7.

Discussion and future prospects

Part of this chapter has been adapted from:

Immunotherapy: Is it different for sarcomas?
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High-grade osteosarcoma is a malignant bone tumor with the highest incidence in young patients. The prognosis for patients with metastasized disease remains dismal, despite aggressive surgery and intensive chemotherapeutic regimens. In **chapters 2** and **3** of this thesis, studies aimed at a better understanding of the etiology and prognostic factors of high-grade osteosarcoma are described. In **chapters 4**, **5** and **6** interactions between high-grade osteosarcoma cells and innate immune cells are studied. Together, these studies can guide the design of clinical studies implementing novel immunotherapeutic regimens. Adding immunotherapy to standard treatment regimens will hopefully result in better survival for osteosarcoma patients.

ETIOLOGY AND PROGNOSTIC FACTORS OF HIGH-GRADE OSTEOSARCOMA

The cell of origin of high-grade osteosarcoma remains elusive, but the strong temporal and spatial relationship with proliferating and differentiating mesenchymal stromal cells (MSCs), suggests that MSCs or early osteoblast precursor cells are likely suspects. Additionally, long term *in vitro* expansion of murine mesenchymal stem cells results in spontaneous oncogenic transformation of the cultured cells which form osteosarcoma-like tumors *in vivo* [113;116;179;183;259]. These transformed cells have a near-tetraploid aneuploid karyotype and show loss of the CDKN2 locus, similar to human spontaneous osteosarcoma [183]. MSCs derived from cynomolgus macaques also transform upon long term *in vitro* culture [107]. Previously, some groups have reported spontaneous transformation of human MSCs, only to later retract these publications because of contamination of the cultures with other cell lines [56;218;221-223;260]. In childhood leukemias, preleukemic alterations have been found in hematopoietic stem cells years before the leukemia developed [164]. The acquisition of additional mutations and subsequent clonal evolution results in the outgrowth of a true leukemic clone [110]. Similarly, in solid tumors, the 'multiple hit' hypothesis postulates that several chronological mutational and cell cycle deregulatory events are necessary for cancer to develop [66]. Alternatively, a single catastrophic event, termed chromothripsis, can result in highly complex chromosomal rearrangements and oncogenic transformation [102;247]. This probably has to occur in a susceptible background, either acquired in specific individual cells or as a somatic genetic predisposition. In **chapter 2**, we hypothesized that MSCs of osteosarcoma patients might harbor one or a few of such 'pre-cancerous' alterations. The added cellular stress of prolonged *in vitro* culture could then result in additional (pre-) oncogenic hits and a higher propensity to spontaneous transformation in osteosarcoma patient derived MSCs than in MSCs harvested from healthy donors. Since MSCs originating from the tumor site but predating tumor formation were obviously not available, we chose to study MSCs harvested from the iliac crest at diagnosis, prior to chemotherapeutic treatment.

On a transcriptional level, downregulation of hematopoietic cell specific Lyn substrate 1 (*HCLS1*) was noted in osteosarcoma patient derived MSCs as compared to healthy donor derived MSCs, the product of which is involved in B-cell receptor signaling and myelopoiesis [75;233]. If downregulation of *HCLS1* in osteosarcoma patient derived MSCs has a functional role in osteosarcomagenesis is as yet unknown. Despite almost two years in culture, none of the samples underwent spontaneous transformation. An increase in binucleation was noted upon increasing passage in both osteosarcoma patient and healthy donor derived MSCs. Perhaps the binucleation is a result of telomere shortening, anaphase bridges and failed cytokinesis [77;123;201]. In any case, functional cell cycle checkpoints were apparently intact in both patients and healthy donors, as no transformation occurred and cytogenetic analysis on metaphases did not reveal any karyotypic abnormalities.

