frustrated by melanoma-derived suppressive factors (e.g. IL-10 and gangliosides).\textsuperscript{12-15} The first lymph node to directly drain the primary tumor, the so-called sentinel lymph node (SLN), is the preferred site of early metastasis\textsuperscript{16-18} and takes the brunt of the tumor-induced immunosuppression.\textsuperscript{15} In early stages of melanoma development SLN already show signs of profound MDC suppression, both in terms of numbers and of phenotypic activation.\textsuperscript{19,20} This will likely cripple specific T cell activation, increasing the chances of tumor immune escape and metastatic spread.\textsuperscript{20,21}

We recently reported on the dendritic cell (DC)-modulatory effects of intradermal (i.d.) injections of granulocyte/macrophage colony-stimulating factor (GM-CSF) around the excision site of Stage I melanoma tumors, resulting in significantly increased numbers and activation state of MDC in the paracortical T cell areas of the SLN.\textsuperscript{22} In view of the critical role of MDC in the initiation of T cell-mediated immunity, we hypothesized that potentiated MDC functions in the GM-CSF-administered group might be reflected in a higher number of tumor-specific CD8\textsuperscript{+} T cells in the SLN. This hypothesis is indeed supported by our finding that local priming of melanoma-specific CD8\textsuperscript{+} T cells, as early as Stage I of melanoma development, is associated with a high MDC content of the SLN, as observed in patients receiving locally administered GM-CSF. We conclude that local GM-CSF administration may offer a valuable adjuvant therapy option for early-stage melanoma patients, aimed at the control of early metastatic events.
METHODS

Patients and GM-CSF administration

Twelve patients with Stage I melanoma according to criteria of the American Joint Committee on Cancer (AJCC), scheduled to undergo a SLN procedure were assigned randomly to preoperative local administration of either recombinant human GM-CSF or NaCl 0.9%. All patients qualified for a SLN procedure, with Breslow thickness ≥ 1mm or with Breslow thickness ≤ 1mm, but with Clark level ≥ IV, regression, ulceration or other high risk factors. Patients who had undergone previous immunotherapy or chemotherapy were excluded as well as patients using 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. Both patient groups received daily i.d. injections, with either 3 μg per kg body-weight rhGM-CSF (Leucomax®; Schering Plough, Maarssen, the Netherlands), dissolved in 1.0 ml NaCl 0.9%, or 1.0 ml plain NaCl 0.9%. These injections were given directly adjacent to the scar of the primary melanoma excision, from day -3 until day 0 (just prior to surgery).

Table 1. Patient and SLN characteristics

<table>
<thead>
<tr>
<th></th>
<th>GM-CSF</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>4:2</td>
<td>3:3</td>
<td>0.575*</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td>56±11</td>
<td>57±15</td>
<td>0.747*</td>
</tr>
<tr>
<td>Breslow thickness (mm, mean±SD)</td>
<td>1.04±0.33</td>
<td>0.84±0.27</td>
<td>0.262*</td>
</tr>
<tr>
<td>Positive SLN</td>
<td>0</td>
<td>0</td>
<td>1.000*</td>
</tr>
<tr>
<td>Time from primary excision to SLN procedure (day, mean±SD)</td>
<td>49±32</td>
<td>66±50</td>
<td>0.470*</td>
</tr>
<tr>
<td>HLA-A1</td>
<td>2/6</td>
<td>1/6</td>
<td>1.000†</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>3/6</td>
<td>1/6</td>
<td>0.545†</td>
</tr>
<tr>
<td>HLA-A3</td>
<td>0/6</td>
<td>3/6</td>
<td>0.182†</td>
</tr>
</tbody>
</table>

P-value: *two-sample Mann-Whitney U T-test; †two-tail Fisher’s Exact Test

Triple-technique SLN procedure and isolation of viable SLN cells

On day 0, patients underwent a triple-technique SLN procedure as described previously. In short, the day before surgery patients underwent a dynamic and static lymphoscintigraphy to determine the lymphatic drainage pattern. Just prior to surgery, a blue inert dye was injected adjacent to the site of the primary melanoma excision. During surgery, guided by the blue staining of the draining tissues, the radioactivity and the preoperatively made lymphoscintigraphy, the SLN was removed. Immediately
after removal the SLN was collected in sterile ice-cold complete medium, comprising IMDM supplemented with 25 mM Hepes buffer (BioWhittaker, Verviers, Belgium), 50 IU/ml penicillin-streptomycin, 1.6 mM L-glutamine, and 0.05 mM β- mercaptoethanol, supplemented with 10% FCS. Before routine histopathological examination of the SLN, viable cells were isolated using a previously described cytological scraping method. In short, after measuring the size of the SLN, it was bisected crosswise and the cutting surface of the SLN was scraped 10 times with a surgical blade (Swann Morton Ltd. England, size no. 22). SLN cells were rinsed from the blade with IMDM containing 0.1% DNase I, 0.14% Collagenase A (Boehringer, Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently in phosphate-buffered saline (PBS) with 5 mM EDTA for 10 minutes on ice. Finally, the SLN cells were washed twice in complete medium, counted and further processed. After isolation of viable SLN cells, the bisected SLN was examined by the pathologist according to routine diagnostic procedures.27

Isolation of peripheral blood mononuclear cells (PBMC) and flowcytometry

On day 0, before surgery, from each patient 40-50 ml of blood was drawn. PBMC were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Cells were washed twice with sterile PBS with 0.1% BSA. To determine the patients’ HLA-A1, -A2, or –A3 status, PBMC were stained with mAbs BB7.2 and MA 2.1 (HLA-A2), GAP A3 (HLA-A3), (American Tissue Culture Collection [ATCC], Rockville, MD), and A1/A36 (HLA-A1, One Lambda, Canuga Park, CA), while CD4/CD8 status was checked with directly labeled anti-CD4 and –CD8 (BD, San Jose, CA). Freshly isolated SLN cells were directly stained with PE-labeled CD1a (Pharmingen, San Diego, CA) and FITC-labeled CD83 (Immunotech, Marseille, France) antibodies and analyzed by flowcytometry at 100,000 events per measurement, as previously described.26

T cell expansion

To obtain sufficient cells for functional analysis, and for the sake of uniformity, T cells from both the SLN and PBMC were expanded as described previously.26 Cells were incubated for 1 hour on ice with 2 μg anti-CD3 and 0.4 μg anti-CD28 per 1 x 10^6 cells (kindly provided by Dr. René van Lier, CLB, Amsterdam, the Netherlands) in 100-200 μl complete medium with 5% FCS. After incubation and washing, the cells were placed on 24-well plates, coated with affinity-purified goat-anti-mouse immunoglobulin (1:100; Dako, Glostrup, Denmark) in complete medium with 10% Human Pooled Serum (HPS, CLB, Amsterdam, The Netherlands) at a concentration of 1 x 10^6 cells/ml/well for one
hour at 4°C. The cells were cultured for 48 hours in a humidified 5% CO₂ incubator at 37°C. After 48 hours the cells were resuspended and the contents of each well was divided over four new uncoated wells at a volume of 250 µl per well. To each new well 750 µl fresh culture medium was added and rhIL-2 (CLB, Amsterdam, the Netherlands) to a final concentration of 10 IU/ml. The cells were cultured for another five days, after which they were harvested and counted. This expansion cycle was repeated at least once more or until sufficient numbers were obtained: T cells from four patients in the control group and five patients in the GM-CSF administered group underwent two expansion cycles, from one patient in both the control and the GM-CSF administered group three expansion cycles, and from one patient in the control group four expansion cycles. Finally, the expanded T cells were harvested, counted, frozen and stored for functional analysis at a later date.

**Culture and adenovirus infection of monocyte-derived dendritic cells (MoDC)**

The MoDC were generated according to previously described methods.²⁸ Plastic-adherent monocytes from 3-5 x10⁶ PBMC per ml CM were cultured for seven days in complete medium with 10% FCS in the presence of 100 ng/ml GM-CSF (Schering-Plough, Maarssen, The Netherlands) and 1000 IU/ml IL-4 (CLB, Amsterdam, the Netherlands). DC phenotype (CD1a+/CD14-) was confirmed by flowcytometry as described.²⁹ For each patient autologous MoDC were infected with E1-deleted adenoviral (type-5) vectors encoding the melanoma associated Mart-1 protein (Ad-Mart-1) or (as a negative control) green fluorescent protein (Ad-GFP) (both kindly provided by Dr D.T. Curiel, University of Alabama, Birmingham, AL). MoDC were infected at a multiplicity of infection (MOI) of 100, in a CD40-targeted fashion, using a bispecific antibody conjugate, as previously described by Tillman et al.²⁹ The day following infection, MoDC were washed and further used in a CD8+ T cell activation assay.

**Peptide loading of MoDC and T2 stimulator cells**

A panel of HLA-A1, -A2, or –A3-binding peptides, derived from various melanoma-associated tumor antigens and containing previously described CD8+ T cell epitopes (Table 2), was used for CD8+ T cell reactivity testing. Peptide-loaded T2 cells were employed as stimulator cells in ELISPOT read-outs for HLA-A2+ patients. Additional inclusion of HLA-A1+ and -A3+ patients necessitated the use of immature autologous MoDC for this purpose as HLA-A1- or -A3-transduced T2 cell lines were not available to us at that time. T2 cells or MoDC were loaded overnight in serum-free medium with β2-microglobulin (5µg/ml, SIGMA, St Louis, MO) and melanoma-associated or control peptides (50 µg/ml) at 37°C in a humidified 5% CO₂ incubator. For A2- and A3-binding
Table 2. Peptides used for CD8+ T cell IFNγ ELISPOT analysis

<table>
<thead>
<tr>
<th>Binding to</th>
<th>CD8+ T cell epitope and tumor antigen origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A1</td>
<td>Tyrosinase 145-156</td>
</tr>
<tr>
<td></td>
<td>Tyrosinase 243-251</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>Mart-1 26-35</td>
</tr>
<tr>
<td></td>
<td>Tyrosinase 369-377</td>
</tr>
<tr>
<td></td>
<td>gp-100 154-162</td>
</tr>
<tr>
<td></td>
<td>Mage-A3</td>
</tr>
<tr>
<td></td>
<td>NY-ESO 157-165</td>
</tr>
<tr>
<td>HLA-A3</td>
<td>Mage-A1 96-104</td>
</tr>
<tr>
<td></td>
<td>gp-100 17-25</td>
</tr>
</tbody>
</table>

melanoma CD8+ T cell peptides, A2- and A3-binding control peptides were used, derived from the Human Papillomavirus type-16 E7 and Bcr-abl protein sequences, respectively. As no appropriate control peptide was available for A1-binding melanoma peptides, unloaded MoDC were used as negative control stimulators. 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 IFNγ ELISPOT analysis**

To functionally test the expanded CD8+ T cells for melanoma-specific reactivity, an IFNγ ELISPOT assay was performed. As effector cells, anti-CD3/anti-CD28-stimulated and expanded CD8+ T cells from the SLN and PBMC were used. CD8+ T cells were isolated from the expanded T cell population using the untouched CD8+ mini MACS selection kit, according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation, CD8+ T cells were washed and resuspended in complete medium with 10% FCS and added directly to the transduced or peptide-loaded MoDC or T2 stimulator cells in anti-IFNγ pre-coated Multiscreen 96-well filtration plates (Millipore, Molsheim, France). Plates were seeded with 10,000 stimulator (S)-cells (MoDC or T2 cells) per well and 100,000 or 50,000 effector (E) CD8+ T cells, resulting in E:S ratios of 10:1 and 5:1, respectively. Assays with Ad-transduced MoDC were tested in quadruplicate. All other assays were tested in quadruplicate where possible, but at least in duplicate at each of the E:S ratios. After overnight (18h) incubation the cells were flicked off and an ELISPOT assay was performed as previously described, using a commercially available anti-IFNγ mAb pair (Mabtech, Nacka, Sweden). After development of the plates, spots were counted by an automated ELISPOT-reader (AID Diagnostika, Strassberg, Germany). CD8+ T cell ELISPOT activity was expressed either as the number of spots per well or as the number of specific effector CD8+ T
cells per 1x10^6 CD8+ T cells (obtained by subtracting frequencies of spot-forming CD8+ T cells in control conditions from the spot-forming CD8+ T cell frequencies in the test conditions, based on results from the highest [10:1] E:S ratio). ELISPOT CD8+ T cell responses were considered positive when 1) 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), comparing values obtained from both of the tested E:S ratios and recalculating the number of spots per 100,000 CD8+ T cells 2) the mean number of spots for both E:S ratios of the test condition exceeded the number of spots for the corresponding E:S ratios of the control condition by at least two-fold; and 3) the absolute difference in number of spots between the test and control condition in the highest [10:1] E:S ratio was at least five.

**HLA-A2-tetramer binding analysis**

In all HLA-A2+ patients (n=4), expanded T cells were stained with HLA-A2/peptide tetramers, comprising the same HLA-A2-binding melanoma-associated peptides that were used in the ELISPOT analysis (Table 2). APC-conjugated HLA-A*0201 tetramers were generated as described previously. Expanded T cells from PBMC and SLN (consisting of only T cells, as checked by flowcytometric analysis with a cocktail of lineage-specific mAbs against CD14, CD15, CD16, CD56 and CD19; BD Biosciences, San Jose, CA) were stained with the APC-labeled tetramers (40 µg/ml) by incubation for 15 minutes in CM at 37°C in a humidified 5% CO2 atmosphere, after which the cells were washed and kept at 4°C. The cells were subsequently analyzed by flowcytometry, double-staining (after tetramer binding) for CD8 (PE-labeled, BD Biosciences, San Jose, CA) and gating out dead cells by propidium iodide uptake. The total number of analyzed T cells was at least 1,000,000 events per measurement, as a detection limit of 0.01% of CD8+ T cells was assumed.

**Statistical analysis**

Differences in patient characteristics between test groups were analyzed using the two-sample Mann-Whitney U test and in HLA-status and T cell response rates using the Fisher’s Exact test. Differences were considered significant when P<0.05.
RESULTS

Patients
There were no significant differences in patient characteristics between the GM-CSF and the control group (Table 1). Intradermal administration of GM-CSF was tolerated well and none of the SLN showed metastatic tumor cells on routine histopathological testing. HLA typing was done using flowcytometry and in both treatment groups two patients were negative for either HLA-A1, HLA-A2, or HLA-A3, which made them ineligible for IFNγ ELISPOT testing and HLA-tetramer staining for melanoma-specific epitopes restricted by these HLA types.

