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Innate Immune Responses of Natural Killer cells and Macrophages against Bone Sarcomas

Towards Cellular Immunotherapy

PhD Thesis

Jens Pahl

**Innate Immune Responses of Natural Killer cells
and Macrophages against Bone Sarcomas**

Towards Cellular Immunotherapy

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CONTENTS OF THIS THESIS

OUTLINE AND SCOPE OF THIS THESIS

The research performed in this thesis demonstrates that human natural killer cells and macrophages are able to mediate anti-cancer immune responses against osteosarcoma and Ewing sarcoma. Implications for harnessing natural killer cells and macrophages for potential anti-cancer immunotherapy are described.

Osteosarcoma and Ewing sarcoma are the most common bone cancers in children and young adults. Despite advanced surgical techniques and multi-drug chemotherapy, one-third of the patients still succumb to recurrent disease with poor prognosis. Likewise, patients with metastatic and chemotherapy-resistant disease have a poor outcome. Thus, novel targeted therapies are needed that combine potent and specific anti-cancer activity with limited toxicity toward normal tissues. Previous research lines have provided evidence that natural killer (NK) cells and macrophages, both cell types of the innate immune system, are able to contribute to anti-cancer responses against osteosarcoma and Ewing sarcoma cells.

The research in this thesis is performed with the aim to characterize cellular interactions of NK cells and macrophages with osteosarcoma and Ewing sarcoma cells in order to achieve favorable effects on anti-cancer immune cell functions. It is explored in preclinical studies, how the anti-cancer potential of especially NK cells but also macrophages can be enhanced and directed to cancer cells. Modulation of tumor–immune cell interactions and the cytolytic activity of NK cells and macrophages may help to design novel immunotherapeutic approaches to harness anti-cancer functions of (innate) immune cells against osteosarcoma and Ewing sarcoma.

In the following introductory chapter 1, clinical and biological properties of osteosarcoma and Ewing sarcoma are described, followed by an overview of cancer immunology and immunotherapy with the primary focus on innate immunity of NK cells and macrophages. The chapters 2 to 6 compose the main body and are further outlined in section 3 of chapter 1. The thesis is concluded by chapter 7 with a general discussion of the key findings.

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INTRODUCTORY CHAPTER 1

1. **Osteosarcoma and Ewing Sarcoma**

2. **Cancer Immunology and Immunology**

3. **Outline of the Research Chapters**

CONTENTS

1. OSTEOSARCOMA AND EWING SARCOMA

1.1 Incidence and Anatomic Distribution

Bone cancers of osseous and chondromatous origin are considered rare diseases, representing 0.2% of cancers in the adult population (≥25 years of age) with an annual incidence rate of 8.1 per 1×10^6 (Table 1) [1;2]. However, in children, adolescents and young adults (<25 years of age), bone cancers occur more frequently with a peak annual incidence of 16.5 per 1×10^6 in individuals between 15 and 19 years of age, representing up to 7.6% of all cancers (Table 1). In this age group (15–19 years), osteosarcoma and Ewing sarcoma are the most common malignant bone tumors (54% and 36% of bone cancers; 4.1% and 2.8% of all cancers) with an annual incidence of 9 and 6 per 1×10^6, respectively, in the United States (Table 1) [1-4]. In the Netherlands, the annual incidence of osteosarcoma can be more frequent with 10–16 per 1×10^6 in patients of 11–18 years of age [5]. Primary osteosarcoma and Ewing sarcoma predominantly occur in the second decade of life (Table 1), suggesting an association with the adolescent growth spurt and bone remodeling. A second peak for osteosarcoma occurs in the elderly (often considered a secondary bone disease), while the occurrence of chondrosarcoma increases in patients with increasing age [3;4;6-8] (Table 1). Male individuals are more often affected than female individuals; Ewing sarcoma is hardly found in the African American population.

Osteosarcoma commonly arises in the metaphysis of the long bones of the extremities (>80% of cases), in particular, in the distal femur, proximal tibia and proximal humerus [9;10]. The majority of patients (80%) presents with localized disease at diagnosis, while the remaining patients exhibit pulmonary (>90%) or bone metastases. Ewing sarcoma mainly occurs in the diaphysis of long bones of the appendicular skeleton (>50% of cases), in particular, in the femur, but can additionally affect bones of the axial skeleton, such as the pelvis or ribs, and is frequently associated with a large soft tissue mass [9;11]. Approximately 20–25% of patients harbor pulmonary (50%), bone (25%) or bone marrow (25%) metastases at diagnosis.

1.2 Cancer Cell Characteristics

Osteosarcoma cases, and sarcomas of long bones and skull in general, have been documented since, at least, the first half of the 19th century and were extensively reviewed by Henry Meyerding in 1938 [12-17]. In 1957, his successors Mark Coventry and David Dahlin classified osteosarcoma (often named osteogenic sarcoma) as a rare, radiotherapy-insensitive malignant bone tumor producing osteoid, involving primarily the metaphysis of long bones and predominantly affecting adolescents and young adults with poor prognosis [18]. Morphologically, osteosarcoma is composed of a diverse mixture of epithelioid, plasmacytoid, spindle-like and small-round cells and can be grouped in histological subtypes which, however, does not affect treatment or prognosis [1]. In many patients, osteosarcoma cells bear alterations in the p53 gene, retinoblastoma-like gene or MDM2 gene and display aberrant expression/activation of growth factor receptors (e.g., insulin-like growth factor receptor) [9;19].

In 1921, James Ewing reported a rapidly progressing, radiotherapy-sensitive, round cell sarcoma with low cytoplasm content and frequently arising in children and adolescents in the shaft of bones of the extremities [20]. Before that time, presumably, Ewing sarcoma had generally been grouped among other small-round cell malignancies [21]. Subsequently, Ewing sarcoma was

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	age ≤14			age 15–19			age 20–24			age 25–29			age 30–34			age 35–39			age ≥40			all age groups		
	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers	a) incidence	b) % of bone cancers	c) % of all cancers
bone cancer	8,5		5,5	16,5		7,6	10,0		2,8	7,5		1,4	6,0		0,7	6,5		0,5	12,5		0,1	9,6		0,5
osteosarcoma	4,0	47,1	2,6	9,0	54,5	4,1	4,5	45,0	1,3	2,0	26,7	0,4	1,5	25,0	0,2	2,0	30,8	0,2	2,5	20,0	0,0	3,6	37,8	0,2
chrondosarcoma	0,0	0,0	0,0	1,0	6,1	0,5	2,0	20,0	0,6	2,5	33,3	0,5	2,5	41,7	0,3	2,5	38,5	0,2	6,0	48,0	0,1	2,7	27,6	0,1
Ewing sarcoma	3,5	41,2	2,3	6,0	36,4	2,8	3,0	30,0	0,8	2,0	26,7	0,4	2,0	33,3	0,2	1,0	15,4	0,1	1,0	8,0	0,0	2,7	27,5	0,1
leukemia	50,0		32,5	31,0		14,3	26,5		7,4	27,0		4,9	31,5		3,7	38,0		2,9	248,0		2,4	64,6		3,2
lymphoma	15,5		10,1	50,0		23,0	69,0		19,3	75,0		13,7	86,5		10,1	100,5		7,6	460,5		4,4	122,4		6,2
CNS	30,5		19,8	20,5		9,4	22,5		6,3	26,0		4,7	30,5		3,6	37,0		2,8	113,0		1,1	40,0		2,0
soft tissue sarcoma	9,5		6,2	14,5		6,7	18,0		5,0	25,5		4,7	35,0		4,1	45,5		3,5	107,5		1,0	36,5		1,8
germ cell & tropho.	5,5		3,6	28,0		12,9	60,0		16,8	74,0		13,5	72,0		8,4	58,0		4,4	21,5		0,2	45,6		2,3
melanoma & skin	2,0		1,3	14,0		6,4	41,0		11,5	72,0		13,2	98,5		11,6	138,5		10,5	498,0		4,8	123,4		6,2
carcinoma	5,5		3,6	39,0		17,9	104,0		29,1	231,5		42,3	478,5		56,1	865,0		65,7	8441		80,5	1452		73,0
others	26,5		17,2	4,0		1,8	5,5		1,5	8,5		1,6	14,0		1,6	27,5		2,1	545,0		5,2	90,1		4,5

Table 1: Incidence of osteosarcoma and Ewing sarcoma at different age groups in comparison to other cancers

(a) Incidence rates of the different cancer types are depicted per 1,000,000 individuals (males and females and all races combined) of the United States 'Vintage' 2009 population. (b) Incidence rates for osteosarcoma, Ewing sarcoma and chondrosarcoma in comparison to the overall bone cancer incidence rate is depicted as % of bone cancers per age group. (c) The incidence rate of each cancer type and subtype in comparison to the overall cancer incidence rate is depicted as % of all cancers per age group. Original incidence rates per age groups were extracted from the extensive 'Surveillance Epidemiology and End Results (SEER) Cancer Statistics Review (1975-2009)' public database (section Adolescents and Young Adults Cancers, tables 32.1 and 32.4) in March 2013 (see reference 2). For the modified presentation in this table, SEER incidence rates were adapted for the depiction of incidence rates per 1,000,000, combination of male and female incidence rates, and calculation of percentages. CNS, central nervous system; tropho, trophoblast.

further reviewed and categorized as a distinct bone cancer, markedly different to radiotherapy-insensitive osteogenic cancers (i.e., osteosarcoma) [7;21]. The uniform, small-round blue cells (high content of eosin-staining nuclei) of Ewing sarcoma are characterized by their distinctive strong membranous CD99 expression, whereas the small-round cell subtype of osteosarcoma only exhibits intracellular CD99 [1].

1.3 Clinical Presentation and Diagnosis

Osteosarcoma and Ewing sarcoma is commonly recognized subsequent to physical activity-related injuries and initially diagnosed by non-specific symptoms such as intermittent and later persistent severe pain, swelling, a palpable mass, limited mobility and rarely spontaneous fractures [1;22]. In contrast to osteosarcoma patients, Ewing sarcoma patients especially with advanced tumors may also present with systemic inflammatory symptoms like mild fever. Lactate dehydrogenase levels, leukocyte counts or erythrocyte sedimentation rates may be elevated in Ewing sarcoma patients. Alkaline phosphatase levels may be elevated in osteosarcoma patients. Diagnosis of osteosarcoma or Ewing sarcoma is further evidenced by plain X-ray radiographs, illustrating mixed radiolucent and radiodense periosteal bone-forming lesions with associated calcified soft tissue masses [22]. Magnetic resonance imaging is employed to determine the size, intramedullary and soft tissue extent of the bone tumor to facilitate subsequent complete surgical resection as well as limb-preserving surgery [22;23]. Presence of pulmonary and skeletal metastases is assessed by computed tomography of the thorax and whole-body radionuclide bone scans plus bilateral bone marrow aspirations, respectively. Biopsy of the tumor (core) by trephine needle puncture or incision is essential to establish the definite diagnosis on the basis of the histological and immunohistochemical cell characteristics [22]. In addition, diagnosis of Ewing sarcoma can be confirmed by the presence of distinctive spontaneous chromosomal translocations, detected by RT-PCR or fluorescence *in situ* hybridization (FISH) techniques [24]. In the majority of cases, the transcriptional activation domain of the EWS gene on chromosome 22 is fused in-frame to the DNA-binding domain of the FLI1 gene on chromosome 11 (t(11;22)(q24;q12)) or other members of the ETS family of transcription factors [25]. In contrast, osteosarcoma cells do not harbor such specific cytogenetic characteristics. The generated fusion proteins in Ewing sarcoma cells possess enhanced transcription-modulating (activating and inhibiting) properties and are involved in tumor development, increasing tumor cell proliferation and survival as well as escape from growth inhibition and apoptosis [26;27].

1.4 Treatment, Prognosis and Outcome

Prior to the availability of chemotherapy (<1970), the principal treatment of patients with bone tumors consisted of dubious toxic chemicals (e.g., mercury), Coley's toxin (see below, section 2.2) and particularly surgical resection or amputation, and later partly effective radiotherapy techniques [6-9;15;28;29]. The prognosis of patients even without overt metastatic disease was considered poor with a five-year survival rate of <25% [6;30]. Most patients rapidly developed recurrent disease and pulmonary metastases which led to the hypothesis of the presence of subclinical micro-metastases at the time of diagnosis of localized disease [18;30;31]. Superior imaging and surgical techniques, advanced detection and removal of lung metastases and especially the introduction of tumor cell-cytotoxic chemotherapeutic agents (e.g., doxorubicin) led to significant improvement of patient outcome [9;29;30;32-36]. Systemic post-operative

(i.e., adjuvant) multi-drug chemotherapy was subsequently confirmed to delay the development of metastases and substantially improved event-free and overall survival in osteosarcoma and Ewing sarcoma patients [37-40].

The current treatment of osteosarcoma patients includes pre-operative (i.e., neoadjuvant) chemotherapy, to initially reduce tumor viability and micro-metastases, followed by resection of the primary tumor and overt metastatic lesions and subsequent (maintenance) adjuvant chemotherapy, to ablate residual tumor cells and micro-metastases [22;41;42]. The chemotherapeutic regimen consists of a three drug cocktail (methotrexate, doxorubicin and cisplatin) or a comparably effective four drug cocktail (+ ifosfamide) [37;43]. Ewing sarcoma patients are treated with vincristine, ifosfamide, doxorubicin and etoposide (+/- cyclophosphamide +/-dactinomycin), followed by local control using surgery +/- radiotherapy, and adjuvant chemotherapy [22;42]. Radiotherapy is less commonly used due to the risk of secondary cancers [9]. Patients achieving remission may be subjected to surgical reconstruction of the affected limb or prosthetics [23].

Good prognostic factors include: no or few metastatic lesions at diagnosis; response to neoadjuvant chemotherapy and radiotherapy; low levels of serum-alkaline phosphatase at diagnosis; small tumor size; (resectable) metastasis in the lung only and not at other sites like bone marrow; tumor location in the appendicular skeleton [10;11;22;44;45]. Adverse factors are metastatic lesions at diagnosis and recurrent disease. Despite their merits, certain chemotherapeutic drugs can result in considerable adverse effects, such as infertility (ifosfamide), renal impairment (ifosfamide), hearing disorders (cisplatin), cardiomyopathy (doxorubicin), secondary cancers (ifosfamide, cisplatin, cyclophosphamide) [9;42]. Chemotherapy can inflict adverse effects such as lymphopenia (cyclophosphamide) but also beneficial effects on anti-cancer immunity such as immunogenic cell death (e.g., doxorubicin) and regulatory T cell depletion (cyclophosphamide) [46].

With current therapies, up to 70% of patients with localized disease accomplish continuous remission, achieving 5-years event-free and overall survival rates of 55–80% and 10-years survival rates of 50–60% [43;47-51]. Notably, up to 30% of these patients develop local relapses or distant metastases [44;52;53]. In spite of intensification of modern multi-drug chemotherapy regimens, patient prognosis has not significantly further improved during the last two to three decades [37;49;54]. In particular, patients with metastatic, recurrent and chemotherapy-refractory disease have a very poor prognosis and survival rates of less than 30%, highlighting the need for novel therapeutic strategies.

2. CANCER IMMUNOLOGY & IMMUNOTHERAPY

Conventional chemotherapy primarily aims at proliferating cells. Cancer cells which are dormant or acquiring resistance to cytotoxic drugs, such as cancer stem cells, may escape chemotherapy, a scenario presumably involved in metastatic and recurrent disease [55;56]. Thus, novel therapeutic strategies are warranted that potently target chemotherapy-refractory cancer cells and have a favorable toxicity profile toward normal cells.

In 2011, Hanahan and Weinberg refined their concept of the hallmarks of cancer [57;58]. These hallmarks envisage the complex factors involved in transforming homeostatic cells to aberrantly-

proliferating cells, local tumor growth and dissemination: sustained proliferative signaling; evasion of growth suppression; resistance to cell death; replicative immortality; angiogenesis; invasion, metastatic spread and colonization; and reprogramming of the energy metabolism. The authors included the phenomenon that the cancer microenvironment can evolve mechanisms to modulate the immune system in such a way that anti-cancer immune responses become suppressed and that immune cells ignore or even support tumor development [58]. Noteworthy, this notion implies that immune cells play a fundamental role in protecting from initial cancer growth. The existence of natural cancer immunosurveillance was originally proposed by Paul Ehrlich (1909) and later by Macfarlane Burnet, Lewis Thomas and contemporaries and was based on the discrimination between 'self' and 'non-self' by means of specific tumor antigens expressed on cancer cells [59-61]. However, cancer immunosurveillance has not been fully appreciated for a long time (e.g., due to the lack of inbred mouse strains and tumor antigens of non-viral cancers at that time) and only evidenced in the last decades [62;63]. The concept of immune evasion by cancer cells, termed 'cancer immunoediting' by Gavin Dunn, Robert Schreiber and colleagues in 2002, describes that initial tumor cell elimination by immune cells results in the selection of less immunogenic tumor cell variants which can then escape immunosurveillance and destruction (Figure 1, panel A) [62-64]. Comprehension of the interaction of cancer cells and immune cells has generated a multitude of immunotherapeutic strategies to exploit, induce, enhance and restore anti-tumor immune cell functions or to (re)-sensitize tumor cells to immune cell recognition [65-67].

2.1 A Rationale for inducing Anti-Cancer Immune Responses by mimicking Acute Infections in the Tumor Microenvironment

Similar to the concept of 'cancer immunoediting' (Figure 1, panel A), viruses establishing chronic infections, such as hepatitis C virus (HCV), evolve genetic variants under the pressure of the immune system. These virus variants may have acquired selected adaptations to evade and exhaust innate and adaptive immune responses, and cause an immunosuppressive milieu that subverts immunological protection of the host [68;69]. As a consequence, chronic viral infections (e.g., Epstein-Barr virus, HCV and human papilloma virus (HPV)) and chronic bacterial infections (e.g., *Helicobacter pylori*) can trigger or predispose to cancer development, respectively [70]. Furthermore, chronic infections, chronic inflammation and accelerated tissue regeneration can impair anti-cancer immune responses and provoke tumor formation, which can actually be supported by certain immune cells such as tumor-promoting M2-type macrophages, myeloid-derived suppressor cells and regulatory T cells (Figure 1, panel A) [64;71-73]. On the other hand, for a long time acute infections have been associated with spontaneous cancer regression and a lower risk for cancer development [70]. While elimination of cancer cells by immune cells becomes suppressed during cancer development, acute infections may rescue anti-cancer immunosurveillance due to non-specific activation of immune cells in response to harmful pathogens [63]. Similar to certain chemotherapeutic agents, acute infections in the tumor microenvironment may trigger immunogenic cell death and improve cancer cell detection by immune cells (Figure 1, panel B)[74]. Hence, acute infections may cause the release and display of 'danger' and 'stress' molecules such as calreticulin, high-mobility group box 1 protein (HMGB1), heat-shock proteins, NK cell activating-receptor ligands, microbial toll-like receptor (TLR) ligands, nucleotide-binding oligomerization domain (NOD)-like receptor
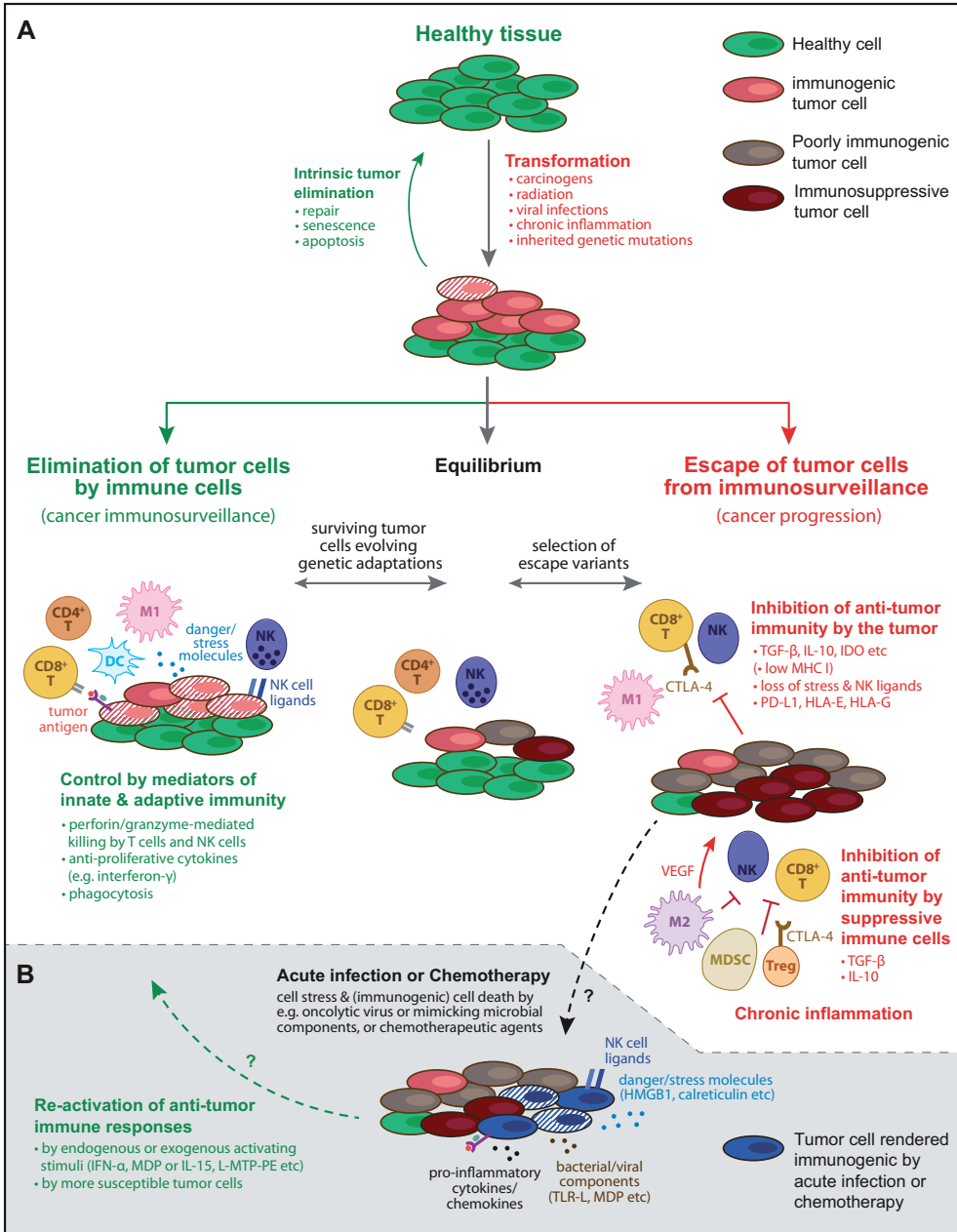
Figure 1: Cancer Immunosurveillance, Cancer Immunoediting and Re-activation of Anti-Tumor Immunity

(A) taken and adapted from Vesely *et al.*, 2011, Annual Review of Immunology [reference 64].
(B) Hypothetical model of the re-activation of anti-tumor immune responses after an acute infection or infection-mimicking agents in the cancer microenvironment, or chemotherapy in the presence of endogenous/exogenous immune cell-activating stimuli.
NK: natural killer cell, T: T cell, M1/2: M1/M2 macrophage, DC: dendiritc cell, MDSC: myeloid-derived suppressor cell, Treg: regulatory T cell, TLR-L: TLR ligands.

(NLR) ligands like muramyl dipeptide (MDP), and secretion of pro-inflammatory cytokines and chemokines [64;74-79]. As a hypothesis, local and attracted innate immune cells such as NK cells, macrophages and dendritic cells may then be activated to combat infected cells. Due to enhanced tumor cell recognition and cytotoxic function, NK cells and macrophages could contribute to the destruction of infected and bystander cancer cells and thus interfere with cancer progression. Ideally, cancer cell destruction by innate immune cells may also liberate potential cancer-associated antigens which could be (cross-)presented by dendritic cells or macrophages to CD4 helper T cells and CD8 cytotoxic T cells. This may favor the induction of a primary or previously suppressed anti-cancer Th1 response. Hence, (re-)activation of anti-cancer innate immune responses of NK cells and macrophages by an acute infection may contribute to the generation of an adaptive anti-cancer T cell response, an immunological prerequisite for systemic, long-lasting and effective immunity.

Several immunotherapeutic approaches have emerged to mimic acute infections in the tumor microenvironment and to mobilize anti-cancer (innate) immune responses. In the following sections, examples of anti-cancer immune responses and their applications for cancer immunotherapy are illustrated with the primary focus on innate immunity of NK cells and macrophages; some highlights of adaptive immunity-related immunotherapy are additonally noted.

2.2 Coley's Toxin and Acute Infections as the Onset for Modern Immunotherapy

As early as in the middle of the 19th century, physicians noted that some cancer patients spontaneously experienced cancer regression and remission if contracting acute erysipelas [80], an infectious skin inflammation [81]. Therefore, Wilhelm Busch (<1866) attempted to transfer the (at that time) undetermined infectious agent of erysipelas to cancer patients, for instance, by cauterizing the tumor surface and covering it in the sheets of a patient who had naturally contracted the infection [80]. Temporary tumor regression (called absorption) was noted to be associated with severe or even lethal febrile symptoms and local influx of leukocytes at the site of infection; it was hypothesized that the severity of fever contributed to the anti-tumor effect. The causative bacteria of erysipelas infection, *Streptococcus pyogenes,* were identified in ~1882, propagated and tested in rabbits and first used in the treatment of patients with inoperable cancers by Friedrich Fehleisen and contemporaries [82].

Inspired by cases of spontaneous tumor regression after acute erysipelas, the bone surgeon William Coley injected sarcoma patients with live *Streptococcus pyogenes* in 1891 and beyond [28]. Despite dramatic tumor regression in a few patients, the infection was difficult to control or even fatal due to the lack of antibiotics at that time [83]. Hence, a safe heat-inactivated preparation of gram-positive *Streptococcus pyogenes* and gram-negative *Serratia marcescens* was developed which conferred anti-tumor activity particularly subsequent to tumor resection. The anti-tumor effect of this preparation, referred to as Coley's toxin, was essentially attributed to the strong immunostimulatory effect of *Serratia marcescens* containing, for instance, relatively heat-resistant endotoxin [84;85]. Treatment of hundreds of patients with Coley's toxin achieved at least in a part of the patients, with local and metastatic disease, considerable tumor regression and long-time remission [83;85;86].

However, William Coley's work was heavily disputed due to inconsistencies in his documentation, multiple refinements of the bacterial preparations and the lack of a standardized treatment schedule because it required individual dosing, administration and patient monitoring over a long time. Eventually, treatment with Coley's toxin was superseded by the introduction of more feasible and broadly applicable radiotherapy and later chemotherapy [87]. Nevertheless, Coley's work has contributed to the development of some modern immunotherapeutic treatments as presented below.

2.3 Enhancing Innate Immunity by Immunomodulators

As one of the most successful adjuvant treatments, live-attenuated *Mycobacterium bovis*-based Bacillus Calmette-Guérin (BCG) is included in the treatment of patients with bladder cancer and significantly reduces cancer progression and the risk for recurrent disease [75;88]. The anti-cancer effects of Coley's toxin and BCG have been linked, at least in part, to the bacterial cell wall constituents lipopolysaccharide (LPS, endotoxin) and peptidoglycan subunits like muramyl dipeptide, which were identified in these bacterial preparations as minimal active moieties to trigger inflammatory responses [75;85;89-91]. Furthermore these bacterial constituents can induce tumoricidal activity and production of pro-inflammatory and immunoregulatory cytokines by macrophages such as tumor-necrosis factor-α (TNF-α) and interleukin-12 (IL-12) (this thesis, chapter 5), which furthermore may stimulate anti-tumor responses of NK cells [75;92;93].

In fact, stimulation of mice with BCG in combination with Coley's toxin or LPS led to the discovery of TNF-α; TNF-α was described as the factor induced in the serum of these treated mice which presumably mediated 'hemorrhagic necrosis' of murine fibrosarcoma *in vivo* and anti-tumor effects *in vitro*, and was suggested to be implicated in the anti-tumor effects of LPS-activated macrophages [94]. Since purified TNF-α was subsequently found to be cytotoxic against certain cancer cells [95], melanoma patients have been treated with recombinant TNF-α, improving the response to chemotherapy but not survival [46;96]. Notably, in certain conditions TNF-α and other pro-inflammatory cytokines support tumor development [64;72]. Interferon-α (IFN-α) was originally discovered to be produced upon virus infection and interfering with virus spread [97]. In addition, IFN-α was found to mediate anti-tumor effects in patients with, in particular, virus-derived cancers and inhibit growth of osteosarcoma cells *in vitro*; a cancer at that time (~1980) believed to be of viral origin [98;99]. Therefore, IFN-α has been included in the treatment of osteosarcoma patients in Sweden, and its clinical benefit to conventional therapy is currently evaluated in the European/American Euramos-1 trial [98;100]. In addition to its direct anti-tumor effect, IFN-α has been shown to enhance the cytotoxic activity of NK cells against osteosarcoma cells *in vitro* [101]. Other cytokines such as IL-2 and IL-15 have been used to enhance or maintain the viability and anti-tumor cytotoxic activity of endogenous or adoptively-transferred *ex vivo*-activated NK cells [102] (see below, section 2.5).

As an alternative to bacterial infections, oncolytic viruses such as genetically-modified poxviruses and adenoviruses are currently explored to selectively infect and directly kill cancer cells which may also induce anti-tumor innate and adaptive immune responses [103-108]. Of note, some but not all osteosarcoma and Ewing sarcoma cell lines are susceptible to lysis by wild-type or naturally selected mutant oncolytic reovirus [109] (and unpublished observation).

Upon release into the circulation, crude bacterial preparations as well as purified LPS have the potential to cause excessive inflammatory responses, culminating in severe adverse affects such as septic shock and extensive tissue damage [110]. As a 'less toxic' alternative, innate immune cells can be activated by less stimulatory equivalents of bacterial and viral constituents such as imiquimod (TLR7/8 ligand), Poly I:C (TLR3 ligand), unmethylated CpG-DNA motifs (TLR9 ligand) [78;111]. TLR ligands can improve maturation and the antigen-presenting capacity of dendritic cells and thus the generation of adaptive T cell responses. Dendritic cell-based protein/peptide vaccines such as applied in prostate cancer generally confer low clinical activity [112]. Hence, TLR ligands as well as IL-12 and granulocyte–macrophage colony stimulating factor are investigated for adjuvant effects on dendritic cell-based vaccines like in HPV-induced cervical cancer [65;113;114]. Notably, to mimic an acute bacterial infection in osteosarcoma, liposomal muramyl tripeptide, presumably activating the intracellular NOD2 receptor in myeloid cells, has recently been introduced in the treatment of osteosarcoma patients to induce anti-tumor cytotoxicity of monocytes and macrophages although with limited clinical efficacy [43;65;115] (and this thesis, chapter 5).