As was discussed in **chapter 2**, there are several possible explanations for the observed similarities between MSCs derived from osteosarcoma patients and healthy donors. It is possible that the hypothesis that osteosarcoma derives from MSCs is incorrect. Alternatively, MSCs are

the precursor cells, but (pre-)oncogenic alterations are not present in MSCs harvested from the iliac crest. This does not preclude the presence of preoncongenic alterations in MSCs at other sites. For example, there could be somatic mosaicism, similar to what has been shown for the enchondromatosis syndromes Ollier disease and Maffucci syndrome [202]. Another possibility is that prolonged *in vitro* culture is insufficient for oncogenic transformation to occur. According to the 'multiple hit hypothesis', several oncogenic alterations are necessary for transformation to occur. Perhaps growth factor signaling (endocrine and paracrine stimuli in the microenvironment where osteosarcoma arises, *i.e.* near the growth plate) or additional oncogenic 'hits' such as loss of cell cycle control are a prerequisite for oncogenic transformation of susceptible MSCs. For example, prolonged culture of MSCs on a background of downregulated CDKN2/p16 may yield transformed cells. P53 is essential for preventing cell cycle progression in case of failed cytokinesis. Loss of p53 might result in cell cycle progression in case of failed cytokinesis with tetraploidy and aneuploidy as a result [77]. A better understanding of what drives oncogenic transformation in human MSCs will help elucidate the mechanisms important in osteosarcomagenesis, which in turn has the potential to identify targets for therapy.

Pulmonary metastatic disease is the main cause of death for osteosarcoma patients [24]. About one in five osteosarcoma patients present with clinically evident pulmonary metastatic disease at diagnosis and two in five develop pulmonary metastatic disease during treatment or follow up of their disease. Risk factors associated with the development of pulmonary metastases include a poor histological response to pre-operative chemotherapy and the presence of a primary tumor not amenable to local resection. Despite aggressive multimodal therapy, about four in five patients with pulmonary metastatic disease succumb to their disease. It is these patients that would benefit most from novel adjuvant (immuno-)therapies.

In **chapter 3**, prognostic factors related to the survival of patients with pulmonary metastasized high-grade osteosarcoma were studied. Higher metastatic tumor burden (*i.e.* larger number of pulmonary nodules), presence of vital metastases upon resection and male sex were associated with an increased risk of death. In **chapter 4**, genome-wide expression studies were performed to identify genes associated with a risk for pulmonary metastatic disease. High expression of macrophage-associated genes was associated with a lower risk of metastatic disease (discussed in paragraph 7.2.1). Together, the results of **chapters 3** and **4** show that osteosarcoma patients with morphologically vital metastases and a high metastatic tumor burden may benefit from immunotherapeutic strategies exploiting migration and activation of monocytes/macrophages towards the tumor site.

INTERACTIONS BETWEEN HIGH-GRADE OSTEOSARCOMA CELLS AND INNATE IMMUNE CELLS

Tumor-associated macrophages in high-grade osteosarcoma

The Janus-faced roles of macrophages in cancer imply both tumor-suppressive and -stimulating actions of these innate immune cells. Whereas the balance is toward tumor promotion in most

epithelial cancers, in **chapter 4** we show that osteosarcoma metastasis seems to be inhibited by the presence of macrophages in the tumor microenvironment.

It is not a coincidence that the 'Father of Immunotherapy,' William B. Coley, was a bone sarcoma surgeon. The first successful example of immunotherapy was in 1891 when Coley's toxins, a mixture of toxins of streptococcal bacteria was injected into an unresectable sarcoma. The resulting immunological reaction led to tumor regression, similar to what has been observed in osteosarcoma patients suffering from post-operative infection following resection of their primary tumor [49]. The few known permanent responses to Coley's toxins in carcinoma were in those cases of mesodermal origin [246]. Are sarcomas and other tumors of mesodermal origin more immunogenic than carcinomas? Or do immune cells have an effect that is different between sarcomas and carcinomas?

The tumor promoting effect of macrophages in carcinomas is well established. Epithelial tumors with high numbers of infiltrating immune cells have a poor prognosis as compared with cases with few infiltrating cells. This is attributed to a number of properties of the immune cells, especially macrophages, which have been shown to be involved with tumor initiation, invasion, migration, intravasation and angiogenesis [210]. Especially the stimulating effect on tumor invasion and migration of in origin non-motile epithelial cells that are the progenitors of carcinomas can be well comprehended. However, it is different for mesenchymal cells, which are much less dependent on contact with adjacent cells and thus more motile. These cells probably do not need the guidance that immune cells seem to give to carcinoma cells in the circulation. Instead, mesenchymal tumor cells might be inhibited in their motility by macrophages, which then act as impediment, instead of promoter for invasion. This is of course speculative, but it has been reported that macrophage inhibitory factor, MIF, which is produced by macrophages, inhibits migration of mesenchymal stem cells [69].