CD8+ T cell reactivity in the SLN and peripheral blood
T cell responses against Mart-1 and the tested melanoma peptides (Table 2) were evaluated by IFNγ ELISPOT assay. So as not to interfere with routine diagnostic procedures and to ensure sufficient numbers of T cells for functional analyses, T cells were non-specifically expanded by anti-CD3 and anti-CD28 stimulation, after which untouched CD8+ T cells were isolated by negative selection, using a magnetic bead cocktail, and tested overnight.26 The number of required expansion rounds for sufficient yields of CD8+ T cells was not significantly different between the control and the GM-CSF-administrated group. Also, the mean pre- and post expansion CD4/CD8 ratios were equivalent for the control group (pre expansion CD4/CD8 ratio: 7.33±3.94; post expansion CD4/CD8 ratio: 17.0±14.3) and the GM-CSF-administered group (pre expansion CD4/CD8 ratio: 8.03±4.67; post expansion CD4/CD8 ratio: 16.2±14.5). CD8+ T cell responses against Mart-1 were evaluated independently of HLA status for all patients, using autologous MoDC infected with an adenoviral vector encoding full-length Mart-1. Two GM-CSF-administered patients, but none of the NaCl 0.9%-administered patients, showed a positive Mart-1-specific SLN CD8+ T cell response (Fig. 1). This was not significant in a two-sided Fisher's Exact test (p=0.227).
CD8+ T cell reactivity against defined melanoma epitopes was tested in the HLA-A1, HLA-A2, or HLA-A3 positive patients and the obtained response rates in the SLN were 1/4 for the control group and 3/4 for the GM-CSF-administered group. Results for the positively responding patients, including the calculated specific CD8+ T cell frequencies, are shown in Figure 2A. One GM-CSF-administered patient (Patient 8) showed a T cell response against both full-length Mart-1 and melanoma peptides (including the HLA-A2-restricted peptide Mart-1 26-35; Fig. 2A). Overall SLN CD8+ T cell response rates, to either full-length Mart-1 or the melanoma-associated peptides, were 1/6 for the control group and 4/6 for the GM-CSF-administered group (Table 3A). Higher melanoma-specific CD8+ T cell reactivity was consistently found in the SLN than in peripheral
Chapter 4

Figure 1. CD8+ T cell responses against full-length Mart-1 were tested in a 1-day IFNγ ELISPOT analysis in sentinel lymph node (SLN) and peripheral blood (PB) from Stage I melanoma patients receiving intradermally injected NaCl 0.9% (no GM-CSF) or GM-CSF (+GM-CSF). ELISPOT reactivity is expressed as number of spots per 100,000 CD8+ effector T cells. For each patient autologous MoDC were infected with an adenoviral type-5 vector encoding melanoma associated full-length Mart-1 (Ad-Mart-1; closed bars) or with an adenovirus encoding Green Fluorescent Protein (Ad-GFP; negative control; open bars) and used to stimulate expanded and isolated CD8+ T cells. Two GM-CSF administered patients (patient 8 and 11) showed a positive Mart-1-specific CD8+ T cell response in the sentinel lymph node (specific CD8+ T cell frequencies [per 1x10⁶ total CD8+ T cells] are listed).

Figure 2. Melanoma-specific CD8+ T cells from Stage I melanoma patients recognize HLA-A1 and -A2-binding epitopes from multiple melanoma-associated antigens; responses are more prominent in the sentinel lymph node (SLN) than in peripheral blood (PB). A) IFNγ ELISPOT reactivity is expressed as number of spots per 100,000 CD8+ effector T cells. CD8+ T cell responses against peptides derived from various melanoma-associated antigens were tested in a 1-day ELISPOT analysis in SLN and PB. Melanoma-specific and control peptides were loaded on to autologous MoDC (in case of HLA-A1 or –A3 positivity) or T2 cells (in case of HLA-A2 positivity), which were used to stimulate expanded and isolated CD8+ T cells. Data are only shown from positively responding patients; patient number, treatment allocation, and HLA-status are indicated. Positive responses (against the listed epitopes) are denoted by the calculated specific CD8+ T cell frequencies (per 1x10⁶ total CD8+ T cells). B) In HLA-A2+ patients, expanded T cells from SLN were stained with HLA-A2/peptide tetramers, comprising the same HLA-A2-binding melanoma associated full-length Mart-1 that were previously tested in the ELISPOT analysis. Cells were analyzed by flowcytometry after double staining for CD8 and HLA-A2 tetramers; results are shown for patient 9, who previously did not respond in the IFNγ ELISPOT assay to any of the indicated epitopes from Mart-1, Tyrosinase, GP100, MAGE-A3, and NY-ESO. C) For all three positively responding HLA-A2+ patients melanoma-specific CD8+ T cell reactivity determined by IFNγ ELISPOT-assay (open bars) could be confirmed by HLA-A2-tetramer binding analysis (closed bars), although not for all tested epitopes. Nor were the calculated frequencies of specific CD8+ T cells (per 1x10⁶ total CD8+ T cells) always equivalent between both methods. ND: not detectable.
blood. Only one patient (Patient 6) showed any specific CD8+ T cell reactivity in the peripheral blood (Fig. 2A).

For all HLA-A2 positive patients (n=4), HLA-A2-tetramer binding analysis was also performed. Cells were analyzed by flow cytometry after double staining for CD8 and HLA-A2 tetramers (show-cased in Fig. 2B for Patients 9 [a negative responder in the ELISPOT assay] and 12 [a positive responder]). A detection limit of 0.01% was assumed and HLA-A2 tetramer+ populations exceeding this percentage of the total tested CD8+ T cell population were considered to signal positive reactivity against a given epitope. Even though the detected frequencies were not always equivalent, melanoma-specific CD8+ T cell reactivity as determined by IFNγ ELISPOT-assay, could generally be confirmed for all three positively responding HLA-A2+ patients by HLA-A2-tetramer binding analysis (Fig. 2C). Of the 17 peptide tests carried out between the four HLA-A2+ donors, CD8+ T cell reactivity was found both with ELISPOT and by tetramer-binding in seven tests, no reactivity was found with either method in eight tests, whereas ELISPOT reactivity was found which could not be confirmed by tetramer binding in two instances (overall concordance between tests: 88%).

**CD1a+ SLN-MDC content in relation to melanoma-specific CD8+ T cell reactivity**

Previously, we found a significant increase in the frequency of mature CD1a+ CD83+ SLN-MDC after GM-CSF administration.\(^{22}\) Mean percentages of SLN-MDC were 0.68%.

Table 3. CD8+ T cell reactivity rates in the sentinel lymph node (SLN) and peripheral blood (PB) of patients tested for stage I melanoma

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLN</td>
<td>PB</td>
</tr>
<tr>
<td>Ad-Mart-1</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>CD8+ T cell peptides</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Overall CD8+ T cell reactivity</td>
<td>1/6</td>
<td>1/6</td>
</tr>
</tbody>
</table>

**B) CD1a+ SLN-DC content**

<table>
<thead>
<tr>
<th></th>
<th>&lt;0.33% SLN-DC*</th>
<th>&gt;0.33% SLN-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLN</td>
<td>PB</td>
</tr>
<tr>
<td>Ad-Mart-1</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>CD8+ T cell peptides</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Overall CD8+ T cell reactivity</td>
<td>0/6†</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*SLN-DC rate: percentage CD1a+ cells of total SLN cell population; overall median SLN-DC rate=0.33%
†Significantly lower than the corresponding SLN CD8+ T cell reactivity rate in the “>0.33% SLN-DC” group (5/6) in a two-sided Fisher’s Exact test (P=0.015)
Tumor-specific CD8+ T cells and DC in melanoma SLN in the GM-CSF group and 0.15% in the control group ($P=0.006$). When patients were ordered according to the percentage of CD1a+ SLN-MDC, an association with positive melanoma antigen-specific CD8+ T cell reactivity became apparent (Fig. 3). Melanoma-specific CD8+ T cell reactivity was found in 5/6 patients with a percentage of SLN-MDC above median (i.e. 0.33 % SLN-MDC -based on all twelve tested patients), whereas no reactivity was detected in any of the patients with a percentage of SLN-MDC below median (Table 3B). This T cell reactivity was significant in a two-sided Fisher's Exact test ($p=0.015$).

**DISCUSSION**

Cutaneous melanoma is the most aggressive type of skin cancer, for which complete surgical excision at an early stage remains the only curative treatment option. Adjuvant therapy options are limited and show no survival benefits. Naturally occurring melanoma-specific T cell responses have mostly been described in advanced-stage melanoma.
patients, but are already detectable in early stages of melanoma development.\textsuperscript{35} This underlines the immunogenicity of this particular tumor type and raises the possibility to boost these early immune responses by novel immunotherapeutic approaches in order to curb early metastatic events. Unfortunately, melanoma-infiltrating T cells and T cells in melanoma SLN have been described to often be in a state of tolerance due to tumor-induced immune suppression.\textsuperscript{5,36} This is effected both indirectly at the level of DC and their interactions with T cells, and directly on the T cells themselves.\textsuperscript{37,38} As shown in adoptive transfer studies this state of functional tolerance is reversible \textit{in vitro} and after activation and expansion tumor-derived T cells can mediate significant tumor regression in patients with refractory melanoma.\textsuperscript{39-41} DC-based vaccination studies in advanced-stage melanoma patients have also resulted in long-lasting anti-melanoma T cell responses but generally with limited clinical effects.\textsuperscript{35,42} The ability to generate or boost melanoma-specific T cell responses \textit{in vivo} and at earlier, more localized stages of melanoma development, might have clinical benefit through the clearance of early occurring micrometastases.

Lymphatic mapping and selective SLN excision in melanoma patients is a minimally invasive procedure, which allows for the identification of patients at risk for lymph node metastasis who should undergo a full therapeutic TDLN dissection. Recent studies have confirmed the SLN procedure to be safe and to offer a possible survival benefit.\textsuperscript{23,24,43} Routine application of this procedure in early stage melanoma patients presents a unique translational setting to study adjuvant therapies \textit{in vivo} aimed at potentiation of immune reactivity within the SLN. We therefore set up a small-scale clinical trial to investigate the effects of GM-CSF, injected around the scar of the previously removed primary melanoma, on the SLN immune status.

We hypothesized that a superior activation state of MDC in the melanoma SLN would be effected through the local administration of GM-CSF and that such a microenvironment would be more conducive to the generation of T cell-mediated anti-tumor immunity.\textsuperscript{44,45} We previously reported a significant increase in the number, maturation and activation state of CD1a\textsuperscript{+} SLN-MDC after local GM-CSF treatment.\textsuperscript{22} We also found a significant increase in the numbers of T cells bound to the SLN-DC upon GM-CSF administration and concluded that this might well be a reflection of protracted periods of binding between DC and T cells upon specific antigen recognition. In keeping with this we now report that melanoma-specific CD8\textsuperscript{+} T cell response rates were higher in the GM-CSF-administered patients and correlated with CD1a\textsuperscript{+} SLN-MDC content. Moreover, a more robust melanoma-specific CD8\textsuperscript{+} T cell reactivity was observed in the SLN of the Stage I melanoma patients compared to the peripheral blood.

To investigate melanoma-specific CD8\textsuperscript{+} T cell response rates in the SLN, viable T cells were obtained by scraping the cutting surface of bisected SLN. Previous validation of
this technique demonstrated that viabilities and phenotypic characteristics of SLN cells obtained by scraping were entirely comparable to SLN cells obtained by dissociation of the total lymph node (LN). T cell functionality was also comparable between both methods, with equal T-cell expansion factors and similar frequencies of CD8+ T cells specifically recognizing the M1 matrix protein of Influenza Haemophilus or the tumor antigen Her-2/neu.

To facilitate standardized functional testing of tumor-specific CD8+ T cells from all SLN samples, polyclonal T cell expansion was required. The employed polyclonal expansion method was previously shown to efficiently induce the proliferation of tumor-specific T cells from tumor-draining LN, while maintaining specificity at the clonal level, even after three months of culture.

The number of HLA-A2 positive patients was more prevalent in the GM-CSF cohort, with three HLA-A2 positive patients in the GM-CSF- and only one in the saline-administered patient group. As this was a relatively small study, it was important to, as much as possible, avoid any bias in the T cell read-outs that might result from this imbalance in HLA-A2 status between the two test groups. We therefore also included HLA-A1 and -A3 binding melanoma antigen-derived epitopes in our panel to enable us to also test HLA-A1+ and -A3+ patients. This resulted in a more balanced distribution with four evaluable patients in both test groups. Although more evaluable peptides were available for HLA-A2 (Table 1), the number of peptides tested per patient was not solely dictated by HLA status but also by the number of CD8+ T cells that were isolated upon T cell expansion. Overall, the total number of ELISPOT assays per group was balanced with 16 peptides tested in four GM-CSF-administered patients and 13 peptides tested in four saline-administered patients. Of note, in a separate study carried out subsequent to this trial, we have tested six HLA-A2+ Stage-1/2 melanoma patients that were similarly administered saline and none of these displayed any reactivity against the same panel of tested peptides used in this study. This supports our assumption that the under-representation of HLA-A2+ patients in the saline control group of this study is not related to the lack of detection of tumor-specific CTL reactivity in this group. Moreover, as a measure of CTL reactivity independent of HLA status, we also included full-length MART-1 in our assays.

The higher melanoma-specific CD8+ T cell reactivity rate found in the SLN compared to the peripheral blood is consistent with literature and is indicative of local priming. No external source of antigen was provided in the currently applied i.d. GM-CSF administration scheme. This suggests that the detected CD8+ T cells were primed against endogenous tumor-derived antigen sources, despite the fact that at the time of GM-CSF administration no discernable tumor load remained in these patients. Interestingly in this context, recent studies from the Boon/Coulie lab demonstrated that even in the case of melanoma antigen-specific vaccination, reinvigorated pre-existent
anti-tumor cytotoxic T lymphocytes (CTL), rather than newly primed anti-vaccine CTL, were likely responsible for subsequent anti-tumor effects.\textsuperscript{8,50} By way of explanation they suggested that a temporary break of tumor-induced immunosuppression by the vaccination (possibly due to immuno-activating signals from the newly primed anti-vaccine CTL) might reactivate previously primed anti-tumor CTL and set in motion an anti-tumor response. A similar effect may be achieved by breaking immunosuppression with DC-activating agents such as GM-CSF. Our observation of an association between GM-CSF administration, high MDC frequencies, and increased anti-melanoma CD8+ T cell frequencies certainly seems in line with this notion. The fact that we did not add an external tumor antigen source for new T cells to be primed against, suggests that either sufficient tumor-derived antigen traces lingered in the SLN (possibly in the context of residing macrophages or DC) to stimulate the melanoma antigen-reactive CD8+ T cells or that previous activation of anti-tumor CD8+ T cells had already induced a shift in the T cell repertoire resulting in increased anti-tumor T cell frequencies in the SLN, which may have been further reinforced by the general immunostimulation provided by GM-CSF. Importantly, no relationship between T cell reactivity rates and the length of interval between primary melanoma excision and the sentinel lymph node procedure was found: in 3/6 patients with a below median interval and in 2/6 patients with an above median interval melanoma-specific CD8+ T cells were detected. In line with this, the interval length between the responding and non-responding patients did not differ significantly either (64±46 versus 49±35 days, respectively; P=0.371 in the Mann-Whitney U test). Thus, the difference in T cell reactivity rates observed between the two test groups was not attributable to a possible difference in the retention of melanoma-derived antigens due to testing at varying time points subsequent to the removal of the primary tumor (i.e. the melanoma antigen source).

Although this is a small study and these data await confirmation in a larger trial, they are nevertheless important in that they show that 1) anti-tumor CD8+ T cells can already be primed \textit{in vivo} in the earliest stages of melanoma development at a relatively low Breslow thickness and that 2) these CD8+ T cell responses appear to be enhanced by immunostimulatory agents such as GM-CSF. In future studies we aim to also include patients with higher Breslow thickness, who are at greater risk for SLN metastasis. SLN metastases in these patients will increase the risk of immune suppression and further metastatic spread, but will also provide a ready source of tumor antigens for immune priming or boosting. Local GM-CSF administration in these patients will not only allow the further study of such processes as reversal of immune suppression and tumor-specific T cell priming, but also of any clinical benefit in terms of (disease-free) survival.
ACKNOWLEDGEMENTS

We thank Schering Plough for their kind provision of rhGM-CSF.