2.4   Natural Anti-Cancer Activity of NK cells

NK cells have originally been described by Rolf Kiessling and contemporaries as a distinct subset of lymphocytes (other than T and B cells) able to kill virus-induced murine leukemia cells in a contact-dependent manner but without the need for prior sensitization to these target cells [116-118]. NK cells were further characterized by Klaas Kärre and colleagues as cytotoxic cells which can respond to cells with incompatible or down-regulated expression of MHC class I molecules like allogeneic ('non-self') and some virus-infected/tumor-transformed cells, escaping from MHC class I-restricted cytotoxic CD8 T cells; in contrast, NK cells spare normal MHC class I-sufficient ('self') cells [119]. This concept was termed ''missing-self hypothesis', elegantly reviewed in [120]. Currently, NK cells are proposed to be grouped together with the emerging population of innate lymphoid cell subtypes [121].

NK cells are considered to bridge innate and adaptive immunity by their capability to produce significant amounts of IFN-γ [122]. IFN-γ increases MHC class I and II expression on abnormal cells and antigen-presenting cells, respectively, which supports the initiation of Th1 responses. In addition, IFN-γ enhances the bactericidal and tumoricidal activity of macrophages. Thus, in interplay with macrophages and dendritic cells, NK cells are involved in initial responses to virus-infected and tumor-transformed cells but may also contribute to the induction of adaptive anti-cancer (memory) T cell responses [123-125] (and this thesis, chapter 2 and 3). The cytotoxic activity of matured (i.e., 'educated') human NK cells is regulated by the balance of activating and inhibitory signals perceived from target cells via germ-line–encoded inhibitory and activating receptors [79;126-129]. Thus, NK cell-mediated lysis of cancer cells is determined by the combination of a) low expression of ligands for NK cell inhibitory receptors such as killer cell immunoglobulin-like receptors (KIR) (ligands: HLA-A, B and C alleles) [130] and NKG2A/CD94 (HLA-E) [131] and b) high expression of ligands for NK cell activating receptors such as NKG2D (MICA, MICB, ULBP1-6) [132-137], DNAM-1 (CD112 and CD115) [138] and NKp30 (B7-H6) [139]. Osteosarcoma and Ewing sarcoma cells have been shown to be susceptible, in particular, to the cytotoxic potential IL-15–activated NK cells, involving NKG2D,

DNAM-1 and NKp30 interactions [140-143] (and this thesis, chapter 4). As one of the most effective NK cell-activating receptors [144], ligation of FcγIIIa/CD16 triggers substantial NK cell cytotoxicity against antibody-coated target cells (and against cells carrying thus far unidentified ligands [145]), eliciting antibody-dependent cellular cytotoxicity (ADCC) [146-148] (and this thesis, chapter 3).

Human NK cells have been suggested to interfere with cancer development since individuals with low NK cell cytotoxic activity have a higher incidence of cancer [149]. Antibody-based immunotherapy has been reported to improve the outcome of cancer patients carrying the high-affinity gene polymorphism in FcγIIIa/CD16 (-158V vs -158F) [150-154], which mediates stronger antibody-binding to FcγIIIa/CD16 and enhances NK cell cytolytic function [155-157]. These observations suggest that CD16+ NK cells (and CD16+ macrophages) and ADCC are involved in the clinical effects of rituximab (anti-CD20), cetuximab (anti-EGFR) and trastuzumab (anti-Her2/neu). The biological relevance of NK cells to recognize human tumor cells by the absence of a complete 'self' HLA-I repertoire was highlighted in the clinical setting of KIR-mismatched haploidentical (T cell-depleted) hematopoietic stem cell transplantation: a subset of alloreactive NK cells, generated from the donor graft in the recipient, presumably mediated the reduced risk for graft rejection, acute graft-versus-host disease and leukemia relapse, but contributed to graft-versus-leukemia effects [158]. These results renewed the interest in NK cells for immunotherapeutic strategies. Moreover, the clinical response to antibody-based and tyrosine kinase inhibitor-based immunotherapy has recently been shown to positively correlate with NK cell activation, NK cytotoxicity and tumor infiltration by NK cells in some cancer patients [159-163], implying a direct role for NK cells in anti-cancer responses in humans.

2.5 Enhancing NK cell Function by Antibodies, Cytokines and Adoptive Cell Transfer

Antibody-based immunotherapy with rituximab, cetuximab and trastuzumab has successfully been included in the treatment of B cell malignancies, colorectal cancer, head-neck cancer and breast cancer [164-169]. In addition to direct anti-cancer effects, the clinical effect of these therapeutic antibodies may involve CD16+ NK cells as well as CD16+ macrophages by the association with the high-affinity polymorphism in FcγIIIa/CD16 (-158V vs -158F) as noted above. A role for macrophages has additionally been suggested by the association with high-affinity polymorphism in FcγIIa/CD32 (-131H vs -131R) reported in some studies. Furthermore, patients with higher numbers of tumor-infiltrating macrophages had a better clinical outcome in response to antibody therapy [153;154;170]. Therapeutic antibodies may mobilize the cytotoxic potential of NK cells which are functionally sufficient for FcγIIIa/CD16-dependent lysis but deficient for antibody-independent lysis; the latter as a result of NK cell education (due to the lack of inhibitory KIR receptors against 'self' ligands) or tumor encounter [171;172] (and this thesis, chapter 4).

In fact, NK cells (and T cells) are frequently rendered hyporesponsive or functionally exhausted in an inhibitory cancer microenvironment, presumably to escape from immunosurveillance [143;171;173-179]. To lower the threshold for NK cell cytotoxicity, counteract NK cell inactivation during cancer progression and exploit 'missing-self' recognition, blockage of inhibitory KIR receptors with antagonistic antibodies is currently explored [180]. Similarly, blockage of the T cell inhibiting receptors CTLA-4 (ipilimumab) and PD-1 has been pursued

to disrupt T cell tolerance which has partly achieved encouraging clinical responses as well as significant autoimmune effects in some patients [181;182]. Depletion of M2-type macrophages or regulatory T cells has been suggested to counteract pro-tumor properties and inhibitory effects of these cells on anti-cancer functions of NK cells and other immune cells [183-185]. Using agonistic antibodies, NK cell functionality can be directly augmented by cross-linking of activating receptors like CD137 (induced upon ADCC *in vivo*), which has been shown to potentiate rituximab-mediated and trastuzumab-mediated ADCC in preclinical studies [186;187]. Similarly, agonistic antibodies against CD40 can improve the co-stimulatory activity of dendritic cells and the anti-tumor activity of macrophages, respectively [188;189].

In view of enhancing the function of endogenous NK cells by activating cytokines, infusion of high-dose IL-2 can result in adverse reactions and activation of regulatory T cells, while low-dose IL-2 may fail to sufficiently enhance NK cell cytotoxicity [102]. As an alternative, NK cells can be (re-)activated *ex vivo* and used for adoptive cell transfer therapy [102;143]. In the case of T cells, the potential of adoptive transfer using autologous tumor-reactive T cells (anti-MART-1 or cancer-testes antigens) and chimeric antigen receptor (CAR) T cells (anti-CD19-CD3ζ-CD28/CD137, MHC class I-independent) has been highlighted; this approach resulted in remarkable cancer regression and remission in up to half of the patients with advanced melanoma or B cell malignancies [190-195]. Still, these T cells fail to control epitope-negative escape variants and have the potential for long-time adverse effects on epitope-positive normal cells [191;195]. Similar to CAR T cells, genetically-engineered CAR NK cells (anti-CD19-CD3ζ-CD137) are currently developed; CAR NK cells may possess enhanced cytotoxicity more specifically directed toward target cells (even if these target cells are normally insensitive to natural NK cell cytotoxicity) while retaining natural cytotoxicity against epitope-negative cells [196;197].

As already alluded to, several studies have investigated to introduce highly functional NK cells into cancer patients by adoptive cell transfer of (a) autologous and (b) haploidentical NK cells in combination with (a,b) *ex vivo* or (b) *in vivo* IL-2 stimulation [171;198-201]. Adoptive transfer of haploidentical NK cells, with the potential for 'missing-self' recognition, has been shown to mediate complete remission with limited adverse effects in patients with advanced hematological malignancies [198-201]. Importantly, haploidentical NK cells did not cause graft-versus-host disease in the recipients. In contrast to patients with hematological malignancies, so far, objective clinical responses in patients with solid cancers have not been reported after adoptive transfer of haploidentical or autologous NK cells [171;198]. In fact, in melanoma patients, even IL-2–activated (autologous) NK cells have been reported to lose cytotoxic activity after adoptive transfer, possibly as a consequence of trafficking through an inhibitory cancer microenvironment [171]. Recent studies in mice have demonstrated that NK cell functionality and viability after adoptive transfer is augmented and prolonged by pre-activation with a cytokine cocktail of IL-12, IL-15 and IL-18 and further enhanced by endogenous IL-2 production of recipient CD4 T cells [202;203]. Thus, these recent studies indicate potential mechanisms to improve persistence and anti-tumor function of NK cells in the recipient after adoptive transfer, which has remained challenging so far.

As pointed out above, a multitude of research lines is currently devoted to the improvement of NK cell cytotoxicity and the elucidation of (inhibitory) NK cell–cancer cell interactions to

more effectively exploit the cytotoxic potential of NK cells for anti-cancer immunotherapeutic strategies.

3. OUTLINE OF THE CHAPTERS 2–6

In the following research chapters it is demonstrated in preclinical studies, how the anti-cancer potential of especially NK cells but also macrophages can be enhanced and directed to osteosarcoma and Ewing sarcoma cells. First an experimental model for measuring NK cell activation *in vitro* is explored.

In **chapter 2**, phenotypical and functional activation of NK cells is examined in response to a prototypical viral infection by adenovirus types 5 and 35. Cellular prerequisites and the requirement for interactions between NK cells and other immune cells (plasmacytoid dendritic cells and T cells) for NK cell activation are addressed.

In **chapter 3** it is investigated how sarcoma–NK cell interactions can be improved to enhance and direct the cytotoxic potential of NK cells to osteosarcoma and Ewing sarcoma cells. The potential of antibody-dependent cytotoxicity by NK cells against sarcoma cells is highlighted by targeting sarcoma cells with a tumor cell-reactive antibody.

NK cells can exert cytolytic activity against osteosarcoma and Ewing sarcoma cell lines in short-term assays as shown in research chapter III. However, as illustrated in **chapter 4**, prolonged tumor–NK cell interactions, potentially occuring in an immunosuppressive cancer microenvironment, can alter NK cell functionality. It is investigated how some of the sarcoma cell lines mediate significant down-regulation of NK cell activating receptors and NK cell cytolytic activity during two-day tumor–NK cell co-cultures. Furthermore, it is demonstrated that this inhibition can be prevented and bypassed by NK cell activation prior to tumor contact and presence of a tumor cell-reactive antibody, respectively.

The presence of tumor-infiltrating macrophages has recently been shown to positively correlate with survival of osteosarcoma patients, suggesting a role for macrophages in anti-cancer responses. In **chapter 5** it is demonstrated that human macrophages are able to interfere with osteosarcoma cell growth. It is determined whether the anti-cancer activity of macrophages can be enhanced and directed to osteosarcoma cells using immunostimulatory bacterial derivates (such as liposomal muramyl tripeptide) and a tumor cell-reactive antibody, respectively.

In the final **chapter 6,** the expression of the immunoregulatory protein CD70 is documented on osteosarcoma and Ewing sarcoma cell lines and in tumor tissue, as a potential mediator in tumor–immune cell interactions or as a potential target for anti-cancer immunotherapy.

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CHAPTER 2

Adenovirus type 35, but not type 5, stimulates NK cell activation via plasmacytoid dendritic cells and TLR-9 signaling

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ABSTRACT

In hematopoietic stem cell transplant (HSCT) recipients, disseminated adenoviral infections during the first two months after HSCT can lead to severe complications and fatal outcome. Since NK cells are usually the first lymphocytes to reconstitute after HSCT and have been implicated in the clearance of adenovirus-infected cells, it was investigated whether NK cells are activated by adenovirus *in vitro*.

Exposure of PBMC to human adenovirus type 5 (HAdV5) or HAdV35 resulted in up-regulation of the activation marker CD69 on NK cells and enhanced cytolytic activity of NK cells. HAdV5-induced NK cell activation relied on the contribution of T cells as depletion of T cells from PBMC abolished NK cell activation. In contrast, NK cell activation in response to HAdV35 occurred in the absence of T cells. Plasmacytoid dendritic cells (pDC) were necessary and sufficient to mediate NK cell activation. HAdV35 induced significantly more interferon-α (IFN-α) production by pDC than HAdV5. The increased IFN-α production and NK cell activation correlated with a higher infection efficiency of viruses with the type 35 fiber. The IFN-α response of pDC was enhanced by the presence of NK cells, suggesting a reciprocal interaction between pDC and NK cells. Incubation with a toll-like receptor 9 (TLR-9) antagonist impaired the IFN-α production by pDC as well as NK cell activation, implying that TLR-9 signaling is critically involved in the IFN-α response of pDC and NK cell activation after HAdV35 exposure.

In conclusion, two human adenovirus serotypes from two different species differ considerably in their capacity to stimulate pDC and NK cells.

INTRODUCTION

Human adenoviruses (HAdV) are non-enveloped double-stranded DNA viruses that are not considered a major risk for immunocompetent hosts. Adenoviral infections commonly occur in early childhood, are self-limiting and typically associated with asymptomatic or mild disease [1]. Among the human adenovirus family, 52 serotypes have been identified on the basis of neutralizing antibodies and are categorized in six HAdV species (A-F) according to their capacity to agglutinate erythrocytes [2].

In the first line of immune defense, dendritic cells are considered the major sentinels for viral pathogens [3;4]. Plasmacytoid dendritic cells (pDC) selectively express the intracellular Toll-like receptor 9 (TLR-9), which detects unmethylated DNA with CpG-motifs frequently found in bacteria and in double-stranded DNA viruses [5-11]. Stimulation of TLR-9 initiates a signaling cascade in association with MyD88, resulting in type I interferon (IFN-I) production as well as enhanced expression of co-stimulatory molecules such as CD80, CD86 and CD40 [10;12]. IFN-α exhibits a direct antiviral effect and has a broad impact on cellular functions, bridging innate and adaptive immunity [12-16].

In immunocompromised hosts, such as hematopoietic stem cell transplant (HSCT) recipients, adenoviral infections can disseminate and lead to severe complications and fatal outcome [17-19]. Previously, it has been reported that in pediatric HSCT patients with disseminated HAdV infection the elimination of adenovirus relied on the recovery of immunity, i.e., lymphocytes [20;21]. HAdV-specific IFN-γ–producing CD4$^+$ or CD8$^+$ T cells were detected after virus clearance from blood [20;22]. However, evidence has been provided for the clearance of HAdV in the presence of NK cells only, and since NK cells constitute the majority of lymphocytes early after HSCT [23;24], we set out to study the requirements to activate NK cells in response to adenoviruses *in vitro*. We focused on the human wild type, replication-competent adenovirus serotypes HAdV5 (species C) and HAdV35 (species B) which utilize two distinct pathways of cell entry and considerably differ in their capacity to infect cells, such as DC [2;25-29]. We hypothesized that this difference has a major influence on the IFN-α production by pDC and, therefore, determined the differential cellular requirements for these two viruses to activate NK cells.

MATERIALS AND METHODS

Cell isolations and depletions

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of healthy adult donors (Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. NK cells were purified from PBMC by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the "Human NK cell Isolation Kit" (Miltenyi Biotech, Bergisch Gladbach, Germany); NK cell purity was >95 % as determined by flow cytometry. NK cells were defined as NK cells as CD56$^+$, CD3$^-$, CD14$^-$ and CD19$^-$ cells. pDC were isolated from PBMC by negative selection using the "human Plasmacytoid Dendritic Cell Isolation Kit" (Miltenyi Biotech); no other cell types than pDC specifically identified by the extracellular

expression of BDCA-2 (CD303) (BDCA-2$^+$ pDC) were detectable by flow cytometry in the cell isolate; pDC purity was >99 %. pDC were depleted from PBMC by positive selection using APC-conjugated anti-BDCA-2 antibodies and anti-APC MicroBeads (Miltenyi Biotech), resulting in the depletion of >99 % of pDC. T cells were depleted from PBMC (TCD PBMC) by positive selection using anti-CD3 MicroBeads (Miltenyi Biotech); TCD PBMC contained less than 0.5 % of contaminating CD3$^+$ T cells as analyzed by flow cytometry.

Cell cultures and stimulations

All cell cultures were performed in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FCS and penicillin/streptomycin at 37 ºC under humidified and 5 % CO_2-containing conditions. PBMC or TCD PBMC were cultured in round-bottomed 96-well plates ($2x10^5$ per 200 µl) to study NK cell activation, or in 24-well plates ($2x10^6$ per ml) to study pDC intracellular IFN-α production and maturation. Isolated NK cells and purified pDC were co-cultured in round-bottomed 96-well plates in RPMI medium at indicated cell ratios and cell concentrations. PBMC, isolated PBMC subsets and the co-cultures were stimulated with human wildtype adenovirus serotype 5 (HAdV5) and HAdV35 or with recombinant rAd5f35 at multiplicity of infection (MOI) 1 (at least one infectious virus particle per cell), 10 and 100. In rAd5f35 the type 5 shaft and head sequences are replaced by the type 35 shaft and head sequences, which are fused to the type 5 fiber tail [27;30]. In addition, PBMC were stimulated with the recombinant replication-deficient, E1-deleted, GFP-encoding adenovirus variants rAd5-GFP, rAd35-GFP and rAd5f35-GFP at MOI 10 and 100 [27;31]. All adenovirus preparations were kindly provided by Drs. Jan de Jong (Erasmus Medical Centre, Rotterdam, Netherlands), Menzo Havenga (TNO Biosciences, Leiden, The Netherlands), and purified on a CsCl-gradient. Additional exogenous stimuli were as follows: IL-15 (10 ng/ml, Bender Med. Sys, Vienna, Austria); IFN-α (ranging from 125 to 25,000 IU/ml (Roche, Basel, Switzerland); inhibitory CpG TTAGGG (iCpG) '5 -ttt agg gtt agg gtt agg gtt agg g -3' (12.6 µM, Invivogen, San Diego, CA, USA). Blocking experiments were performed with the following antibodies: rabbit polyclonal anti-IFN-α, mouse monoclonal anti-interferon-Alpha/Beta Receptor 1 (both PBL Interferon Source, Piscataway, NJ, USA; cat.no. 31101-1 and 21385-1, respectively) and mouse monoclonal anti-IL-2 (R&D Systems, Minneapolis, MN, USA; clone 5334). As the kinetics of activation between pDC (i.e., intracellular IFN-α production and maturation) and NK cells (i.e., considerably induced levels of CD69 expression) in response to HAdV were different, pDC and NK cells were analyzed 18 h and 40 h following virus exposure, respectively.

Flow cytometry

pDC and NK cells were analyzed phenotypically by staining with fluorescently labeled antibodies and flow cytometry. The following antibodies were applied according to the manufacturer's instructions: BDCA-2/CD303^{APC}, BDCA-1/CD1c^{APC} (Miltenyi Biotech); CD3$^{PerCP-Cy5.5}$, CD11c^{PE}, CD14$^{PerCP-Cy5.5}$, CD19$^{PerCP-Cy5.5}$, CD20$^{PerCP-Cy7}$, CD40^{FITC}, CD69^{PE}, HLA-DR^{FITC} (Becton Dickinson, Franklin Lakes, NJ, USA); CD19$^{Pacific Blue (PB)}$ (Dako, Glostrup, Denmark); CD3^{PB} (BD Biosciences, San Jose, CA, USA); CD56^{APC},CD56^{PE-Cy7} (IOTEST Immunotech, Marseille, France). After extracellular staining, intracellular staining of IFN-α with IFN-α^{FITC} (PBL Interferon Source; cat.no. 21112-3) was performed in PBMC using the "FoxP3 Staining Buffer Set" (eBiosciences, San Diego, CA, USA). FACS measurements were performed

with the FACSCalibur or the LSR II (BD Biosciences) and analyzed with the "BD Cell Quest Pro[TM]" software (version 5.2.1) or "BD FACSDiva[TM]" software (version 6.1.2.).

Cytolytic activity of NK cells – [51]chromium release assay

Cytolytic activity of NK cells against the cell lines K562 (sensitive to unstimulated and activated NK cells) and Daudi (sensitive to activated NK cells only) was measured in 4-hour [51]chromium (^{51}Cr) release assays. Effector cells were cultured for 72 h under conditions as indicated. K562 and Daudi target cells (ATCC, Manassas, VA, USA) were labeled with 100 µl Na-chromate (^{51}Cr, 3.7 MBq) for 1 hr. After washing, 2.5×10^3 target cells were added to effector cells in triplicate at the indicated effector-target (E:T) ratios and incubated for 4 h at 37°C. Supernatants were collected, and the release of ^{51}Cr was measured with a gamma-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and maximum release were obtained by incubation with medium and 2N HCl, respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 x (experimental release–spontaneous release / maximum release–spontaneous release).

### Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IFN-α were determined in supernatants from cultures of PBMC, TCD PBMC or purified PBMC subpopulations using the "ELISA assay for human interferon-α pan" (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions.

RESULTS

Adenovirus serotype 5 and type 35 stimulate activation and cytolytic activity of NK cells in unseparated PBMC

To investigate the capacity of wildtype adenovirus serotypes 5 (HAdV5) and 35 (HAdV35) to induce activation of NK cells in PBMC *in vitro*, CD69 expression and the cytolytic activity against the HLA class I negative cell lines Daudi and K562 were assessed. Analogous to the effect of interleukin-15 (IL-15), 40 h stimulation of PBMC with escalating viral loads of HAdV5 and HAdV35 increased the percentage of NK cells expressing the activation marker CD69 as compared to the unstimulated medium control (Figure 1, panel A, i). HAdV5 induced CD69 expression only at MOI 100, whereas HAdV35 produced appreciable activation of NK cells at a 10-fold lower MOI. Similarly, HAdV35 has previously been shown to be more effective than HAdV5 in eliciting T cell responses [25]. Notably, stimulation with HAdV35 at MOI 100 appeared to exhibit cytopathic effects because the percentage of CD14^{+} monocytes in PBMC was strongly reduced, whereas lower viral loads of HAdV35 or HAdV5 did not alter the percentage of monocytes (Figure 1, panel A, ii). For that reason, virus stimulations were performed with HAdV5 at MOI 100 and HAdV35 at MOI 10 in subsequent experiments.

Stimulation of PBMC with either HAdV5 (MOI 100) or HAdV35 (MOI 10) induced NK cell cytolysis of Daudi cells, a target cell line which is naturally not lysed by resting but only by activated NK cells [32-34] (Figure 1, panel B). Additionally, NK cell-mediated lysis of K562 cells was slightly enhanced in HAdV5-stimulated PBMC (Figure 1, panel B).
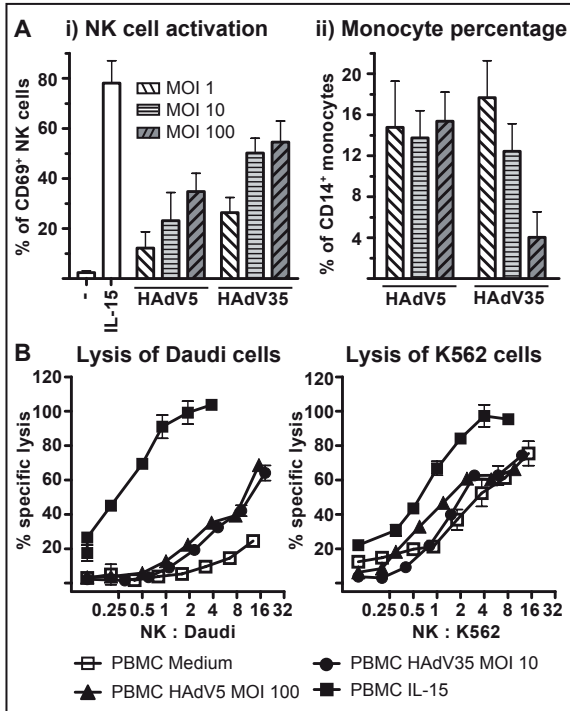
**Figure 1: HAdV5 and HAdV35 induce NK cell activation in PBMC**

(A) PBMC were stimulated with HAdV5 and HAdV35 at MOI 1, 10 and 100 and controls (unstimulated medium as negative control or IL-15 as positive control) for 40 h. (i) NK cell activation was measured by the increased expression of the activation marker CD69 on NK cells. NK cells were gated as lymphocytes based on FSC/SSC plots and defined as the cell population positive for CD56 expression but devoid of CD3, CD14 and CD19 expression. Data depicted are combined from 3 independent experiments (error bar represents SEM). (ii) The percentage of viable $CD14^+$ monocytes was compared among virus stimulations. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(B)** After stimulation of PBMC with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 72 h, the cytolytic potential of NK cells against Daudi and K562 cells was tested in triplicate in a 4 h ^{51}chromium release assay. Effector:target (E:T) ratios were two-fold dilutions of effectors, starting with $5x10^5$ PBMC and $2.5x10^4$ target cells. PBMC E:T ratios were adjusted to the frequency of NK cells in PBMC measured by FACS to obtain actual NK:T ratios. Data are representative for 2 independent experiments.

Hence, stimulation of PBMC with HAdV5 and HAdV35 resulted in enhanced expression of the NK cell activation marker CD69 and concomitantly increased the cytolytic activity of NK cells.

HAdV5-induced NK cell activation requires the presence of T cells

To study whether the activation of NK cells by HAdV was due to a direct effect or required other cell populations of blood mononuclear cells, purified NK cells were stimulated with HAdV5 and HAdV35 for 40 h. On purified NK cells alone CD69 expression was not increased after HAdV5 or HAdV35 exposure as compared to NK cells in HAdV-stimulated PBMC (Figure 2, panel A, i and ii). In contrast, IL-15 stimulation induced CD69 up-regulation, demonstrating that the purified NK cells were functional and responsive. Therefore, it can be concluded that at least one additional cell type of blood mononuclear cells was essential to mediate HAdV-induced NK cell activation.

Since most healthy individuals have HAdV-specific T cells recognizing multiple serotypes, we reasoned that the activation of NK cells in HAdV-stimulated PBMC might be induced via the production of IL-2 by HAdV-specific T cells [29;35-38]. In order to investigate whether NK cell activation requires T cells, PBMC were depleted of $CD3^+$ T cells (TCD PBMC). CD69 up-regulation on NK cells in response to HAdV5 was abolished by T cell depletion. In contrast, HAdV35 still induced CD69 up-regulation in the absence of T cells (Figure 2, panel A, iii). Notably, HAdV5 and HAdV35 exposure of PBMC induced CD69 expression on both the CD56^{dim} and the CD56^{bright} NK cell population, whereas in the absence of T cells CD69 up-regulation was mainly observed in the CD56^{dim} NK cell population after HAdV35 exposure.
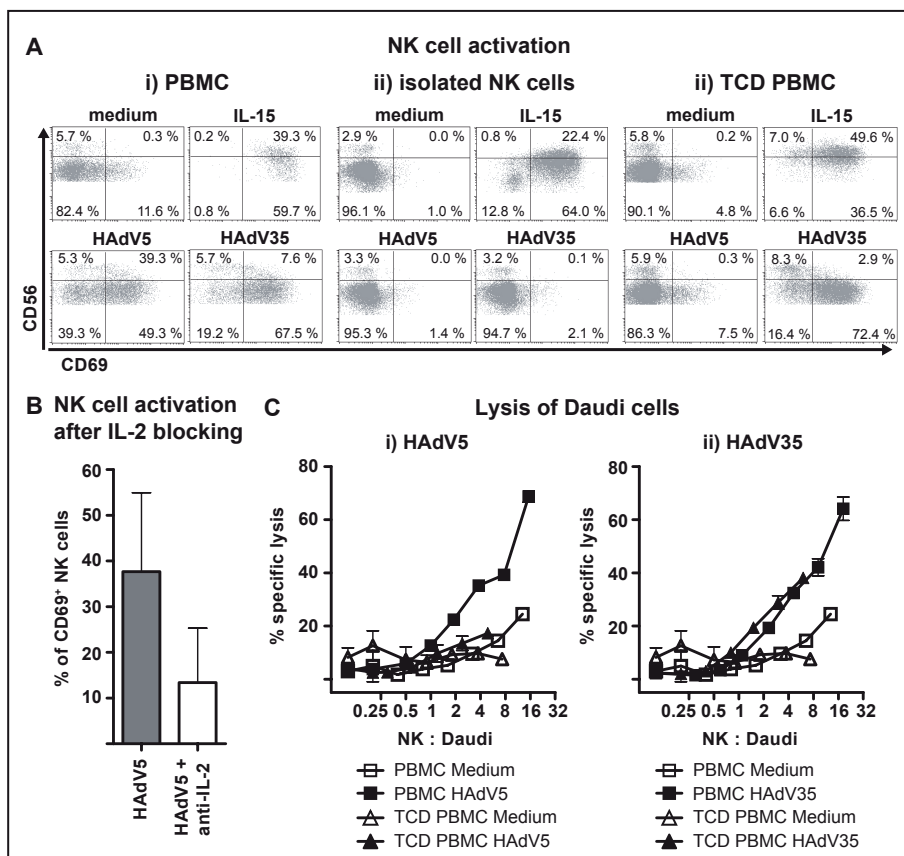
Figure 2: HAdV5, but not HAdV35, requires T cells to activate NK cells
(A) NK cells were isolated from PBMC by negative selection, i.e., depleting non-NK cells, with a purity of >95%. T cells were depleted from PBMC by positive selection (TCD PBMC); TCD PBMC contained less than 0.5% of contaminating $CD3^+$ T cells. PBMC ($2x10^5$) (panel i), isolated NK cells ($1x10^5$) (panel ii) and TCD PBMC ($2x10^5$) (panel iii) were stimulated with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 40 h. NK cell activation was measured by up-regulation of CD69 expression on CD56^{dim} and CD56^{bright} NK cell subsets. NK cells were gated as lymphocytes based on FSC/SSC plots and defined as the cell population positive for CD56 expression but devoid of CD3, CD14 and CD19 expression. Data are representative for 3 independent experiments. **(B)** CD69 expression on NK cells was assessed in PBMC exposed to HAdV5 with or without the addition of neutralizing antibodies against IL-2. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(C)** Cytolytic potential of NK cells against Daudi cells was tested after stimulation of PBMC and TCD PBMC with HAdV5 (panel i) and HAdV35 (panel ii) and compared to unstimulated medium controls. The [51]Chromium release assay was performed in triplicate as described in Figure 1.