In **chapter 4**, an expression profiling study in a relatively large series of high-grade osteosarcomas was performed, with results corroborating a metastasis inhibiting role for macrophages. The 'expression profile' associated with non-metastatic behavior of osteosarcoma surprisingly consisted of a large number of genes associated with macrophage function, such as antigen processing and presentation or pattern recognition, as well as specific monocyte and macrophage markers such as *CD14* and *MSR1*. Also a large number of genes with other immunological functions, such as cytokine production and phagocytosis were found to be upregulated. Expression of the macrophage-associated genes was confined to primary tumor tissue and not detected in a panel of 19 osteosarcoma cell line RNA samples, indicating that infiltrating immune cells were responsible for this expression profile. Furthermore the results were confirmed at the protein level by immuno histochemical staining on a larger patient cohort.

A role for macrophages to prevent or reduce metastases of osteosarcoma is corroborated by one of the few efficacious new therapeutic agents that were tested since the successful introduction of conventional chemotherapy for osteosarcoma, i.e., liposomal muramyl tri-peptide (MTP), also known as Mepact® or mifamurtide. This proprietary drug elicits activation of macrophages. Although the clinical trial that included adjuvant treatment with mifamurtide was initially denounced because of a presumed interaction with one of the chemotherapeutic compounds, it eventually appeared to give an improvement from 70 to 78% survival in patients

with non-metastatic osteosarcoma, which was the best achievement in improving outcome in decades [169;170]. Our finding that macrophages are associated with less metastases now provides a valid biological rationale for the efficacy of this drug. However, concerns regarding the design of the trial has prevented wide-scale clinical adoption of the compound in therapeutic regimens. Additional supportive evidence for the effectiveness of immune-stimulation in osteosarcoma is the use of interferon(IFN)-α as adjuvant therapy, with encouraging results in historical cohorts of Scandinavian osteosarcoma patients [249]. *In vitro* data suggests that the positive effect may involve both immunological (as shown in **chapter 6**) and direct anti-tumor effects [190]. In the recently completed EURAMOS-1 clinical trial this drug was included in one of the randomized arms [278]. Preliminary data suggests no benefit, but follow-up of patients is ongoing [25].

Neither the mechanism of metastasis suppression by intratumoral macrophages in osteosarcoma is clarified, nor the contrast with epithelial tumors. It may be sought in the different flavors of macrophages that are distinguishable by specific markers. M1 are tumor suppressive, M2 support invasion, metastasis and angiogenesis of tumor cells. We assessed the nature of the tumor associated macrophages in osteosarcoma clinical samples using HLA-DRα, associated with M1 macrophages and CD163, a marker to distinguish M2. Surprisingly, both types of macrophages were present in the tumor tissues analyzed (Fig. 7.1). Recent perceptions on good vs. bad macrophages are more nuanced. Macrophages are flexible cells that polarize to a certain direction, but are not destined to stay that way (Fig. 1.5).

To complicate things even more, there was a recent report that macrophage infiltration in another primary bone tumor, Ewing sarcoma, predicts a poor prognosis [78]. Tumor associated macrophages are also associated with a poor prognosis in leiomyosarcoma and gastrointestinal stromal cell tumors [138;266]. The tumor microenvironment is conducive towards the generation of pro-tumor, immunosuppressive and pro-angiogenic M2 macrophages in many epithelial cancers and apparently also in some sarcoma types [208]. On the other hand, a pro-inflammatory tumor microenvironment can skew macrophage polarization towards M1 type macrophages with anti-tumor properties. Similar to epithelial cancers, high macrophage infiltration was associated with increased microvessel density in osteosarcoma, suggesting a similar role for M2 type macrophages in the promotion of angiogenesis. However, in the case of osteosarcoma, the influx of pro-angiogenic macrophages may be similar to a "Trojan horse." Perhaps macrophages are attracted by the tumor to support angiogenesis, but following chemotherapeutic treatment, the release of endogenous danger signals by dying tumor cells causes the macrophages to become polarized toward an M1, anti-tumor phenotype (Fig. 7.2). This proposed mechanism is supported by the fact that the survival benefit of high macrophage infiltration as determined in **chapter 4** was partly dependent on the histological response to chemotherapy.