REFERENCES


Matched skin and sentinel lymph node samples of melanoma patients reveal exclusive migration of mature dendritic cells

American Journal of Pathology 2005;167:1301-7

BG Molenkamp
RJCLM Vuylsteke
PAM van Leeuwen
S Meijer
W Vos
PGJTB Wijnands
RJ Scheper
TD de Gruijl
ABSTRACT

In secondary lymphoid tissues mature and immature myeloid dendritic cells (DC) are thought to differentially modulate T cell responses. While under pro-inflammatory conditions mature DC are believed to induce T cell activation, under steady state conditions immature DC are believed to maintain a state of T cell tolerance. Yet, little is known about the actual activation state of human DC under these different conditions. Here, we compared the frequency and activation state of human DC between matched skin and sentinel lymph node (SLN) samples, following intradermal administration of either granulocyte/macrophage colony-stimulating factor (GM-CSF) or saline, at the excision site of Stage-I primary melanoma. While DC remained immature (CD1a+CD83- ) and mostly situated in the epidermis of the saline-injected skin (fully consistent with a quiescent steady state), mature (CD1a+CD83+) DC frequencies were significantly increased in the GM-CSF-injected skin and correlated with the number of mature DC in the SLN, indicative of increased DC migration. Interestingly, irrespective of GM-CSF or saline administration, all CD1a+ myeloid DC in the SLN were found to be phenotypically mature (i.e. CD83+). These data are indicative of migration of small numbers of phenotypically mature DC under steady state conditions.
INTRODUCTION

Antigen-specific cutaneous immune responses are initiated by epidermal and dermal dendritic cells (DC). The majority of DC in the skin are Langerhans cells (LC), residing in the epidermis in an immature state. These immature LC (iLC) derive from CD34+ haematopoietic progenitor cells. LC precursors home from the bone marrow to the skin, where they differentiate to LC that are actively phagocytic. When an antigen is encountered under pro-inflammatory conditions, LC are activated and start migrating to regional lymph nodes (LN), synthesizing new MHC molecules and up-regulating CD80, CD86 and other co-stimulatory and adhesion molecules. On arrival in the LN they have become mature DC (mDC). Expression of the chemokine receptor CCR7 facilitates their migration to the paracortical areas where T cells reside and may then be primed. Recently it has been suggested that immature DC (iDC) also migrate to the LN to induce peripheral T cell tolerance in the steady state, this way preventing autoimmunity. Data which support iDC migrating to the LN to induce peripheral T cell tolerance, originate mainly from murine studies. In humans iLC were reported in skin-draining LN only under chronic inflammatory conditions. So far, evidence for the presence of iDC in LN under normal steady state conditions is lacking. In melanoma, LC take up and transport tumor-associated antigens (TAA) to tumor-draining lymph nodes (TDLN). In order to subsequently activate melanoma-specific T cells, the migrated LC need to become activated. DC development and activation can both be frustrated by inhibitory factors commonly associated with melanoma, such as IL-10 or gangliosides. iDC with ready access to TAA from the tumor may induce specific tolerance through inappropriate or abortive T cell activation. DC in TDLN were similarly reported to display immature characteristics. The degree of immunosuppression in TDLN is directly related to their distance to the primary tumor, indicating the causative agents to be tumor-derived. The first LN to directly drain the primary tumor, the so-called sentinel lymph node (SLN), is the preferential site of early metastasis and shows the most pronounced immunosuppression. Clearly, this crippling of DC functions in the first line of immunological defense will frustrate specific T cell activation and increase the chance of tumor immune escape and metastatic spread. To overcome this suppression, we recently administered intradermal (i.d.) injections of granulocyte/macrophage colony-stimulating factor (GM-CSF) around the excision site of primary melanoma tumors and found increased numbers and activation state of DC in the paracortical areas of the SLN. In the same study, the absence of iDC in the TDLN of the saline control group seemed to contrast with the currently dominant school of thought that holds iDC in the TDLN to be primarily responsible for cancer-associated immune tolerance. Patients included in this study underwent re-excision of the scar of the primary melanoma excision...
at the same time as the SLN procedure. This gave us the unique opportunity to compare the phenotype of DC in the skin to their phenotype in the exactly corresponding SLN after local administration of either recombinant human GM-CSF or saline. The results reported here are consistent with increased migration of large numbers of mature CD83+ LC through the dermis of GM-CSF-injected skin to the corresponding SLN. In contrast, a quiescent steady state prevails in the control group with iLC scattered throughout the epidermis and only small numbers of isolated CD83+ LC in the dermis. Nevertheless, iDC (CD1a+CD83-) are completely absent in the SLN under both these conditions. We conclude that small numbers of mDC migrate to LN under steady state conditions and that these are apparently responsible for a maintained state of tolerance under these conditions.

**METHODS**

**Patients**

Twelve patients with Stage-I melanoma according to criteria of the American Joint Committee on Cancer (AJCC) (Breslow thickness ≤ 1.5 mm, aged 18-70 years) were included in this single-blinded Phase II study. All patients were scheduled to undergo a SLN procedure and re-excision of the scar of the primary melanoma excision. Re-excision of the scar of the primary tumor in all cases took place subsequent to SLN excision, during the same operative procedure. An excision margin of 1 cm was applied, as routine for melanoma with a Breslow thickness ≤ 2 mm in our hospital. They were randomly assigned to preoperative local administration of either recombinant human GM-CSF or saline. Patients who had undergone previous immunotherapy or chemotherapy were excluded as well as patients receiving immunosuppressive medication or suffering from any autoimmune disorder. The medical ethical committee of the VU University Medical Center approved the study and written informed consent was obtained from each patient before treatment. There were no significant differences between the patient groups in terms of gender, age (average 57±12 years), or Breslow thickness (average 0.94±0.30 mm). None of the patients had a tumor-positive SLN. In the saline and GM-CSF group respectively four and five of the primary tumors were located on the trunk and respectively two and one were located on the extremities. For the saline group this resulted in the excision of the SLN from the groin area in two patients and from the axilla in four patients. In the GM-CSF group all SLN were located in the axilla.
GM-CSF administration
Both patient groups received daily i.d. injections, with either 3 μg per kg body-weight rhGM-CSF (Leucomax®; Schering Plough, Maarssen, the Netherlands), dissolved in 1.0 ml saline, or 1.0 ml plain saline. These injections were given directly adjacent to the scar of the primary melanoma excision, from day -3 until day 0 (just prior to surgery).

Triple-technique SLN procedure and isolation of viable SLN cells
On day 0, patients underwent a triple-technique SLN procedure as described previously. Immediately after removal, SLN were collected in sterile ice-cold complete medium, comprising IMDM supplemented with 25 mM Hepes buffer (BioWhittaker, Belgium) with 10% FCS, 50 IU/ml penicillin-streptomycin, 1.6 mM L-glutamine and 0.05 mM β- mercaptoethanol. Before routine histopathological examination of the SLN, viable cells were isolated using a previously described cytological scraping method. In short, after measuring the size 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 Ltd. England, size no. 22). SLN cells were rinsed from the blade with medium containing 0.1% DNase I, 0.14% Collagenase A (Boehringer Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently in PBS with 5 mM EDTA for 10 minutes on ice. Finally, the SLN cells were washed twice in complete medium, counted, and further processed. After isolation of viable SLN cells, the bisected SLN was examined meticulously by the pathologist according to routine diagnostic procedures.

Flow cytometry
Freshly isolated SLN cells were directly stained with antibodies labeled with either PE or FITC, and analyzed by flow cytometry at 100,000 events per measurement, as previously described. Monoclonal antibodies against CD1a, CD86 (Pharmingen, San Diego, CA) and CD83 (Immunotech, Marseille, France) were used.

Immunocytochemistry
Cytospin preparations of SLN cells were acetone-fixed and stained immunocytochemically as described previously. Monoclonal antibodies against CD1a, CD3, CD86 (Becton Dickinson, San Jose, CA), CD83 (Immunotech, Marseille, France) and S100 (DAKO A/S, Glostrup, Denmark) were used. The number of positively stained DC was determined using an interactive video morphometry system (Q-PRODIT®, Leica, Cambridge, UK). The outer border of each cytospot was demarcated at a 100-fold magnification and 40 fields of vision (FOV) were randomly selected in an automated manner for subsequent evaluation. The total number of CD3⁺ T cells was counted in these 40 FOV and used to
correct for cell density of the cytospots of each patient. In each FOV the number of DC was counted on the basis of positive staining of specific markers and DC morphology. Results are listed as total number of DC, normalized per 600 CD3+ T cells (i.e. the mean number of T cells detected per 40 FOV).

**Immunohistochemistry of the skin**

Paraffin-embedded re-excision skin biopsies were available for 11 of the 12 patients. Paraffin sections were mounted on Superfrost Plus glass slides and dried overnight at 37°C. After deparaffination, 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 10mM citrate (pH 6) in an autoclave for 21 minutes at 121°C (for Langerin, CD83, CD14 and an isotype-matched IgG1 control antibody, MOPC21) or in a microwave at 100°C for 10 minutes (CD1a and CD68). Other tissue sections were pretreated with 10mM tris/ 1mM EDTA (pH9) in a microwave at 100°C for 10 minutes (CD3), or with protease (Ventana, Tucson, AZ) (S100). All antibodies (except CD68 and S100) were applied and incubated at room temperature for one hour. Detection and visualization (with DAB/hydrogen peroxide) were performed with the DAKO Chemmate™ Envision™ detection kit (Dakopatts, Glostrup, Denmark) as described for CD1a, CD83, CD14 and CD3 or with NeoMarkers™ Labvision™

<table>
<thead>
<tr>
<th>Table 1. Markers tested and immunohistochemical detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marker</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>CD1a*</td>
</tr>
<tr>
<td>Langerin*</td>
</tr>
<tr>
<td>CD83*</td>
</tr>
<tr>
<td>CD14*</td>
</tr>
<tr>
<td>CD68†</td>
</tr>
<tr>
<td>S100†</td>
</tr>
<tr>
<td>CD3†</td>
</tr>
<tr>
<td>MOPC21 TT</td>
</tr>
</tbody>
</table>

*Novocastra, Newcastle upon Tyne, UK, †Dakopatts, Glostrup, Denmark, §Organon Teknika-Cappel, Boxtel, The Netherlands, †Ventana, Tuscon, AZ
labvisionkit (NeoMarkers, Fremont, USA) for Langerin. For the CD68 antibody and the S100 antibody an automated immunostainer (Ventana, Tucson, AZ) was used for pretreatment (protease, S100), incubation, detection and visualization steps according to standard procedures. Sections were counterstained with haematoxylin, dehydrated, and mounted. The used antibodies and pretreatments are summarized in table 1. Tonsillar tissue sections were used as positive control samples.

**Quantitation**

All slides were coded and counted by two independent observers. The number of CD1a, Langerin, CD83, CD14, CD68, S100 and CD3 positive cells in the epidermis, the superficial papillary dermis and the deep reticular dermis were evaluated by direct counting of stained nucleated cell bodies per 400x magnification microscopic field (i.e. high power field (HPF)). Each observer counted 10 HPF in the epidermis, in the superficial dermis, defined as the HPF adjacent to the epidermis, and in the deep dermis for each slide. For CD3, 5 adjacent HPF were counted, in the epidermis, superficial and deep dermis. Counts were expressed as mean number (averaged between the independent observers) of positive cells per HPF.

**Statistical analysis**

Differences between patient study groups were analyzed using the two-sample Mann-Whitney U test and considered significant when $P<0.05$. Correlations were calculated using Spearman’s rho test and also considered significant when $P<0.05$.

**RESULTS AND DISCUSSION**

**Intradermal injection of GM-CSF induces maturation and migration of skin DC**

Immunohistochemical analysis of the skin was performed in order to study the effects of pre-operative i.d. injections of GM-CSF and saline on the DC of the epidermis, superficial dermis and deep dermis (Fig. 1). We found significantly more CD1a$^+$, CD83$^+$ and S100$^+$ DC in the epidermis of the GM-CSF administered patients (Fig. 2A). Increased numbers of CD1a$^+$ epidermal DC and the presence of CD83$^+$ and S100$^+$ DC, scattered throughout the epidermis, is highly suggestive of an ongoing recruitment of LC (precursors) and a simultaneous migration of maturing LC from the epidermis, both under the influence of pre-operative GM-CSF administration for four consecutive days. Our results differ from those reported by Smith and colleagues$^1$ who studied the effect of GM-CSF on
Figure 1. DC phenotype and localisation in primary tumor re-excision skin samples after intradermal administration of saline or GM-CSF. Immunohistochemical analysis for the indicated DC markers from two representative melanoma patients (Stage-I) after intradermal injections of either saline or GM-CSF (400x magnification).
LC in normal and healthy atopic volunteers and found that i.d. injections of GM-CSF led to a reduction of CD1a\(^+\) DC in the epidermis in parallel with increased numbers in the dermis. This may be explained by the difference in administered GM-CSF dosages: i.e. 0.05 µg in their study versus 3 µg/kg body weight in our study. The relatively high doses in our study may have led to significantly more CD1a\(^+\) cells in the epidermis resulting from an ongoing recruitment of new LC (precursors).

A significant increase in the amount of CD1a\(^+\), Langerin\(^+\), CD83\(^+\) and S100\(^+\) DC was found in the superficial dermis of the GM-CSF injected skin (Fig. 2B). The equal amount of cells in the superficial dermis expressing CD1a, Langerin and CD83, as well as their co-localization in consecutive sections (Fig. 1), suggests that these are all CD83\(^+\) mature LC in the process of GM-CSF-induced migration.
In the deep dermis of both the saline and the GM-CSF-administered patients, only low amounts of CD1a+, Langerin+, CD83+ and S100+ DC were found (Fig. 2C). This is consistent with DC entering and migrating via lymph vessels, which are mainly situated in the superficial dermis. Significantly increased numbers of CD68+ macrophages were found throughout the skin, including the deep dermis, under the influence of i.d. injections of GM-CSF. In line with the recruitment of immune effector cells by the activated DC and by macrophages, we also found significantly more CD3+ T cells infiltrating the superficial- and the deep dermis upon GM-CSF administration (Fig. 2B, C).

**Strong correlation between frequencies of mature DC in the superficial dermis and the SLN**

The numbers of CD1a, CD83, and S100 positive DC correlated strongly and significantly between the superficial dermis and the SLN (Fig. 3D-F). A less perfect correlation was observed between the numbers of CD1a, CD83, and S100 positive DC in the epidermis and the SLN (Fig. 3A-C). These observations are consistent with the contiguous nature of the superficial dermis and the SLN, with mature DC migrating between these compartments through the lymph vessels. The higher numbers of mature DC in both compartments after GM-CSF administration (Fig. 3A-F) is consistent with DC maturation induction and an increased DC migration rate.

**Only mature DC migrate to the SLN**

The correlation between the number of CD1a+ DC and of CD83+ DC in the SLN is very strong and near linear (r=0.836, p=0.001, Fig. 4A). Flowcytometric analysis of SLN single cell suspensions supports these findings (Fig. 4B), revealing all CD1a+ DC to be CD83+, irrespective of GM-CSF administration. This shows a lack of CD1a+, CD83− immature DC in the SLN, which implies that only CD83+ mDC migrate to the skin-draining LN. This is contrary to the currently prevailing idea that iDC in the LN induce peripheral T cell tolerance in a steady state.3,4 It is also in contrast with findings by Geissmann et al.,7 who reported that DC in the human skin-draining LN were largely immature Langerin+ cells. An explanation for this apparent discrepancy might be that all patients included in the study of Geissmann et al.,7 were suffering from dermatopathic lymphadenitis. Chronic inflammation of the skin may have led to a high local turn-over rate of DC, leading to abnormal iDC migration. Human lymph from normal skin (steady state) was sampled by Brand et al.,27 by means of microsurgical lymph cannulation. It was found that virtually all migrating CD1a+ cells under these conditions co-expressed CD80. Although the expression of CD83 was not tested, these data nevertheless indicate that skin-originating DC in afferent lymph have a mature phenotype, which is in line with our own observation that the expression of CD80 is closely linked to that of CD83 and CCR7
Exclusively mature state of human lymph node DC

In a recent murine study by Stoitzer et al., it was also shown that LC trafficking into the skin-draining LN in the steady state express the maturation marker 2A1, the co-stimulatory molecules CD86 and CD40, and high levels of MHC class II. After application of contact allergen, a small but consistent increase in the expression of CD86 and CD40 was seen. This study thus confirms our findings that even in the steady state, LC migrate from the epidermis in a mature state. Further activation (e.g. by GM-CSF) only leads to higher numbers of mature LC that migrate to the SLN with a coinciding up-regulation of co-stimulatory markers such as CD86 and CD40.