Neutralization of IL-2 reduced CD69 expression on NK cells of HAdV5-exposed PBMC, suggesting that NK cell activation in response to HAdV5 is, at least partially, induced by IL-2 production (Figure 2, panel B).

Cytolysis of Daudi cells by NK cells after exposure to HAdV5 was strongly reduced in TCD PBMC as compared to non-depleted PBMC (Figure 2, panel C, i), whereas HAdV35-stimulated lysis of Daudi cells was unaffected after T cell depletion (Figure 2, panel C, ii). These results suggest that HAdV5 and HAdV35 can induce NK cell activation in PBMC by different mechanisms. HAdV5 activates NK cells via a T cell-dependent mechanism, whereas the activation of NK cells in response to HAdV35 can occur independently of T cells.
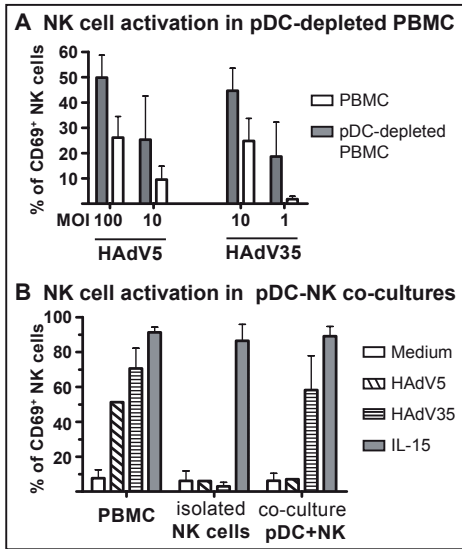
Figure 3: Plasmacytoid dendritic cells directly mediate NK cell activation after HAdV35 exposure

(A) pDC were depleted from PBMC by positive selection, resulting in the depletion of >99% of pDC. PBMC and pDC-depleted PBMC (each 2x10^5) were stimulated with HAdV5 (MOI 10 and 100) and HAdV35 (MOI 1 and 10) for 40 h. NK cell activation was measured by up-regulation of CD69 expression. Data depicted are combined from 4 independent experiments (error bar represents SEM). (B) pDC were purified from PBMC by negative selection with a purity of >99 % and no CD3$^+$, CD14$^+$, CD19$^+$ cells detectable. PBMC, isolated NK cells (1x10^5) and co-cultures of NK cells (1x10^5) and pDC (1x10^4) (10:1 ratio) were stimulated with HAdV5 (MOI 100), HAdV35 (MOI 10) and controls for 40 h. NK cell activation was measured by up-regulation of CD69 expression. Data depicted are combined from 4 independent experiments (error bar represents SEM).
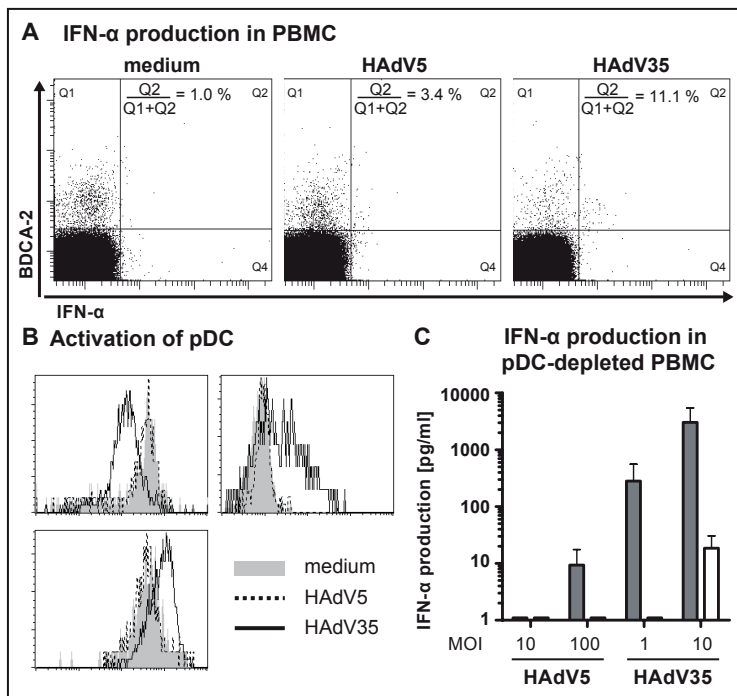
Plasmacytoid dendritic cells mediate HAdV35-induced NK cell activation

NK cell activation in response to HAdV35 occurred in the absence of T cells but still required another cell population of PBMC as purified NK cells were not activated. Since pDC are known to sense viruses, it was investigated whether pDC were required for NK cell activation following HAdV35 exposure. Depletion of pDC from PBMC led to reduced CD69 expression on NK cells after both HAdV5 and HAdV35 stimulation, in particular at lower viral loads, indicating that pDC were involved (Figure 3, panel A). To explore whether pDC were sufficient to elicit NK cell activation following HAdV35 exposure, pDC were isolated by negative selection, added to purified NK cells and stimulated with both HAdV serotypes. HAdV35, but not HAdV5, was capable of inducing NK cell activation in the presence of pDC and, apparently, did not require the contribution of other cell types (Figure 3, panel B). Consequently, pDC were both necessary and sufficient for the activation of NK cells following the exposure to HAdV35.

HAdV35 induces IFN-α production by plasmacytoid dendritic cells

As pDC were able to mediate NK cell activation by HAdV35, activation of pDC was assessed by analyzing intracellular IFN-α production following virus exposure. In contrast to HAdV5, HAdV35 exposure of PBMC resulted in a higher percentage of IFN-α–producing cells which expressed the pDC marker BDCA-2 (Figure 4, panel A). There were practically no IFN-α$^+$ cells detectable in the non-BDCA-2$^+$ cell fraction. Detailed analysis of the phenotype of all IFN-α$^+$ cells (Q2 + Q4; Figure 4, panel A) revealed a specific increase of pDC after HAdV35 exposure (Table 1). The percentage of other PBMC subsets, such as myeloid dendritic cells (mDC), T cells, B cells, NK cells and monocytes, did not alter or decreased after HAdV35 exposure. In addition, HAdV35, but not HAdV5, induced up-regulation of HLA-DR and CD40 as well as down-regulation of BDCA-2 on pDC, illustrating activation and maturation of pDC by HAdV35 (Figure 4, panel B). HAdV35 exposure resulted in considerable IFN-α levels in culture supernatants of unseparated PBMC (Figure 4, panel C). The depletion of pDC from

Figure 4: HAdV35 induces IFN-α production and maturation of plasmacytoid dendritic cells

(A) PBMC (2×10^6) were cultured with HAdV5 (MOI 100), HAdV35 (MOI 10) and the medium control for 18 h. pDC were identified in PBMC as BDCA-2$^+$ cells by extracellular staining followed by intracellular staining for IFN-α. The percentage of IFN-α$^+$ cells was determined within the BDCA-2$^+$ pDC population. Data are representative for 3 independent experiments. **(B)** Activation and maturation of pDC was assessed by the expression of CD40 and HLA-DR as well as down-regulation of BDCA 2 on BDCA 2$^+$ purified pDC after the exposure to HAdV5 (MOI 100) and HAdV35 (MOI 10) for 18 h. Data are presented as histograms, depicting the mean fluorescent intensity, MFI, of the four markers and the three conditions: medium, grey filled histogram, HAdV5, dotted line; HAdV35, black solid line. Data are representative for 3 independent experiments. **(C)** IFN-α levels were measured in culture supernatants of HAdV5 (MOI 10 and 100) and HAdV35-exposed (MOI 1 and 10) PBMC and pDC-depleted PBMC by ELISA. Data depicted are combined from 4 independent experiments (error bar represents SEM).

Table 1: The percentage of pDC within the IFN-α$^+$ cells increases following HAdV35 exposure

	Percentage of PBMC subsets within IFN-α$^+$ cells		
	Medium	HAdV5	HAdV35
pDC	7.7	11.1	73.0
mDC	5.1	0.0	0.2
T cells	43.6	13.9	13.5
B cells	10.3	30.6	0.0
NK cells	18.0	11.1	3.8
monocytes	2.6	13.8	0.0

PBMC were cultured with HAdV5 (MOI 100), HAdV35 (MOI 10) and medium for 18 h. Extracellular stainings of PBMC subsets were followed by intracellular staining for IFN-α. Within all IFN-α$^+$ cells, pDC were identified as BDCA-2$^+$ cells which were negative for CD11c$^-$ and devoid of CD3$^+$/14$^+$/19$^+$/20$^+$/56$^+$ cells; mDC as cells double positive for BDCA-1 and CD11c and devoid of CD3$^+$/14$^+$/19$^+$/20$^+$/56$^+$ cells; T cells as CD3$^+$ cells devoid of CD20$^+$/56$^+$ cells; NK cells as CD56$^+$ cells devoid of CD3$^+$/19$^+$ cells; B cells as CD19$^+$ and CD20$^+$ cells devoid of CD3$^+$/56$^+$ cells; monocytes as cells double positive for CD14 and CD11c. The percentage of the various PBMC subpopulations within IFN-α$^+$ PBMC (equals 100%) was determined.
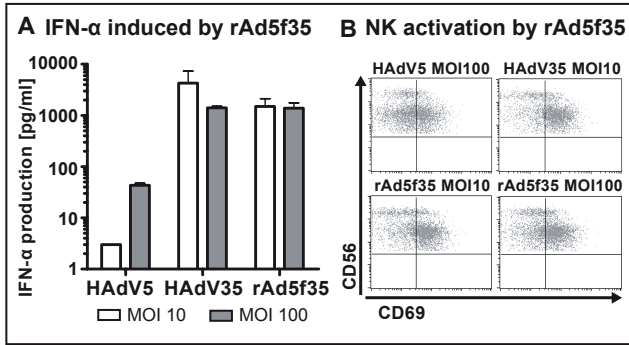
Figure 5: rAd5f35 induces high IFN-α production and activates NK cells

(A) PBMC were stimulated with HAdV5, HAdV35 and rAd5f35 (all MOI 10 and 100) for 40 h. IFN-α levels were measured in culture supernatants by ELISA. Data depicted are combined from 3 independent experiments (error bar represents SEM). **(B)** PBMC were stimulated with HAdV5 at MOI 100, HAdV35 at MOI 10 and rAd5f35 at MOI 10 and 100 for 40 h. NK cell activation was measured by up-regulated expression of CD69 on NK cells. Data are representative for 3 independent experiments.

PBMC substantially diminished the IFN-α levels, confirming that IFN-α production was almost exclusively attributed to pDC (Figure 4, panel C).

Adenovirus strains from different species utilize distinct surface molecules to enter cells. Internalization of HAdV5 involves binding of the HAdV5 fiber to the CAR receptor on target cells, while the HAdV35 fiber engages CD46 for cell entry [2]. To investigate the role of the fiber and its potential role in the way of entry into pDC, a recombinant rAd5f35 virus, which is a HAdV5 virus equipped with a HAdV35 fiber [27;30], was tested for its ability to induce IFN-α production and NK cell activation. At identical MOI, IFN-α production following rAd5f35 stimulation of PBMC was similar to the induction by HAdV35 and much higher than IFN-α production after HAdV5 exposure (Figure 5, panel A). Correspondingly, exposure of PBMC to rAd5f35 resulted in CD69 expression on NK cells comparable to HAdV35 exposure (Figure 5, panel B). To investigate whether the higher NK cell activation of type 35 fiber-equipped viruses was dependent on a higher infection rate of PBMC and in particular pDC, PBMC were stimulated with recombinant, replication-deficient rAd5-GFP, rAd35-GFP and rAd5f35-GFP and the percentage of GFP-expressing cells was assessed per PBMC subset. Exposure of PBMC with rAd5f35-GFP and rAd35-GFP resulted in a higher percentage of GFP-expressing pDC than rAd5-GFP in particular at high viral loads (Table 2). Infection of mDC and monocytes was also higher by rAd35-GFP and rAd5f35-GFP viruses, whereas only a minor percentage of T cells, NK cells and B cells (except for rAd5f35-GFP at MOI 100) was infected by both viruses.

Table 2: rAd5f35 and rAd35 but not rAd5 are able to infect pDC

	Percentage of GFP+ cells among PBMC subsets						
	Medium	rAd5-GFP		rAd35-GFP		rAd5f35-GFP	
MOI		10	100	10	100	10	100
pDC	0.0	0.0	0.2	4.5	6.1	0.5	6.1
mDC	0.0	22.3	22.5	17.8	30.4	42.5	70.9
T cells	0.0	0.1	0.4	0.5	0.8	1.0	4.5
B cells	0.0	0.2	1.8	1.4	1.7	3.5	11.6
NK cells	0.0	0.1	0.4	0.2	0.8	0.6	1.8
monocytes	0.0	2.2	16.5	8.9	26.0	31.8	70.8

PBMC were cultured with replication-deficient GFP-encoding rAd5-GFP, rAd35-GFP and rAd5f35-GFP at MOI 10 and 100 for 18 h. pDC, mDC, T cells, B cells, NK cells and monocytes were identified in PBMC as described in Table 1. To determine infection by the adenoviruses, the percentage of GFP-expressing cells per PBMC subset was assessed.
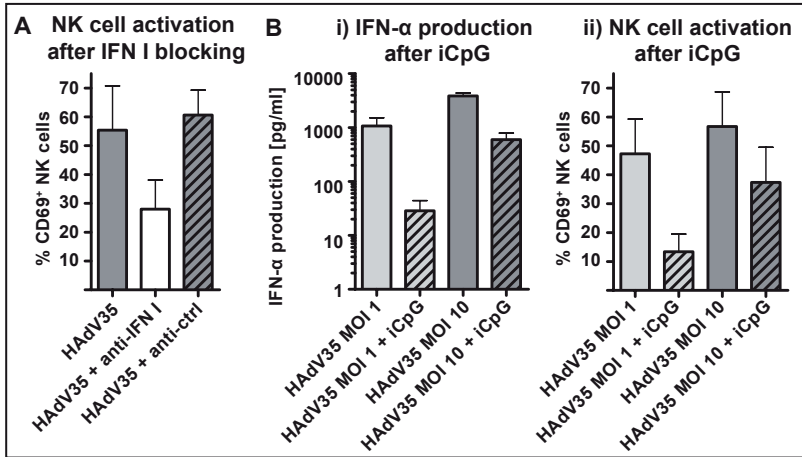
Figure 6: Both NK cell activation and IFN-α production require TLR9 signaling
(A) TCD PBMC were stimulated with HAdV35 at MOI 10 for 40 h in the presence or absence of neutralizing antibodies against IFN-α and the IFN I receptor or an irrelevant antibody. CD69 expression on NK cells was measured. Data depicted are combined from 4 independent experiments (error bar represents SEM). **(B)** TCD PBMC were stimulated with HAdV35 at MOI 1 and MOI 10 for 40 h in the presence or absence of iCpG. (i) IFN-α levels in supernatants of these cultures as well as (ii) CD69 expression on NK cells was measured. Data depicted are combined from 5 independent experiments (error bar represents SEM).

Therefore, the type of fiber and, likely, the rate of infection appeared to be responsible for the higher IFN-α production after exposure to HAdV35 than to HAdV5.

IFN-α production in response to HAdV35 relies on TLR-9 signaling and mediates NK cell activation

To further confirm whether IFN-α did mediate NK cell activation, CD69 expression on NK cells was assessed in the presence of neutralizing antibodies against soluble IFN-α and the IFN-I receptor [14;39]. In TCD PBMC exposed to HAdV35 at MOI 10, blockage of IFN-I reduced CD69 expression on NK cells by approx. 50 %, indicating that IFN-I was at least in part responsible for this activation (Figure 6, panel A). As far as other cytokines are involved, the presence of IL-12 or IL-15 in supernatants of HAdV35-exposed pDC-NK cell cultures was excluded (data not shown).

To explore whether IFN-α production by pDC requires sensing of adenovirus via the intracellular double-stranded DNA receptor TLR-9, the effect of inhibitory CpG (iCpG) was addressed. iCpG neutralizes the stimulatory effect of the TLR-9 agonist CpG and blocks IFN-α production by pDC in response to CpG and viruses [40-43]. IFN-α levels in supernatants of HAdV35-stimulated TCD PBMC were considerably reduced by iCpG (Figure 6, panel B, i). Likewise, iCpG inhibited NK cell activation following exposure to HAdV35 (Figure 6, panel B, ii). The inhibiting effect of iCpG was most apparent when TCD PBMC were exposed to lower viral loads of HAdV35, i.e., MOI 1 compared to MOI 10.

Overall, TLR-9 signaling appeared to be involved in the IFN-α production by pDC in response to HAdV35 which could mediate the activation of NK cells in response to HAdV35.

Figure 7: IFN-α production by plasmacytoid dendritic cells is enhanced in the presence of NK cells
(**A**) Purified pDC, purified NK cells and pDC-NK cell co-cultures (pDC: NK cells = 1:4) were stimulated with identical virus concentrations as for pDC-NK cell cultures (MOI 10) for 18 h and 40 h, respectively. IFN-α levels were measured in the supernatants of these cultures. Data depicted are combined from 2 independent experiments (error bar represents SEM). (**B**) Purified NK cells were stimulated with decreasing amounts of recombinant IFN-α with or without the addition of HAdV35 (MOI 10) for 40 h, and CD69 expression on NK cells was analyzed. Data depicted are combined from 2 independent experiments (error bar represents SEM).
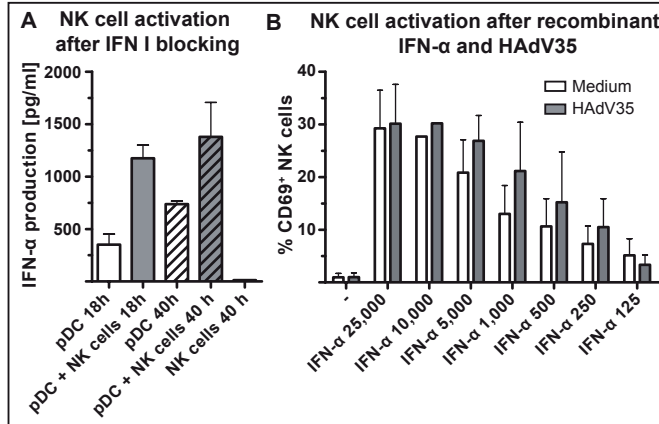
The IFN-α production by plasmacytoid dendritic cells is amplified by NK cells

It was examined whether NK cells contribute to the IFN-α response of pDC [14]. Indeed, IFN-α levels were augmented in HAdV35-stimulated co-cultures of pDC and NK cells as compared to pDC alone (Figure 7, panel A). NK cells alone failed to produce IFN-α. After 18 h stimulation with HAdV35, the increase of IFN-α levels by the presence of NK cells was superior to the increase after 40 h stimulation, suggesting that the reciprocal interaction between pDC and NK cells boosted the IFN-α response of pDC in particular early after exposure to HAdV35.

To investigate whether the increased response could be due to the NK cells becoming directly responsive to virus after exposure to pDC derived IFN-α, purified NK cells were stimulated with different concentrations of recombinant IFN-α in the presence and absence of HAdV35. However, HAdV35 hardly increased the CD69 expression on IFN-α–induced NK cells, suggesting that NK cells themselves cannot sense HAdV35 (Figure 7, panel B).

It can be concluded that there was cross-talk between NK cells and pDC characterized by the activation of NK cells through pDC-derived IFN-α and the amplification of IFN-α production by pDC through NK cells.

DISCUSSION

In this study we demonstrate that HAdV35 exposure results in considerable production of IFN-α by pDC, leading to NK cell activation. Conversely, under the same conditions HAdV5 does not induce significant IFN-α production and requires the contribution of T cells to activate NK cells. The difference in IFN-α production is specifically related to the virus fiber and, therefore, most likely due to the way of cell entry and infection efficiency of the virus. The IFN-α response of pDC to HAdV35 is mediated by TLR-9 signaling and is further enhanced by the reciprocal interaction of NK cells and pDC.

In patients post HSCT, the interplay between pDC and reconstituting NK cells may play a role in the immune response to HAdV early after HSCT, especially in the period when T lymphocytes are not yet detectable [20]. The presumed NK cell response to adenovirus in humans, however, has not yet been described as detailed as the NK cell response to other viruses, such as herpes simplex virus type 1, influenza virus or reovirus [14;39;44;45]. In mice, IFN-I and dendritic cell-dependent activation of NK cells has been implicated in the clearance of cells infected with adenoviral vectors [46;47]. However, since human adenovirus is known to result in only low yields of productive virus after infection of murine cells due to limited viral replication, imperfect protein synthesis and an abortive virus life cycle, mouse models are not optimal for studying immune responses to human adenovirus [48-51]. After infection with recombinant adenoviral vectors, human fibroblasts were more susceptible to lysis by NK cells in association with the NK cell-activating receptor NKG2D [52]. Inspired by these observations, we set out to study the activation of NK cells in PBMC of healthy adult donors in response to human adenovirus.

Interestingly, the mechanisms of NK cell activation in response to the two tested HAdV serotypes, which belong to different species of the HAdV family, differed substantially. Most healthy individuals possess a highly cross-reactive T cell repertoire, recognizing a variety of different HAdV serotypes and species [29;35-37]. The addition of HAdV to PBMC results in T cell activation and, thereby, is likely to cause IL-2 production. As the T cell repertoire is highly cross-reactive this T cell activation occurs in response to both HAdV5 as well as HAdV35 [36;53]. It is known that T cell-derived IL-2 stimulates NK cell proliferation, activation and IFN-γ production [38;54-56]. Neutralization of IL-2 indeed inhibited at least partially the NK cell activation in response to HAdV5. A similar mechanism was shown to account for NK cell activation after exposure to influenza, a virus that also elicits a strong T cell response in most healthy donors [38].

Surprisingly, although the depletion of T cells did abrogate NK cell activation in response to HAdV5, it did not abrogate HAdV35-induced NK cell activation. Accordingly, an additional mechanism for HAdV35-induced NK cell activation is operational that is independent of T cells. Compared to HAdV5, HAdV35 evoked considerably stronger IFN-α production by pDC which may be sufficiently high to induce NK cell activation via pDC [14;25].

IFN-I production by pDC in response to viral DNA or stimulatory CpG motifs requires TLR-9 signaling [15]. IFN-α production in response to HAdV35 was also mediated by TLR-9 as most of the response was eliminated by the TLR-9 antagonist iCpG [41;57]. Since IFN-α was almost exclusively derived from pDC upon HAdV35 exposure, TLR-9-independent pathways of other cells, such as mDC or monocytes, do not seem to contribute to the IFN-α response as supported by the data on intracellular IFN-α in various subsets of mononuclear cells [14;39;58]. NK cell activation following HAdV35 exposure was at least partly mediated by IFN-α as the presence of neutralizing antibodies against IFN-α and the IFN-I receptor reduced CD69 expression on NK cells by approx. 50 % [14;39]. The lack of total inhibition suggested that either IFN-α was produced in large excess or that besides IFN-α additional (soluble) factors may be involved in mediating NK cell activation by pDC. While incubation of purified NK cells with IFN-α–containing supernatants of HAdV35-stimulated pDC-NK cell co-cultures induced NK cell activation, neither IL-12 nor IL-15, two other NK cell-activating cytokines, were detected in these supernatants (data not shown) [14].

Finally, we observed that cross-talk between NK cells and pDC boosted the IFN-α production of pDC in response to HAdV35, suggesting a reciprocally amplified antiviral innate immune response. In previous studies, cross-talk between NK cells and DC via direct cell-cell contact, such as NKG2D-NKG2D ligand interaction, has been associated with NK cell activation and can enhance IFN-α production [14;39;47]. Interestingly, the DNAM-1 ligand CD112 was expressed but not up-regulated on pDC after virus exposure whereas NKG2D ligands were not detectable on pDC (data not shown). Hence, interaction between NK cells and pDC could theoretically occur via DNAM-1 and CD112.

Our findings raised the question why HAdV35 was so much more effective than HAdV5 in inducing IFN-α production and maturation of pDC. The two virus serotypes differ in the type of cellular receptor necessary for cell entry. The HAdV5 fiber binds to the CAR (coxsackie adenovirus receptor) receptor which is absent from pDC, while the type 35 fiber binds to the cellular surface protein CD46 (complement regulatory protein [59]) which is abundantly expressed on pDC [2;25]. As reported previously, especially myeloid cells such as mDC and monocytes were infected by rAd5-GFP, rAd35-GFP and rAd5f35-GFP (rAd5 equipped with the type 35 fiber), while lymphocytes such as T cells, B cell and NK cells were weakly infected [25-28]. Moreover, we show that rAd5f35 induced IFN-α levels similar to HAdV35 and likewise HAdV35 and rAd5f35 were able to infect pDC at comparable efficiencies, whereas HAdV5 failed to infect pDC. Thus, the increased IFN-α production of rAd5f35 may be a result of higher infection rates of pDC due to the type 35 fiber. Correspondingly, the much higher infection efficiency of HAdV5f16 (HAdV5 equipped with the species B type 16 fiber) than HAdV5 has been associated with high IFN-α production by pDC [41]. The percentage of IFN-α producing pDC was higher than the percentage of GFP-expressing pDC, suggesting that also non-infected pDC might produce IFN-α (data not shown).

The clinical relevance of NK cells with respect to HAdV clearance in humans is unresolved. In pediatric HSCT patients, the kinetics of lymphocyte recovery is different, i.e., NK cells usually reconstitute earlier than T cells. During the early post-HSCT period, HAdV may (re)activate and potentially cause a disseminated infection with a fatal course [36;60;61]. HAdV5 and other species C HAdV are predominantly encountered in these patients in our center and elsewhere [62-64]. In comparison to HAdV5, antibodies specific for the species B virus HAdV35 are rarely detected in sera of healthy donors, suggesting that the virus is not ubiquitously present in the human population [65;66]. In immunocompromised patients, HAdV35 causes disease only infrequently although it has been reported in HSCT centers in the USA [67]. The low prevalence of the virus, absence of functional immune evasion mechanisms or a strong response of the innate immune system to HAdV35 may provide an explanation for the low occurrence of disseminated HAdV35 infections in HSCT patients.

The phenomenon that these two adenovirus serotypes exhibit substantial differences in their capacity to evoke an innate immune response is relevant because HAdV35-derived vectors are recently being explored for the delivery of vaccines [66;68]. Most experience with adenovirus-derived vectors has been acquired in experimental models or clinical trials using HAdV5-derived vectors. Based on the present findings, it may be anticipated that the HAdV35-derived vectors behave differently in their efficacy and/or their degree of side effects in clinical trials.

ACKNOWLEDGMENTS

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CHAPTER 3

Anti-EGFR antibody cetuximab enhances the cytolytic activity of Natural Killer cells toward osteosarcoma

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ABSTRACT

Osteosarcoma and Ewing sarcoma are the most common bone tumors in children and adolescents. Despite intensive chemotherapy, patients with advanced disease have a poor prognosis, illustrating the need for alternative therapies. Sarcoma cells are susceptible to the cytolytic activity of resting natural killer (NK) cells which can be improved by interleukin(IL)–15 stimulation. In this study, we explored whether the cytolytic function of resting NK cells can be augmented and specifically directed toward sarcoma cells by antibody-dependent cellular cytotoxicity (ADCC).

Epidermal growth factor receptor (EGFR) expression was examined on osteosarcoma and Ewing sarcoma cell lines by flow cytometry and in osteosarcoma biopsy and resection specimens by immunohistochemistry. Cetuximab-mediated ADCC by NKcells from osteosarcoma patients and healthy controls was measured with 4-hour ^{51}Cr release assays.

EGFR surface expression was shown on chemotherapy-sensitive and chemotherapy-resistant osteosarcoma cells (12/12), most primary osteosarcoma cultures (4/5), and few Ewing sarcoma cell lines (2/7). In the presence of cetuximab, the cytolytic activity of resting NK cells against all EGFR-expressing sarcoma cells was substantially increased and comparable with that of IL-15–activated NK cells. Surface EGFR expression on primary osteosarcoma cultures correlated with EGFR expression in the original tumor. The cytolytic activity of osteosarcoma patient-derived NK cells against autologous tumor cells was as efficient as that of NK cells from healthy donors.

Our data show that the cytolytic potential of resting NK cells can be potentiated and directed toward osteosarcoma cells with cetuximab. Therefore, cetuximab-mediated immunotherapy may be considered a novel treatment modality in the management of advanced osteosarcoma.

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INTRODUCTION

Osteosarcoma and Ewing sarcoma most frequently arise in adolescents and young adults and represents the majority of all malignant primary bone tumors in this patient group [1–3]. The current treatment consists of a combination of systemic multi-drug chemotherapy and complete surgical resection [3–5]. In cases with localized disease, up to 70% of the patients achieve persistent remission. In contrast, patients with advanced, metastasized, and recurrent disease experience a very poor prognosis, which has not improved during the last decades despite intensification of chemotherapy regimens. Therefore, novel treatment strategies with a favorable toxicity profile are warranted. In this perspective, we and others have recently reported on the potential exploitation of cellular immunotherapy against sarcomas by natural killer (NK) cells [6–8].

NK cells can respond to and kill cells undergoing cellular stress due to virus infection or malignant transformation. The cytotoxic activity of NK cells is regulated by the equilibrium of inhibiting and activating signals conveyed by target cells. On tumor cells, MHC class I expression (NK cell inhibitory signal) may be down-regulated to evade cytotoxic T-cell recognition. Conversely, the expression of stress ligands (NK cell–activating signal) may be upregulated on tumor cells. Both of these processes may lead to increased sensitivity of tumor cells to NK cells [9,10]. In addition, the interplay with other immune cells and the pro- or anti-inflammatory microenvironment may modulate the function and activity of NK cells [10,11]. Recently, we and others have shown that sarcoma cell lines are moderately susceptible to the cytolytic potential of resting NK cells [6–8]. The cytolytic activity of NK cells can be directly potentiated by activating cytokines, such as interleukin(IL)–15, leading to increased lysis of sarcoma cells [6–8].