Coley's toxins were denounced by another famous bone sarcoma expert, the pathologist James Ewing who gave his name to the second aggressive pediatric bone tumor [65]. Ewing was not charmed by the medieval treatment developed by Coley, he was a fervent proponent of radiation therapy, which was effective for many tumors, but not for osteosarcoma.

The relatively good response of sarcomas to immune stimulation and the favorable prognostic effect of tumor associated macrophages as opposed to carcinomas suggests that

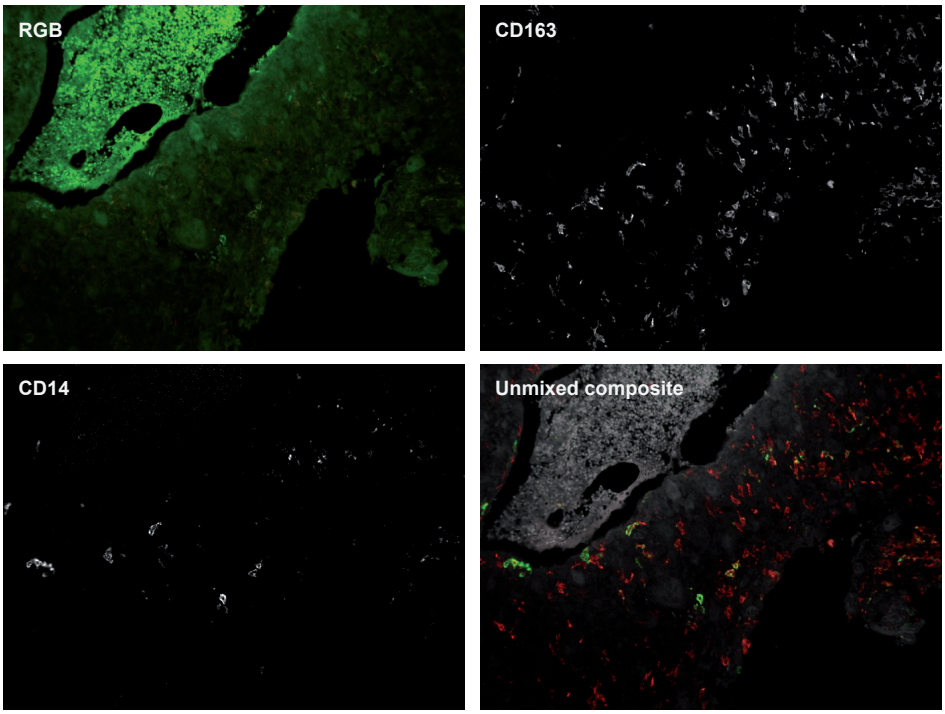

Fig. 7.1 Osteosarcoma samples are infiltrated with CD14 and CD163 single and double positive macrophages. Spectral imaging was used to reduce autofluorescence of osteosarcoma cells. In the composite image, CD14-positive cells are represented in green, CD163-positive cells are represented in red, and CD14/CD163 double positive cells are represented in yellow. Background autofluorescence of tumor cells is represented in gray.

tumor immunology is different for sarcomas. This does not seem attributable to a particular macrophage subtype, but lies in the nature of this tumor type. Few clinical trials have been conducted on immunotherapy in sarcomas. Given our findings that macrophages are associated with less metastases in osteosarcoma, tumor immunotherapy specifically targeted at this tumor type should be evaluated.

Activation of natural killer cells in immunotherapy of osteosarcoma

The ability of natural killer (NK) cells to lyze tumor cells without the need for prior sensitization is an attractive prospect for anticancer immunotherapy [148;186]. In **chapters 5** and **6** we show that osteosarcoma cells are sensitive to lysis by both autologous and allogeneic NK cells. The antitumor activity of NK cells could be further augmented by activation with interleukin(IL)-15 and IFN-α. Importantly, chemotherapy resistant osteosarcoma cells retained their susceptibility to NK cell mediated lysis. Lysis of osteosarcoma cells by NK cells was dependent on Natural Killer Group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1) but not on CD95. In many cancer types, systemic immunosuppression appears to influence the phenotype and effector capability of immune cells [27;51;52;62]. In contrast, osteosarcoma patient derived NK cells have normal