Figure 3. Correlation between DC numbers in the epidermis or superficial dermis and in the sentinel lymph node (SLN). The numbers of DC positive for CD1a, CD83 and S100 in the SLN (expressed as number per 600 T cells: quantified on cytospins from SLN cell suspensions), correlated to the number of DC positive for A) CD1a, B) CD83 and C) S100 in the epidermis and D) CD1a, E) CD83 and F) S100 in the superficial dermis (all shown as mean numbers per 10 high power fields, HPF at 400x magnification). ◊ patients treated with saline, ▲ patients treated with GM-CSF. Correlations were calculated using Spearman’s rho test: r and P values are displayed.
Figure 4. Myeloid DC in skin-draining sentinel lymph nodes (SLN) have a CD83+ mature phenotype, irrespective of intradermal GM-CSF or saline administration. A) Correlation between the number of CD1a+ DC and CD83+ DC in the SLN (expressed as number per 600 T cells) of patients treated with saline (○) and patients treated with GM-CSF (▲) reveals a linear relationship (Spearman’s rho r=0.836, P=0.001). B) Flowcytometric analysis of CD83 expression on myeloid DC in SLN single cell suspensions from all saline- and GM-CSF-treated melanoma patients. Myeloid DC were gated by CD1a positivity and high side scatter levels.
Small numbers of mature DC may maintain tolerance in the steady state LN

In conclusion, our findings do not support the hypothesis that iDC induce peripheral T cell tolerance in the steady state. On the contrary, we have found evidence to suggest that small numbers of mDC that migrate to the LN are responsible for the maintenance of tolerance. We do realize that our data are from Stage I melanoma patients and as such may not qualify as “normal” steady state. However, the primary melanoma was previously resected (on average 89 days prior to the re-excision and SLN procedure) and none of the tested patients harbored metastases in the SLN, minimizing any tumor-associated effects at the time of testing.

The observed mature phenotype of DC in SLN under these quiescent conditions (as established in the corresponding skin samples) is in keeping with a previous study by Albert et al., who reported the necessity of DC maturation for the cross-tolerance of cytotoxic T lymphocytes (CTL). CTL activation required further CD40-mediated activation of the DC by Th cells, which is accompanied by the release of cytokines essential for CTL activation, such as IL-12. Thus, as previously suggested by Shortman and Heath, small numbers of short-lived, phenotypically mature but quiescent DC may induce tolerance, while activated mature DC induce immunity. Our findings from matched melanoma skin and SLN samples certainly seem to underline this notion.

ACKNOWLEDGMENTS

We thank Schering Plough (Maarssen, the Netherlands) for their kind provision of recombinant human GM-CSF.

REFERENCES


Intradermal CpG-B activates both plasmacytoid and myeloid dendritic cells in the sentinel lymph node of melanoma patients

Clinical Cancer Research 2007;13:2961-9

BG Molenkamp
PAM van Leeuwen
S Meijer
BJR Sluijter
PGJTB Wijnands
A Baars
AJM van den Eertwegh
RJ Scheper
TD de Gruijl
ABSTRACT

Purpose: A decrease in the frequency and activation state of dendritic cells (DC) in the sentinel lymph node (SLN) has been observed in early stages of melanoma development. This may hinder the generation of effective anti-tumor 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 (TLR9) and activate plasmacytoid DC (PDC). CpG-activated PDC in turn release interferon-α (IFNα) and may thus boost T and natural killer cell (NK cell) responses as well as activate conventional myeloid DC (MDC).

Experimental design: We studied the effects of pre-operative local administration of the CpG B-type oligodeoxynucleotide (ODN) PF-3512676 (formerly known as CPG 7909) on DC and T cell subsets in the SLN of 23 Stage I-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⁺CD123⁺CD83⁺TRAIL⁺ mature SLN-MDC subset, an increased release of a variety of inflammatory cytokines, and lower frequencies of CD4⁺CD25⁺CTLA-4⁺FoxP3⁺ regulatory T cells (Treg) 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.
INTRODUCTION

Early melanoma development is accompanied by impaired immune effector functions in the initial tumor-draining lymph node (TDLN), the so-called sentinel lymph node (SLN).\cite{1-4} Most notably, the observed reduced maturation state of professional antigen-presenting cells, the dendritic cells (DC), may interfere with the presentation of tumor-associated antigens (TAA) to specific anti-tumor cytotoxic T lymphocytes (CTL) and T-helper (Th) cells in the SLN.\cite{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 (GM-CSF) administration on the number and activation state of myeloid DC (MDC) and melanoma-specific CTL reactivity in the SLN of early-stage melanoma patients, demonstrating the clinical feasibility and immunopotentiating effects of this approach, specifically targeting MDC subsets.\cite{6,7} Plasmacytoid DC (PDC) constitute another important DC subset in lymph nodes (LN) with potential antigen presenting and T cell activating capabilities, which may present an additional immunotherapeutic target.

PDC were first identified as a DC subset distinct from conventional MDC in peripheral blood and secondary lymphoid organs and were found to be responsible for most of the type I interferon (IFN) that is produced in response to viral infection.\cite{8} PDC have a CD11c\(^{-}\)CD123\(^{hi}\) phenotype and differentiate from CD34\(^{+}\) precursors under the influence of IL-3.\cite{8} In blood and LN immature PDC are characterized by their expression of the C-type Lectin BDCA-2.\cite{9,10} Maturation induction \textit{in vitro} results in an upregulation of costimulatory molecules and the maturation marker CD83 and a complete down-regulation of BDCA-2.\cite{10} L-Selectin- and CXCR3-mediated homing and migration allow PDC to travel from the blood to LN.\cite{8} PDC bind microbial products through specific receptors such as Toll-like receptor-9 (TLR9). TLR9 expression in human immune effector cells appears to be restricted to PDC and B cells.\cite{11,12} Bacterially derived unmethylated Cytosine-phosphate-Guanine oligodeoxynucleotides (CpG ODN) directly stimulate PDC through TLR9 triggering. TLR- and/or CD40L-activated PDC preferentially release large amounts of IFN\(\alpha\),\cite{13-15} which may facilitate direct activation of CD8\(^{+}\) T cells and natural killer cells (NK cells) as well as promote the differentiation and maturation of neighboring MDC (precursors) and thus also indirectly stimulate T cell activation,\cite{9,16-18} all of which are effects with potential anti-tumor benefits.

In studies of murine tumor models CpG ODN administration was found to increase the size of vaccine-draining LN, to elevate their type-1 T cell content, and to enhance tumor rejection.\cite{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, while immunization against a tumor-associated epitope in the presence of CpG-ODN resulted in a strong CD8+ T cell response and an increased survival.\textsuperscript{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.\textsuperscript{23} In a recent trial PF-3512676 was administered in combination with Incomplete Freund’s Adjuvant (IFA) to vaccinate melanoma patients against a MART-1 peptide and shown to rapidly induce strong and specific CD8+ T cell responses.\textsuperscript{24} However, in vitro data also point to less desirable effects, such as an increased generation of regulatory T cells (T\textsubscript{reg}) by PF-3512676-activated PDC.\textsuperscript{25}

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

**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, Wellesley, MA) or saline (NaCl 0.9%). Pathological examination revealed six patients with Stage III melanoma based on the presence of tumor cells in the SLN (2/11 patients in the PF-3512676 administered group and 4/12 patients in the saline administered group). In 4/6 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 (LND) and all LN in the additional LND 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
Effects of CpG on the melanoma sentinel lymph node 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 (NCI CTC Toxicity scale Version 2.0) approximately 80% of the PF-3512676 administrated patients had mild constitutional symptoms (grade 1 fatigue, fever and rigors) and grade 1 myalgia. Injection site reaction was present in all PF-3512676 administrated patients with approximately 85% grade 1 and 15% grade 2 reactions. No toxicity was observed in the saline administered patient population.

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

<table>
<thead>
<tr>
<th></th>
<th>PF-3512676</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>6:5</td>
<td>8:4</td>
<td>0.561*</td>
</tr>
<tr>
<td>Age (y)</td>
<td>51±13</td>
<td>55±13</td>
<td>0.424*</td>
</tr>
<tr>
<td>Breslow thickness (mm)</td>
<td>1.51±0.88</td>
<td>1.68±1.20</td>
<td>0.758*</td>
</tr>
<tr>
<td>Volume SLN (mm³)</td>
<td>1805.3±1519.6</td>
<td>727.1±492.0</td>
<td>0.052*</td>
</tr>
<tr>
<td>Weight SLN (g)</td>
<td>1.1±0.8</td>
<td>0.49±0.2</td>
<td>0.038*</td>
</tr>
<tr>
<td>Yield scraping (x10⁶)</td>
<td>36.0±30.6</td>
<td>15.6±6.1</td>
<td>0.023*</td>
</tr>
<tr>
<td>Tumor cells in the SLN</td>
<td>2</td>
<td>4</td>
<td>0.640†</td>
</tr>
<tr>
<td>Additional lymph node dissection</td>
<td>2</td>
<td>2</td>
<td>1.000†</td>
</tr>
</tbody>
</table>

NOTE: Values in table expressed as n or mean±SD; *From Mann-Whitney U test; †Volume: height x width x length; † †By Fisher’s Exact test

PF-3512676 administration and triple-technique SLN procedure

Both patient groups received an i.d. 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. To identify and retrieve the SLN, the triple technique was used as described previously. In short, the day before surgery patients underwent a dynamic and static lymphoscintigraphy to determine the lymphatic drainage pattern. Just prior to surgery, Patent Blue V (Laboratoire Guerbet, Aulnay-sous-Bois, France) was i.d. injected next to the excision scar 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 and, after isolation of viable SLN cells, examined meticulously by the pathologist.

Isolation of viable SLN cells

Immediately after removal, SLN were collected in sterile ice-cold complete medium, comprising IMDM supplemented with 25 mM Hepes buffer (BioWhittaker, Verviers,
Belgium) with 10% FCS, 50 IU/ml penicillin-streptomycin, 1.6 mM L-glutamine and 0.05 mM β-mercaptoethanol. Before routine histopathological examination of the SLN, viable cells were isolated using a previously described scraping method. 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 Ltd. England, size no. 22). SLN cells were rinsed from the blade with medium containing 0.1% DNase I, 0.14% Collagenase A (Boehringer, Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently in PBS with 5 mM EDTA for 10 minutes 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 either FITC, PE, PE-CY5.5, PerCP-CY5.5 or APC and analyzed by flow cytometry at 100,000 events per measurement, as previously described. Monoclonal antibodies against CD3, CD4, CD8, CD11c, CD14, CD25, CD123, CCR7, (BD, San Jose, CA), CD1a, CD14, CD40, CD80, CD86, CD123, TRAIL, CTLA 4 (Pharmingen, San Diego, CA), CD11c, CD40, CD83 (Immunotech, Marseille, France), BDCA2 (Miltenyi Biotec, Bergisch Gladbach, Germany) and FabalgM (Southern Biotechnology, Birmingham, AL) and matching isotype control antibodies were used. Intracellular FoxP3 staining was performed using the eBioscience PE anti-human FoxP3 staining set, following the manufacturer’s instructions (eBioscience, San Diego, CA).

**Cytokine profiling**

Fresh and viable SLN leukocytes were incubated overnight (1x10e5 per 100 ul) in 10% FCS-containing complete culture medium. The supernatants were harvested and stored at -20°C and analyzed by BD-cytometric bead array (CBA) (BD, San Jose, CA) for sensitive FACS detection of the levels of the inflammatory cytokines IL-1β, -6, -8, -10, -12p70, and TNFα. Supernatants were also taken from overnight anti-CD3/-CD28 stimulated SLN-T cell cultures (prior to 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, Amsterdam, The Netherlands) 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 BD, San Jose, CA).
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% hydrogenperoxide in methanol. Tissue sections were pre-treated with 10mM citrate (pH 6) in an autoclave for 21 minutes at 121°C (for CD83 [1:25]; Novocastra, Newcastle upon Tyne, United Kingdom) or in a microwave at 100°C for 10 minutes (for CD68 [1:400]; DAKO, Glostrup, Denmark). The CD83 antibody was applied and incubated at room temperature for one hour. Detection and visualization were performed with the DAKO Chemmate™ Envision™ detection kit (DAKO, Glostrup, Denmark; Cat. no. K5007) according to manufacturers’ instructions. For the CD68 antibody an automated immunostainer (Ventana, Tuscon, AZ) was used for all incubation, detection and visualisation steps according to the manufacturer’s instructions. Sections were counterstained with haematoxylin, 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 tolerated well by all patients with transient and mild flu-like symptoms and mild to moderate fevers between eight and 24 hours after PF-3512676-administration, all easily controlled by paracetamol administration. Induration at the injection site remained palpable for 3-5 days after injection. Metastatic melanoma cells were detected in the SLN of 2/11 patients in the PF-3512676-administered group and 4/12 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, B; Table 1). Also, significantly higher SLN cell yields were obtained after scraping the cutting surface of the SLN from PF-
Chapter 6

3512676-administered patients, as compared to saline-administered patients (p=0.023). Histological architecture of the SLN was conserved upon conditioning by CpG, without an obvious increase in the number of follicles (Fig. 1C, 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). Also, a normal distribution was observed of CD68⁺ macrophages (preferentially in the marginal sinuses, Fig. 1C, D) and of CD83⁺ DC (in the paracortical T cells areas, Fig. 1E, F).

Figure 1. Sentinel lymph nodes (SLN) from PF-3512676 administered patients were increased in volume and weight as compared to 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). Histological 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⁺ DC in the paracortical areas was observed on saline (E) or PF-3512676 (F) administration (magnification 100x).
**Activation of both PDC and MDC upon PF-3512676 administration**

SLN-derived PDC (SLN-PDC, defined as CD123^{hi} and BDCA-2^{+}) were analyzed by flow cytometry for the expression of DC maturation (CD83) and activation markers (CD86 and CD40; Fig. 2A). Although expression of BDCA-2 and CD83 on in vitro matured 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-2^{+} SLN-PDC to be associated with i.d. 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 co-stimulatory 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 to 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 described previously,^{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%).

![Figure 2](image-url)
The expression of activation markers on both the PDC and the CD1a⁺ MDC, was also compared between the SLN tumor positive and 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⁺CD123⁺ myeloid cells (phenotypically distinct from CD11c⁺CD123⁺ 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⁺CD123⁺CD1a⁻CD14⁻ cells through 4-parameter flow cytometry.

![Figure 3](image-url).