In this study, we set out to explore whether the cytolytic activity of resting NK cells can be improved and directed specifically toward sarcoma cells. Therefore, we intended using a monoclonal antibody (mAb) of the human IgG1 subtype which recognizes an antigen expressed on sarcoma cells and is able to induce antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Osteosarcoma has previously been shown to express the epidermal growth factor receptor (EGFR, erbB1/Her1 [12]), which is recognized by the clinically-approved, chimeric IgG1 type mAb cetuximab [13]. So far, the application of anti-EGFR mAb–targeted therapy in bone sarcomas has not been reported. We focused on exploring whether the cytotoxic potential of allogeneic and autologous NK cells can be specifically directed toward sarcoma cells with cetuximab.

MATERIALS AND METHODS

Patient samples

Formalin-fixed, paraffin-embedded tumor samples were obtained from one biopsy (obtained at the time of diagnosis, prechemotherapy) and 4 resections of local recurrent or metastatic tumors (postchemotherapy) from 4 high-grade osteosarcoma patients (diagnosed between 2008 and 2010) by the Department of Pathology, Leiden University Medical Center. Five short term grown primary osteosarcoma cell cultures (between passage 2 and 13) were generated from the tumor material as previously described [6, 14]. Clinicopathologic details of these patients and samples are summarized in Table 1. Peripheral blood samples from these patients were collected

Table 1: Clinicopathologic details of patient material

Osteosarcoma patient	Sex	Age	Tumor origin	Tumor site	Histologic subtype	Primary cell line
369	Female	31	Local relapsed	Proximal humerus	Fibroblastic	L2792 p3
398	Female	14	Prechemotherapy diagnostic biopsy	Distal femur	Conventional	L2635 p6
404	Male	46	Postchemotherapy resection	Humerus	Conventional	L2826 p9
404	Male	46	Local relapse	Humerus	Conventional	L3312 p13
407	Male	9	Postchemotherapy resection	Proximal tibia	Chondroblastic	L2857 p7

prior to the initiation of chemotherapy after written informed consent approved by the Review Board on Medical Ethics of the Leiden University Medical Center and used for cytotoxicity experiments (Table 1). Tumor specimens were obtained and analyzed according to the ethical guidelines of the national organization of scientific societies (FEDERA, http://www.federa. org/ gedragscodes-codes-conduct-en).

Cell lines

The following extensively characterized sarcoma cell lines were included in this study: osteosarcoma cell lines OHS, OSA (SJSA-1), SAOS-2, U2OS, ZK-58 [15] and the Ewing sarcoma cell lines A673, CADO-ES, SK-ES-1, SK-NMC, STA-ET2.1, TC71 [15] and L1062 [14]. All sarcoma cell lines were obtained from the EuroBoNeT cell line repository (by 2007) and were confirmed for their identity by short-tandem repeat DNA fingerprinting in 2011. The cell line TC71 was maintained in IMDM medium (Invitrogen). All other cell lines were grown in RPMI-1640 medium (Invitrogen). Both media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin (all Invitrogen). All Ewing sarcoma cell lines were grown in 0.1% gelatin-coated tissue culture flasks. The chemotherapy-resistant variant cell lines of SAOS-2 and U2OS [16–18] were cultured in IMDM medium with 10% FCS and penicillin/streptomycin and maintained in chemotherapeutic drugs as follows: SAOS-2–DX580 and U2OS–DX580 with 580 mg/ml of doxorubicin (DX); SAOS-2–MTX1000 and U2OS–MTX300 with 1,000 and 300 ng/ml of methotrexate (MTX), respectively; SAOS-2–CDDP6 (SAOS-2–CDDP6mg) and U2OS–CDDP4 (U2OS–CDDP4μg) with 6 and 4 mg/ml of cisplatin (cis-diamminedichloroplatinum, CDDP), respectively. Drug sensitivities of each cell line were calculated from the drug dose–response curves and expressed as IC50 (drug concentration resulting in 50% inhibition of cell growth after 96 hours of *in vitro* treatment). Fold increases in drug resistance, quantified as the ratio between IC50 of each drug-resistant variant to that of its corresponding parental cell line, were as follows: 315 for U2OS–DX580, 328 for SAOS-2–DX580, 135 for U2OS–MTX300, 281 for SAOS-2–MTX1mg, 63 for U2OS–CDDP4mg, and 112 for SAOS-2–CDDP6mg. All cell lines were negative for Mycoplasma infection as regularly checked by PCR. The primary osteosarcoma cultures were maintained in RPMI-1640 medium supplemented with 20% FCS and penicillin/streptomycin in gelatin-coated culture tissue culture flasks.

Cell isolations and stimulations

Peripheral blood mononuclear cells (PBMC) were isolated from osteosarcoma patients' blood samples (autologous) or buffy coats of healthy adult donors (allogeneic; Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. NK cells were purified by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the "Human NK cell Isolation Kit" (Miltenyi Biotech); NK cell purity was more than 95% as determined by flow cytometry, analyzing NK cells as $CD56^+$, $CD3^-$, $CD14^-$, and $CD19^-$ cells. NK cells were depleted from PBMC (NKD PBMC) by positive selection using anti-CD56 MicroBeads (Miltenyi Biotech); NKD PBMC contained less than 0.1% of NK cells as analyzed by flow cytometry. IL-15–activated NK cells were obtained by incubating purified NK cells in AIM-V medium with 2 mM of glutamine (Invitrogen) supplemented with 10% of pooled human AB serum (Sanquin), penicillin/streptomycin and 10 ng/ml of IL-15 (Peprotech) for 2 to 3 weeks in 24-well format tissue culture plates without feeder cells. To measure NK cell activation after cetuximab crosslinking, upregulation of CD69 expression on NK cells (300,000) was measured after coculture with STA-ET2.1 (150,000), L1062 (80,000), and OSA (75,000) cells for 48 hours in 24-well plates in the absence or presence of cetuximab (10 mg/ml).

Flow cytometry

Determination of NK cell percentages in PBMC, validation of NK cell purity, and expression of the NK cell activation marker (CD69) was analyzed phenotypically by staining with fluorescently labeled antibodies followed by fluorescence-activated cell sorting (FACS). The following antibodies were applied according to the manufacturer's instructions: $CD3^{FITC}$ (SK7), $CD3^{PerCP-Cy5.5}$ (SK7), $CD14^{PerCP-Cy5.5}$ (M5E2), $CD19^{PE}$ (4G7), $CD69^{FITC}$ (L78; Becton Dickinson); $CD56^{APC}$ (N901 NKH1), $NKG2D^{PE}$ (ON72; IOTEST Immunotech). Expression of EGFR on the surface of sarcoma cell lines and primary osteosarcoma cultures was measured using the anti-EGFR mAb cetuximab (Erbitux; Merck KGaA) followed by the Alexa Fluor 647 goat anti-human IgG secondary antibody (A21445; Invitrogen). The anti-CD20 mAb rituximab (MabThera; Roche) was used as an IgG1 isotype-matched negative control for cetuximab. FACS measurements were carried out with the FACSCalibur (BD Biosciences) and analyzed with the "BD Cell Quest Pro" software (version 5.2.1).

^{51}chromium release assay

The cytolytic activity of PBMC, NKD PBMC, and purified NK cells against sarcoma cell lines and primary osteosarcoma cultures was measured in 4-hour ^{51}chromium (^{51}Cr) release assays. Target cells were labeled with 100 ml Na-chromate (^{51}Cr, 3.7 MBq) for 1 hour. After washing, $2.5x10^3$ target cells were added to the effector cells in duplicate or triplicate at the indicated effector–target (E:T) ratios and incubated in the presence or absence of cetuximab (10^{-7} to 10 mg/ml as indicated) or the control mAb rituximab (10 mg/ml) for 4 hours at 37°C. Supernatants were collected, and the release of ^{51}Cr was measured with a beta-counter (Wallac/PerkinElmer). Spontaneous and total release were obtained by incubation with medium and Triton X-100 (2.5%; Merck Chemicals), respectively. The specific lysis was calculated by the following

formula: percentage of specific lysis = 100 − (experimental release − spontaneous release/total release − spontaneous release).

Immunohistochemistry

Sections of 4 mm of representative tumor sections were deparaffinized and pepsin antigen retrieval was done. Expression of EGFR was assessed using a mouse monoclonal anti-EGFR antibody (31G7, 1:10; Zymed; Invitrogen) followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP linker antibody conjugate (DPVO-110HRP; Immunologic) and DAB+Substrate Chromogen System (Dako) detection. The sections were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica).

Statistical analysis

Statistical analyses were carried out with GraphPad Prism version 5.04 or SPSS version 16.0 (IBM) using paired student t tests, comparing means between groups of samples and linear regression analysis. Error bars represent the SEM. A P value less than 0.05 was considered statistically significant. ns, not statistically significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
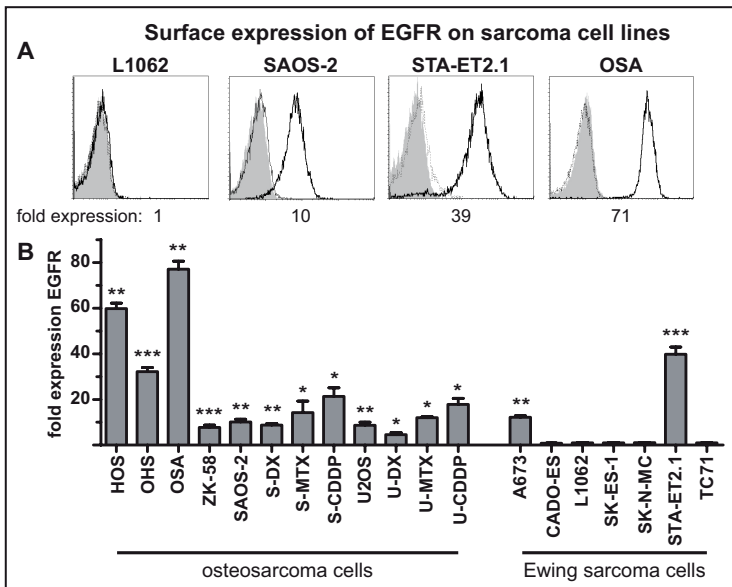
Figure 1: EGFR is expressed on the surface of sarcoma cell lines
(A) Surface expression of EGFR on osteosarcoma and Ewing sarcoma cell lines was measured by flow cytometry using the anti-EGFR mAb cetuximab followed by the Alexa Fluor-647 goat anti-human IgG secondary antibody. A, representative FACS overlay plots, detecting EGFR by cetuximab (bold solid line) and CD20 by the isotype-matched, negative control mAb rituximab (solid line), both followed by secondary antibody and secondary antibody only (grey area). The indicated fold expression of EGFR was calculated by dividing the geometric mean fluorescence intensity of EGFR by the geometric mean fluorescence intensity of the control CD20. **(B)** combined data of the fold change of EGFR expression on all tested sarcoma cell lines of multiple experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

EGFR is expressed on the surface of osteosarcoma cell lines

ADCC by NK cells requires antibody binding to an antigen expressed on the tumor cell surface. Therefore, surface expression of EGFR, as detected by cetuximab, was measured on a panel of osteosarcoma (n=12) cell lines by flow cytometry. All osteosarcoma cell lines expressed EGFR on the cell surface, with the highest expression on the cell lines HOS, OHS, and OSA (Figure 1). The chemoresistant variants of SAOS-2 and U2OS also expressed EGFR. Previously, EGFR has been reported undetectable in Ewing sarcoma cell lines (n=3; [19]). To extend these findings, surface EGFR expression was assessed on a panel of Ewing sarcoma cell lines (n=7). EGFR expression was not detectable on 5 of 7 Ewing sarcoma cell lines. Correspondingly, EGFR expression was not detectable in Ewing sarcoma biopsy and resection specimens, as determined by immunohistochemistry (data not shown).
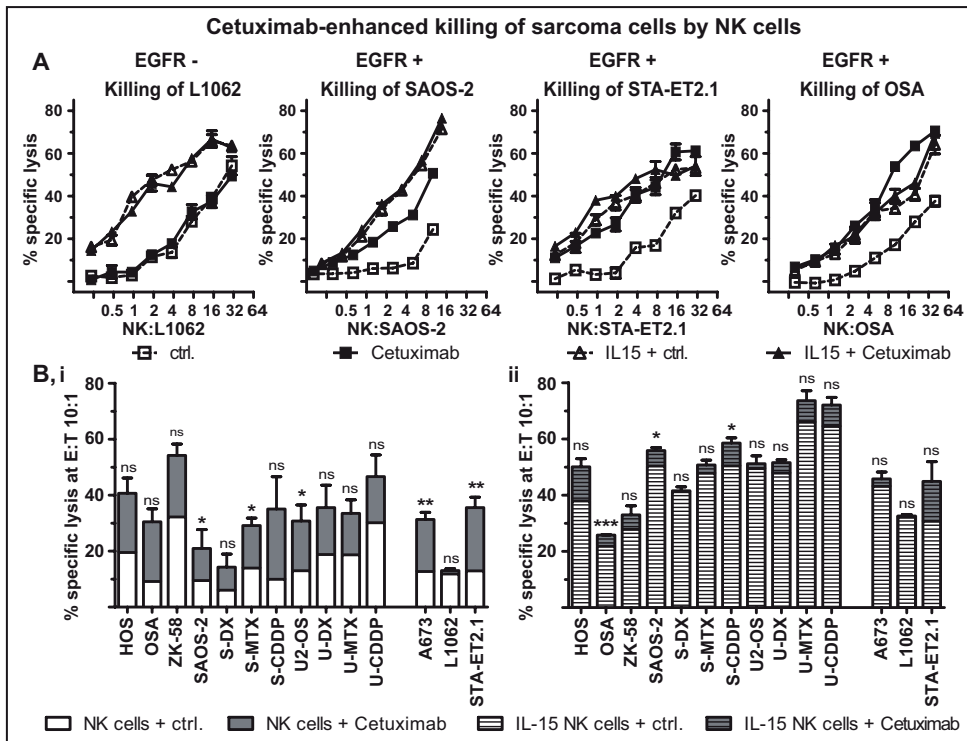
**Figure 2: Cetuximab-enhanced killing of sarcoma cell lines by NK cells**

Lysis of EGFR+ and EGFR- sarcoma cell lines by purified, resting NK cells and IL-15–activated NK cells was measured in triplicate in a 4-hour ^{51}Cr release assay in the presence of cetuximab or the isotype-matched, negative control mAb rituximab. The specific lysis (%) of sarcoma cells in the presence of the control mAb was comparable with killing without mAb addition. **(A)** representative data of the specific lysis of sarcoma cell lines (ranked by increasing EGFR density) by resting NK cells (squares) and IL-15–activated NK cells (triangles) in the presence of cetuximab (filled symbols) and the control mAb (open symbols). **(B)** combined data of the specific lysis of sarcoma cells by resting NK cells (i, no pattern) and IL-15–activated NK cells (ii, horizontal pattern) calculated at a 10:1 E:T ratio. Data represent 3 independent experiments done in triplicate, showing lysis in the presence of the control mAb (open bars) and the respective extra lysis induced by the presence of cetuximab (filled bars, SEM). Significance of cetuximab-induced lysis was calculated by comparing whether the total lysis in the presence of cetuximab (filled bar plus open bar) was statistically different to the lysis in the presence of the control antibody (open bar). ns, not significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
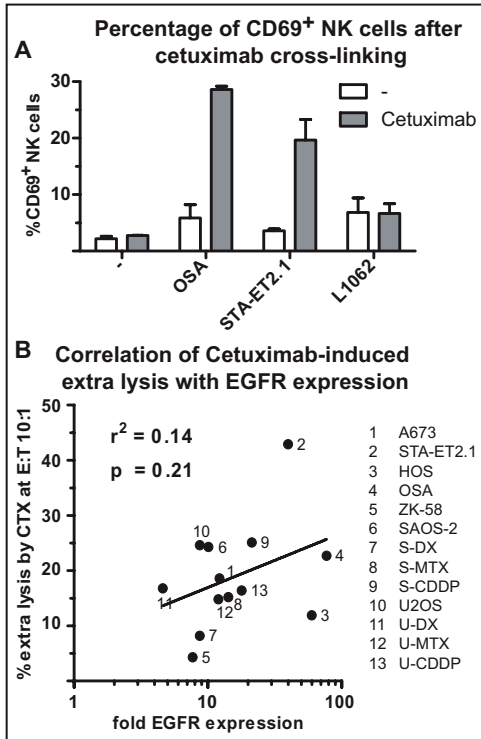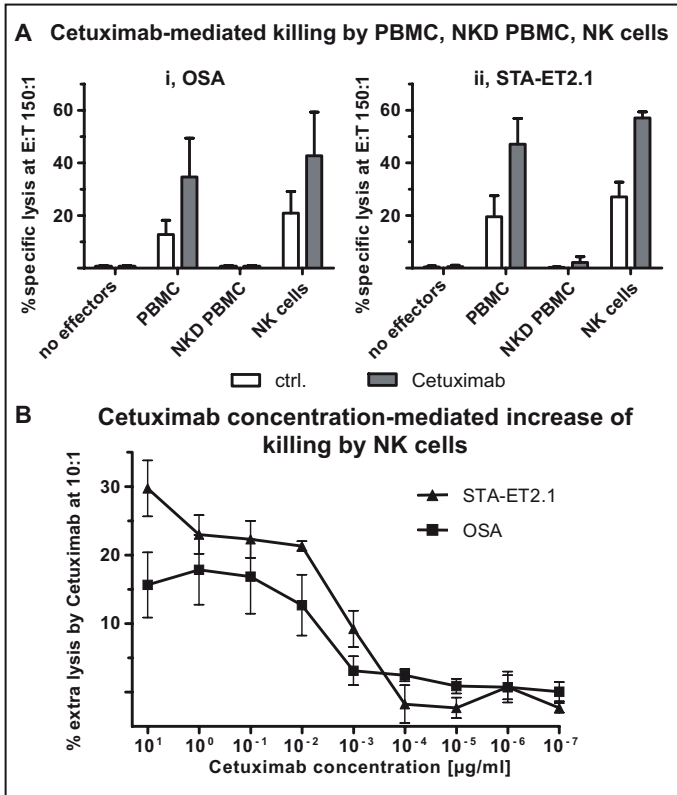
Figure 3: Cetuximab-induced activation of NK cells

(A) EGFR-expressing (OSA and STA-ET2.1) and EGFR-negative cell lines (L1062) were cocultured with purified NK cells in the presence of cetuximab for 48 hours and CD69 expression on NK cells was assessed. The percentage of CD69-positive NK cells is indicated. **(B)** The extra lysis induced by cetuximab, as calculated by subtracting the lysis by the control mAb from the lysis by cetuximab, was correlated with EGFR expression levels on the cell lines. Lysis of EGFR$^+$ sarcoma cell lines (n = 13) by resting NK cells at a 10:1 E:T ratio was measured in the presence of cetuximab or the negative control mAb (Fig. 2). The extra lysis was calculated by subtracting the specific lysis in the presence of the control mAb from the total lysis in the presence of cetuximab, and plotted against the log of the fold change of EGFR expression (Figure 1). The regression coefficient (*r2*) between extra lysis and EGFR expression was 0.14 (*P* = 0.21), as calculated by linear regression analysis.

Cetuximab enhanced cytolysis of EGFR-expressing osteosarcoma cell lines by NK cells

To investigate whether cetuximab can enhance cytolysis of EGFR-expressing osteosarcoma and Ewing sarcoma cell lines by NK cells, resting NK cells were incubated with one EGFR$^-$ and several EGFR* cell lines in the presence of cetuximab or the isotype-matched, nonbinding anti-CD20 control mAb in a 4-hour ^{51}Cr release assay. As compared with cytolysis in the absence of mAb, the negative control mAb did not alter killing of sarcoma cells by NK cells (data now shown). In contrast, the addition of cetuximab increased the lysis of EGFR-expressing sarcoma cells but not of EGFR-negative cell lines (Figure 2, panel A and B, i; data not shown). The lysis of the chemotherapy-resistant variant cell lines of SAOS-2 and U2OS was equally enhanced by cetuximab. Cetuximab-enhanced lysis by resting NK cells was comparable with the lysis by IL-15–activated NK cells (Figure 2, panel A and B, ii). The addition of cetuximab to IL-15–activated NK cells hardly led to a further increase of the cytolytic activity. As an alternative parameter for NK cell activation, it was observed that the percentage of CD69- expressing NK cells increased after coculture of NK cells with EGFR-expressing sarcoma cells in the presence of cetuximab (Figure 3, panel A).

Thus, the cytotoxic function of resting NK cells toward EGFR-expressing sarcoma cell lines as well as their activation status was substantially augmented in the presence of cetuximab.

A Cetuximab-mediated killing by PBMC, NKD PBMC, NK cells

i, OSA

ii, STA-ET2.1

□ ctrl.   ■ Cetuximab

B Cetuximab concentration-mediated increase of killing by NK cells

Figure 4: Cetuximab-mediated killing is dependent on NK cells
(A) The specific lysis of the osteosarcoma cell line OSA (i) and the Ewing's sarcoma cell line STA-ET2.1 (ii) in the presence of cetuximab or the control mAb by resting NK cells was compared with the lysis by total PBMCs, NKD PBMCs, and medium (absence of effector cells). Killing by PBMCs was analyzed at a 150:1 E:T ratio. Killing by NKD PBMCs was analyzed at a 150:1 E:T ratio corrected for the absence of NK cells in accordance to the percentage of NK cells in the corresponding PBMCs. Killing by purified NK cells was extrapolated to a 150:1 E:T ratio of PBMCs in accordance with the percentage of NK cells in the corresponding PBMCs. Combined data of 2 experiments. **(B)** The extra lysis of the two EGFR+ cell lines OSA and STA-ET2.1 by resting NK cells was measured in the presence of increasing concentrations of cetuximab, from 10^{-7} to 10 mg/ml at a 10:1 E:T ratio. Combined data of 3 independent experiments.

Cetuximab-mediated lysis is independent of EGFR expression intensity

Despite different sensitivities to NK cell killing, the magnitude of cetuximab-enhanced lysis by resting NK cells was comparable among most EGFR-expressing cell lines (Figure 2, panel B, i). This increase did not correlate significantly with EGFR surface densities (Figure 3, panel B). Thus, even the minimal EGFR expression levels on some of the sarcoma cells were sufficient for the induction of ADCC.

Cetuximab-mediated lysis is dependent on NK cells

In the absence of effector cells, cetuximab did not elicit cytolytic effects on EGFR-expressing cell lines during the 4-hour cytotoxicity assay (Figure 4). FcγRIIIa/CD16 expression is required to elicit ADCC by NK cells. Because FcγRIIIa/CD16 can also be expressed by monocytes, it was studied whether cetuximab-mediated ADCC by PBMC is dependent on NK cells. In the presence of cetuximab, lysis of EGFR-expressing sarcoma cells by PBMC was comparable with the cetuximab- enhanced lysis by purified NK cells (Figure 4, panel A). In contrast, NK cell depletion abolished killing by PBMC both in the absence and presence of cetuximab, indicating that in this *in vitro* system cetuximab-mediated killing was strictly dependent on NK cells present in the PBMC.

Next, the dependence of cetuximab-mediated lysis on the concentration of cetuximab was investigated in a serial dilution experiment. The lysis induced by cetuximab was comparable

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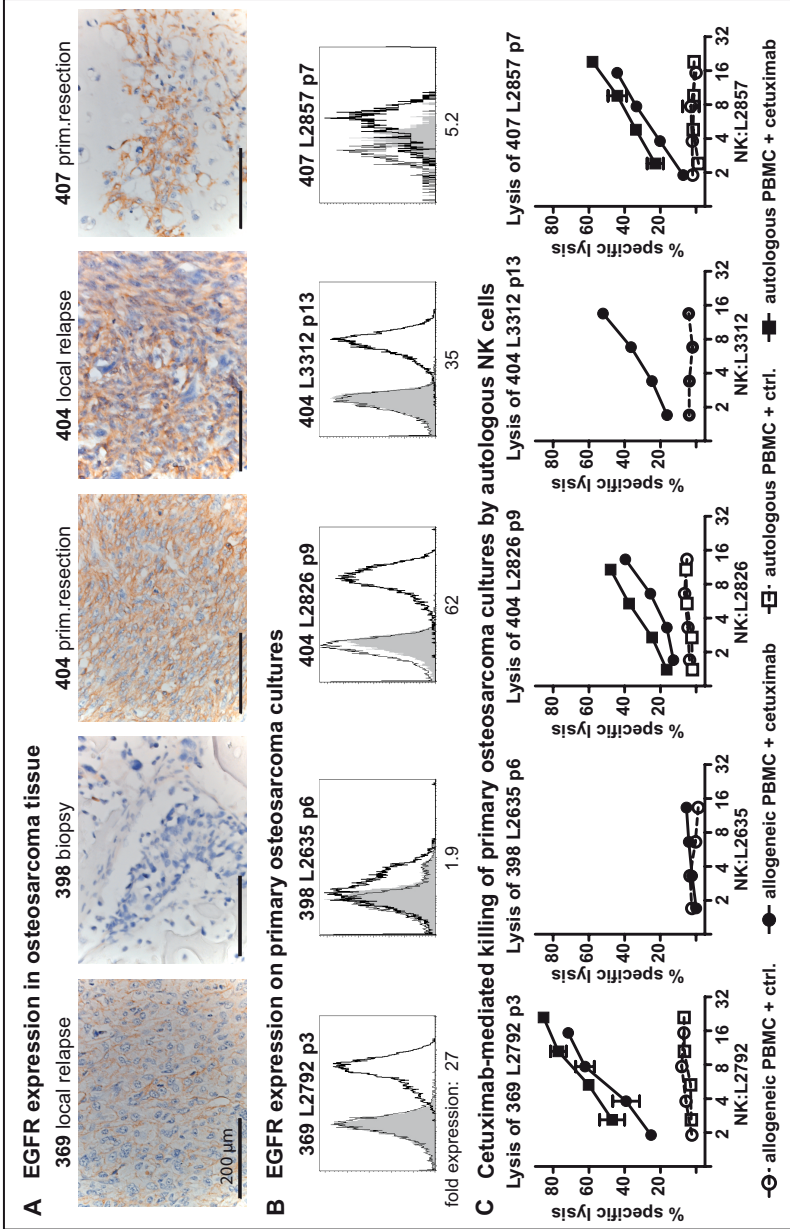
Figure 5: EGFR+ primary osteosarcoma cultures, derived from EGFR+ osteosarcoma specimens, are highly susceptible to cetuximab-mediated lysis by autologous PBMC

(A) EGFR expression in biopsies and resection specimens from osteosarcoma patients was detected using a mouse monoclonal anti-EGFR antibody followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP linker antibody conjugate. Brown-colored EGFR staining patterns and counterstaining with Mayer's hematoxylin are displayed at a 40-fold magnification for the respective tumor origin and patient. **(B)** Primary osteosarcoma cultures were generated from EGFR+ and EGFR biopsies and resections of osteosarcoma patients as indicated. EGFR expression on the primary osteosarcoma cultures was measured as described in Figure 1 at the indicated passage numbers. **(C)** Killing of primary osteosarcoma cultures by autologous PBMC, derived from the same osteosarcoma patient, and allogeneic PBMC (one representative result from 2 healthy donors) was analyzed in duplicate in the presence of cetuximab or the control mAb at the indicated E:T ratios. p, passage number; prim, primary.

between 10 and 1×10^{-2} mg/ml of cetuximab, but it was reduced by at least 50% at a concentration of 1×10^{-3} mg/ml (Figure 4, panel B). Lower cetuximab concentrations failed to enhance lysis. Hence, 0.01 mg/ml of cetuximab was the minimal concentration to substantially enhance cytolysis by NK cells.

EGFR+ primary cultures derived from EGFR+ osteosarcoma tumors are highly susceptible to cetuximab-mediated lysis by autologous PBMC

Primary tumor cell cultures (n=5) were generated from osteosarcoma biopsy (n=1) and resection (n=4) specimens derived from 4 different osteosarcoma patients (Table 1). EGFR expression in these osteosarcoma samples was membranous, as determined by immunohistochemistry, and correlated to the EGFR densities on the corresponding primary cultures, as determined by flow cytometry between passages (p) 3 and 13. Except for osteosarcoma patient 398, in which EGFR was not detectable in the biopsy and only weakly detectable on the corresponding primary culture L2635 p6, all osteosarcoma patients (369, 404, and 407) presented EGFR expression both in the original tumor material and the respective primary cultures (L2792 p3; L2826 p9, and L3312 p13; L2857 p7; Figure 5, panel A and B).

The cytolytic activity of NK cells from patient-derived PBMC toward EGFR-expressing autologous osteosarcoma cultures was substantially enhanced by cetuximab (Figure 5, panel C). Notably, patient-derived NK cells lysed the osteosarcoma cultures as efficient as NK cells from healthy donors. Thus, when sarcoma cells are specifically targeted with cetuximab, their lysis by resting NK cells can be enhanced by ADCC.

DISCUSSION

The identification of antigens specifically expressed on tumor cells has fueled the development of tumor-specific, mAb-based targeted therapies. The introduction of anti- CD20 mAb (Rituximab, MabThera) and anti-Her2 mAb (Trastuzumab, Herceptin) against B cell malignancies and breast cancer, respectively, has improved patient prognosis [20,21]. Surface expression of EGFR has been shown in several tumors, such as colorectal cancer, head and neck squamous cell carcinoma, and is specifically recognized by the chimeric IgG1 mAb cetuximab [13,20]. In numerous clinical phase II and III studies, the addition of cetuximab to conventional multi-drug chemotherapy or radiotherapy has led to a significant improvement of clinical response rates, progression-free survival, and overall survival. Therefore, cetuximab therapy was approved for the treatment of recurrent, refractory, and metastatic colorectal cancer [22–25] as well as head and neck squamous cell carcinoma [26,27] by the U.S. Food and Drugs Administration (FDA).

Previously, we and others have shown the cytolytic potential of NK cells against sarcoma cells [6–8]. In this study, we sought to explore whether this cytolytic activity can be more specifically targeted toward sarcoma cells using a sarcoma-reactive mAb, with a human Fc portion that can bind to FcγRIIIa/CD16 on human NK cells. As several studies have described surface EGFR expression in osteosarcoma tumors and on osteosarcoma cell lines [12,19,28–31], we explored the potential of the anti-EGFR mAb cetuximab to specifically direct NK cell–mediated killing to sarcoma cell lines. In agreement with previous studies on other tumor types, cetuximab induced NK cell–dependent ADCC against EGFR-expressing sarcoma cells. Similar to previous

studies [32–35], we show that 0.01 mg/ml of cetuximab was the minimal concentration to induce cetuximab-mediated lysis by NK cells. These concentrations have been reported in sera of patients treated with cetuximab and in the tumor environment in a xenograft model [36,37], indicating that cetuximab-mediated ADCC could be a functional anticancer mechanism *in vivo*.