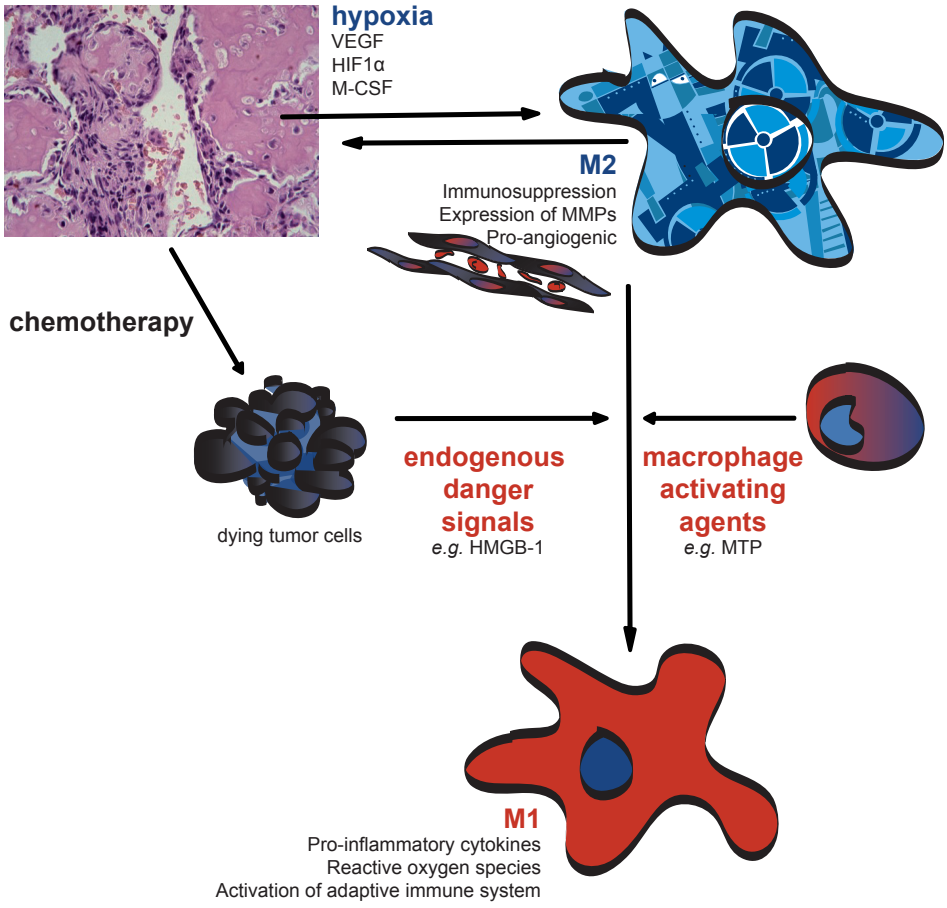

Fig. 7.2 Model depicting the pro-tumor M2 macrophage as a 'Trojan horse' which can polarize towards an anti-tumor M1 type macrophage following activation by danger signals and cytokines.

phenotypic characteristics and unimpaired native cytolytic function (**chapter 5**). In addition, patient derived NK cells can be adequately activated by cytokine treatment with IL-15 (**chapter 5**) or IFN-α (**chapter 6**). Therefore, activation of autologous NK cells (either *in vivo* or *ex vivo*) may be efficacious. However, since patients are treated with lymphodepleting chemotherapy, careful thought needs to be given regarding the optimal timing of adjuvant immunotherapeutic treatment.

A trial in which autologous activated NK cells were infused in melanoma and renal cell cancer patients demonstrated that NK cells persisted in the circulation for days to weeks following adoptive transfer [203]. Disappointingly, no clinical responses were seen, perhaps because of an observed down-regulation of the activating receptor NKG2D *in vivo*. In addition to cytolysis as a result of ligation of activating receptors, NK cells are capable of antibody-dependent cellular cytotoxicity (ADCC). Using the anti-epidermal growth factor receptor (EGFR) antibody cetuximab, osteosarcoma cells are sensitive to ADCC by NK cells [200]. ADCC is not dependent on signaling through the activating

NK cell receptors. Perhaps the combination of the adoptive transfer of autologous NK cells and treatment with humanized monoclonal antibodies will yield the desired clinical results.