**Figure 3.** Identification of a novel PF-3512676-induced CD1a⁻TRAIL⁺ myeloid dendritic cell (MDC) subset in the sentinel lymph node (SLN) of melanoma patients. A) Using 4-parameter flow cytometry we were able to gate and quantify the novel MDC subset as CD11c⁺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⁺CD123⁺CD1a⁻CD14⁻ SLN-MDC as compared to 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⁺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 PF3512676-administered patient). Forward and side scatter (FSC/SSC) plots are also shown for the gated MDC.
Effects of CpG on the melanoma sentinel lymph node (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 DC activation markers (Fig. 3C) as compared to the PF-3512676-administered patient population. In forward scatter (FSC) analyses these \( \text{CD}11c^{\text{hi}}\text{CD}123^{+}\text{CD}1a^{-}\text{CD}14^{-} \) cells were found to be of intermediate size between monocytes and \( \text{CD}1a^{+} \) MDC and in terms of side scatter (SSC) levels showed a granular/dendritic morphology (Fig. 3C). Phenotypic analysis of this novel myeloid DC population, gated as \( \text{CD}11c^{\text{hi}}\text{CD}1a^{-}\text{CD}14^{-} \), demonstrated it to be DC-SIGN negative but positive for CD83, CD40, CD86, CD80, and CCR7 (all indicative of a mature DC phenotype), as well as for TRAIL and CD123 (Fig. 3C). Of note, this phenotype is compatible with previously described in vitro IFN\( \alpha \)-induced monocyte-derived DC.\(^{30,31} \)

**PF-3512676-induced inflammatory cytokine release**

BD-CBA FACS analysis for the detection of cytokines was performed with supernatants from 24h-cultures of the total SLN leukocyte population. Significantly increased levels of IL-6, IL-8, IL-10 and TNF\( \alpha \) were found in the supernatants of the PF-3512676-administered patient population as compared to the supernatants from the saline-administered patient population, while there was no significant difference in the concentration of IL-1\( \beta \) and IL-12p70 between both patient groups (Table 2A). IFN\( \alpha \) was below detection level of the employed ELISA in both PF-3512676- and saline-administered patients (data not shown). This was not unexpected since any PF-3512676-induced IFN\( \alpha \) release by PDC that might be expected is an acute/immediate event whereas both PF-3512676 and saline were administered one week prior to the SLN procedure and subsequent supernatant collection.

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

<table>
<thead>
<tr>
<th>A) Total SLN leukocyte population</th>
<th>IL-1( \beta )</th>
<th>IL-6</th>
<th>IL-8</th>
<th>IL-10</th>
<th>IL-12p70</th>
<th>TNF( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline*</td>
<td>41±33</td>
<td>49±28</td>
<td>908±398</td>
<td>9±4</td>
<td>8±6</td>
<td>12±5</td>
</tr>
<tr>
<td>PF-3512676†</td>
<td>47±31</td>
<td>172±103</td>
<td>1988±1105</td>
<td>22±16</td>
<td>9±4</td>
<td>19±6</td>
</tr>
<tr>
<td>P-value(^{T})</td>
<td>0.602</td>
<td>0.002</td>
<td>0.022</td>
<td>0.001</td>
<td>0.310</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B) SLN-T cells</th>
<th>IFNy</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-10</th>
<th>IFNy/IL-4</th>
<th>IFNy/IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline*</td>
<td>1682±972</td>
<td>61±82</td>
<td>84±81</td>
<td>5528±5169</td>
<td>45±29</td>
<td>33±41</td>
</tr>
<tr>
<td>PF-3512676†</td>
<td>2597±1983</td>
<td>44±40</td>
<td>47±47</td>
<td>5817±5171</td>
<td>74±43</td>
<td>111±119</td>
</tr>
<tr>
<td>P-value(^{T})</td>
<td>0.284</td>
<td>0.884</td>
<td>0.191</td>
<td>0.839</td>
<td>0.099</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Mean±SD (pg/ml) in the saline administered patient population; †Mean±SD (pg/ml) in the PF-3512676-administered patient population; \(^{T}\)By Mann-Whitney U test
T cell cytokine skewing and T\textsubscript{reg} frequencies

Because of the significant rise 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 towards a more suppressive T cell profile, either by increased type-2 cytokine release or by elevated T\textsubscript{reg} frequencies.

In the supernatants from overnight anti-CD3/-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 to the saline-administered patient population, while 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 rise in IL-10 release by the total SLN leukocyte population, a slight bias towards 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\textsubscript{reg} frequencies (defined as CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{hi} and found to co-express high levels of both CTLA4 and FoxP3; Fig. 4A) were determined. Significantly lower SLN-T\textsubscript{reg} frequencies were found in the PF-3512676-administered patients (\(p=0.004\); Fig. 4B), again arguing against a PF-3512676/IL10-induced immunosuppressive microenvironment.

**Figure 4.** Regulatory T cell (T\textsubscript{reg}) frequencies in the sentinel lymph node (SLN) of PF-3512676-administered patients (n=10) compared with saline-administered patients (n=4). A) T\textsubscript{reg} were defined and gated as CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{hi} (the events shown in the dot plot were pre-gated on CD3 positivity) and express characteristically high levels of intracellular CTLA4 and FoxP3, as compared to levels of these markers on CD4\textsuperscript{+}CD25\textsuperscript{+} non-T\textsubscript{reg} cells -also shown here. Mean fluorescence indices (MFI), determined by dividing the mean fluorescence intensity by that of the corresponding isotype control, are listed in the histograms. B) T\textsubscript{reg} frequencies (expressed as percentage of the total CD3\textsuperscript{+}CD4\textsuperscript{+} T cell population) were significantly decreased in the PF-3512676-administered patients as compared with the saline-administered patients (\(p=0.004\)).
DISCUSSION

The first LN to directly drain the primary tumor, the SLN, is the preferential site of early metastasis and shows the most pronounced suppression of DC phenotype and numbers, as early as Stage-I of melanoma development. DC represent a heterogeneous population of bone marrow-derived cells that are both powerful initiators and modulators of immune responses. Beside the conventional MDC, LN contain CD11c- PDC. The PDC's primary task appears to be the production of large amounts of type-1 IFN, most notably IFNα, upon activation by specific TLR and/or CD40L, in order to stimulate components of both the innate (e.g. NK cells, MDC) and the adaptive immune system (e.g. CTL). 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. Moreover, PDC infiltrating head and neck squamous cell carcinomas show low TLR9 expression and a diminished capacity for IFNα production. It may well be that activation of these PDC by appropriate CpG ODN will abrogate their suppressive traits and lead to enhanced anti-tumor 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 demonstrate 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 i.d. 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 DC maturation marker CD83 and of the co-stimulatory marker CD86 as well as a significant upregulation of CD40 levels was induced on PDC upon PF-3512676 administration. In contrast to Dzionek et al., 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-CD123hiCD83+ cell population. But our study design was not entirely comparable with theirs: Dzionek et al studied human blood PDC after IL-3-mediated maturation in culture, 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 upregulation of CD83 and CD86 on CD1a+ MDC. Unlike the observed PDC activation, this finding is more remarkable since 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, appears sufficient for subsequent MDC activation.

IFNα released by CpG-activated PDC is also most likely responsible for the recruitment of the CD1a−CD83+TRAIL+ 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 DC. Parlato *et al.* found blood-derived monocytes, when cultured with IFNα for 3 days, to exhibit a wide spectrum of phenotypic and functional features typical of mature DC.30 They described IFN-induced monocyte-derived DC as negative for CD14 and positive for CD80, CD83, CD86, CD123, and CCR7.30 Santini *et al* found IFN-induced monocyte-derived DC to also express TRAIL on their membranes.31 In keeping with this reported phenotype, our population of PF-3512676-induced MDC was positive for CD11c, CD80, CD83, CD86, CD123, CCR7 as well as for TRAIL. These phenotypic similarities strongly suggest that the PF-3512676-associated DC subset identified by us actually represents IFNα-induced monocyte-derived DC. In support of this we found the PF-3512676 ODN employed 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 origins 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 appears 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 DC 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 1) 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 2) 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 12h of *in vitro* CpG-mediated activation.40 It is therefore not surprising that we were unable to detect any spontaneous IFNα release in 24h supernatants of freshly isolated SLN cells that were collected seven 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
Effects of CpG on the melanoma sentinel lymph node

In addition, as mentioned previously, CpG-B ODN like PF-3512676 are relatively poor IFNα release inducers anyway—in contrast to CpG-A and –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. 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 or from secondarily activated immune effector cells in vivo, possibly providing a negative feedback loop for the pro-inflammatory effects of the administered CpG ODN. There was no difference in IL-1β and IL-12p70 concentrations between both patient groups, which, in the case of IL-12p70, is in keeping with previous reports of a lack of IL-12 production upon CpG-mediated activation of human PDC in vitro. 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.

Finally, we studied the influence of a PF-3512676-conditioned SLN environment on Treg frequencies. Whether human CD4+CD25hi Treg (also strongly positive for CTLA-4 and the Treg-associated transcription factor FoxP3, see Fig. 5A) 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, as well as in human metastatic melanoma lymph nodes and are predictive for a poor prognosis. Treg 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 DC differentiation and maturation. Treg-mediated immune suppression may be overcome by decreasing the number of Treg or by blocking/reversing their influence on DC and/or T cells. In the PF-3512676-administered patients a significant decrease in CD4+CD25hi Treg frequencies was found as compared to the saline control group. Both immature PDC and immature or IL-10-modulated MDC are known to induce Treg. In view of this, the reduced Treg frequencies observed in the SLN of the PF-3512676-administered patients may have resulted either from the directly induced maturation of PDC or indirectly from MDC maturation induction. However, the latter option seems more likely in view of a report by Moseman et al who showed that CpG-activated PDC actually induce CD4+CD25+ Treg 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 Treg frequencies that we observed in vivo.
In conclusion, PF-3512676 certainly seems promising as an adjuvant immunotherapeutic modality for early-stage melanoma, based on the following observations in the SLN: 1) increased PDC and MDC activation status, 2) the induction of a novel TRAIL+ MDC subset with a mature T cell stimulatory phenotype, 3) an increased pro-inflammatory and type-1 cytokine profile, and 4) a reduction in immunosuppressive T\textsubscript{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 DC and T\textsubscript{reg} in the SLN translate into higher frequencies of melanoma-specific CD8\textsuperscript{+} effector T cells, which would further confirm the clinical utility of CpG-ODN in the immunotherapy of melanoma. Altogether these immune effects should then contribute to an increased protection against early metastatic spread.

ACKNOWLEDGMENTS

The authors wish to thank Sinéad Lougheed for the CBA analysis, Dr Joost Oudejans for reviewing SLN histology, Dr Art Krieg for critical invaluable discussions, and Coley Pharmaceutical Group (Wellesley, MA) and Pfizer (New York, NY) for their kind provision of PF-3512676, formerly known as CPG 7909.

REFERENCES


Local administration of PF-3512676 CpG-B instigates tumor-specific CD8+ T cell reactivity in melanoma patients

Submitted Clinical Cancer Research

BG Molenkamp
BJR Sluijter
PAM van Leeuwen
S Meijer
PGJTB Wijnands
AJM van den Eertwegh
RJ Scheper
TD de Gruijl
ABSTRACT

Purpose: Impaired immune effector functions in the melanoma sentinel lymph node (SLN) may conspire to allow for early metastatic events. Local administration of PF-3512676 (formerly known as CpG 7909) has shown immunostimulatory effects of on both dendritic cell (DC) 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+ T cells.

Experimental design: Twenty-four stage I-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 in an IFNγ ELISPOT-assay against a number of HLA-A1/-A2/-A3-restricted epitopes derived from a range of melanoma-associated antigens (MAA) in 21/24 enrolled patients. Frequencies of natural killer (NK) cells and frequencies and maturation state of DC subsets in the SLN were determined by flowcytometry.

Results: Melanoma-specific CD8+ T cell response rates against >1 MAA epitope in the SLN were 0/11 for the saline group versus 5/10 for the PF-3512676 administered group (P=0.012). Of these five responding patients, four 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 DC maturation.

Conclusions: These data demonstrate a rise 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.
INTRODUCTION

Cutaneous melanoma is the most aggressive type of skin cancer, the incidence of which has increased rapidly over the past decades.\(^1\) Fortunately, as a result of early detection, melanoma mortality increases at a slower rate, but melanoma still causes a disproportionate mortality in young and middle-aged patients.\(^1,2\) On average 18.6 potential life years are lost for each melanoma death and this is among the highest rates for adult-onset cancers.\(^2\) Adjuvant therapy options are still limited and complete surgical excision at an early stage remains the only curative treatment option. Paradoxically, melanoma-associated antigens (MAA) have proven relatively immunogenic with specifically reactive T cells already detectable at early stages of tumor development, both in peripheral blood and in tumor-draining lymph nodes (TDLN), while their frequency can be further increased by vaccination.\(^3-7\) These observations raise the possibility to boost anti-melanoma immunity in order to curb early metastatic events. Unfortunately, early melanoma development is accompanied by a tumor-induced inhibition of maturation and activation of professional antigen-presenting cells, the dendritic cells (DC), in the initial TDLN; the so-called sentinel lymph node (SLN).\(^8\) This inhibition may well interfere with effective presentation of MAA to specific anti-tumor cytotoxic T lymphocytes (CTL) and Th cells.\(^9\) Novel therapeutic approaches aiming at the circumvention or reversal of this melanoma-induced immune suppression are therefore urgently needed.

Plasmacytoid DC (PDC) constitute an important DC subset with potential antigen presenting and T cell activating capabilities. PDC reside in the lymph node (LN) and are able to bind microbial products through specific receptors such as Toll-like receptor 9 (TLR9). TLR9 expression in human immune cells appears to be restricted to B cells and PDC.\(^10,11\) Unmethylated Cytosine-phosphate-Guanine oligodeoxynucleotides (CpG ODN) directly stimulate PDC through intracellular TLR9 triggering. TLR- and/or CD40L-activated PDC preferentially release large amounts of interferon-α (IFNα),\(^12-14\) which may facilitate direct activation of CD8\(^+\) T cells and natural killer cells (NK cells) as well as promote the differentiation and maturation of neighboring myeloid DC (MDC) or their precursors, and thus also indirectly stimulate T cell activation.\(^15-19\)

NK cells are powerful innate effector cells 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 CD56\(^{lo}\) subset has been shown to express perforin as well as the killer cell Ig-like receptors (KIR), while the CD56\(^{hi}\) subset does not carry perforin and KIR but seems to exhibit immunoregulatory functions through the secretion of various cytokines (i.e. IFNγ, TNFα or IL-10).\(^20,21\)
We recently reported on the immunostimulatory effects of intradermal (i.d.) injections of the CpG-B ODN PF-3512676 (formerly known as CpG 7909) around the excision site of Stage I-III melanoma tumors, resulting in increased PDC and MDC activation, an increased pro-inflammatory type-1 T cell cytokine profile, and a reduction in immunosuppressive regulatory T cell (T\text{reg}) frequencies.\(^2\) 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 CD56\text{lo} effector NK cell rate, upon i.d. administration of PF-3512676.