Although in other studies the magnitude of cetuximab-induced ADCC correlated with the level of EGFR expression [34,35,38], this correlation was not evident in osteosarcoma, despite the use of highly comparable methods to assess ADCC [32,39]. In fact, minimal EGFR densities were sufficient for the cetuximab-induced lysis of sarcoma cells [32,39]. Cetuximab-induced ADCC was comparable with the maximal killing achieved by IL-15–activated NK cells. In contrast to some other models [32,34,40,41], we did not observe an additive effect of cetuximab on the lysis by cytokine (IL-15)–activated NK cells.

Multiple mechanisms may account for the antitumor effect of cetuximab in patients. Masking of the EGFR extracellular binding site from its natural ligand EGF inhibits the activation of the receptor tyrosine kinase and downstream signaling pathways [13,42]. EGFR blockage has been shown to arrest cell-cycle progression and lead to apoptosis [13]. Cetuximab can inhibit tumor angiogenesis, neovascularization and invasion, and sensitizes tumor cells to radiation and chemotherapy-induced growth inhibition and apoptosis *in vivo* [13]. Finally, cetuximab may induce complement-dependent cytotoxicity or cytolytic effects by immune cells via ADCC [13,32,38,39,43]. An advantage of cetuximab-mediated ADCC is that it would be independent of the EGFR mutation status [34,38] and persistently activated EGFR signaling pathways [13,40].

The primary mode of action of cetuximab *in vivo* is difficult to determine. In a murine model, the anticancer effect of cetuximab was presumed to be mediated by NK cells [41]. Depletion of NK cells in murine osteosarcoma xenograft models or in mice with syngeneic mesenchymal stem cell–induced osteosarcoma could address whether an antitumor effect of cetuximab or murine anti-EGFR mAb relies on the presence of NK cells [44–46]. In humans, the relevance of ADCC has been suggested by the finding that FcγRIIIa/CD16 polymorphism of NK cells correlated with the survival of colorectal cancer patients [47,48], as well as with the efficacy of cetuximab-mediated ADCC by NK cells *in vitro* [34]. Interestingly, the intratumoral NK cells have recently been associated with improved survival when colorectal cancer patients had been treated with cetuximab [49]. In this study, we used a unique combination of tumor specimens, primary tumor cultures, and PBMC from osteosarcoma patients. This allowed us to establish that cetuximab treatment can improve the lysis of EGFR-expressing, autologous primary osteosarcoma cells by patient-derived NK cells via cetuximab-mediated ADCC.

Cetuximab treatment is associated with relatively mild adverse effects and has been approved for clinical usage by the FDA [37,50]. Therefore, in the treatment of osteosarcoma patients, cetuximab-mediated immunotherapy could be scheduled in the presence of endogenous or adoptively transferred NK cells. As such, cetuximab may provide an interesting treatment modality for patients with chemotherapy-resistant or metastatic EGFR$^+$ sarcomas.

ACKNOWLEDGMENTS

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CHAPTER 4

Antibody-dependent cell lysis by NK cells is preserved after sarcoma-induced inhibition of NK cell cytotoxicity

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ABSTRACT

Osteosarcoma and Ewing sarcoma tumor cells are susceptible to IL-15–induced or antibody-mediated cytolytic activity of NK cells in short-term cytotoxicity assays. When encountering the tumor environment *in vivo*, NK cells may be in contact with tumor cells for a prolonged time period. We explored whether a prolonged interaction with sarcoma cells can modulate the activation and cytotoxic activity of NK cells.

The 40-hour co-culture of NK cells with sarcoma cells reversibly interfered with the IL-15–induced expression of NKG2D, DNAM-1 and NKp30 and inhibited the cytolytic activity of NK cells. The inhibitory effects on receptor expression required physical contact between NK cells and sarcoma cells and were independent of TGF-β. Five days pre-incubation of NK cells with IL-15 prevented the down-regulation of NKG2D and cytolytic activity in subsequent co-cultures with sarcoma cells. NK cell FcγRIIIa/CD16 receptor expression and antibody-mediated cytotoxicity were not affected after the co-culture. Inhibition of NK cell cytotoxicity was directly linked to the down-regulation of the respective NK cell–activating receptors.

Our data demonstrate that the inhibitory effects of sarcoma cells on the cytolytic activity of NK cells do not affect the antibody-dependent cytotoxicity and can be prevented by pre-activation of NK cells with IL-15. Thus, the combination of cytokine-activated NK cells and monoclonal antibody therapy may be required to improve tumor targeting and NK cell functionality in the tumor environment.

4

INTRODUCTION

Osteosarcoma and Ewing sarcoma represent the most frequent osseous, malignant tumors in adolescents and young adults. The current treatment consists of a combination of systemic multi-drug chemotherapy and surgical resection [1–3]. Up to 70% of patients with localized disease achieve persistent remission. In contrast, the prognosis of patients with metastasized and recurrent disease has remained dismal, despite advancements in surgical techniques and intensification of chemotherapy regimens during the last decades. In the quest for novel cytolytic therapies, we have previously reported the sensitivity of sarcoma cells to NK cell-based cellular immunotherapy [4–7].

Human NK cells express a broad repertoire of germ-line–encoded inhibitory and activating receptors [8–10]. The proportion of inhibiting and activating signals perceived from target cells tunes the cytotoxic activity of NK cells. The inhibitory 'killer cell immunoglobulin-like receptors' (KIR) recognize HLA-A, B, C alleles of the MHC class I complex [11], whereas the NKG2A/CD94 complex binds HLA-E [12]. The activating receptor NKG2D recognizes the stress ligands MHC class I chain-related protein (MIC) A, MICB and UL-binding proteins (ULBP) 1 to ULBP6 [13–17], and DNAM-1 binds to CD112 and CD155 [9,18]. B7-H6 has recently been identified as a ligand for the 'natural cytotoxicity receptors' (NCR) NKp30 [19], whereas ligands for NKp44 and NKp46 are insufficiently characterized on tumor cells. Loss of MHC class I expression and ligation of NK cell–activating receptors increase the sensitivity of tumor cells to NK cell-mediated lysis [8–10]. In addition to the properties of target cells, NK cell cytotoxicity can be 'primed' by cytokines such as IL-2 and IL-15 [20–22].

Apart from killing tumor cell lines *in vitro*, NK cells have been shown to be involved in the rejection of transplanted hematopoietic tumors or chemically-induced tumors in mice, preventing tumor outgrowth and supporting tumor-specific T cell responses [23–26]. A role of NK cells in anti-cancer responses in humans has been emphasized by the observation that patients with leukemia have a better outcome when receiving KIR-ligand–mismatched allogeneic stem cell transplantations [27]. However, tumor cells may acquire diverse mechanisms to evade NK cell responses. Shedding of NKG2D ligands [28,29], down-regulation of NKG2D and DNAM-1 surface expression [30–33] by sustained ligand–receptor interactions, and secretion of TGF-β [34,35] have been associated with defective NK cell functions, hampering tumor cell recognition and killing.

We and others have established that ligands for the NK cell–activating receptors NKG2D and DNAM-1 are differentially expressed in sarcoma tissue and on sarcoma cell lines. Sarcoma cell lysis by NK cells involves NKG2D and DNAM-1 as measured in short-term cytotoxicity assays [4,5,7]. *In vivo*, however, NK cells may be in contact with tumor cells over a long time period which may alter the cytotoxic function of NK cells. Indeed, we have previously observed that peripheral blood NK cells from Ewing sarcoma patients exerted low cytolytic activity [7]. In the present study we demonstrate that prolonged interactions with sarcoma cells can inhibit the cytotoxic activity of NK cells as well as NK cells receptor expression. Remarkably, IL-15–activated NK cells and antibody-dependent cytotoxicity by NK cells were resistant to this inhibition. Therefore, the combination of IL-15–activated NK cells and monoclonal antibodies may sustain anti-tumor functions of NK cell-based immunotherapy in sarcoma patients.

4

MATERIALS AND METHODS

Cell lines

The following previously characterized sarcoma cells were included in this study: Ewing sarcoma cell lines A673, CADO-ES, SK-ES-1, SK-N-MC, STA-ET2.1, TC71 and L1062; osteosarcoma cell lines HOS, OHS, OSA, SAOS-2 and U2OS. All sarcoma cell lines were obtained from the EuroBoNeT cell line repository (by 2007) and were confirmed for their identity by short tandem repeat DNA fingerprinting in 2011. The TC71 cell line was cultured in IMDM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen); all other sarcoma cell lines as well as the erythroleukemic cell line K562, the Burkitt's lymphoma cell line Daudi and the murine mastocytoma cell line P815 (ATCC, Manassas, VA, USA) were maintained in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin. All Ewing sarcoma cell lines were grown in 0.1% gelatin coated culture flasks. All cell lines were negative for mycoplasma infection as regularly tested by RT-PCR.

T cell depletion and NK cell isolation

PBMC were separated from buffy coats of healthy adult donors (Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. T cells were depleted from PBMC (TCD PBMC) by positive selection using anti-CD3 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) to exclude NK cell activation via T cell–derived IL-2 due to potential alloreactivity toward sarcoma cells. TCD PBMC contained less than 0.5% of contaminating $CD3^+$ T cells as analyzed by flow cytometry. NK cells were purified from PBMC by negative selection, depleting non-NK cells through a combination of biotin-conjugated monoclonal anti-human antibodies and MicroBeads using the "Human NK cell Isolation Kit" (Miltenyi Biotech); NK cell purity was >95% as determined by flow cytometry, analyzing NK cells as $CD56^+$, $CD3^-$, $CD14^-$ and $CD19^-$ cells. IL-15–activated NK cells were obtained by incubating purified NK cells for five days with 10 ng/ml of IL-15 (Peprotech, Rocky Hill, NJ, USA) in RPMI medium (with 10% FCS and penicillin/streptomycin) in 24-well tissue culture plates.

Co-cultures

Sarcoma cell lines were seeded in 24-well plates in 1 ml of RPMI medium and incubated for 18 h. According to cell size and growth rate, the cell lines were seeded at the following cell numbers per well, allowing sub-confluent cell densities at the start of co-cultures: TC71 and HOS at 50,000; OHS, OSA, SAOS-2 and U2OS at 75,000; A673, CADO-ES and L1062 at 80,000; SK-ES-1 and STA-ET2.1 at 150,000; SK-N-MC at 200,000. Co-cultures were assembled by carefully adding $1x10^6$ TCD PBMC, $1.5x10^5$ purified NK cells or 5-day IL-15–activated NK cells to the attached sarcoma cells, achieving NK cell–tumor cell ratios of 1:1 to 3:1 according to the tumor cell numbers seeded. In trans-well experiments, TCD PBMC (upper compartment) were separated from STA-ET2.1 or TC71 cells (lower compartment) by a porous membrane (0.4 μm pore size). Co-cultures were incubated for approximately 40 h with or without IL-15. The following TGF-β inhibitors were applied: TGF-β–neutralizing antibody (10 μg/ml; anti-TGFβ1/TGFβ2/TGFβ3; clone 1D11; R&D Systems, Minneapolis, MN, USA),

ALK (TGF-βRI kinase) small molecule inhibitor SB-431542 (10 μM; kindly provided by Dr. Luuk J.A.C. Hawinkels, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, Netherlands) or recombinant human latency-associated peptide (LAP; 250 ng/ml; R&D Systems). As controls, TCD PBMC or purified NK cells were incubated in the absence of sarcoma cells with or without IL-15, recombinant TGF-β (1 ng/ml; R&D Systems) or TGF-β inhibitors. Subsequently, non-attached cell populations were harvested to analyze the expression of NK cell activation markers by flow cytometry and the cytolytic activity of NK cells in a 4 h ^{51}chromium (^{51}Cr) release assay.

Flow cytometry

Determination of NK cell percentages in TCD PBMC, validation of NK cell purity and T cell depletion, and the expression of NK cell activation markers were analyzed phenotypically with fluorescently labeled antibodies and flow cytometry. The following mouse anti-human monoclonal antibodies were applied according to the manufacturer's instructions: HLA-G^{FITC} (MEM-G/9) (Abcam, Cambridge, UK); CD3^{FITC} (SK7), CD3$^{PerCP-Cy5.5}$ (SK7), CD14$^{PerCP-Cy5.5}$ (M5E2), CD69PE (L78), CD69^{FITC} (L78), DNAM-1^{FITC} (DX11), MICA/B^{PE} (6D4), PD-1 (CD279, MIH4), goat anti-mouse Ig^{APC} (550826) (BD Biosciences, Franklin Lakes, NJ, USA); HLA-E^{PE} (3D12), PD-1L^{APC} (CD274, MIH1) (eBiosciences, San Diego, CA, USA); CD16^{FITC} (3G8), CD56^{APC} (N901 NKH1), CD112^{PE} (R2.477.1), CD155 (PV404.10), NKG2A^{PE} (Z199), NKG2D^{PE} (ON72), NKp30^{PE} (Z25), NKp44^{PE} (Z231), NKp46^{PE} (BAB281) (IOTEST Immunotech, Marseille, France); MICA (159227), MICB (236511), ULBP1 (170818), ULBP2 (165903), ULBP3 (166510) (R&D Systems); perforin^{FITC} (deltaG9, Ancell, Bayport, MN, USA). To analyze ligands for NKG2D, NKp30 and NKp44 on sarcoma cells, freshly harvested tumor cells were incubated with the respective Fc fusion protein constructs for 2 h (2.5 μg/mL; R&D Systems, Minneapolis, MN, USA) followed by the Alexa Fluor 647 goat anti-human IgG secondary antibody (A21445; Invitrogen). The anti-CD20 mAb rituximab (MabThera; Roche, Basel, Switzerland) was used as an IgG1 isotype-matched negative control for fusion proteins. FACS measurements were performed with the FACSCalibur (BD Biosciences) and analyzed with the "BD Cell Quest ProTM" software (version 5.2.1). The indicated fold expression data were calculated by dividing the geometric mean fluorescence intensity after the 40 h co-cultures by the geometric mean fluorescence intensity after 40 h incubation in medium or IL-15 only (as set to 1.00).

^{51}Chromium release assay

The cytolytic activity of NK cells in TCD PBMC or purified NK cells against K562 (sensitive to unstimulated and activated NK cells), Daudi (sensitive to activated NK cells only) and sarcoma cell lines was measured in 4 h ^{51}Cr release assays. Effector cells were unstimulated, non-co-cultured TCD PBMC or purified NK cells, or the non-adherent cell fraction from the 40 h co-cultures. For blocking experiments, effector cells were incubated with 10 μg/ml of mouse anti-human NKG2D (149810, R&D Systems), DNAM-1 (DX11, BD Biosciences) and NKp30 (P30-15, Biolegend, San Diego, CA, USA) for 30 min. Target cells were labeled with 100 μl Na-chromate (^{51}Cr, 3.7 MBq) for 1 hr. To measure antibody-dependent cellular cytotoxicity (ADCC), sarcoma cells were incubated with the chimeric monoclonal antibody cetuximab (anti-epidermal growth factor receptor, 10 μg/ml; Erbitux®, Merck KGaA, Darmstadt, Germany) for

30 min. To measure redirected lysis, murine $FcγR^+$ P815 cells were incubated with 1 μg/ml of mouse anti-human $CD16^{FITC}$ (3G8, 1/100, IOTEST Immunotech), DNAM-1 (DX11, BD Biosciences), NKG2D (149810, R&D Systems), NKp30 (P30-15, Biolegend) and NKp46 (9E2, Biolegend) for 30 min. $2.5x10^3$ target cells +/- antibodies were added to effector cells in triplicate or duplicate at the indicated effector-target (E:T) ratios and incubated for 4 h at 37°C. Supernatants were collected, and the release of ^{51}Cr was measured with a β-counter (Wallac/ PerkinElmer, Waltham, MA, USA). Spontaneous and total release were obtained by incubation in medium and Triton X-100 (2.5%; Merck Chemicals, Darmstadt, Germany), respectively. The specific lysis was calculated by the following formula: percentage of specific lysis = 100 x (experimental release–spontaneous release / total release–spontaneous release).

Statistical analysis

Statistical analyses were performed with Graphpad Prism version 5.04 (La Jolla, CA, USA) or SPSS version 16.0 (IBM, Armonk, NJ, USA) using paired Student's t-tests, comparing means between groups of samples. Error bars represent the standard error of the mean. A P-value of <0.05 was considered statistically significant. ns, not statistically significant; *, P<0.05; **, P<0.01; ***, P<0.001.

RESULTS

Sarcoma cells can interfere with NK cell receptor expression and cytolysis

To explore whether sarcoma cell lines are able to influence NK cell function, T cell–depleted PBMC (TCD PBMC) were co-cultured with osteosarcoma and Ewing sarcoma cell lines for 40 h. After the co-culture, NKG2D and DNAM-1 were down-regulated on resting NK cells by most of the cell lines (8/12 and 6/12 respectively) (Figure 1, panel A and B). Four cell lines significantly down-regulated both NKG2D and DNAM-1 expression.

In addition to the phenotypical changes, it was investigated whether sarcoma cells can affect the cytolytic potential of resting NK cells. TCD PBMC were removed from co-cultures with STA-ET2.1, HOS (relatively strong inhibitors of NKG2D expression) and TC71 cells (relatively weak inhibitor of NKG2D). Afterwards, NK cell-mediated cytolytic activity was tested against non–co-cultured sarcoma and non-sarcoma target cells in 4 h ^{51}Cr release assays. The 40 h co-culture of TCD PBMC with STA-ET2.1 and HOS cells substantially reduced the cytolytic activity against (non–co-cultured) STA-ET2.1 and HOS target cells (Figure 1, panel C and D). Likewise, lysis of the NK cell target cell line K562 was decreased after these co-cultures (Supplementary Figure 1, panel A, page 87), indicating that there was no sarcoma cell-specific inhibitory effect. In contrast, co-culture with TC71 cells had little effect on NK cell cytolysis.

Hence, sarcoma cell lines differed in their efficacy to down-regulate the cytolytic activity of NK cells as well as the expression of the NK cell–activating receptors NKG2D and DNAM-1.

IL-15–induced NK cell activation is impaired by sarcoma cells

IL-15 is a potent NK cell–activating cytokine which enhances the expression of NKG2D and DNAM-1 on NK cells and boosts their cytolytic activity against sarcoma cells [4,7]. It was investigated whether IL-15–induced activation of NK cells can also be inhibited by sarcoma cells.
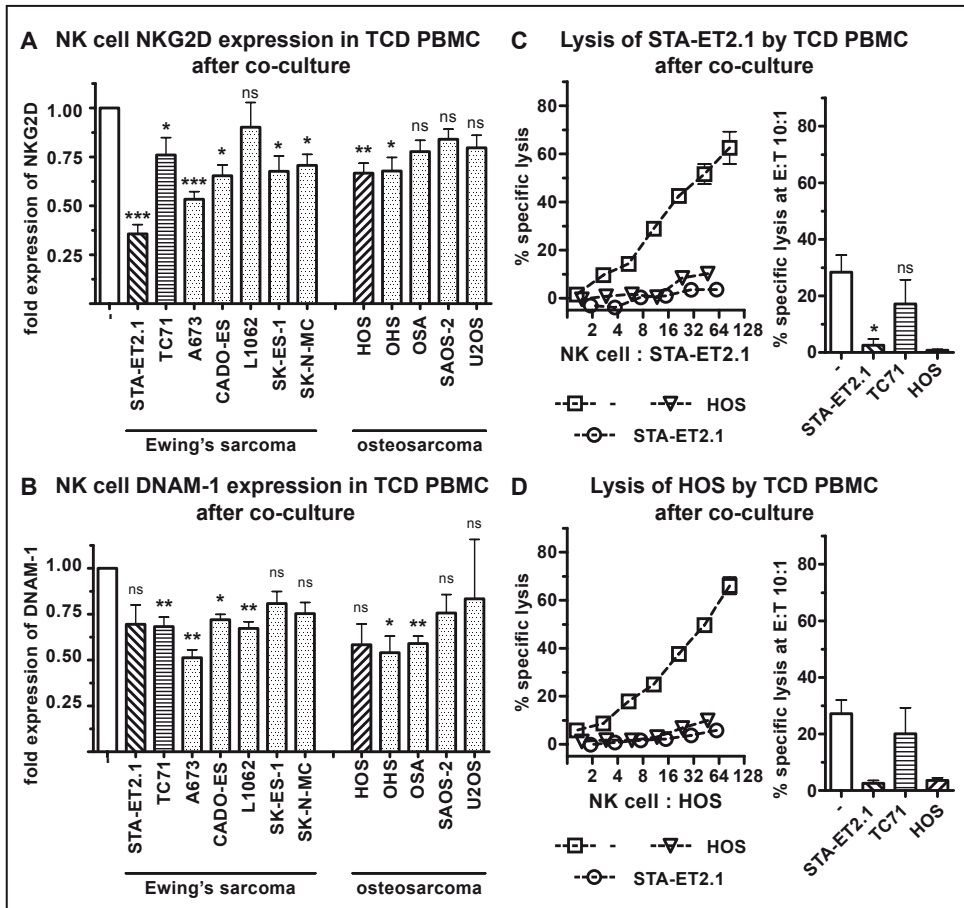
Figure 1: Down-regulation of NK cell NKG2D, DNAM-1 and cytolytic activity by sarcoma cells

Surface expression of NKG2D **(A)** and DNAM-1 **(B)** on NK cells was analyzed after 40 h co-cultures of TCD PBMC with the indicated Ewing sarcoma and osteosarcoma cell lines by flow cytometry. The indicated fold expression was calculated by dividing the geometric mean fluorescence intensity after 40 h co-cultures by the geometric mean fluorescence intensity after 40 h incubation in medium only (as set to 1.00). Within the lymphocyte population of life-gated TCD PBMC, NK cells were identified as CD56^{+} cells devoid of CD3 and CD14 expression. Diagonal and horizontal-lined patterns in bars indicate the co-cultures mainly focused on in the following experiments (relatively strong inhibitors of NKG2D, STA-ET2.1 and HOS; relatively weak inhibitor, TC71). Combined data of the fold expression of multiple experiments are depicted. Lysis of (non-co-cultured) STA-ET2.1 **(C)** and HOS cells **(D)** by NK cells after the 40 h co-culture of TCD PBMC with STA-ET2.1, TC71 and HOS cells or 40 h incubation in medium only was measured in 4 h ^{51}Cr release assays. The specific lysis (%) is depicted as representative data of one experiment and as combined data of at least experiments analyzed at a 10:1 E:T ratio. ns, not statistically significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

When co-cultured with TCD PBMC in the presence of IL-15, sarcoma cell lines significantly interfered with the IL-15–induced up-regulation of NKG2D (12/12) and DNAM-1 (9/12) on NK cells (Figure 2, panel A and B; Figure 3, panel A). In addition, STA-ET2.1 cells inhibited the IL-15–induced expression of CD69, NKp30, NKp44 and NKp46, whereas HOS and TC71 cells inhibited only NKp30 and NKp44 (Figure 3, panel B).

Stimulation of TCD PBMC with IL-15 for 40 h enhanced the lysis of non–co-cultured STA-ET2.1 and HOS target cells (Figure 2, panel C and D). The IL-15–induced lysis of both cell lines was reduced after the co-culture with STA-ET2.1 cells, while the co-cultures with
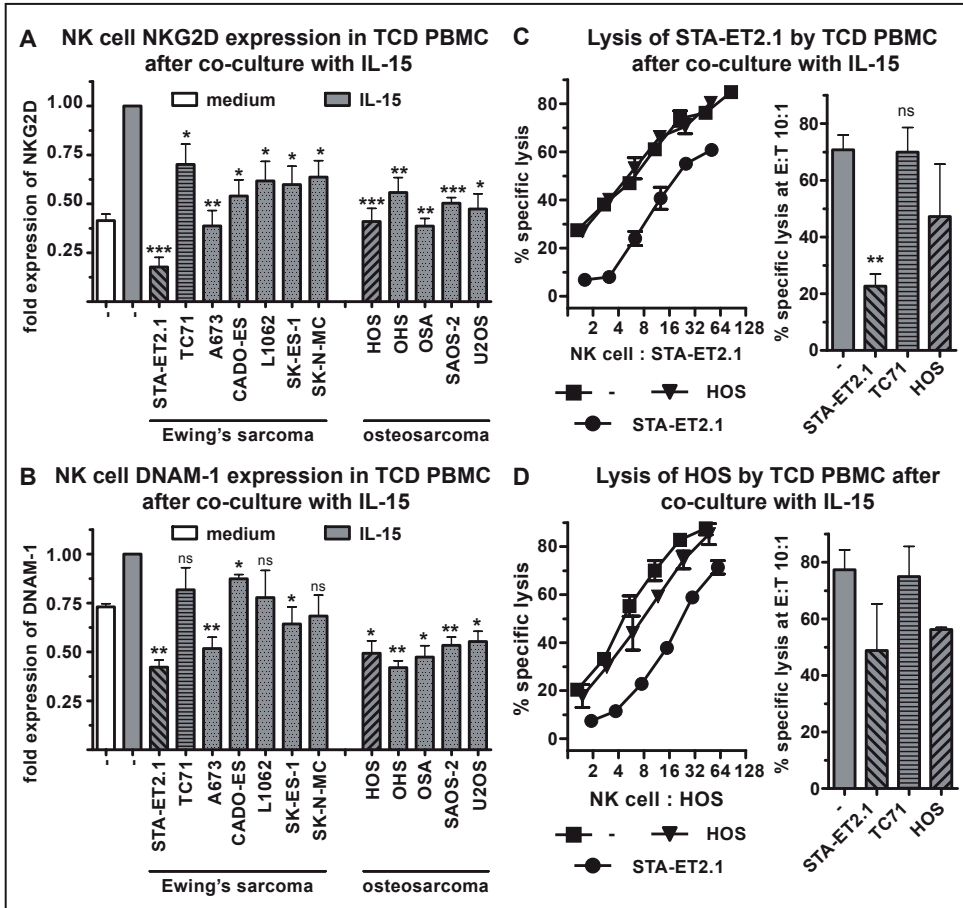
Figure 2: Down-regulation of IL-15–induced NK cell cytolytic activity and NKG2D and DNAM-1 expression by sarcoma cells

NKG2D **(A)** and DNAM-1 **(B)** on NK cells was analyzed after IL-15–stimulated co-cultures of TCD PBMC with Ewing sarcoma and osteosarcoma cell lines. The indicated fold expressions are combined data of multiple experiments, comparing expression levels of IL-15–stimulated 40 h co-cultures with expression levels after incubation with IL-15 only. Gray bars depict TCD PBMC incubated with IL-15 only (no pattern) or IL-15–stimulated co-cultures (diagonal and horizontal-lined patterns with STA-ET2.1, HOS or TC71 cells), and white bars depict TCD PBMC incubated in medium only. The expression of NKG2D after IL-15 stimulation only was set to 1.00. Lysis of STA ET2.1 **(C)** and HOS cells **(D)** by NK cells after IL-15–stimulated co-culture of TCD PBMC with sarcoma cells or incubation in IL-15 only. Representative data of one experiment and combined data of at least two experiments analyzed at a 10:1 E:T ratio are depicted.

HOS or TC71 had little or no effect. Furthermore, the IL-15–induced lysis of Daudi cells, which are only susceptible to activated NK cells, was greatly diminished after the co-culture with STA-ET2.1 cells (Supplementary Figure 1, panel B, page 87).

These results indicate that sarcoma cells could also interfere with NK cell function in the presence of the activating cytokine IL-15.

Figure 3: NKp30 and NKp44 down-regulation on NK cells is not associated with NKp30 and NKp44 ligands expression on sarcoma cells

(A) Representative FACS overlay plots of the geometric mean fluorescent intensity of NKG2D expression on NK cells after 40 h incubation of TCD PBMC with medium (light gray area) and with IL-15 (dark gray area) and after 40 h co-culture (black solid line) with the sarcoma cell lines STA-ET2.1 (left panel), TC71 (middle panel) and HOS (right panel) in the presence or absence of IL-15. **(B)** Expression of the NK cell receptors NKG2D, DNAM 1, FcγRIIIa/CD16, NKG2A, NKp30, NKp44 and NKp46 and of the NK cell activation marker CD69 was investigated after the co-culture of TCD PBMC with STA ET2.1, TC71 or HOS cells in the presence of IL-15. Fold expression data were calculated from geometric mean fluorescence intensities and combined of multiple experiments. **(C)** Representative FACS overlay plots, detecting NKp30 ligands by NKp30-Fc fusion protein (black solid line), NKp44 ligand by NKp44-Fc fusion protein (grey solid line) and isotype-matched negative control by the anti-CD20 mAb rituximab (light grey area), all followed by secondary antibody, on STA-ET2.1, TC71 and HOS cells.