Although osteosarcoma cells were sensitive to lysis by autologous NK cells *in vitro*, there is some theoretical benefit of using allogeneic NK cells. NK cells are inhibited in their cytolytic activity when inhibitory killer cell immunoglobulin-like receptors (KIRs) recognize their cognate major histocompatibility complex (MHC) class I ligands (Fig. 1.6) [118]. KIR-ligand mismatch has been shown to contribute to NK cell cytolysis *in vivo,* for example in the setting of stem cell transplantion in acute myeloid leukemia (AML)-patients [173;270]. In line with this data, studies in mice show that KIR-ligand mismatched NK cells facilitate engraftment of hematopoietic transplants, and have increased graft-versus-leukemia efficacy and reduced graft-versus-host disease in comparison to NK cells without such a KIR-ligand mismatch [224]. Similar results were obtained in a murine breast cancer model employing haploidentical bone marrow and spleen transplantation [76]. In osteosarcoma, there is a relatively high level of MHC class I expression (Fig. 5.2, and [262]). Therefore, application of allogeneic NK cells with a KIR-ligand mismatch (for example in the setting of a haploidentical stem cell transplantation) will possibly result in a higher degree of tumor cell lysis than strategies aimed at the activation of autologous NK cells.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Despite multi-agent chemotherapy and wide margin surgery, high-grade osteosarcoma has a poor prognosis. In this thesis, data demonstrating a role for cells of the innate immune system in controlling and possibly treating high-grade osteosarcoma are presented. Future studies should be aimed at a better understanding of the interaction between tumor and host. Tumor cells may influence migration, differentiation, polarization and activation of innate immune cells. Conversely, immune cells can have direct or indirect effects on viability, motility, invasion and migration of tumor cells. For example, immune cells can express cytokines and chemokines -such as CXCL12- which can bind to the receptors – in this case CXCR4- on tumor cells, with a potential to influence tumor cell viability and motility. There is substantial interconnectivity between these various aspects of tumor-host interactions, which also involve other cells, such as endothelial cells and stromal cells.

As was shown in **chapter 4**, infiltration of macrophages in osteosarcoma is associated with a reduced risk of metastatic disease, but the exact biological mechanism in which macrophages inhibit (metastasis of) osteosarcoma tumor cells remains unclear. Both classically activated M1 type macrophages as well as alternatively activated M2 type macrophages are present in osteosarcoma. Using an *in vitro* co-culture system, the effect of osteosarcoma cells on the differentiation and polarization of monocytes and macrophages can be studied. In addition, cytotoxicity experiments can be performed to study if macrophages are able to lyse osteosarcoma cells and if this is dependent on the activation and polarization status (M1 *vs.* M2) of the effector cells. Upregulation of the SIRPα-ligand CD47 on myeloid leukemia cells resulted in reduced phagocytosis by macrophages, possibly contributing to immune evasion by the tumor [109]. Although the interaction between CD47 and SIRPα was not studied in this thesis, in **chapter 4**, several macrophage genes associated with phagocytosis were shown to be

upregulated in osteosarcoma patients with good prognosis. Perhaps specific blocking of CD47-SIRPα interaction augments macrophage-mediated osteosarcoma cell phagocytosis.

A promising tool to study tumor-host interactions are xenograft models [182]. Freshly isolated tumor cells obtained from diagnostic biopsies can be transplanted in immunodeficient mice. If corresponding peripheral blood mononuclear cells (PBMCs) are available from these patients, these can be used to reconstitute the immune system of the recipient. This will allow for detailed study of tumor-immune cell interaction in an 'autologous' setting. Tumor growth can be monitored in the presence or absence of specific immune cell subsets. Using such an 'autologous xenograft model', several fundamental questions regarding the interaction between monocytes/macrophages and osteosarcoma cells can be answered. Do monocytes/macrophages influence growth of osteosarcoma xenografts? If so, is this effect dependent on the presence of other immune cell subsets such as T cells, implicating a possible role of the adaptive immune system in tumor control? Does treatment with macrophage activating agents influence macrophage polarization and tumor outgrowth in this model? Using a humanized antibody, is there evidence of ADCC by monocytes/macrophages and/or NK cells *in vivo?* Is there an effect of monocytes/macrophages on angiogenesis in osteosarcoma and is this dependent on polarization status of the macrophages?