**METHODS**

**Patients and PF-3512676 administration**

In this single-blinded Phase II study, twenty-four 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 8mg PF-3512676 (Coley Pharmaceutical Group, Wellesley, MA) dissolved in 1.6ml saline or 1.6ml plain saline (NaCl 0.9%). I.d. injections were given directly adjacent to the scar of the primary melanoma excision one week prior to 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 previously reported, PF-3512676 injections were tolerated well by all patients with transient and mild flu-like symptoms and induration at the injection site.\(^2\)

**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 prior to surgery, Patent Blue V (Laboratoire Guerbet, Aulnay-sous-Bois, France) was injected intradermally 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 (CM), comprising IMDM supplemented with 25mM Hepes buffer (BioWhittaker, Verviers,
Belgium) with 10% Fetal Calf Serum (FCS; Hyclone, Amsterdam, The Netherlands),
50IU/ml penicillin-streptomycin, 1.6mM L-glutamine and 0.05mM β- mercaptoethanol.
Before routine histopathological 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 Ltd. England, size
no. 22). SLN cells were rinsed from the blade with medium supplemented with 0.2mg/
ml DNAse I, 1mg/ml Collagenase A (Boehringer, Mannheim, Germany) and 5% FCS
and incubated for 45 minutes at 37°C. Finally, the SLN cells were washed twice in CM,
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 (PBMC) and flow
cytometry
From each patient 40-50ml blood was taken before, one week after and three weeks
after PF-3512676/saline administration. PBMC were isolated from heparinized blood by
density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway).
Cells were washed twice with sterile PBS with 0.1% BSA 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 CTL studies, freshly isolated SLN cells were
directly stained with mAbs 8.L.104 (HLA-A1; USBiological, Swampscott, MA), BB7.2,
MA 2.1 (HLA-A2) and GAP A3 (HLA-A3) (American Tissue Culture Collection [ATCC],
Rockville, MD). Freshly isolated SLN cells were also directly stained with monoclonal
antibodies against CD3, CD4, CD8, CD25, CD56, CD69, CD123, HLA-DR (BD, San
Jose, CA), CD1a, CD40, CD86, CD123, CTLA-4 (Pharmingen, San Diego, CA), CD40,
CD83 (Immunotech, Marseille, France), BDCA2 (Miltenyi Biotec, Bergisch Gladbach,
Germany) 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 previously described.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
hour on ice with 2μg anti-CD3 and 0.4μg anti-CD28 per 1x10⁶ cells (kindly provided by
Dr. René van Lier; CLB, Amsterdam, the Netherlands) in 100-200μl CM with 5% FCS.
After incubation and washing, cells were plated on 24-well plates (Greiner Bio-One,
Frickenhausen, Germany), coated with affinity-purified goat-anti-mouse immunoglobulin
(1:100; Dako, Glostrup, Denmark) in CM with 10% FCS at a concentration of 1x10⁶ cells/
ml/well for one hour at 4°C. The cells were cultured for 48 hours in a humidified 5% CO₂ incubator at 37°C. After 48 hours the cells were resuspended and the contents of each well was divided over 4 new uncoated wells at a volume of 250μl per well. To each new well 750μl CM supplemented with 14IU/ml rhIL-2 (CLB, Amsterdam, the Netherlands) was added, resulting in a final concentration of 10IU rhIL-2 per ml. The cells were cultured for another 5 days, after which they were harvested and counted. All SLN-T cells underwent two expansion cycles. Finally, the expanded T cells were harvested, frozen 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, -A2, or -A3-binding peptides (Peptide Synthesis Facility; IHB-LUMC, Leiden, The Netherlands), derived from various melanoma-associated tumor antigens and containing previously described CD8+ T cell epitopes, was used for CD8+ T cell reactivity testing (Table 2). Peptides were dissolved in 100% dimethylsulphoxide at 50mg/ml and stored at -80°C. From this stock solution, peptide was dissolved in CM without FCS at 5mg/ml and stored at -20°C until use. Non-transfected T2 cells (in case of A2-binding peptides) or HLA-A1 or -A3 transfected T2 cells (in case of A1- or A3-binding peptides; kindly provided by Dr. E.M. Jaffee, The Johns Hopkins University School of Medicine, Baltimore, MD) were loaded overnight in serum-free medium with β2-microglobulin (5µg/ml, SIGMA, St Louis, MO) and melanoma-associated or control peptides (50µg/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 IFNγ ELISPOT analysis**

To test the CD8+ T cells for functional melanoma-specific reactivity, an IFNγ ELISPOT assay was performed.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, Bergisch Gladbach, Germany). After isolation, CD8+ T cells were washed and resuspended in CM with 10% FCS and added directly to the peptide-pulsed T2 cells in anti-IFNγ pre-coated Multiscreen 96-well filtration plates (Millipore, Molsheim, France). Plates were seeded with 10.000 stimulator (S)-cells (peptide-pulsed T2 cells) per well and 100,000 or 50,000 effector (E)-cells (CD8+ T cells), resulting in E:S ratios of 10:1 and 5:1, respectively. Assays were tested in quadruplicate where possible, but at least in triplicate at each of the E:S ratios. After overnight (18h) incubation the cells were flicked off and an ELISPOT assay was performed as previously described,30 using a commercially available anti-IFNγ mAb pair (Mabtech, Nacka, Sweden). After development of the plates, spots were
PF-3512676 instigates anti-tumor T cell response

counted by an automated ELISPOT-reader (AID Diagnostika, Strassberg, Germany). 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 1x10⁶ 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 E:S ratio of 5:1). ELISPOT CD8⁺ T cell responses were considered positive when 1) 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), 2) the mean number of spots of the test condition exceeded the mean number of spots for the corresponding E:S ratio of the control condition by at least two-fold; and 3) the absolute difference in number of spots between the test and control condition was at least five.³¹

Statistical analysis

Differences between patient study groups were analyzed using the two-sample Mann-Whitney U test or the Fisher’s Exact test (all two-tailed). Correlations were calculated using the Spearman’s rho 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 Pathological examination revealed six patients with Stage III melanoma based on the presence of tumor cells in the SLN (2/11 patients in the CpG administered group and 4/13 patients in the saline administered group). In 4/6 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 (LND) and all LN in the additional LND were found to be tumor negative. Based on flowcytometric positivity for HLA-A1, -A2, or -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

Prior to expansion, CD4/CD8 ratios were determined among the T cells from the freshly sampled SLN (i.e. 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, medians 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 upon CpG administration. As a measure of T cell activation, flowcytometric analysis of T cell activation markers on CD4+ and CD8+ T cells in the SLN was also performed prior to T cell expansion (Fig. 1B). No difference was observed in expression of the T cell activation markers CD69 or HLA-DR on either CD4+ or CD8+ T cells. Interestingly, both on CD8+ and on 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).

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
<th>PF-3512676</th>
<th>Saline</th>
<th>P</th>
</tr>
</thead>
<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 LND</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 (day, mean±SD)</td>
<td>52±16</td>
<td>45±18</td>
<td>0.338*</td>
</tr>
</tbody>
</table>

Abbreviations: SLN, sentinel lymph node; LND, lymph node dissection; *two-sample Mann-Whitney U T-test; †two-tailed Fisher’s Exact Test

CD8+ T cell reactivity in the SLN and peripheral blood

Prior to expansion, CD4/CD8 ratios were determined among the T cells from the freshly sampled SLN (i.e. 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, medians 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 upon CpG administration. As a measure of T cell activation, flowcytometric analysis of T cell activation markers on CD4+ and CD8+ T cells in the SLN was also performed prior to T cell expansion (Fig. 1B). No difference was observed in expression of the T cell activation markers CD69 or HLA-DR on either CD4+ or CD8+ T cells. Interestingly, both on CD8+ and on 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).

Figure 1. Local PF-3512676 CpG-B administration leads to increased CD8+ T cell numbers in the sentinel lymph node (SLN) as well as to detectable levels of melanoma-specific CD8+ effector T cells. A) CD4:CD8 T cell ratios in saline control (n=12) versus PF-3512676 modulated SLN samples (n=11) from which CD8+ T cells were expanded for testing of melanoma specific reactivity. B) Expression levels of the indicated T cell activation markers on CD4+ and CD8+ T cells (by % positivity) in the saline control (open bars) or PF-3512676 modulated SLN (closed bars) -determined by flowcytometric analysis. C) Example of IFNγ ELISPOT results for the indicated gp100, FLU, and E7 control peptides in HLA-A2+ patients after administration of either saline or PF-3512676; one representative well out of three or four per condition is shown. Melanoma-specific CD8+ T cells from early stage melanoma patients recognize HLA-A-3 (D) and -A2- (E) binding epitopes from multiple melanoma-associated antigens (MAA); both in the SLN and in the peripheral blood. IFNγ ELISPOT reactivity is expressed as number of spots per 100,000 CD8+ effector T cells. Data from all positively responding patients to >1 MAA in the SLN (all treated with PF-3512676) are presented (indicated as responder 1-5); of these patients both the response in the SLN (d7 SLN) and in the peripheral blood, one week (d7 blood) and three weeks (d21 blood) after treatment is shown. Open bars represent results obtained for the negative control peptides and closed bars for the tested MAA peptides. Asterisks denote positive responses according to criteria as formulated in the Materials and Methods.
CD8+ T cell reactivity in response to a range of melanoma-derived epitopes was measured at three different time points (before, one week after, and three weeks after CpG or saline administration) by an IFNγ ELISPOT read-out (as shown in Fig. 1C). Beside MAA-specific CD8+ T cell reactivity, also reactivity against Influenza epitopes (FLU, see Table 2) was determined as a measure of general immunocompetence (see Fig. 1C). A list of all the tested FLU, MAA, and negative control peptides is presented in Table 2. ELISPOT CD8+ T cell responses were considered positive only after meeting a
Complying with these criteria, post-treatment CTL response rates against at least one of the tested MAA epitopes were 0/11 for the saline control group in both blood and SLN and 7/10 and 5/10 for the CpG-administered group in blood and SLN, respectively (p=0.001 and 0.012, Table 3). Five patients showed specific post-treatment reactivity to more than one MAA-derived epitope, i.e. either in blood-derived CD8+ T cells at one or three weeks after CpG administration or in SLN-derived CD8+ T cells, one week after CpG administration (responding patients to >1 MAA in the SLN shown for the tested MAA and FLU epitopes in Fig. 1D and 1E [HLA-A3 or –A2 positive patients, respectively], positive responses marked by an asterisk). Importantly, none of these, nor any other of the tested patients, showed MAA-specific reactivity against more than one MAA epitope in the blood prior to treatment (Table 3). 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 6/10 patients in the CpG-administered group and 0/11 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 upon CpG administration (saline vs CpG: prior to treatment,
PF-3512676 instigates anti-tumor T cell response

The following text is extracted from the given page:

119

1/11 vs 4/10 \( p=0.149 \); after treatment, 2/11 vs 7/10 \( p=0.030 \); Table 3) are indicative of a generalized increase in CTL activation state resulting from CpG injection.

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 IFNα\(^{12-14}\) 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. 2A), but no such association was found for the expression of CD86 and CD40 on CD1a+ MDC (Fig. 2A). These data suggest a direct casual relationship between PDC and CTL 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 CD8+ T cell reactivity to >1 MAA in the post-treatment blood samples. Fig. 2B clearly demonstrates the relationship between 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 post-injection blood (all upon 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. 2B). Again, neither the expression of activation markers

### Table 3. Melanoma-specific CD8+ T cell responses

<table>
<thead>
<tr>
<th></th>
<th>FLU</th>
<th>Saline</th>
<th>PF-3512676</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC pre</td>
<td>1/11</td>
<td>4/10</td>
<td></td>
<td>0.149</td>
</tr>
<tr>
<td>PBMC post</td>
<td>2/11</td>
<td>7/10</td>
<td></td>
<td>0.030</td>
</tr>
<tr>
<td>SLN</td>
<td>2/11</td>
<td>5/10</td>
<td></td>
<td>0.183</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MAA</th>
<th>Saline</th>
<th>PF-3512676</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC pre</td>
<td>0/11</td>
<td>2/10</td>
<td></td>
<td>0.214</td>
</tr>
<tr>
<td>PBMC post</td>
<td>0/11</td>
<td>7/10</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>SLN</td>
<td>0/11</td>
<td>5/10</td>
<td></td>
<td>0.012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>&gt;1 MAA</th>
<th>Saline</th>
<th>PF-3512676</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC pre</td>
<td>0/11</td>
<td>0/10</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PBMC post</td>
<td>0/11</td>
<td>5/10</td>
<td></td>
<td>0.012</td>
</tr>
<tr>
<td>SLN</td>
<td>0/11</td>
<td>5/10</td>
<td></td>
<td>0.012</td>
</tr>
</tbody>
</table>

Abbreviations: PBMC, peripheral blood mononuclear cells; SLN, sentinel lymph node; MAA, melanoma-associated antigens; P values determined by Fisher’s Exact test.
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). Thus, 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**

IFNα released by activated PDC not only potentially boosts CTL but also NK cell responses.12-14 From 4 saline- and 10 CpG-administered patients frozen stored samples
PF-3512676 instigates anti-tumor T cell response

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. 3A). Increases in the frequency of CD56lo ("cytotoxic effector") and of CD56hi ("regulatory") NK cells were found in the CpG test group as compared to the saline control group (both non-significant, see Fig. 3B). 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. 3C), revealed a significant association between MAA CD8+ T cell reactivity and high frequencies of CD56lo cytotoxic effector NK cells. Altogether these data clearly demonstrate a simultaneous increase in melanoma-specific CD8+ effector T cells and

Figure 3. Activation of plasmacytoid dendritic cells (PDC) and melanoma-specific CD8+ T cell reactivity are associated with increased frequencies of natural killer (NK) cells in the sentinel lymph node (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); shown r and P value from Spearman rho correlation. Frequencies of CD56lo cytotoxic effector NK cells and CD56hi regulatory NK cells in relation to B) PF-3512676 (closed bars) or saline administration (open bars) and C) melanoma-specific CD8+ T cell reactivity (negative: open bars, positive: closed bars) in the SLN of PF-3512676 administered patients. Shown P values were determined by Mann Whitney U test.
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. 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. We recently reported immunostimulatory effects of local administration of the CpG-B ODN 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 pro-inflammatory type-1 T cell cytokine profile, and reduced Treg frequencies. Our hypothesis that these CpG-induced immunostimulatory effects on both DC 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 paper. Even in this is relatively small study of CD8+ T cells obtained from blood or by scraping the cutting surface of bisected SLN we found convincing evidence that melanoma-specific CD8+ T cell responses (detected by specific IFNγ release) were enhanced by i.d. injection of PF-3512676 around the excision site of the primary melanoma. Thus, as previously reported for GM-CSF, PF-3512676 is able to (re-)activate 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 upon 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 ODN. It is known that PF-3512676 activates PDC through TLR9 triggering and that activated PDC release IFNα. The rapid release of large amounts of IFNα may enable PDC to boost CD8+ T cell responses as well as promote the differentiation and maturation of neighboring MDC (precursors) and thus also indirectly stimulate T cell activation. However, the relatively poor induction of IFNα release by CpG-B ODN like PF-3512676, 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/IFNα-induced CD83+TRAIL+ MDC subset, data not shown), is
PF-3512676 instigates anti-tumor T cell response

123

suggestive of direct activation of specific CD8+ T cells through PDC-mediated antigen presentation, as previously reported.17 Indeed, expression levels on SLN-resident PDC of the co-stimulatory 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 in 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 as compared to 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 DC maturation and/or differentiation, resulting in immature and/or partially differentiated DC.39,40 Tumor-associated PDC have been reported to induce CD8+ regulatory T cells41-43 and tumor-associated immature MDC are capable of promoting T_{reg} proliferation.44-47 We previously demonstrated that a single dose of PF-3512676 was sufficient to both increase the PDC and MDC activation state and decrease the T_{reg} 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 signal immunosuppressive, regulatory functions that may be induced in memory T cells by tumor-modulated PDC or MDC.9,41-43 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 suggest that beside the down-regulation of natural T_{reg}s,22 CpG may also contribute to increased anti-melanoma T cell reactivity through down-modulation of negative feedback loops in activated effector T cells, maintained by inhibitory molecules such as CTLA-4. 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 between T_{reg}s and tumor-conditioned DC, leading to respective upregulation of the immunosuppressive transcription factor FoxP3 and the T cell-inhibitory enzyme Indoleamine-2,3-dioxygenase (IDO).48 On the other hand, systemic CpG administration has been reported to result in increased IDO expression by PDC;49 determination of the net effect of local PF-3512676 administration on IDO expression in the studied SLN awaits the results of ongoing immunohistochemical analyses. Finally, Ghiringhelli et al. found an inverse correlation between NK cell activation and T_{reg} frequency in tumor-bearing patients, indicating a role for T_{reg} frequencies in blunting the NK cell arm of the innate immune system.50 Therefore, PF-3512676 might not only boost the innate immune system by triggering PDC to release large amount of IFNγ13,18 but also by reducing T_{reg} frequencies.50
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 the 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.