NKG2D down-regulation on NK cells requires direct contact with sarcoma cells and is independent of TGF-β

The mechanism responsible for NK cell receptor down-regulation by certain sarcoma cells could be based on sustained ligand–receptor interactions. Although the STA-ET2.1 cell line had the strongest inhibitory effect on NK cell NKG2D and DNAM-1 expression, the NKG2D ligands MIC-A, ULBP1 and ULPB3 were hardly detected on STA-ET2.1 cells, while MIC-B, ULBP2, the NKG2D-Fc fusion construct and the DNAM-1 ligands CD112 and CD155 were detected at lower intensities as compared to other sarcoma cell lines (Supplementary Figure 2, page 88). Notably, the STA-ET2.1 cell line down-regulated NKp30 and NKp44 expression on NK cells but ligands for NKp30 and NKp44 were barely detected on STA-ET2.1 cells by Fc fusion constructs as compared to TC71 and HOS cells (Figure 3, panel C). Ligands for NKp30 and NKp44 were only expressed on a few Ewing sarcoma and osteosarcoma cell lines (Supplementary Table,

Figure 4: Down-regulation of NKG2D on NK cells requires cell-cell contact and is independent of TGF-β

(A) TCD PBMC (upper compartment) were separated from sarcoma cells (lower compartment) by a porous membrane (0.4 μm) in trans-well experiments. NKG2D on NK cells was investigated after the unseparated co-culture and the trans-well co-culture, both in the presence of IL-15 and incubated for 40 h. **(B)** (i) NKG2D expression on NK cells was investigated after incubation of TCD PBMC with IL-15 in the presence or absence of recombinant TGF-β and TGF-β inhibitors (TGF-β neutralizing antibody, SB 431542 and recombinant LAP) and (ii) after the IL-15–stimulated co-culture with sarcoma cells in the presence or absence of TGF-β inhibitors. **(C)** NKG2D on NK cells was measured after co-culture of purified NK cells with sarcoma cells in the presence of IL-15. All data were combined from at least two experiments. In all panels, IL-15–stimulated samples are indicated by gray bars, white bars depict control incubations in medium only. Expression of NKG2D after IL-15 stimulation was set to 1.00.

page 89). Overall, when comparing surface densities of NKG2D, DNAM-1, NKp30 and NKp44 ligands on the various sarcoma cell lines, differences in surface densities and expression profiles did not appear to correspond to the level of NKG2D, DNAM-1, NKp30 or NKp44 receptor down-regulation by the respective cell lines (Supplementary Table, page 89). Thus, variability in ligand surface densities alone did not explain the differences among sarcoma cell lines in down-regulating NK cell receptors. In addition, despite the strong inhibitory effect of STA-ET2.1 cells, expression levels of the known NK cell–inhibitory surface ligands HLA-E, HLA-G and PD-1L were not particularly high on STA-ET2.1 cells (Supplementary Figure 2,

page 88). PD-1 expression was not specifically increased on NK cells after co-culture with STA-ET2.1 cells (Supplementary Figure 3, panel A, page 89).

To investigate the role of soluble factors and/or cell–cell contact in the down-regulation of the NKG2D receptor, it was tested whether cell-free supernatants of long-term, high-density cultures of STA-ET2.1, TC71 and HOS cells influenced NK cell NKG2D and DNAM-1 expression. Incubation of NK cells with supernatant of sarcoma cell cultures did not alter NKG2D or DNAM-1 expression on NK cells (Supplementary Figure 1, panel C, page 87). When STA-ET2.1 or TC71 cells (lower compartment) were separated from TCD PBMC (upper compartment) in trans-well cultures, both cell lines failed to interfere with IL-15–induced NKG2D expression on NK cells (Figure 4, panel A). Thus, cell–cell contact was either required directly or because it resulted in the production of inhibitory soluble factors mediating NKG2D down-regulation. Incubation of TCD PBMC with supernatant of co-cultures of TCD PBMC and STA-ET2.1 or TC71 cells did also not down-regulate NKG2D expression on NK cells (Supplementary Figure 1, panel C, page 87). Altogether, these data indicate that the down-regulation of NKG2D and DNAM-1 was mediated by close cell–cell contact between sarcoma cells and NK cells and not by soluble mediators derived from sarcoma cell lines or produced after cell–cell contact during the co-cultures.

In previous studies, membrane-associated TGF-β has been described to interfere with the activation of NK cells in a cell–cell contact dependent-manner [36-38]. Therefore, it was investigated whether neutralization of TGF-β can abolish the inhibitory effect of sarcoma cells. The inhibition of IL-15–induced up-regulation of NKG2D on NK cells by recombinant TGF-β could be prevented by the addition of a TGF-β–neutralizing antibody, the small molecule inhibitor SB-431542 (TGF-βRI kinase inhibitor) or recombinant LAP (inactivator of TGF-β). In contrast, addition of the TGF-β inhibitors to the co-cultures of TCD PBMC with STA-ET2.1 or HOS cells did not prevent the interference with the IL-15–induced NKG2D expression on NK cells, suggesting that TGF-β did not play a role in the inhibitory effects of the sarcoma cell lines (Figure 4, panel B, ii).

Since until now all experiments have been performed using TCD PBMC, sarcoma cell–dependent inhibition of NK cells could have been indirect and mediated by non-NK, non-T cell populations in PBMC. Therefore NK cells were purified and co-cultured with sarcoma cells. Similar to NK cells in TCD PBMC, also on purified NK cells the IL-15–induced up-regulation of NKG2D was inhibited after the co-culture with, in particular, STA-ET2.1 cells (Figure 4, panel C). Consequently, sarcoma cells could influence NK cells by direct cell–cell interaction without requiring the presence of other PBMC populations.

Pre-activation of NK cells with IL-15 prevents NK cell inhibition

To explore whether the inhibitory effect of sarcoma cells resulted in irreversible alterations in NK cells, TCD PBMC were removed from STA-ET2.1 cells after the 40 h co-culture and subsequently incubated with IL-15 alone. The down-regulated expression of NKG2D and DNAM-1 after the 40 h co-culture was restored when the TCD PBMC were incubated for three additional days with IL-15 in the absence of STA-ET2.1 cells (Figure 5, panel A and data not shown). NK cell NKG2D and DNAM-1 expression remained inhibited when STA-ET2.1 cells were present during the additional three days of incubation with IL-15 (Figure 5, panel A).
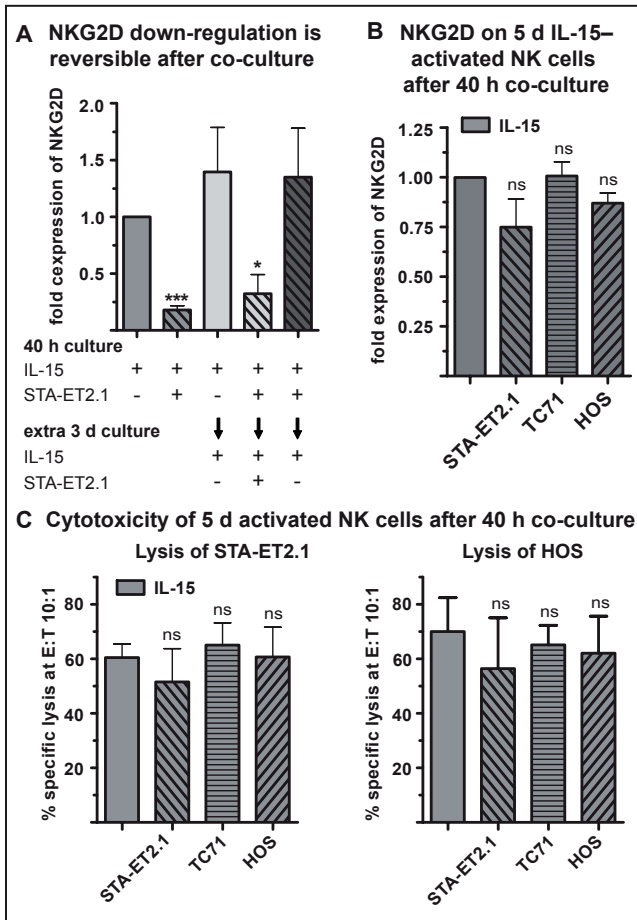
Figure 5: NKG2D expression and cytotoxic activity of 5-day IL-15–activated NK cells is not inhibited by sarcoma cells

(A) After the IL-15–stimulated 40 h co-culture (second bar), TCD PBMC were removed from STA-ET2.1 cells, as indicated by the arrows, and subsequently incubated with IL-15 for three additional days in the presence (fourth bar) or absence (fifth bar) of fresh STA-ET2.1 cells. NKG2D expression on NK cells was analyzed after the 40 h co-culture and after the 40 h plus 3 days co-culture. Data are combined of multiple experiments. **(B)** Purified NK cells were activated with IL-15 for 5 days and afterwards co-cultured with sarcoma cells for 40 h (gray bars, IL-15–stimulated samples). Data of NKG2D expression are combined of multiple experiments. **(C)** Lysis of non-co-cultured STA-ET2.1 and HOS cells after the co-culture of 5-day IL-15–activated NK cells with sarcoma cells. Data are analyzed at a 10:1 E:T ratio and combined of three and two experiments, respectively.

Next it was investigated whether NK cells activated with IL-15 prior to the co-culture are still susceptible to sarcoma cell-induced inhibition. Remarkably, when using five days pre-activated NK cells neither NKG2D expression nor the cytolytic activity of the NK cells was altered by subsequent 40 h co-cultures with sarcoma cells (Figure 5, panel B and C).

Overall, the sarcoma cell-induced inhibitory effect on NK cell activation was reversible and could be prevented by pre-activation of NK cells with IL-15.

Antibody-dependent cytotoxicity by NK cells is not affected by sarcoma cells

In the previous experiments, the co-culture with the STA-ET2.1 cell line significantly down-regulated NKG2D, DNAM-1 and NKp30 expression as well as the cytolytic activity against sarcoma cell lines. These receptors play an important role in sarcoma cell lysis by NK cells as illustrated in ^{51}Cr release assays performed in the presence of specific neutralizing antibodies against the respective receptors. Lysis of STA-ET2.1, TC71 and HOS cells by resting NK cells was significantly reduced by antibodies against DNAM-1 and fully inhibited by combined blocking of DNAM-1 and NKG2D while lysis was only partly dependent on NKG2D alone (Supplementary Figure 1, panel D, page 87). Lysis by IL-15–activated NK cells could only be
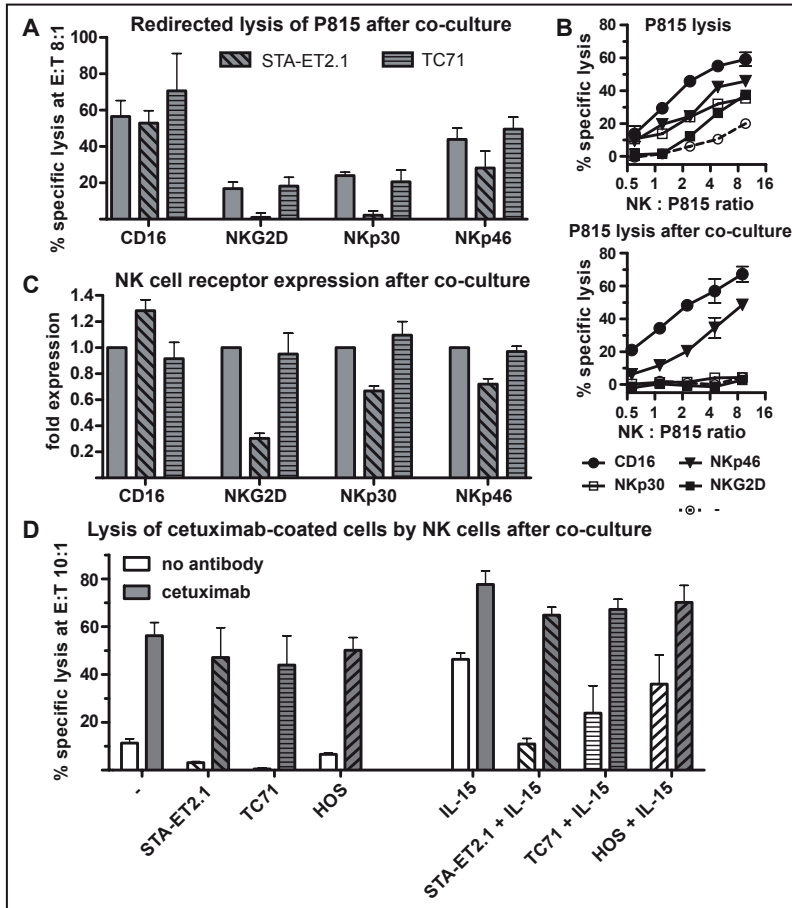
Figure 6: Lysis of antibody-coated tumor cells by NK cells is not affected after the co-culture

(A) Redirected lysis of P815 cells coated with FcγRIIIa/CD16, NKG2D, NKp30 and NKp46 antibodies by NK cells after for two days incubation of purified NK cells with IL-15 only (bars without pattern) and in the presence or absence of STA-ET2.1 or TC71 cells (bars with pattern), depicted as combined data of three experiments analyzed at a 8:1 E:T ratio (lysis of P815 in the absence of antibodies was subtracted from the total lysis in the presence of antibodies). (B) Representative data of redirected lysis of P815 cells with or without antibody coating without (upper panel) or after co-culture with STA-ET2.1 cells (lower panel). (C) FcγRIIIa/CD16, NKG2D, NKp30 and NKp46 expression on NK cells after co-culture used in redirected lysis experiments above. Data are combined of three experiments. (D) Lysis of non-co-cultured, uncoated (white bar, no antibody addition) and cetuximab-coated (gray bar) STA-ET2.1 cells by NK cells after co-culture of purified NK cells with STA-ET2.1, TC71 or HOS cells in the presence or absence of IL-15. Data are combined of two experiments.

significantly reduced by the combination of DNAM-1 and NKG2D antibodies and was fully inhibited by the addition of NKp30 antibodies.

Therefore, down-regulation of these NK cell receptors may be an explanation for the reduced cytolytic activity. To investigate whether down-regulation of a single receptor resulted in reduced lysis mediated by this receptor, NK cell cytotoxicity after co-culture was analyzed in redirected lysis assays against the murine FcγR+ P815 cell line. Lysis of P815 cells by two days IL-15–stimulated NK cells was enhanced when P815 cells were coated with mouse anti-human FcγRIIIa/CD16, NKG2D, NKp30 or NKp46 but not DNAM-1 antibodies (Figure 6, panel A and B

and data not shown). After the IL-15–stimulated co-culture with STA-ET2.1 cells, however, redirected lysis through NKG2D and NKp30 was severely diminished (Figure 6, panel A and B). Lysis through NKp46 was partly reduced, whereas FcγRIIIa/CD16–mediated lysis remained intact. These results were closely correlated with the down-regulation of NKG2D and NKp30 expression and the partly reduced levels of NKp46 on these NK cells, whereas FcγRIIIa/CD16 expression remained intact (Figure 6, panel C). In contrast, after co-culture with TC71 cells, redirected lysis via CD16, NKG2D, NKp30 and NKp46 as well as expression of the respective receptors was unaffected (Figure 6, panel A and C). These data demonstrate that the inhibition of NKG2D-, NKp30- and partly NKp46-dependent NK cell cytotoxicity were directly linked to the down-regulation of the respective NK cell receptors. As indicated by functional FcγRIIIa/CD16–mediated redirected lysis, the components of the cytolytic machinery appeared to be intact after the co-culture. This was further corroborated by the fact that intracellular perforin expression in NK cells remained normal (Supplementary Figure 3, panel B, page 89).

Since FcγRIIIa/CD16–mediated redirected lysis as well as FcγRIIIa/CD16 receptor expression was intact after the co-culture, this raised the question whether the FcγRIIIa/CD16–mediated antibody-dependent cytotoxic function of NK cells can be exploited to preserve sarcoma cell lysis after the co-culture. Coating of STA-ET2.1 cells with the anti-epidermal growth factor receptor antibody cetuximab substantially enhanced their lysis by resting NK cells and, moderately, by IL-15–stimulated NK cells (Figure 6, panel D) [6] (this thesis, chapter 3). Remarkably, lysis of antibody-coated STA-ET2.1 cells by NK cells was not inhibited after the co-culture with STA-ET2.1, TC71 or HOS cells in the absence or presence of IL-15.

Thus, despite the inhibitory effects of certain sarcoma cells, the FcγRIIIa/CD16–mediated, antibody-dependent cytotoxicity of NK cells was preserved after the co-culture.

DISCUSSION

Tumor escape from NK cell immunosurveillance may occur by selection of tumor cells with no or low expression of ligands for NK cell activating receptors or increased expression of ligands for inhibitory receptors. Alternatively, tumor cells may produce factors which inhibit NK cell activity [39]. In our study, the prolonged, i.e., 40 hours, co-culture of sarcoma cells with NK cells resulted in a sarcoma cell line-specific modulation of NK cell activating receptors, in particular reducing (IL-15–induced) expression of NKG2D and DNAM-1. Most remarkably, the STA-ET2.1 cell line reduced the IL-15–induced expression of NKG2D even to lower levels than expressed on resting NK cells. In addition, the IL-15–induced cytolytic activity of NK cells was reduced against sarcoma and non-sarcoma cell targets after the co-culture. The down-regulation of NK cell activating receptors such as NKG2D, NKp30 and NKp46 was directly linked to the reduction in cytotoxicity mediated through these receptors as shown in redirected lysis experiments. This may offer an explanation for the inhibited sarcoma cell lysis which was dependent on NKG2D, DNAM-1 and NKp30.

It was notable that the different sarcoma cell lines were differentially able to down-regulate NK cell phenotype and function. We attempted to define whether one or more of the described mechanisms were responsible for this effect. Sustained interactions of NKG2D with its ligands expressed on tumor cells have been described to induce receptor internalization and degradation,

resulting in NK cell hyporesponsiveness [33,40–43]. However in our study, the degree of down-regulation of NK cell receptors by different sarcoma cell lines did not correlate with the expression of the cognate ligands or known NK cell inhibitory molecules, such as HLA-E, HLA-G and PD-1L on these tumor cells. In particular, the sarcoma cell line with the strongest inhibitory potential (STA-ET2.1) exhibited only few NKG2D ligands (MICB and ULBP2), had relatively low intensities of NKG2D and DNAM-1 ligands and had undetectable expression of NKp30 and NKp44 ligands. Thus, the differences in ligand on the sarcoma cell lines do not sufficiently explain the differences in down-regulation of NK cell receptors. This suggests that other inhibiting factors than sustained ligand–receptor interactions are involved. Another potential mechanism of NK cell receptor down-regulation could be through soluble mediators as has been demonstrated for melanoma cells [44,45]. However, NKG2D and DNAM-1 down-regulation after co-culture with sarcoma cells was not mediated by soluble factors. Instead, direct cell–cell contact between sarcoma cells and NK cells was required as similarly shown for other tumor cell types [30,38,45,46]. This requirement for cell–cell contact could be mediated by membrane-associated TGF-β as has been shown for various other tumor cells perturbing NK cell NKG2D expression [36–38]. However, in our experiments TGF-β, either membrane-associated or soluble, was not involved since blockage of TGF-β or its signaling pathway during the co-culture did not prevent NKG2D down-regulation on NK cells. Hence, we have determined that the inhibition of NK cell cytotoxicity was linked to the down-regulation of the respective NK cell activating receptors. However, the mechanism of NKG2D and DNAM-1 down-regulation by some of the sarcoma cell lines remains to be further explored. It is tempting to speculate that certain sarcoma cells express a membrane-bound, inhibiting factor other than TGF-β that can globally impair NK cell receptor expression and thus NK cell cytolytic activity but leaving FcγRIIIa/CD16–mediated lysis intact.

Several studies have reported on defective cytotoxic functions of tumor-associated and peripheral blood NK cells from patients with cancer (prior to the start of anti-cancer therapies) in association with reduced expression of NK cell–activating receptors [30,47–50]. As an exception to these studies, we have previously shown that peripheral blood NK cells from patients with Ewing sarcoma exerted low cytolytic activity despite higher levels of NKG2D, suggesting that another mechanism may be responsible for the *in vivo* down-regulation of NK cell reactivity [7]. Interestingly, when the NK cells were activated with IL-15 for five days prior to the co-culture, NKG2D expression and cytolytic activity were no longer affected by sarcoma cells. Conversely, the impaired expression of NKG2D and DNAM-1 could be reversed when the NK cells were removed from the co-culture and subsequently stimulated with IL-15 [32,38,40,42]. Thus, the reduction of NK cell receptor expression requires the permanent presence of tumor cells. *Ex vivo* stimulation with IL-15 of NK cells from patients with Ewing sarcoma restored the cytolytic activity [7], indicating that the inhibited cytolytic function of patient's NK cells can also be reversed. Similarly, it has been shown that the decreased cytolytic activity of NK cells from patients with leukemia normalizes when patients have achieved complete remission [48].

In vivo, the excess of tumor cells, imperfect NK cell activation and incomplete tumor cell elimination may result in prolonged tumor–NK cell interactions, rendering NK cells non-functional over time. In this perspective, adoptively transferred NK cells have been shown to lose antibody-independent cytolytic activity in murine cancer models and cancer patients but retain

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antibody-dependent cytotoxic activity in patients [51,52]. Importantly, in our experiments antibody-dependent target cell lysis remained functional after the interaction with sarcoma cells, whereas FcγRIIIa/CD16–independent cytotoxicity of NK cells was inhibited. In several other studies, defective antibody-dependent cytotoxicity of NK cells from patients with cancer was associated with reduced expression of FcγRIIIa/CD16 on NK cells, whereas in our experiments FcγRIIIa/CD16 expression was unaffected and mediated antibody-dependent target cell lysis. Different tumors apparently employ different mechanisms to interfere with cytolytic pathways of NK cells [30,41,53]. Hence, the co-culture with sarcoma cells neither exhausted the cytolytic potential nor caused overall NK cell hyporesponsiveness. The inhibition of both ITAM-dependent (NKp30 and NKp46) and ITAM-independent (NKG2D) NK cell cytotoxicity appeared to be directly linked to the down-regulation of the respective NK cell receptors. Thus, target cell recognition was presumably impaired while other prerequisites for target cell lysis, such as perforin expression, remained intact.

In our study, neither the cytotoxicity of five-day IL-15–activated NK cells nor the antibody-dependent cytolytic function of resting and IL-15–stimulated NK cells was affected by the inhibitory effects of sarcoma cells. Thus, a combination of adoptively transferred, IL-15–activated NK cells with infusion of tumor-reactive antibodies may improve tumor targeting and conserve NK cell functionality *in vivo* in a potentially NK cell–inhibiting tumor environment.

ACKNOWLEDGMENTS

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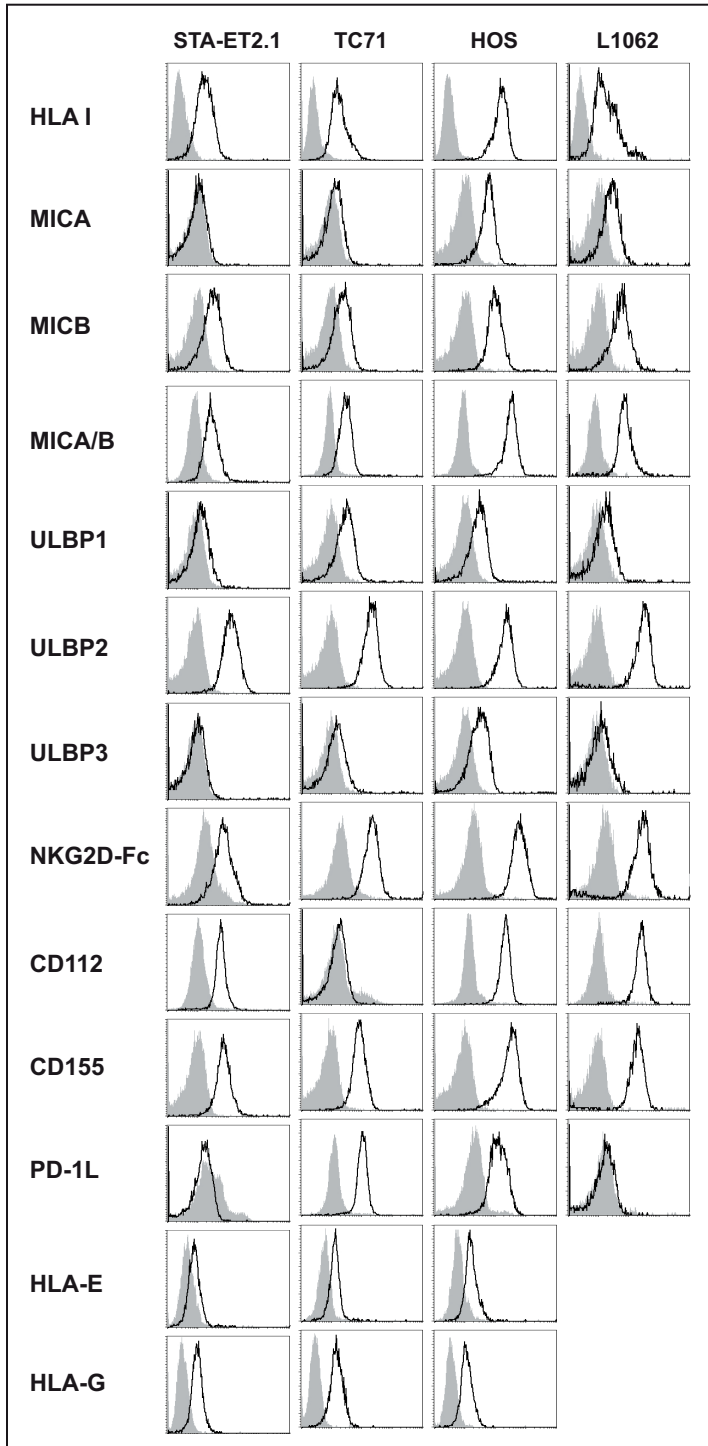
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Supplementary Figure 1: Lysis of K562 (**A**) and Daudi (**B**) cells by NK cells after the IL-15–stimulated 40 h co-culture of TCD PBMC with sarcoma cells or incubation in medium or IL-15 only. (**C**) Expression (MFIgeo) of NKG2D (left) and DNAM-1 (right) on NK cells after incubation with STA-ET2.1 cells or in medium containing 50% of cell-free supernatants (sup) of long-term, high density cultures of STA-ET2.1, TC71 and HOS cells; or on NK cells after incubation of TCD PBMC in medium containing 33% of supernatant of co-cultures of TCD PBMC and STA-ET2.1 or TC71 cells or IL-15 only. (**D**) Lysis of STA-ET2.1, TC71 and HOS cells by resting NK cells (left) or one week IL-15–pre-activated NK cells (right) +/- 10 μg/ml of blocking antibodies against DNAM-1, NKG2D, NKp30 or combinations. Representative data of at least two experiments.
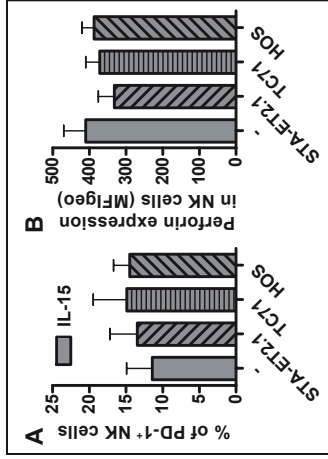
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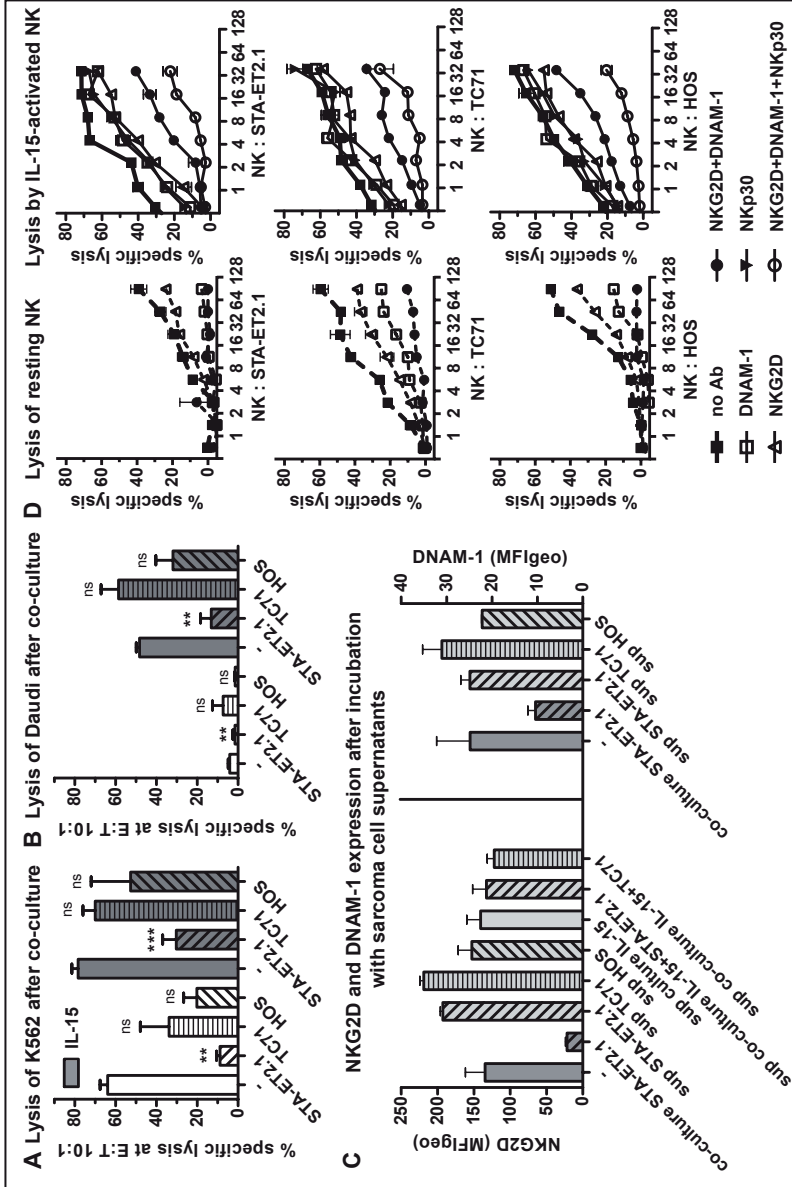
Supplementary Figure 2: Expression of NK cell receptor ligands on sarcoma cells

Supplementary Figure 3:

(A) Percentage of PD-1+ NK cells and **(B)** intracellular perforin expression in NK cells (MFIgeo) after the IL-15–stimulated 40 h co-culture with STA-ET2.1, TC71 or HOS cells. For intracellular stainings, cells fixed with 4% paraformaldehyd and permeabilized with 0.1% saponin. Combined data of at least two experiments.

