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.[1] 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,[2-4] 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.[5] Exposure to solar UV radiation, fair skin, dysplastic nevi syndrome, and a family history of melanoma are major risk factors for melanoma development.[6] 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.[7] 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.[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[1] and early surgical excision being the only curative treatment option,[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.[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[12-15] but only after a recent publication of Morton *et al* the SLN procedure was accepted as standard of care for melanoma patients.[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 *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.[17,18] In order to subsequently activate melanoma-specific T cells, the migrated MDC need to be sufficiently activated.[19] DC development and activation can both be frustrated by inhibitory factors commonly associated with melanoma.[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,[22-24] the SLN shows the most pronounced immunological deficits.[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.[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.[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.[28] 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.[29] 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.[29] 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.[29,30] 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,[31-33] 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.[34-38] 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 $CD4^+CD25^{hi}CTLA\text{-}4^+FoxP3^+$ 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[16] 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 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.

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