ACKNOWLEDGMENTS

The authors wish to thank Hugo Bakker for technical assistance and Coley Pharmaceutical Group (Wellesley, MA) and Pfizer (New York, NY) for their kind provision of PF-3512676 (formerly known as CpG 7909).

REFERENCES

Summary, discussion and future prospects
Summary & Discussion

Melanoma is an aggressive, therapy-resistant malignancy of melanocytes, the incidence and mortality rates of which have been increasing in Europe over the past decades. In the Netherlands melanoma accounts for 3% of all new cancer cases per year with an incidence of 14 per 100,000 and this incidence rate is currently increasing at 5% per year, resulting in a considerable public health problem. The absolute total number of new cases of melanoma in the Netherlands is expected to be more than 4800 in 2015, as compared to around 2400 in 2000. Exposure to solar UV radiation, fair skin, dysplastic nevi syndrome, and a family history of melanoma are major risk factors for melanoma development. The avoidance of UV radiation and screening of high-risk patients have the potential to reduce the population burden of melanoma. Mortality among melanoma patients with metastatic disease is high since adjuvant therapy options are limited and show low survival benefits. Therefore, early detection and treatment of melanoma are of great importance.

Doctors treating patients with pigmented skin lesions will always worry about missing the diagnosis of melanoma, since complete surgical excision at an early stage remains the only curative treatment option. To accurately diagnose cutaneous melanoma a diagnostic biopsy is needed. Different diagnostic biopsy types can be distinguished: wide excisional biopsy (lateral clearance ≥2 mm), narrow excisional biopsy (lateral clearance <2 mm), excisional biopsy with positive margins, and incisional (including punch) biopsy. The influence of a non-radical diagnostic biopsy on melanoma patient survival is an ongoing topic of discussion. Chapter 2 investigates both the influence of diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen on disease free and overall survival. After (partial) removal of a pigmented skin lesion 471 patients were diagnosed with clinically stage I/II melanoma and underwent re-excision and a sentinel lymph node (SLN) procedure. Patients were divided into groups according to their diagnostic biopsy type (wide excision biopsy, n=279; narrow excision biopsy, n=109; excision biopsy with positive margins, n=52; incisional biopsy, n=31) or the presence of residual tumor cells in their re-excision specimen (n=41). Survival analysis was performed using the Cox’s proportional hazard model, adjusted for eight important confounders of melanoma patient survival, and revealed that neither the diagnostic biopsy type nor the presence of tumor cells in the re-excision specimen negatively influenced melanoma patient survival. Nevertheless, the routine use of incision biopsies is not recommended. Incisional biopsies often consist of only a small percentage of the pigmented skin lesion surface area, making it difficult to sample a representative area within the tumor. Furthermore, when melanoma is diagnosed, attempting to evaluate the depth of invasion in an incisional biopsy is treacherous and may lead to over- or
underestimation of the invasion.\textsuperscript{8,9} On the other hand, these problems are less prominent in excision biopsies with positive margins, where the majority of the lesion has been removed and only the outer borders are compromised making a sampling error highly unlikely. With melanoma incidence rates rising\textsuperscript{1} and early surgical excision being the only curative treatment option,\textsuperscript{10} it is important for all physicians to feel confident about removing a suspect pigmented skin lesion. Incisional biopsies are not recommended but there is no cause for concern when an excision biopsy turns out to have positive margins.

Approximately 16\% of all melanoma patients will develop metastases and in 50\% of these patients the first metastases are found within the regional lymph nodes.\textsuperscript{11} In order to prevent melanoma patients without nodal metastases from undergoing an elective lymph node dissection and identify those that might benefit from it, the SLN procedure was developed. The SLN concept is based on the theory of an orderly progression of initial tumor cell metastasis within the lymphatic system. It assumes that early lymphatic metastases, if present, are always found first within the most proximal tumor draining lymph node, the SLN. Several studies have validated this assumption\textsuperscript{12-15} but only after a recent publication of Morton et al the SLN procedure was accepted as standard of care for melanoma patients.\textsuperscript{16} Morton et al found that the SLN status: 1) was the most important prognostic factor and 2) identified patients with nodal metastases whose survival could be prolonged by immediate lymphadenectomy.

Since the SLN not only constitutes the first expected site of metastasis, but also the first point of contact between tumor-associated antigens (TAA) and the adaptive immune system, routine application of the SLN procedure presents a unique translational setting to study the \textit{in vivo} effects of adjuvant therapies aimed at immunopotentiation of the SLN. Dendritic cells (DC) are antigen-presenting cells that are critical to the initiation of T cell-mediated immunity. In melanoma, skin-resident myeloid dendritic cells (MDC) take up and transport TAA to tumor-draining lymph nodes.\textsuperscript{17,18} In order to subsequently activate melanoma-specific T cells, the migrated MDC need to be sufficiently activated.\textsuperscript{19} DC development and activation can both be frustrated by inhibitory factors commonly associated with melanoma.\textsuperscript{17,20,21} The degree of such immunosuppression in the tumor draining lymph node is directly related to their distance to the primary tumor. Indeed, as the preferential site of early metastasis,\textsuperscript{22-24} the SLN shows the most pronounced immunological deficits.\textsuperscript{25,26} The frequency of SLN-DC is reduced, and most SLN-DC lack dendritic morphology and display lower expression levels of co-stimulatory molecules as compared to non-SLN-DC.\textsuperscript{25,26} Such a paralysis of DC in the first line of immunological defense likely facilitates the metastatic spread of melanoma cells to more distal tumor-draining lymph nodes.\textsuperscript{25,27} To study the effect of local immunotherapy on immune effector cells in the SLN a special technique was developed to investigate DC
and T cell functions without interfering with routinely performed diagnostic procedures. SLN were bisected cross-wise and the cutting surface scraped with a surgical blade to obtain viable cells for phenotypic and functional testing. Using this technique in Chapter 3 a small-scale phase II study was performed in which Stage-I melanoma patients were randomized to receive intracutaneous injections, either with Granulocyte/Macrophage Colony-stimulating Factor (GM-CSF) or plain saline around the scar of the primary excision, preceding re-excision and SLN dissection. Flowcytometric analysis showed a significant increase in the number and maturation state of the CD1a+ MDC in the SLN to be associated with GM-CSF treatment and a concomitant increase in DC–T cell clustering. In view of the critical role of MDC in the initiation of T cell-mediated immunity, it was hypothesized that potentiated MDC functions in the GM-CSF-administered group might be reflected in a higher number of tumor-specific CD8+ T cells in the SLN. This hypothesis was tested and supported by findings reported in Chapter 4, showing that local priming of melanoma-specific CD8+ T cells was associated with a high MDC content of the SLN, as observed in patients receiving locally administered GM-CSF. Together, Chapters 3 and 4 demonstrate that local administration of GM-CSF may offer a valuable adjuvant therapy option for early-stage melanoma patients, aimed at the immune control of early metastatic events.

In secondary lymphoid tissues mature and immature DC are thought to differentially modulate T cell responses. While under pro-inflammatory conditions mature DC are believed to induce T cell activation, under steady state conditions immature DC are believed to maintain a state of T cell tolerance. Yet, little is known about the actual activation state of human DC under these different conditions. Chapter 5 compares the frequency and activation state of human CD1a+ DC between matched skin and SLN samples, following intradermal administration of either GM-CSF or saline, at the excision site of Stage-I primary melanoma. While skin DC remained immature (CD1a+CD83-) and mostly situated in the epidermis of the saline-injected skin (fully consistent with a quiescent steady state), mature (CD1a+CD83+) DC frequencies were significantly increased in the GM-CSF-injected skin and correlated with the number of mature DC in the SLN, indicative of increased DC migration. Of note, under both steady state (i.e. saline control) and GM-CSF-induced pro-inflammatory conditions, all CD1a+ DC displayed a CD83+mature phenotype in the SLN. These data are indicative of migration of small numbers of phenotypically mature DC under steady state conditions that may be molecularly equipped to maintain peripheral T cell tolerance, while GM-CSF increases both the number and the immunostimulatory phenotype of MDC migrating to the draining lymph nodes, thus inducing T cell activation.

Chapters 3-5 describe the effects of GM-CSF administration on the number and activation state of MDC and melanoma-specific CTL reactivity in the SLN of early-stage
melanoma patients, demonstrating the clinical feasibility and immunopotentiating effects of this approach, specifically targeting MDC subsets. Plasmacytoid dendritic cells (PDC) constitute another important DC subset in lymph nodes with potential antigen presenting and T cell activating capabilities. PDC are able to bind microbial products through specific receptors such as Toll-like receptor 9 (TLR9). Unmethylated Cytosine-phosphate-Guanine oligodeoxynucleotides (CpG ODN) directly stimulate PDC through intracellular TLR9 triggering. Activated PDC preferentially release large amounts of IFNα, which may facilitate direct activation of CD8+ T cells and natural killer cells (NK cells) as well as promote the differentiation and maturation of neighboring MDC or their precursors, and thus also indirectly stimulate T cell activation. In Chapter 6 we report the effects of pre-operative local administration of the CpG B-type ODN, PF-3512676 (formerly known as CpG 7909), on DC and T cell subsets in the SLN of 23 patients with clinically Stage I-II melanoma, randomized to receive either PF-3512676 (8 mg) or saline. Intradermal PF-3512676 administration around the primary tumor excision site resulted in 1) an increased PDC and MDC activation status, 2) the induction of a newly identified TRAIL+ MDC subset with a mature T cell stimulatory phenotype, 3) an increased pro-inflammatory type-1 T cell cytokine profile, and 4) a reduction in immunosuppressive CD4+CD25hiCTLA-4+FoxP3+ regulatory T cell frequencies. We hypothesized that these PF-3512676-induced immunostimulatory effects on both DC and T cell subsets in the SLN would translate into higher frequencies of melanoma TAA-specific CD8+ T cells. Indeed, this hypothesis was supported by findings presented in Chapter 7 showing increased CD8+ T cell responsiveness to melanoma associated epitopes upon treatment with PF-3512676. CD8+ T cells from SLN and peripheral blood were tested for reactivity in an IFNγ ELISPOT-assay against a number of HLA-A1/-A2/-A3-restricted epitopes derived from a range of melanoma-associated antigens (MAA). Melanoma-specific CD8+ T cell response rates against more than one MAA epitope in either the SLN or the post-injection blood were 0/11 for the saline control group and 6/10 for the PF-3512676 administered group. 4/6 of the responding patients had a significant response to more than one MAA epitope in both the post-injection blood and the SLN. Furthermore, a clear relationship was found between increased frequencies in the SLN of both melanoma-specific CD8+ T cells, NK cells and PF-3512676-induced PDC maturation. These data demonstrate a simultaneous increase in melanoma-specific CD8+ T cells and innate NK effector cells upon PF-3512676 treatment. Furthermore, the significant correlation between activated SLN-PDC on the one hand and NK cells and melanoma-specific CD8+ T cell reactivity in both the SLN and the post-treatment blood samples on the other, strongly suggests local and systemic protection against tumor spread to specifically result from direct PF-3512676-induced PDC activation rather than from indirect (e.g. IFNα-mediated) MDC activation. Altogether, these data demonstrate
the utility of local PF-3512676 administration as adjuvant treatment in early-stage melanoma to try and halt metastatic spread.

**FUTURE PROSPECTS**

With the availability of cheap charter flights to the tropics, current fashion trends, a changing climate, and a growing melanoma awareness, the incidence of melanoma is expected to continue to rise. General practitioners will see more and more patients with pigmented skin lesions worried about the possibility of melanoma. And even though immunotherapy for melanoma shows promise, early diagnosis and surgical treatment is still the only curative treatment at this moment. Keeping this in mind and adding a general tendency towards a more defensive type of medicine, we expect general practitioners to take an increasing amount of diagnostic biopsies. As discussed in Chapter 2, radical excisional biopsies are preferred but incisional biopsies (including punch) are acceptable for example facial areas. All biopsies will be examined for the presence of melanoma but only a small percentage of patients will be diagnosed with melanoma.

In this thesis we have demonstrated that both GM-CSF and PF-3512676 are potent boosters of the immune system. Even without the administration of TAA, we have found increased frequencies of melanoma-specific CD8+ T cells in both the SLN and the peripheral blood after local administration of GM-CSF or PF-3512676 around the excision site of the primary tumor. This clearly indicates the presence of primed T cell responses in the SLN at early stages of melanoma development that can be (re)activated by an immunostimulatory “push” such as can be provided by pro-inflammatory cytokines and/or TLR-L, leading to increased local and systemic frequencies of functionally active CD8+ T cells. Recent publications by Morton et al made the SLN procedure for patients with intermediate thickness melanoma (Breslow thickness 1.2 to 3.5 mm) the accepted standard of care and precisely this patient population might also benefit most from immunomodulation of the SLN. The prognosis for patients with early melanoma (Breslow thickness <1mm) is already excellent and patients with more advanced melanoma (Breslow thickness >4mm) may rather need systemic treatment to counteract the pervasive immunosuppression associated with a higher tumor load and accompanying clinical and distant metastases.

Simultaneous activation of both conventional MDC and PDC through the combined administration of GM-CSF and PF-3512676, may lead to even stronger anti-tumor CTL activation, while also strengthening innate immunity. This hypothesis is currently being tested in the VU Medical Center as a sequel to the trials described in this thesis. A single intradermal injection of GM-CSF and PF-3512676 is relatively cheap, easy to
administer, and has no serious side effects. We therefore envision a future course of action whereby general practitioners, after diagnosing an intermediate thickness melanoma, might administer a single shot of an immunopotentiating cocktail around the tumor excision site before referral to a surgical oncologist. Since, according to our findings in Chapter 7, tumor-specific CD8+ T cell (re)activation in the SLN and subsequent expansion takes 1-3 weeks after PF-3512676 administration to result in detectable frequencies in the blood, we propose a delay of 4 weeks between boosting the SLN and the SLN procedure. This is certainly within the currently practicable time frame as the period between excision and SLN procedure averages 48 days ± 17 days—see also Chapter 7. This proposed treatment schedule for melanoma patients is based on the diagnostic outcome of the pathological examination of the excision biopsy and incorporates adjuvant immunopotentiation as studied in this thesis, see also Figure 1. To irrevocably demonstrate the benefit of such a non-specific and generally applicable

**Figure 1.** Proposed treatment schedule for melanoma patients based upon Breslow thickness. *or Breslow thickness <1mm, but with a Clark’s level of invasion >III and/or ulceration. †An ultrasound of the SLN basin can result in no suspect LN (LN-) or suspect LN followed by fine needle aspiration with a negative cytology (LN-) or a positive cytology (LN+). Abbreviations: SLN: sentinel lymph node; LN: lymph node; ELND: elective lymph node dissection.
immunopotentiation, large-scale, randomized Phase III trials are needed. Until then, more easily feasible small-scale Phase II trials (as presented in this thesis) are urgently needed to test the relative efficacy of different cytokines, TLR-L, or novel modulators of tumor-related immune suppression (e.g. small-molecule inhibitors of indoleamine-2,3-dioxygenase or STAT3 phosphorylation), in terms of DC and melanoma-specific T cell activation. These studies may ultimately lead to the identification of the most promising immunopotentiating cocktails to be further developed for routine clinical application in the adjuvant treatment of intermediate thickness and possibly more advanced melanoma.