Supplementary Table: NK cell receptor down-regulation after IL-15–stimulated co-culture is not associated with ligand expression on sarcoma cells

	MICA	MICB	ULBP1–3	NKG2D after co-culture	CD112	CD155	DNAM-1 after co-culture	NKp30-Fc	NKp30 after co-culture	NKp44-Fc	NKp44 after co-culture
STA-ET2.1	-	+	+++	17.7%	++	++	42.3%	-	57.3%	-	71.0%
A673	+++	++	+++	38.7%	+++	+++	51.8%	-	n/d	-	n/d
OSA	+	-	++	38.7%	+++	++	47.3%	-	n/d	+	n/d
HOS	++	+++	+++	41.0%	+++	+++	49.3%	+	59.2%	+	74.6%
U2OS	+++	-	+++	47.3%	+++	+++	55.3%	-	n/d	-	n/d
SAOS-2	+++	-	+++	50.3%	++	++	53.3%	-	n/d	-	n/d
CADO-ES	+	+	++	54.0%	+++	+++	87.3%	-	n/d	-	n/d
OHS	-	-	++	55.8%	+	++	42.0%	-	n/d	+	n/d
SK-ES-1	++	+	++	59.8%	+++	+++	64.3%	-	n/d	+	n/d
L1062	+	++	+++	61.7%	+++	+++	77.8%	-	n/d	-	n/d
SK-N-MC	+	+	++	63.7%	+++	+++	68.3%	+	n/d	-	n/d
TC71	-	+	+++	70.1%	++	+++	81.8%	+	86.5%	+	77.2%

Surface expression of ligands for the NK cell receptors NKG2D (MICA, MICB and ULBP1–3) and DNAM-1 (CD112 and CD155) as derived from Supplementary Figure 2 and previous data (4, 5, 7). In this study, Nkp30 and NKp44 ligand expression, detected by Fc fusion constructs, was investigated. Ligand expression (geometric mean fluorescence intensity (MFI) ratio between ligand staining and isotype control <2 is [-]; MFI ratio >2<5 is [+]; MFI ratio >5<10 is [++]; MFI ratio >10 is [+++]) and expression of respective NK cell receptors (from Figure 2 and Figure 3; not determined is [n/d]) after IL-15–stimulated co-culture (IL-15 incubation alone set to 100%) are depicted.

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CHAPTER 5

Macrophages inhibit human osteosarcoma cell growth after activation with the bacterial cell wall derivative liposomal muramyl tripeptide in combination with interferon-γ

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ABSTRACT

In osteosarcoma, the presence of tumor-infiltrating macrophages positively correlates with patient survival in contrast to the negative effect of tumor-associated macrophages in patients with other tumors. Liposome-encapsulated muramyl tripeptide (L-MTP-PE) has been introduced in the treatment of osteosarcoma patients, which may enhance the potential anti-tumor activity of macrophages. Direct anti-tumor activity of human macrophages against human osteosarcoma cells has not been described so far. Hence, we assessed osteosarcoma cell growth after co-culture with human macrophages.

Monocyte-derived M1-like macrophages inhibited osteosarcoma cell growth after two-day co-culture when activated with LPS+IFN-γ. Likewise, stimulation of M1-like macrophages with liposomal muramyl tripeptide (L-MTP-PE) inhibited tumor growth, but only when combined with IFN-γ. Addition of the tumor-reactive anti-EGFR antibody cetuximab did not further improve the anti-tumor activity of activated M1-like macrophages. The inhibition was mediated by supernatants of activated M1-like macrophages, containing TNF-α and IL-1β. However, specific blockage of these cytokines, nitric oxide or reactive oxygen species did not inhibit the anti-tumor effect, suggesting the involvement of other soluble factors released upon macrophage activation. While LPS+IFN-γ–activated M2-like macrophages had low anti-tumor activity, IL-10–polarized M2-like macrophages were able to reduce osteosarcoma cell growth in the presence of the anti-EGFR cetuximab involving antibody-dependent tumor cell phagocytosis.

This study demonstrates that human macrophages can be induced to exert direct anti-tumor activity against osteosarcoma cells. Our observation that the induction of macrophage anti-tumor activity by L-MTP-PE required IFN-γ may be of relevance for the optimization of L-MTP-PE therapy in osteosarcoma patients.

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INTRODUCTION

Osteosarcoma is the most frequent malignant bone tumor in adolescents and young adults. Of patients with localized, non-metastatic disease, up to 70% achieve persistent remission [1]. In contrast, prognosis of patients with advanced, metastatic and recurrent disease is as low as 20% despite intensive chemotherapy and surgery. Recently, we have demonstrated that the presence of tumor-infiltrating macrophages at the time of diagnosis is positively correlated with a favorable outcome of patients with osteosarcoma [2]. Hence, targeting tumor-associated macrophages in osteosarcoma with macrophage-activating agents is an attractive option to complement current anti-tumor treatments.

Macrophages are mononuclear phagocytic cells that are involved in homeostatic, pro-inflammatory and immune regulatory responses in the tissue [3, 4]. While macrophages can originate from blood monocytes under inflammatory conditions, as in the classical model for macrophage development, it has recently been revealed that under non-inflammatory conditions tissue macrophages primarily originate from the yolk sac and fetal liver and are maintained independently of hematopoietic precursors [5]. Macrophages possess great functional and phenotypic plasticity which is often simplified by classification in M1 and M2 phenotypes [6]. M1 macrophages are involved in host defense through their bactericidal and tumoricidal activity and pro-inflammatory cytokine production if 'classically-activated' by interferon-γ (IFN-γ) and Toll-like receptor ligands such as bacterial lipopolysaccharide (LPS) [7, 8]. M2 macrophages can exhibit many different phenotypes in response to diverse stimuli such as polarization with interleukin-10 (IL-10) or LPS. M2 macrophages are involved in scavenging cell debris and bacteria, antibody-dependent phagocytosis, tissue remodeling, angiogenesis, wound healing and immune regulation. In contrast to 'classically-activated' M1 macrophages, macrophages with an M2-like phenotype are often detected in solid tumors and considered to promote tumor progression [6-9].

Macrophages constitute the majority of tumor-infiltrating immune cells in solid tumors including osteosarcoma [2, 10]. In most tumors, the presence of macrophages represents an unfavorable prognostic factor [11]. In contrast, in osteosarcoma as well as colorectal cancer higher numbers of tumor-infiltrating macrophages correlate with better survival [2, 12, 13]. In osteosarcoma, there was no clear association of good survival with an M1-like or M2-like phenotypic polarization of macrophages [2].

Monocytes and macrophages activated with LPS have been implicated in anti-tumor responses for a long time [14-19]. But while canine macrophages have been reported to have anti-tumor activity against canine osteosarcoma cells, comparable evidence for anti-tumor activity of human macrophages against human osteosarcoma cells is not available. The anti-tumor activity of canine macrophages was shown to be dependent on stimulation with LPS or another bacterial cell wall constituent, i.e. muramyl dipeptide (MDP) or the lipophilic derivative muramyl tripeptide phoshatidylethanolamine (MTP-PE) [20]. Application of liposome-encapsulated MTP-PE (L-MTP-PE) *in vivo* improved survival of dogs with osteosarcoma [21]. This observation encouraged the addition of L-MTP-PE to the treatment of osteosarcoma patients as a macrophage-activating agent but did not increase event-free survival of non-metastatic or metastatic osteosarcoma patients [1, 22].

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Therefore, we set out to investigate the anti-tumor activity of human macrophages against human osteosarcoma cells and determine whether this activity can be manipulated. We set up an *in vitro* model in which the effect of human macrophages on the growth of osteosarcoma cells can be directly assessed by counting residual tumor cells after a two-day co-culture with macrophages. Using this model we demonstrate how anti-tumor activity of M1-like macrophages and M2-like macrophages can be induced by bacterial stimuli like L-MTP-PE and the therapeutic anti-EGFR antibody cetuximab, respectively.

MATERIALS AND METHODS

Cell lines

The osteosarcoma cell lines HOS, HOS-143b, OHS, OSA, SAOS-2 and U2OS were obtained from the EuroBoNeT cell line repository (2007) [23]. Cell line identity was confirmed by short tandem repeat DNA fingerprinting in 2012. All cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen) and 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen). All cell lines were negative for mycoplasma infection as regularly tested by RT-PCR.

Preparation of liposomal MTP-PE

Liposomes (multi-lamellar vesicles) were prepared from a mixture of the synthetic phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 850457P) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, 840035P) (both from Avanti Polar Lipids, Alabaster, Al, USA) at a 7:3 molar ratio in chloroform by mechanical agitation on a vortex mixer. MTP-PE (M_r 1237.5 g/mol; Mifamurtide; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in chloroform:methanol:water 60:36:4 (v/v/v). 5 mg of liposomes (M_r 775 g/mol) were loaded with 0.02 mg of MTP-PE (1:250 ratio). The organic solution was dried in a rotary evaporator under reduced pressure for one hour to obtain a dry lipid film. Afterwards, the lipid film was rehydrated in 2.5 ml sterile PBS, resulting in a final concentration of 6.45 nmol MTP-PE per 2 μmol/ml liposome preparation (L-MTP-PE). The liposomes were passed four times through a 1 μm unipore polycarbonate filter (Nuleopore). Control liposomes (L-PBS) were prepared by the same procedure except without MTP-PE addition. The z-average diameter of the liposomes was ~350 nm with a mean zeta potential of -97 mV as measured on a Zetasizer (version 6.01) (Malvern Instruments, Worcestershire, UK).

Monocyte Isolation and differentiation to macrophages

PBMC were separated from buffy coats of healthy adult donors (Sanquin Blood bank, Region Southwest, Rotterdam, the Netherlands) by Ficoll-Hypaque density gradient centrifugation. Monocytes were isolated from PBMC by positive selection using anti-CD14 MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). For M1-like and M2-like macrophage differentiation, monocytes ($1,5x10^6$ per well per 3 ml of a 6-well tissue culture plate) were incubated with GM-CSF (80 ng/ml; Peprotech, Rocky Hill, NJ, USA) and M-CSF (20 ng/ml, R&D Systems, Minneapolis, MN, USA) for seven days as previously described [9;27]. In some conditions, M1-like and M2-like macrophages were additionally stimulated during the

last day of differentiation with combinations of LPS (10 ng/ml; *E. coli* strain 0111:B4; Sigma-Aldrich), IFN-γ (100 U/ml; Boehringer, Mannheim, Germany), empty control liposomes (250 nmol) (L-PBS) or liposomes (250 nmol) containing MTP-PE (0.8 nmol, i.e., 1 µg) (L-MTP-PE) per 3 ml culture medium. M2-like macrophages were alternatively stimulated with IL-10 (10 ng/ml; Peprotech) during the last two days of differentiation. The phenotype of macrophage populations was tested in each experiment. Macrophages were devoid of the monocyte-derived dendritic cell marker CD1a (data not shown).

Macrophage-tumor cell co-cultures

After differentiation, culture supernatants of macrophages were collected. Adherent macrophages were washed in cold PBS, detached by incubation in accutase (Sigma-Aldrich) for 30 min at 37 °C and combined with the non-adherent cell fraction. Cell scraping of firmly adherent macrophages was avoided to maximize macrophage viability. Macrophages were seeded in 96-well flat-bottom plates in RPMI medium at 3,000 (cell conjugate formation assay) or 30,000 cells (tumor cell survival assay) per well (four wells per condition) and incubated for cell attachment. After two hours 3,000 osteosarcoma cells were added and macrophage-tumor cell co-cultures were incubated for two hours in cell conjugate formation assays at a 1:1 ratio in 50 µl medium and for two hours, one day and two days in tumor cell survival assays at a 10:1 ratio in 100 µl medium. In some experiments, tumor cells were coated with the chimeric monoclonal antibody cetuximab (anti-epidermal growth factor receptor, 1 µg/ml final concentration in co-cultures; Erbitux; Merck KGaA, Darmstadt, Germany) or with the non-binding anti-CD20 antibody rituximab (1 µg/ml; MabThera; Roche, Basel, Switzerland) prior to the co-culture. In blocking experiments, co-cultures were performed in the presence of the soluble tumor-necrosis factor–α (TNF-α) receptor etanercept (10 µg/ml; Enbrel; Wyeth; Madison, NJ, USA) and TNF-α–neutralizing antibody adalimumab (10 µg/ml; Humira; Abbot; North Chicago, IL, USA), the IL-1 receptor antagonist anakinra (10 µg/ml; Kineret; Amgen; Thousand Oaks, CA, USA), nitric oxide species inhibitor Nω-Nitro-L-arginine methyl ester (10 µM; L-NAME; Sigma-Aldrich), reactive oxygen species inhibitors catalase (186 µg/ml; Sigma-Aldrich) and superoxide dismutase (4.2 µg/ml; Sigma-Aldrich).

Anti-Tumor Activity Assay

The effect of macrophages on tumor cell survival was assessed by enumerating tumor cells by flow cytometry [13, 25]. Adherent and non-adherent cells were harvested after co-culture using accutase (if necessary supported by cell scraping) and stained with anti-CD56 and anti-CD32 to distinguish tumor cells and macrophages, respectively. The complete tumor cell-macrophage suspension was analyzed by flow cytometry. Live-gated tumor cells present at the end of the co-culture were quantified and in each experiment compared to the number of tumor cells grown in the absence of macrophages. In some experiments viable tumor cell numbers were measured after their incubation in medium with 50% (v/v) of macrophage cell-free supernatant or after their incubation with inhibitors in the presence of macrophages. Single measurements from multiple independent experiments were combined as indicated in figure legends.

Cell conjugate formation

Tumor cell lines were labeled with CFSE (1 μM; Invitrogen) and incubated overnight to allow leakage of excess CFSE. IL-10–stimulated M2-like Macrophages were co-cultured with CFSE-labeled HOS-143b cells for two hours at 1:1 ratio. All cells were harvested from the culture by cell scraping and macrophages were labeled with APC-labeled anti-CD32 antibodies. Cell conjugate formation between macrophages and tumor cells was analyzed by flow cytometry, assessing the percentage of CD32$^+$ macrophages acquiring high CFSE fluorescence from tumor cells.

For an indication of phagocytosis, after the cell conjugate formation assay, CD32$^+$ macrophages which have acquired the fluorescent signal of CFSE$^+$ tumor cells were sorted by flow cytometry in one experiment. The cells were stained with mouse anti-human HLA-DR (TAL.1B5; Dako, Glostrup, Denmark) followed by the Alexa-Fluor-594 goat anti-mouse IgG1 secondary antibody (Invitrogen) and embedded in Vectashield mounting medium containing DAPI (Vectorlabs, Burlingame, CA, USA). Cell conjugates were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica, Solms, Germany), detecting nuclei in blue, HLA-DR$^+$ macrophages in red and CFSE$^+$ tumor cells in green.

Flow cytometry

The following fluorochrome-labeled mouse anti-human monoclonal antibodies were used: CD32^{APC} (clone FLI8.26), CD86^{PE} (FUN-1), CD163^{PE} (GHI/61), HLA-DR^{FITC} (L243) (BD Biosciences, Franklin Lakes, NJ, USA); CD56^{PE} (NKH-1) (Beckman Coulter, Brea, CA, USA); CD16^{FITC} (3G8), CD64^{FITC} (22) (IOTEST Immunotech, Marseille, France). Measurements were performed with the FACSCalibur (BD Biosciences) and analyzed with the BD Cell Quest ProTM software (version 5.2.1). Fold-expression data indicated in histogram plots were calculated by dividing the geometric mean fluorescence intensity (geoMFI) of antibodies by the geoMFI of the PBS control.

Luminex assay

Cytokine production in cell-free supernatants of macrophage cultures was measured using the Bio-Plex Pro Human Cytokine 27-plex group 1 panel according to the manufacturer's description (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Paired student t-tests were performed to compare the means two samples. One-way analysis of variance (ANOVA) was performed to compare the means of three or more samples followed by Dunnett's or Bonferroni's multiple comparison post test to compare samples of interest with a control sample as described in the figure legends. Error bars represent the standard error of the mean. A P-value of <0.05 was considered statistically significant. All statistical analyses were performed using Graphpad Prism version 5.04 (La Jolla, CA, USA).

RESULTS

M1-like macrophages inhibit osteosarcoma cell growth if activated with LPS+IFN-γ

The potential of human macrophages to inhibit osteosarcoma cell growth in vitro was investigated. M1-like and M2-like macrophages were differentiated from blood monocytes with or without the polarization stimuli LPS+IFN-γ (for M1 and M2) or IL-10 (for M2) as previously established [8, 24, 26]. The various macrophage subtypes were co-cultured with osteosarcoma cell lines and after two days the residual number of viable tumor cells was assessed by flow cytometry [13, 25]. In particular M1-like macrophages pre-stimulated with LPS+IFN-γ were able to significantly reduce tumor cell numbers of HOS-143b and OHS cells to as low as 50% as well as of four other osteosarcoma cell lines (Figure 1, panel A-C). This inhibition of tumor cell growth as a consequence of macrophage addition was not yet apparent after 2 and 24 hours of co-culture but became pronounced after two days of co-culture (Figure 1, panel D). M2-like macrophages stimulated with LPS+IFN-γ showed less anti-tumor activity than LPS+IFN-γ–stimulated
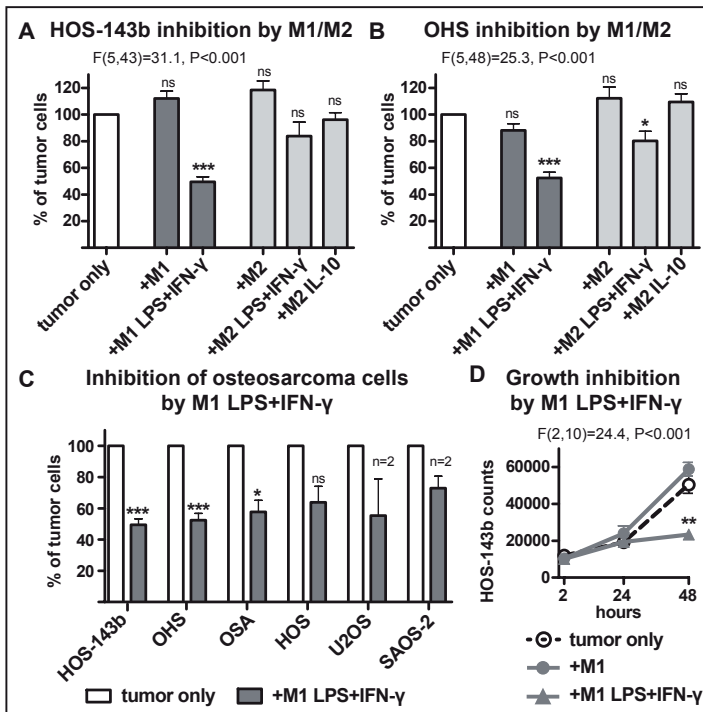
Figure 1: Inhibition of osteosarcoma cell growth by LPS+IFN-γ–activated M1-like macrophages

Human M1-like macrophages (dark shade) and M2-like macrophages (light shade) were pre-activated with or without LPS+IFN-γ (M1 and M2) or IL-10 (M2) and afterwards incubated with **(A)** HOS-143b cells (n= 4–11) and **(B)** OHS cells (n= 5–12) and **(C)** four other osteosarcoma cell lines (n= 2–12). After two days of co-culture, tumor cell numbers were counted by flow cytometry. Differences between one macrophage–tumor co-culture and the control, i.e. tumor cell recovery after incubation in the absence of macrophages (white bar, set to 100%), as in panel C were statistically analyzed by paired student t-tests, *** is P<0.001, ** is P<0.01, * is P<0.05, ns is not statistical significant. Differences between multiple groups as in panel A and B were statistically analyzed by ANOVA as indicated followed by Dunnett's post test for differences (p<0.05) between individual co-cultures and the control as indicated by asterisks. **(D)** HOS-143b cell counts after 2, 24 and 48 hours co-culture with M1-like macrophages +/- LPS+IFN-γ (n=5). All data are means of multiple experiments as indicated (n).
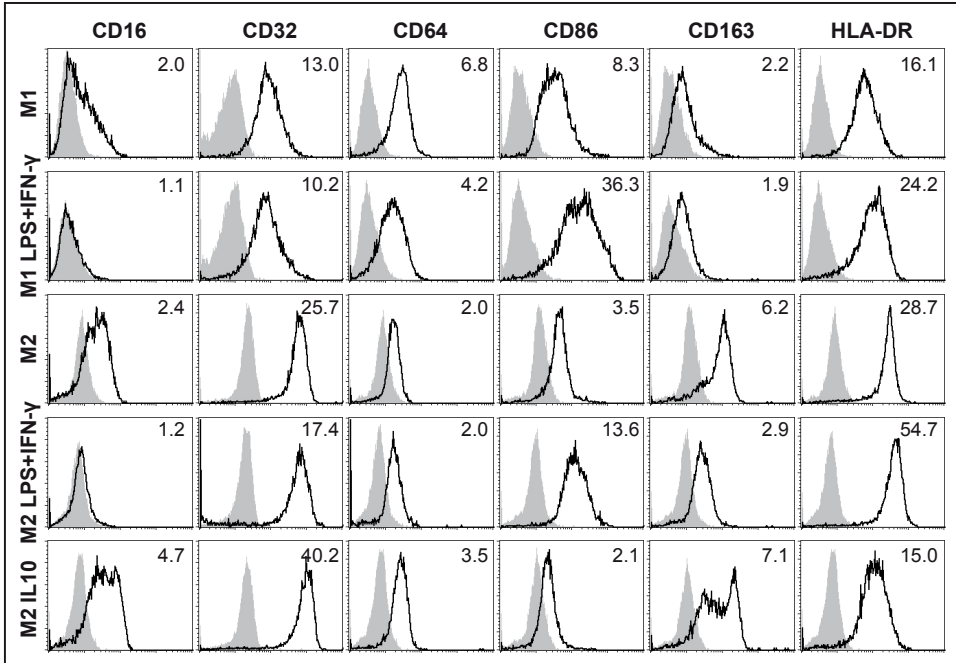
Figure 2: Phenotypes of M1-like and M2-like macrophages
Surface expression of Fcγ receptors (CD16, CD32 and CD64), HLA-DR, CD86 and CD163 on the various M1-like and M2-like macrophage populations was analyzed by flow cytometry. Fold change of geoMFI of specific antibody stainings (bold solid line) compared to PBS control (light shade) is indicated in the FACS histogram plots. Representative data of three experiments are depicted.

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M1-like macrophages, while IL-10–polarized M2-like macrophages were not able to inhibit tumor cell growth (Figure 1, panel A and B). Incubation of tumor cells with LPS+IFN-γ alone had no inhibiting effect (data not shown).

Induction of anti-tumor activity by M1-like macrophages after stimulation with LPS+IFN-γ was associated with a more activated phenotype as indicated by the up-regulation of CD86 and HLA-DR expression (Figure 2). In contrast to M1-like macrophages, M2-like macrophages expressed CD163, a marker frequently described for tumor-associated M2-like macrophages. LPS+IFN-γ–stimulated M2-like macrophages showed phenotypic similarities to LPS+IFN-γ–stimulated M1-like macrophages, exhibiting reduced levels of CD163 and increased levels of CD86 and HLA-DR expression. Notably, in particular when stimulated with IL-10, M2-like macrophages displayed high levels of FcγRII (CD32) in addition to FcγRI (CD64) and FcγRIIIa (CD16), suggesting that IL-10–polarized M2-like macrophages could exert antibody-dependent functions as described below.

Overall, of the different macrophage populations tested, LPS+IFN-γ–activated M1-like macrophages, resembling 'classically-activated' M1-like macrophages [6-8], were the most capable of inhibiting osteosarcoma cell growth.
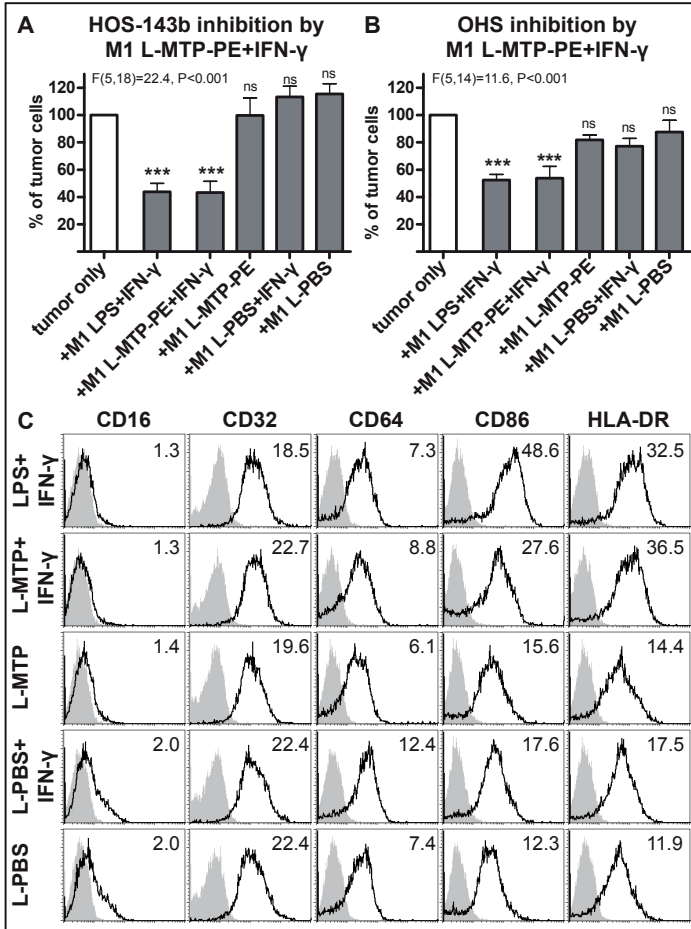
Figure 3: Anti-tumor activity by M1-like macrophages stimulated with L-MTP-PE requires IFN-γ

M1-like macrophages were stimulated with LPS+IFN-γ, L-MTP-PE+IFN-γ, L-MTP-PE alone, L-PBS+IFN-γ or L-PBS alone. Macrophages were incubated with **(A)** HOS-143b (n= 3–5) and **(B)** OHS (n= 2–4) tumor cells. After two-day co-culture, tumor cell numbers were assessed and statistically analyzed relative to the control by ANOVA and Dunnett's post test as indicated. **(C)** Surface expression of Fcγ receptors (CD64, CD32 and CD16), HLA-DR and CD86 was analyzed on M1-like macrophages after activation with LPS+IFN-γ, L-MTP-PE+IFN-γ, L-MTP-PE alone, L-PBS+IFN-γ or L-PBS alone. Representative data of two experiments are depicted.

Liposomal muramyl tripeptide only induces anti-tumor activity of M1-like macrophages in the presence of IFN-γ

In clinical applications, infusion of macrophage-activating bacterial products can cause severe, detrimental toxic reactions. This can be circumvented by incorporation of very lipophilic, synthetic MTP-PE into liposomes (L-MTP-PE), which results in rapid uptake by myeloid cells and low toxicity [19]. In spite of the inclusion of L-MTP-PE in clinical trials of osteosarcoma patients, direct evidence of L-MTP-PE to induce anti-tumor activity by human macrophages against human osteosarcoma cells is lacking. Therefore, it was investigated whether M1-like macrophages can be activated by L-MTP-PE to reduce tumor cell growth. Remarkably, only if co-stimulated with IFN-γ, L-MTP-PE–stimulated M1-like macrophages significantly inhibited tumor cell growth such as of HOS-143b cells and OHS cells to as low as 45% (Figure 3, panel A and B). The inhibition by L -MTP-PE+IFN-γ–stimulated M1-like macrophages was as potent as by LPS+IFN-γ–activated M1-like macrophages. In contrast, M1-like macrophages stimulated with MTP-PE-loaded liposomes alone, empty liposomes (L-PBS) or empty liposomes in combination with IFN-γ, failed to inhibit tumor cell growth. Moreover, only if co-stimulated with IFN-γ,

L-MTP-PE–stimulated M1-like macrophages exhibited an activated phenotype by CD86 and HLA-DR up-regulation similar to LPS+IFN-γ–activated M1-like macrophages (Figure 3, panel C). In conclusion, L-MTP-PE stimulation induced substantial anti-tumor activity of M1-like macrophages but only after co-stimulation with the pro-inflammatory cytokine IFN-γ.

Soluble factors produced by M1-like macrophages after LPS+IFN-γ and L MTP PE+IFN-γ activation inhibit tumor cell growth

Next, the mechanisms involved in the strong anti-tumor effect of LPS+IFN-γ–activated and L-MTP-PE+IFN-γ–activated M1-like macrophages were investigated. Incubation of osteosarcoma cells in medium with cell-free supernatant of activated M1-like macrophages reduced tumor cell growth to similar levels as activated M1-like macrophages themselves (Figure 4, panel A). In contrast, supernatant from non-activated M1-like macrophages did not reduce tumor cell growth. Thus, both LPS+IFN-γ–activated and L-MTP-PE+IFN-γ–activated M1-like macrophages produced soluble factors that inhibited osteosarcoma cell growth. Therefore we measured the levels of soluble factors produced by activated macrophages alone and after two-day co-culture with the tumor cells. Activation of M1-like macrophages with LPS+IFN-γ enhanced the production of the pro-inflammatory cytokines IL-1β, IL-6, IL-12p70, TNF-α, CXCL10 (IP-10) and CCL5 (Rantes), while CCL2 (MCP-1), CCL3 (MIP-1α) and CCL4 (MIP-1β) remained unchanged (Figure 4, panel B). L-MTP-PE+IFN-γ–activated M1-like macrophages displayed a similar cytokine profile except for lower levels of IL-12p70.

Since TNF-α was reported to be able to confer anti-tumor effects [27] and was also produced by both LPS+IFN-α–activated and L-MTP-PE+IFN-α–activated M1-like macrophages during the co-culture (Figure 4, panel C), a role for TNF-α in the inhibition of osteosarcoma cell growth was examined. Blocking of TNF-α during the co-culture of macrophages and tumor cells by the soluble TNF receptor etanercept combined with the TNF-α neutralizing antibody adalimumab did not prevent the inhibiting effects of LPS+IFN-γ–activated M1-like macrophages or supernatants derived from these macrophages (Figure 4, panel D and data not shown). Blocking of TNF-α did also not prevent the inhibiting effects of L-MTP-PE+IFN-γ–activated M1-like macrophages (data not shown). Moreover, blocking of IL-1 receptor, combined blocking of TNF-α and IL-1 receptor, or inhibition of nitric oxide and reactive oxygen species did not significantly interfere with the inhibition of tumor cell growth by activated macrophages (Figure 4, panel D). None of the tested inhibitors affected tumor cell growth as compared to tumor cells incubated in the absence of inhibitors.

These results indicate that the inhibition of osteosarcoma cell growth by activated M1-like macrophages was mediated by soluble factors induced by macrophage activation in a TNF-α/IL-1–independent manner.