In **chapters 5 and 6**, pre-clinical data regarding the efficacy of NK cells in the treatment of osteosarcoma are presented. As was discussed in paragraph 7.2.2, there is a theoretical advantage of the adoptive transfer of KIR-ligand mismatched allogeneic NK cells as opposed to the activation of autologous NK cells. Additional *in vitro* cytolytic experiments can determine if KIR-ligand mismatch also contributes to increased NK cell mediated cytotoxic activity in osteosarcoma. Xenograft models using immunodeficient mice with human NK cell reconstitution can be used to study efficacy of NK cells and NK cell activating agents in osteosarcoma lysis *in vivo*. Another potentially efficacious approach in augmenting NK cell mediated cytotoxicity is blocking the effect of non-KIR co-inhibitory receptors. For example, NK cells can express the inhibitory receptor PD-1 [289]. Perhaps blocking of this receptor might result in a better lysis of PD-1 ligand expressing tumor cells.

A hurdle in successful application of NK cell activation or adoptive transfer in the immunotherapy is the infiltration of sufficient numbers of activated NK cells in the tumor. Zebrafish or murine xenografts with live cell imaging of labeled immune cells can be used to study migration of immune cells towards the tumor site.

In addition to the preclinical studies, the results as presented in this thesis justify translation to clinical trials. Treatment with mifamurtide -a macrophage activating agent-, yielded promising clinical results [169;170], but it remains unknown which patient group would benefit most from treatment with macrophage activating agents. Perhaps treatment with a macrophage activating agent is only beneficial in patients that already have relatively large numbers of macrophages present in the tumor. Alternatively, activation of monocytes circulating in the peripheral blood could also result in enhanced intratumoral infiltration and subsequent antitumor activity of intratumoral macrophages and will thus (also) benefit patients who present with low numbers of macrophages in the primary tumor. To better understand

what is happening *in situ* during treatment with a monocyte/macrophage activating agent, the migration and activation status of monocytes/macrophages before, during and after treatment needs to be monitored. Patients with bilateral metastatic disease, eligible for multi-step surgery, could undergo resection of metastatic lesions in one lung, followed by several weeks of treatment with a macrophage activating agent such as mifamurtide. Subsequently, the metastases in the contralateral lung could be resected. In this way, the numbers and activation status of macrophages infiltrating the tumor can be determined, both before and after treatment with a macrophage activating agent. PBMCs and plasma can be collected at several time-points to monitor the activation status of monocytes and cytokine levels during this process. This will yield important information regarding the mechanism of action of macrophages in inhibiting metastasis in osteosarcoma.

The safety and efficacy of NK-cell based immunotherapy in osteosarcoma should be addressed in a phase I/II clinical trial. A possible strategy could be the adoptive transfer of cytokine-activated donor NK cells in an allogeneic (especially haploidentical) stem cell transplantation setting. In this scenario, KIR-ligand mismatch will hopefully result in maximal efficacy. Alternatively, autologous NK cells can be activated by the administration of cytokines, either *in vivo* or *ex vivo*. IFN-signaling was unimpaired in PBMCs of osteosarcoma patients and osteosarcoma cells were efficiently killed by IFN- α activated NK cells (**chapter 6**). Preliminary results of the EURAMOS-1 trial however, did not show a clinical benefit of monotherapy with IFN-α in osteosarcoma patients with good histological response to neo-adjuvant chemotherapy [25]. The *in vivo* activation of NK cells might not be as effective as the *ex vivo* activation, possibly due to being able to achieve higher concentrations *in vitro*. Perhaps the adoptive transfer of cytokine-activated NK cells is more efficacious than the administration of cytokines to patients in an attempt to activate immune cells *in vivo*.

In conclusion, the activation of innate immune cells such as macrophages and NK cells is a promising new adjuvant treatment strategy to treat patients with high-grade osteosarcoma. Using xenograft models, the interactions between tumor and host can be examined in more detail. Further studies should be aimed at the translation of pre-clinical data towards clinical trials exploiting the potential of the innate immune system in controlling high-grade osteosarcoma.