REFERENCES


Publications
PUBLICATIONS


Nederlandse samenvatting
NEDERLANDSE SAMENVATTING

Melanoom van de huid (moedervlekkanker) is een zeer agressieve en steeds vaker voorkomende vorm van huidkanker, waarbij uitzaaiing al in een vroeg stadium van de ziekte plaatsvindt. De afgelopen decennia is het voorkomen van melanoom fors gestegen. Gelukkig wordt het melanoom steeds vaker in een relatief vroeg stadium herkend en behandeld, waardoor de sterfte in mindere mate is toegenomen. De levensverwachting van patiënten met een melanoom wordt vooral bepaald door eventuele metastasering (tumoruitzaaiing), omdat de behandelmogelijkheden in dit stadium beperkt zijn. Vroege diagnostiek en behandeling van het melanoom zijn dus van groot belang voor de prognose van melanoompatiënten.

De diagnose melanoom kan pas worden gesteld na het nemen van een diagnostisch biopt. Het geldende advies is om gepigmenteerde huidafwijkingen verdacht voor melanoom te verwijderen door middel van een radicale excisie biopsie met een minimale tumorvrije marge van 2 mm. In de praktijk worden er echter ook krap-radicale excisies (tumorvrije marge < 2 mm), niet-radicale excisies en stans-/incisiebiopten genomen. De invloed van zowel het type diagnostische biopsie als de aanwezigheid van tumorcellen in het re-excisiepreparaat op de overleving van melanoompatiënten wordt onderzocht in hoofdstuk 2.

Daarin wordt beschreven dat beide niet van invloed te zijn op de overleving van melanoompatiënten. Toch blijft het belangrijk om de gehele tumor in één keer te verwijderen.

Sinds een aantal jaren is er een procedure waarbij de eerste tumor-drainerende lymfeklier in het regionale lymfeklierstation geïdentificeerd en vervolgens geëxcludeerd (uitgesneden) wordt. Deze klier wordt ook wel de schildwachtklier (SWK) genoemd. Wanneer de SWK positief bevonden wordt door de patholoog, dat wil zeggen dat er tumorcellen in de lymfeklier aanwezig zijn, dan komt de patiënt in aanmerking voor een regionale lymfeklierdissectie. Dit komt in ongeveer 20% van alle stadium I/II melanoompatiënten voor. Dat tumorcellen de kans krijgen om te metastaseren komt vermoedelijk mede voort uit het feit dat het immuunsysteem van de patiënt de tumor-geassocieerde antigenen niet als voldoende "lichaamsvreemd" en gevaarlijk beschouwt; er bestaat immunologische tolerantie. Het principe van immuuntherapie is dan ook om het immuunsysteem van de patiënt te activeren tegen de tumor. De belangrijkste afweercellen hierbij zijn de zogenaamde dendritische cel (DC) en de T-cel. DC kunnen lokaal tumoreiwitten opnemen uit het tumorweefsel en vervolgens naar de drainerende lymfeklier migreren. Als de DC voldoende geactiveerd raken, kunnen zij hier de tumoreiwitten aanbieden aan een groot aantal T-cellen dat constant recirculeert door het bloed en de lymfebanen. Na herkenning van de tumoreiwitten,
kunnen de geactiveerde tumor-specifieke T-cellen vervolgens naar de tumor migreren en daar de tumorcellen selectief doden. DC spelen zo een cruciale rol in het starten van een succesvolle immuunrespons tegen de tumor. Helaas scheiden melanomen suppressieve stoffen af die DC kunnen remmen in hun activatie en functioneren. De plek waar de eerste afweercellen geactiveerd moeten worden, is wrang genoeg ook de plek waar deze tumor-geïnduceerde immuunsuppressie het eerst en het hardst toeslaat: de SWK. Zo wordt het immuunsysteem ondernijdend en kan metastasering optreden. Dit proefschrift laat zien dat het mogelijk is om het immunologisch functioneren van de SWK te stimuleren door middel van het injecteren van immuunstimulerende stoffen op de plek van het primaire melanoom.

In de eerste klinische studie kregen 12 melanoompatiënten GM-CSF of fysiologisch zout toegediend door middel van een intracutane injectie rond de primaire tumorplaats. GM-CSF staat bekend als een DC activerende stof. In de SWK van de met GM-CSF behandelde patiëntpopulatie werd niet alleen een significante toename van het aantal DC in de SWK gevonden, maar de aanwezige DC waren ook beter van kwaliteit (meer gedifferentieerd en geactiveerd) en dus theoretisch ook beter in staat om T-cellen tot een specifieke anti-tumorrespons aan te zetten; zie hoofdstuk 3. Het aantal melanoom-specifieke T-cellen in de SWK van de met GM-CSF of fysiologisch zout behandelde patiëntpopulatie werd getest, zoals beschreven in hoofdstuk 4, en er werd inderdaad een significant verband gevonden tussen het aantal DC in de SWK en de aanwezigheid van melanoom-specifieke T-cellen. Samen laten de resultaten beschreven in hoofdstukken 3 en 4 zien dat het lokaal toedienen van GM-CSF de kans op een effectieve immuunrespons tegen melanoom verhoogt en dus mogelijk bescherming biedt tegen het ontstaan van (micro-)metastasen. Verder hebben we in hoofdstuk 5 het aantal en de activatie staat van DC in de huid vergeleken met de DC in de corresponderende SWK voor zowel de fysiologisch zout als GM-CSF toegediende patiëntpopulatie. Terwijl de DC in de huid ingespoten met fysiologisch zout in een rustende staat verkeerden waren de DC in de GM-CSF ingespoten huid geactiveerd en toegenomen in aantal, wat exact correleerde met de gevonden aantallen in de SWK.

GM-CSF is vooral van invloed op de vroegst beschreven, klassieke myeloïde DC maar er bestaat nog een belangrijke groep DC, namelijk de meer recent geïdentificeerde plasmacytoïde DC. Ook plasmacytoïde DC komen voor in de SWK en zodra ze geactiveerd raken geven ze grote hoeveelheden van de stof IFNα af, wat direct melanoom-specifieke T-cellen kan activeren. Daarnaast heeft IFNα een maturerend effect op nabij gelegen myeloïde DC en kan op deze manier dus ook indirect melanoom-specifieke T-cell activeren. Bacterieel verkregen ongemethyleerd CpG, zoals PF-3512676, bindt en activeert plasmacytoïde DC.
In de tweede klinische studie werd het effect van PF-3512676 op de verschillende DC en T-cel subgroepen van de SWK bestudeerd. Vierentwintig patiënten met klinisch stadium I/II melanoom werden gerandomiseerd voor preoperatieve toediening van PF-3512676 (n=11) of fysiologisch zout (n=13), beide werden een week preoperatief toegediend door middel van een intracutane injectie rond de primaire tumorplaats. Zoals in hoofdstuk 6 staat beschreven werd PF-3512676 door alle melanoompatiënten goed verdragen. De SWK van de met PF-3512676 behandelde patiëntenpopulatie was significant groter en zowel de plasmacytoïde DC als de myeloïde DC waren beter gedifferentieerd en geactiveerd. Verder werden er significant minder regulatoire T-cellen gevonden in de PF-3512676 behandelde patiëntenpopulatie. Dit is belangrijk omdat het hebben van veel immuunsuppressieve, regulatoire T-cellen bij kankerpatiënten voorspellend is voor een slechte prognose. Het immunstimulerende effect van PF-3512676 werd ook terug gezien in een significante toename van het aantal melanoom-specifieke T-cellen. Opvallend genoeg werden er niet alleen meer melanoom-specifieke T-cellen gevonden in de SWK, maar ook in het bloed van de met PF-35312676 behandelde patiëntenpopulatie; zie hoofdstuk 7. Daarnaast was ook het aantal NK (natural killer) cellen in de SWK van de met PF-3512676 behandelde patiëntenpopulatie significant toegenomen. NK cellen maken deel uit van het niet-specifieke (of aangeboren) afweersysteem dat een eerstelijns defensie vormt tegen bijvoorbeeld tumoren en virale infecties. Samen tonen de resultaten beschreven in hoofdstuk 6 en 7 dat een eenmalige toediening van PF-3512676 zowel een niet-specifieke (of aangeboren) als een specifieke (of adaptieve) afweerrespons tegen tumorcellen in de SWK kan induceren en/of versterken. Bovendien werd de immuniteit niet alleen lokaal verbeterd in de SWK maar ook systemisch, door het toenemen van het aantal melanoom-specifieke T-cellen in het perifere bloed. Deze universeel toepasbare en laagdrempelige therapie biedt zo ook mogelijk bescherming tegen tumoruitzaaiingen op afstand.

Of het lokaal injecteren van immuunstimulerende middelen zoals GM-CSF en PF-3512676 uiteindelijk leidt tot een verbeterde overleving van melanoompatiënten moet gaan blijken uit grote gerandomiseerde fase III studies. Tot die tijd blijft het belangrijk om in kleinere studies verschillende middelen - alleen of in combinatie - te testen op hun immuunstimulerend vermogen: een zoektocht naar de meest potente cocktail.
Dankwoord
DANKWOORD

Het is af: mijn proefschrift! De afgelopen vier jaar waren intensief, maar ik heb er met veel plezier aan gewerkt. Een proefschrift schrijven doe je gelukkig niet alleen, en er zijn dan ook velen die ik moet bedanken. Allereerst natuurlijk de melanoompatiënten die in bange en onzekere tijden bereid waren om mee te werken aan onze studies. En dan al die mensen binnen de afdelingen Heelkunde, Immunologie, Pathologie en Oncologie van het VU Medisch Centrum die bereid waren om mij wegwijzen te maken binnen de verschillende disciplines. Een aantal mensen wil ik graag in bijzonder bedanken:

Prof.dr. P.A.M. van Leeuwen, promotor. Beste Paul, jij hebt mij deze unieke kans gegeven binnen de wereld van de wetenschap. Als gedreven en origineel mens wist je altijd te verrassen en te motiveren.

Prof.dr. S. Meijer, promotor. Beste Sybren, het is heerlijk om samen te werken met iemand die altijd de grote lijnen in de gaten houdt. “De oplossing” voor gemetastaseerde ziekte hebben we niet gevonden, wellicht toch een stap in de juiste richting.

Prof.dr. R.J. Scheper, promotor. Beste Rik, zelden heb ik zo’n enthousiast mens ontmoet. Bedankt voor alle kritische, maar altijd opbouwende kritiek.

Dr. T.D. de Gruijl, co-promotor. Beste Tanja, natuurlijk was jouw kennis van de immunologie onmisbaar, en wist je met jouw enorme gevoel voor taal al mijn stukken net dat beetje extra te geven…maar ik vond het vooral héél erg gezellig.

Leden van de promotiecommissie: Prof.dr. E. van der Wall, Prof.dr. Y. van Kooyk, Prof.dr. A.M.M. Eggermont, Prof.dr. G.J. Adema, Prof.dr. B.B.R. Kroon, Prof.dr. P.J. van Diest, Dr. A.J.M. van den Eertwegh. Bedankt voor het bestuderen van het manuscript en het voeren van de oppositie.

Dr. E. Ph. Steller, opleider St Lucas Andreas ziekenhuis, bedankt voor alle steun bij het vinden van een promotieplek, ik had mijn onderzoeksjaren niet willen missen.

Dr. M.P. van den Tol, chirurg VUMC. Beste Petrousjka, bedankt voor het includeren, opereren en het geven van alle wijze raad. Ik hoop dat we Los Angeles nog een keer over kunnen doen.

Ronald Vuylsteke, voorganger. Beste Ronald, op een solide basis is het gemakkelijk bouwen. Bedankt voor de gestructureerde overdracht en goede adviezen.

Berbel Sluijter, opvolgster. Beste Berbel, de onderzoekslijn is bij jou in goede handen. Heel veel succes met het afronden van je promotie.

Pepijn Wijnands, analist. Beste Pepijn, bedankt voor het verzetten van bergen! Zonder jou was het mij niet gelukt om zo snel te promoveren.


Benny Oosterhof, student. Beste Benny, je hebt je ontpopt als een ware database expert. Je hulp was onmisbaar. Bedankt!

De onderzoeksgang Heelkunde: Frank Termaat, Robert-Jan Derksen, Babs Zonderhuis, Milan Richir, Steven Oosterling, Michiel Siroen, Rita van der Hem-Stokroos, Petra Boelens en Matthijs Elzinga. Bedankt voor jullie gezelligheid.


De afdeling Pathologie: Sinéad, Hetty, Anita, Adrie, Wim Vos, en alle andere medewerkers die mijn verblijf op de oude 2de etage zo plezierig hebben gemaakt. Bedankt voor alle (technische) ondersteuning.

De afdeling Heelkunde, stafleden, fellows, CHIVO’s en arts-assistenten van het VU medisch centrum, bedankt voor alle interesse en steun.

Familie en vrienden, bedankt voor de (morele) steun! Ik ben blij dat we elkaar weer wat vaker zullen zien.

En dan mijn ouders…die mij met zoveel liefde en geborgenheid omringen. Jullie steun en vertrouwen vormen de basis van mijn bestaan. Ik ben blij dat ik jullie dochter ben.

En tot slot mijn allerliefste Bram, die mij elke keer weer achter de computer zag verdwijnen. Maar het is af…ook zeker dankzij jou!
Curriculum vitae
CURRICULUM VITAE

Barbara Molenkamp was born on the 7th of April 1976 in Hoogezand-Sappemeer, the Netherlands. A few years later her family moved to Sneek, where she attended high school (gymnasium at the Rijksscholengemeenschap Magister Alvinus). At the age of sixteen, she received a two-year scholarship to study at the United World College of the Atlantic in Wales, where she passed the International Baccalaureate in 1994. After graduation, she moved to India for a year and worked as a volunteer teaching Health & Hygiene for the Barnes and Daniels foundation. Having decided that she wanted to become a medical doctor, she returned to the Netherlands to study Medicine at the Rijksuniversiteit Groningen. During her undergraduate studies she went to Mexico to investigate the nutritional state of liver disease patients in Monterrey, under the supervision of Prof.dr. C.H. Gips and Prof.dr. L.E. Munoz-Espinoza. After graduating and obtaining her MD in 2002, she first worked as cardiothoracic surgery resident at the Onze Lieve Vrouwe Gasthuis (head: Prof.dr. L. Eijsman) and later as a surgical resident at the Sint Lucas Andreas Ziekenhuis (head: Dr. E.Ph. Steller), both in Amsterdam. In 2004 she started working as a research-fellow at the department of Surgical Oncology (head: Prof.dr. S. Meijer) at the VU University Medical Center in Amsterdam, where the research was performed described in this thesis under supervision of Prof.dr. P.A.M. van Leeuwen. In 2006 she returned to the Sint Lucas Andreas Ziekenhuis to start her surgical training under supervision of Dr. E.Ph. Steller and this training will be continued at the Academic Medical Center in Amsterdam under supervision of Dr. O.R.C. Busch.