IL-10–stimulated M2-like macrophages can inhibit growth of osteosarcoma cells in an antibody-dependent manner

Both M1-like and M2-like macrophages have been detected in osteosarcoma lesions [2]. Hitherto, IL-10–stimulated M2-like macrophages were unable to inhibit osteosarcoma cell growth. In a previous study it has been shown that IL-10-polarized M2-like macrophages internalized antibody-coated B cell lymphoma cells [26]. Since IL-10–stimulated M2-like
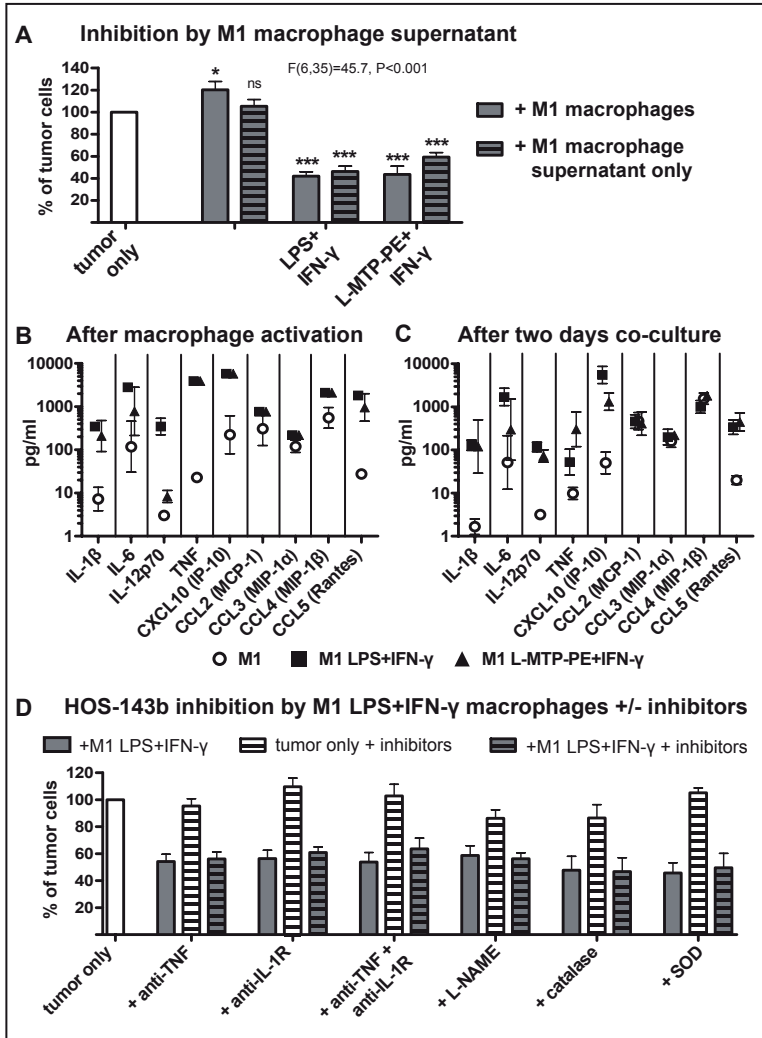
Figure 4: Tumor cell growth inhibition by activated M1-like macrophages is mediated by soluble factors

(A) HOS-143b cells were incubated with M1-like macrophages (pre-activated +/- LPS+IFN-γ or L-MTP-PE+IFN-γ) (no pattern) or with cell culture supernatant from these macrophages (hatched pattern) for two days and tumor cell numbers were analyzed relative to the control by ANOVA and Dunnett's post test as indicated (n= 3–8). **(B)** The cytokine and chemokine profile of M1-like macrophages was assessed in cell-free supernatants obtained after macrophage activation +/- LPS+IFN-γ or L-MTP-PE+IFN-γ or **(C)** after two-day co-culture with HOS-143b cells (subsequent to macrophage activation and washing). Data of IL-1β, IL6, IL-12p70, TNF-α, CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (Rantes) were acquired by Luminex assays (n= 2–3). There was no cytokine/chemokine production by tumor cells alone. Compared to activated macrophages alone the co-culture with tumor cells did not enhance cytokine/chemokine production (data not shown). **(D)** HOS-143b cells were incubated with or without LPS+IFN-γ-activated M1-like macrophages in the presence or absence of inhibitors against TNF-α (neutralizing antibody and soluble TNF receptor), IL-1 receptor (i.e., IL-1Ra), TNF-α and IL-1 receptor, nitric oxide (i.e., L-NAME) or reactive oxygen species (i.e., catalase and SOD). In each set of inhibitor experiments (n= 3–6), HOS-143b cell numbers of activated M1-like macrophage co-culture (filled bar without pattern), tumor cells only with inhibitors (white bar with hatched pattern) and activated M1-like macrophage co-culture with inhibitors (filled bar with hatched pattern) are depicted and analyzed relative to the control (tumor cells only without inhibitors set to 100%, white bar without pattern). There was no statistical significant difference between co-cultures without inhibitors and co-cultures with inhibitors, or between tumor cells only with inhibitors and co-cultures with inhibitors, whereas differences between tumor cells only without inhibitors and co-cultures with/without inhibitors were statistical significant (P<0.05) as analyzed by ANOVA and Bonferroni's post test.
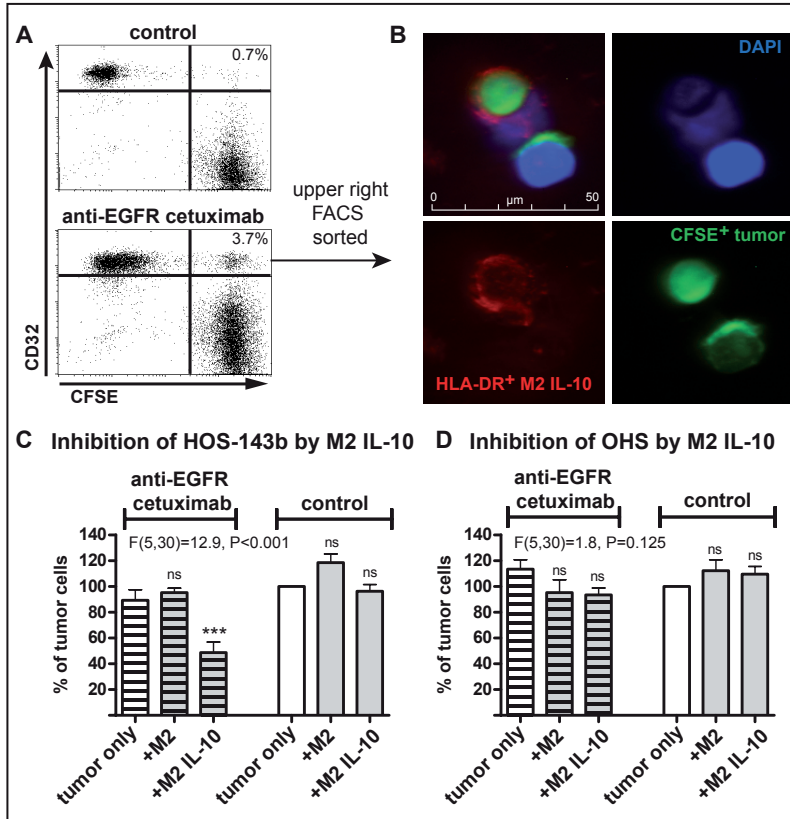
Figure 5: Antibody-dependent tumor cell growth inhibition by IL-10–polarized M2-like macrophages

(A) CFSE-labeled HOS-143b cells coated with anti-EGFR cetuximab or non-binding anti-CD20 rituximab were incubated with IL-10–stimulated M2-like macrophages for two hours. Cell conjugate formation was evaluated by flow cytometry, assessing CD32+ macrophages acquiring high CFSE fluorescence of the tumor cells. Representative data of two experiments are depicted. (B) In one experiment, CD32+CFSE+ cells (upper right quadrant in lower panel A) were sorted by flow cytometry and examined by Immunofluorescence microscopy, detecting HLA-DR-stained macrophages in red (lower left), CFSE+ tumor cells in green (lower right) and DAPI-stained cell nuclei in blue (upper right) and composites (upper left). (C) HOS-143b (n= 4–7) and (D) OHS (n= 5–8) cells were coated with cetuximab (hatched pattern) or rituximab (control, no pattern) and incubated with M2-like macrophages pre-stimulated with or without IL-10. After two days tumor cell numbers were analyzed by ANOVA and Dunnett's post test.

macrophages exhibited the highest expression of FcγR in our experiments, we investigated whether these macrophages are able to form cell conjugates with and internalize osteosarcoma cells in an antibody-dependent manner as a potential anti-tumor mechanism. After two-hour co-culture of IL-10–stimulated M2-like macrophages and CFSE-labeled HOS-143b cells, CD32+ M2-like macrophages acquiring the fluorescent signal of HOS-143b cells were analyzed by flow cytometry, as similarly described before [26, 28]. If HOS-143b cells were coated with the anti-EGFR antibody cetuximab, the percentage of CD32+CFSE+ macrophages was increased, indicative for cell conjugate formation between macrophages and tumor cells (Figure 5, panel A). Furthermore, FACS-sorted CD32+CFSE+ macrophages (upper right quadrant) were analyzed by immunofluorescence microscopy and contained tumor cells bound to macrophages as well as tumor cells internalized by macrophages (Figure 5, panel B).

Next, it was examined whether the antibody-dependent interaction between tumor cells and macrophages can result in inhibition of tumor cell growth. Indeed, IL-10–stimulated M2-like macrophages substantially inhibited growth of half of the osteosarcoma cell lines (3/6) such as HOS-143b to as low as 50% if coated with cetuximab (Figure 5 panel C and data not shown). There was not such an inhibiting effect when HOS-143b cells were treated with the isotype-matched, non-tumor-binding anti-CD20 antibody rituximab. In contrast, IL-10–stimulated M2-like macrophages failed to inhibit cell growth of (or form cell conjugates with) cetuximab-coated OHS cells despite high levels of EGFR expression [29], indicating that additional cell characteristics play a role in determining the sensitivity to antibody-dependent anti-tumor activity (Figure 5, panel D and data not shown). In the absence of macrophages, there was no inhibitory effect by cetuximab on osteosarcoma cells (Figure 5, panel C and D) [29]. Inhibition of tumor cell growth by LPS+IFN-γ–stimulated M1-like macrophages was not further increased by cetuximab (data not shown).

Hence, IL-10–stimulated M2-like macrophages have the potential to inhibit osteosarcoma cell growth in an antibody-dependent manner with similar efficacy as antibody-independent inhibition by activated M1-like macrophages.

DISCUSSION

In this report, we describe for the first time that human macrophages can interfere with the growth of human osteosarcoma cells. Significant induction of anti-tumor activity of human M1-like macrophages by liposomal muramyl tripeptide required co-stimulation with pro-inflammatory IFN-γ. Inhibition of osteosarcoma cell growth by activated M1-like macrophages was mediated by soluble factors which were induced upon macrophage activation before interaction with tumor cells. In addition, we report that IL-10–polarized M2-like macrophages could exert anti-tumor activity against some osteosarcoma cell lines in an antibody-dependent manner.

More than 100 years ago it has been observed by Busch, Fehleisen, Bruns and others that bacterial infections can result in tumor regression accompanied by febrile inflammatory responses which presumably mediated the anti-tumor effects [30, 31]. These findings pioneered the first extensive immunotherapy of bone sarcoma patients by Coley, administering heat-inactivated bacterial preparations with considerable but disputed remission rates. The anti-tumor effect was probably, at least in part, linked to the pro-inflammatory response of innate immune cells such as macrophages to bacterial constituents like LPS [31, 32]. Another bacterial cell wall component, muramyl dipeptide (MDP) has originally been discovered as the minimal (synthetically-derived) moiety of peptidoglycan which can substitute for mycobacteria in Freund's complete adjuvant [33]. MTP-PE is a lipophilic, synthetic derivate of MDP which has low toxicity and enhanced macrophage-activating properties if incorporated in liposomes (L-MTP-PE) [19]. To mimic bacterial infections and trigger macrophage activation, L-MTP-PE has been included in the treatment of osteosarcoma patients [1]. Our observation that the anti-tumor effect of L-MTP-PE–stimulated macrophages was dependent on IFN-γ is noteworthy in this respect. IFN-γ was originally described as macrophage-activating factor [34]. 'Priming' of macrophages by IFN-γ may enhance liposome uptake and improve the response to bacterial components by, for instance, intracellular NOD2, which is the receptor for MDP and presumably MTP-PE [19,

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35-37]. The significance of IFN-γ observed in our experiments reproduces previous studies using different tumor cells which showed that activation of human/murine monocytes/macrophages by L-MTP-PE was enhanced by simultaneous or preceding stimulation with IFN-γ [15, 19, 36]. Furthermore, addition of IFN-γ to L-MTP-PE was reported to improve survival and inhibit metastases in murine renal adenocarcinoma [38]. Altogether, the clinical efficacy of L-MTP-PE addition in the treatment of osteosarcoma patients may be improved by the inclusion of a macrophage-priming signal like IFN-γ.

This raises the question how such a macrophage-priming factor could be safely introduced in the osteosarcoma microenvironment. IFN-γ levels could be increased by local or systemic IFN-γ therapy as applied in patients with cancers or mycobacterial infections [39, 40]. To target the same macrophages with IFN-γ as with L-MTP-PE, IFN-γ could be incorporated in MTP-PE–containing liposomes which are then both efficiently internalized by phagocytic cells such as tumor-resident or tumor-infiltrating macrophages. This approach is supported by murine studies in which the incorporation of IFN-γ into MTP-PE–containing liposomes enhanced the tumoricidal activity of macrophages as compared to liposomal MTP-PE alone [19, 41]. Alternatively, lymphocytes such as NK cells activated to secrete IFN-γ and recruited to tumor sites might enhance local IFN-γ production [42].

In our experiments, bacterial and pro-inflammatory stimuli induced the strongest inhibition of tumor cell growth by M1-like macrophages. Therefore, such macrophage-activating therapies may primarily be effective in tumor types that contain M1-like macrophages [12, 13]. Most tumors contain high numbers of potentially 'pro-tumor' immune regulatory M2-like macrophages. Therefore, several studies have considered depleting macrophage numbers or inhibiting macrophage recruitment to the tumor [9, 43, 44]. Instead, in our experiments, IL-10–polarized M2-like macrophages could be induced to inhibit osteosarcoma cell growth if the tumor cells were coated with the therapeutic anti-EGFR antibody cetuximab. Antibody-dependent cell conjugate formation and inhibition of tumor cell growth were only observed for half of the osteosarcoma cell lines despite significant EGFR expression [29]. Hence, to improve antibody-dependent anti-tumor activity by M2-like macrophages, it would be required to elucidate additional parameters besides surface antigen expression that determine inhibition of tumor cell growth by macrophages. The potential of antibody-dependent anti-tumor activity by macrophages has been shown to mediate anti-tumor responses in murine lymphoma models [45, 46]. In humans, the addition of rituximab therapy to patients with follicular lymphoma can counteract the non-favorable prognostic factor of high macrophage counts in the tumor [47]. We have previously demonstrated that the cytotoxic activity of NK cells can be enhanced and directed to osteosarcoma cells by anti-EGFR cetuximab [29]. Since macrophages abundantly infiltrate osteosarcoma lesions, antibody-dependent inhibition of osteosarcoma cell growth by macrophages may be an additional anti-tumor mechanism of cetuximab.

The recent finding that anti-CD40 therapy can induce anti-tumor activity in mice and humans independently of T cells but presumably via activating macrophages has revived the role of macrophages in anti-tumor responses [48]. Overall, activation of macrophages by e.g. L-MTP-PE in the presence of IFN-γ, and/or treatment with tumor-reactive antibodies may in particular be advantageous in tumors like osteosarcoma that have a high content of infiltrating macrophages.

ACKNOWLEDGMENTS

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CHAPTER 6

Expression of the immune regulation antigen CD70 in osteosarcoma

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ABSTRACT

Osteosarcoma is the most frequent bone cancer in children and young adults. The outcome of patients with advanced disease is dismal. Exploitation of tumor–immune cell interactions may provide novel therapeutic approaches. CD70–CD27 interactions are important for the regulation of adaptive immunity. CD70 expression has been reported in some solid cancers and implicated in tumor escape from immunosurveillance. In this study, expression of CD70 and CD27 was analyzed in osteosarcoma cell lines and tumor specimens.

CD70 protein was expressed on most osteosarcoma cell lines (5/7) and patient-derived primary osteosarcoma cultures (4/6) as measured by flow cytometry. In contrast, CD70 was detected on few Ewing sarcoma cell lines (5/15) and was virtually absent from neuroblastoma (1/7) and rhabdomyosarcoma cell lines (0/5). CD70^{+} primary cultures were derived from CD70^{+} osteosarcoma lesions. CD70 expression in osteosarcoma cryosections was heterogeneous, restricted to tumor cells and not attributed to infiltrating CD3^{+} T cells as assessed by immunohistochemistry/immunofluorescence. CD70 was detected in primary (1/5) but also recurrent (2/4) and metastatic (1/3) tumors. CD27, the receptor for CD70, was neither detected on tumor cells nor on T cells in CD70^{+} or CD70^{-} tumors, suggesting that CD70 on tumor cells is not involved in CD27-dependent tumor–immune cell interactions in osteosarcoma. CD70 gene expression in diagnostic biopsies of osteosarcoma patients did not correlate with the occurrence of metastasis and survival (n=70).

Our data illustrate that CD70 is expressed in a subset of osteosarcoma patients. In patients with CD70^{+} tumors, CD70 may represent a novel candidate for antibody-based targeted immunotherapy.

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INTRODUCTION

Osteosarcoma is the most frequent bone cancer in children and adolescents. Despite intensive chemotherapy, patients with recurrent, metastatic and chemotherapy-refractory osteosarcoma have a poor prognosis [1]. Osteosarcoma is frequently infiltrated by immune cells such as macrophages and T cells [2–4]. Insight in tumor–immune cell interactions may be instrumental to develop novel treatment approaches like targeted immunotherapy. Aside from its role in adaptive immunity, CD70 is expressed on certain solid tumors and reported to be involved in tumor cell escape from immunosurveillance [5].

CD70 is the natural ligand for the tumor necrosis factor (TNF) superfamily member CD27 and has originally been described as a co-stimulatory molecule for B cell and T cell activation [5,6]. Expression of CD70 in non-malignant tissue is primarily confined to cells of the hematopoietic system, yet mostly transient and tightly regulated [6–9]. CD70 is absent from human and murine naïve T cells, immature B cells and Natural Killer (NK) cells, but induced by T cell and B cell receptor triggering, B cell transformation or NK cell activation by interleukin-15 (IL-15) [10–14]. While absent from immature dendritic cells and neutrophils [13], CD70 expression can be induced on myeloid and plasmacytoid dendritic cells as well as Langerhans cells by e.g. toll-like receptor ligands in combination with CD40 ligation [9,15–17]. Constitutive CD70 expression has only been described on a population of antigen-presenting cells in the gut [18]. Interaction of CD70 with its receptor CD27 has originally been demonstrated to enhance the expansion, interferon-γ (IFN-γ) and IL-2 production and alloreactive cytotoxicity of T cells [11,12,19–21]. In addition, CD70 promotes survival of T cells responding to low-affinity or low-dose antigens [22,23].

Accordingly, CD27 is expressed on naïve and central memory CD4 and CD8 T cells as well as on thymocytes [5,21,24]. In addition, CD27 is expressed on mature B cells and CD70–CD27 signaling results in B cell expansion, differentiation into plasma cells and IgG synthesis [6,10,17,25,26]. In innate immunity, CD70–CD27 interactions induce proliferation and IFN-γ production (but not direct cytotoxicity) of human and murine NK cells in vitro and have been shown to mediate NK cell-dependent tumor rejection in mice [27–29]. CD27 is expressed on nearly all murine NK cells [27]. On human NK cells, CD27 is acquired during maturation in lymphoid organs but down-regulated in terminal maturation stages as for effector memory CD45RA^{+} T cells and plasma cells [24,25,30]. In peripheral blood, CD27^{+} NK cells are functionally closely related to CD56^{bright} NK cells [14].

In addition to its function and transient expression limited to innate and adaptive immune cells, abundant CD70 expression has been documented in B cell malignancies, chronic myelogenous leukemic stem and progenitor cells, and renal cell carcinoma [7,31–33]. Hence, CD70–CD27 interactions may possess additional functions in cancer cells such as triggering tumor progression or escape from immunosurveillance [34,35]. In addition, CD70 on cancer cells is an attractive candidate for targeted immunotherapy due to its restricted expression on non-malignant cells.

In this study, we sought to determine the expression of CD70 and CD27 in osteosarcoma as well as other (pediatric) solid cancers, and the correlation with clinical outcome.

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MATERIALS AND METHODS

Patient samples

Tumor samples derived from biopsies (obtained at the time of diagnosis, pre-chemotherapy) and resections of primary, local recurrent and metastatic tumors (all post-chemotherapy) from ten high-grade osteosarcoma patients were freshly frozen in 2-methylbutane at the Department of Pathology, Leiden University Medical Center. From five of these patients, six primary osteosarcoma cell cultures were generated from the tumor material as previously described [36,37]. An overview of tumor samples and primary cultures as well as clinicopathological details of osteosarcoma patients is summarized in Table 1. Tumor specimens were obtained and analyzed according to the ethical guidelines of the national organization of scientific societies (FEDERA, http://www.federa.org/gedragscodes-codes-conduct-en). CD70 gene expression was analyzed from a genome-wide gene profiling data base consisting of diagnostic biopsies of 83 high-grade osteosarcoma patients as previously published [2] (accessible online at http://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

Cell lines

Established osteosarcoma cell lines HOS, HOS-143b, OHS, OSA (SJSA-1), SAOS-2, U2OS, ZK-58 and Ewing sarcoma cell lines A673, CADO-ES, ET10, EW3, RD-ES, SK-ES-1, SK-N-MC, STA-ET1, STA-ET2.1, TC32, TC71, VH64 and WE68 were obtained from the EuroBoNeT cell line repository [38]. The Ewing sarcoma cell line IOR/BER was kindly provided by K. Scotlandi (Rizzoli Orthopaedic Institute, Bologna, Italy), L1062 was derived as previously described [37] and TC32 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). TC71 cells were cultured in IMDM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FSC), 100 U/ml penicillin and 100 ug/ml streptomycin (P&S) (all Invitrogen). All other osteosarcoma and Ewing sarcoma cell lines as well as the EBV-transformed B-LCL cell line 107 (established in our laboratory) were cultured in RPMI-1640 medium (Invitrogen) supplemented with FCS and P&S. Ewing sarcoma cell lines were grown in 0.1% gelatin coated tissue culture flasks. The neuroblastoma cell lines SJNB8, SKNFI, SKNBE, IMR32 (obtained from ATCC) were cultured in DMEM Glutamax I medium (high glucose) supplemented with FCS, P&S and 1% MEM-non-essential amino acids (Invitrogen); UKF NBL1 and UKF NBL4 (kindly provided by U. Koehl, Medical University of Hannover, Germany) in IMDM medium with FCS and P&S; and CHP126 (obtained from ATCC) in RPMI medium with FCS and P&S. The rhabdomyosarcoma cell lines RD (obtained from ATCC) and A204, TE671, RH30 and RH41 (obtained from DSMZ, Braunschweig, Germany) were cultured in DMEM medium with the above indicated supplements). All cell lines were negative for mycoplasma infection.

CD70 protein expression by flow cytometry

The following mouse anti-human monoclonal antibodies and mouse isotype control antibodies were used: CD70[PE] (Ki-24) (BD Biosciences, Franklin Lakes, NJ, USA) (this antibody produced comparable results to 2F2); CD70 2F2 (0.2 μg/ml; kindly provided by R.A.W. van Lier) and IgG1 (0.2 μg/ml; R&D Systems, Minneapolis, MN, USA) followed by the goat-anti mouse Ig[APC] secondary antibody (BD Biosciences). FACS measurements were performed with the

Table 1: CD70 expression and clinicopathological details of patient material

Patient code	Primary Tumor Biopsy			Primary Tumor Resection			Local Relapse Resection			Pulmonary Metastasis Resection			Patient clinicopathological data of primary tumor			
	code	tissue CD70	cell line CD70	code	tissue CD70	cell line CD70	code	tissue CD70	cell line CD70	code	tissue CD70	cell line CD70	Sex	Age	Primary Tumor site	Primary Histological subtype
88	L1372	+	n/a										M	16	femur	telangiectatic
363							L2792	-	-				M	17	femur	osteoblastic
369							L2531	+	+				F	31	humerus	fibroblastic
398	L2635	n/a	+	L2826	+	+	L3312	+	+				F	14	femur	conventional
404				L2857	n/a	-							M	46	humerus	conventional
407													M	9	tibia	chondroblastic
1				L47	-	n/a				L1021	-	n/a	M	25	humerus	n/a
6				L458	-	n/a	L1072	-	n/a				F	45	femur	periosteal/juxtacortical
29				L1045	-	n/a				L1020	-	n/a	M	23	femur	telangiectatic
47				L1046	-	n/a				L437	+	n/a	M	28	tibia	telangiectatic

n/a, not available; +, positive for CD70 expression; -, negative for CD70 expression

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FACSCalibur (BD Biosciences) and analyzed with the "BD Cell Quest ProTM" software (version 5.2.1). Indicated fold expression data are the ratio between the geometric mean fluorescence intensity (geoMFI) of CD70 2F2 and the geoMFI of the isotype control.

CD70 gene expression

CD70 gene expression was analyzed from genome-wide gene profiling data of osteosarcoma cell lines and patient's tumor specimens as previously published [2].

Immunohistochemistry (IHC) and Immunofluorescence (IF)

Sections of 4 µm of representative tumor cryosections of resection specimens (Table 1) and of B cell lymphoma control tissue were fixed in acetone at -20°C for 10 min (for IHC: supplemented with 0.3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) to inactivate endogeneous peroxidase), followed by incubation in 10% normal goat serum (Dako, Glostrup, Denmark) in PBS buffer to block non-specific antibody binding.

Immunohistochemical expression of CD70 was assessed using the mouse monoclonal anti-CD70 2F2 (IgG1, 0.16 µg/ml) antibody followed by a polyclonal goat anti-mouse/rabbit/rat IgG HRP-linker antibody conjugate (Brightvision, DPVO-110HRP; Immunologic, Duiven, the Netherlands) and DAB+Substrate Chromogen System (Dako) detection. All sections were examined with an Olympus BX41 microscope and Cell^B acquisition software (Olympus, Tokyo, Japan).

Immunofluorescent double-staining for CD3 and CD70 or CD3 and CD27 was performed with rabbit polyclonal anti-human CD3 (2.4 µg/ml; Dako), CD70 2F2 and mouse monoclonal anti-human CD27 137B4 (IgG1, 1:200; Novocastra, Leica Microsystems, Wetzlar, Germany) followed by goat anti-rabbit Alexa 488 or goat anti-mouse IgG1 Alexa 546 (1:300; Invitrogen, Carlsbad, CA, USA). All sections were examined with a Leica DM5000 fluorescence microscope and LAS-AF acquisition program (Leica, Solms, Germany).

Statistical analysis

Statistical analyses were performed with Graphpad Prism version 5.04 (La Jolla, CA, USA). A P-value of <0.05 was considered statistically significant.

RESULTS

Osteosarcoma cell lines exhibit highest expression of CD70 among pediatric solid cancer cell lines

CD70 membrane protein expression was investigated on established osteosarcoma, Ewing sarcoma, neuroblastoma and rhabdomyosarcoma cell lines by flow cytometry. Expression of CD70 was detected on five out of seven established osteosarcoma cell lines, on HOS, HOS-143b, OSA, SAOS-2 and U2OS cells but not OHS or ZK-58 cells (Figure 1, panel A). The cell line OSA exhibited the highest expression of CD70 amongst all cell lines tested which was of similar intensity as detected on EBV-transformed B cells (EBV–B-LCL). In addition, four out of six primary osteosarcoma cultures derived from five osteosarcoma patients (Table 1) were positive for CD70 expression (Figure 1 panel B).
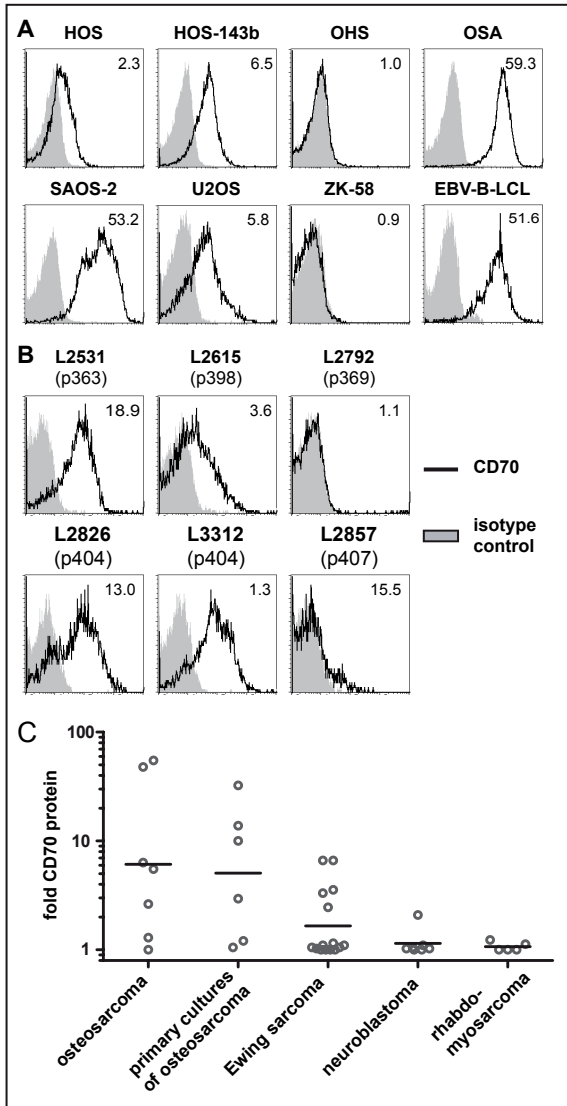
Figure 1: CD70 protein is abundantly expressed on osteosarcoma cells

CD70 protein expression on the surface of **(A)** established osteosarcoma cell lines and EBV-transformed B cell lymphoma (EBV-B-LCL) cells and **(B)** patient-derived primary osteosarcoma cultures (Table 1) was analyzed by flow cytometry. Fold change of geoMFI of specific antibody stainings (bold solid line) compared to geoMFI of isotype control (light shade) is indicated in the representative FACS histogram plots. **(C)** CD70 protein expression (fold change geoMFI) on the surface of established osteosarcoma cell lines and patient-derived primary osteosarcoma cultures (geometric mean of three experiments), and of established Ewing sarcoma (geometric mean of two experiments), neuroblastoma (one experiment) and rhabdomyosarcoma (one experiment) cell lines. In addition to the indicated osteosarcoma cell lines, CD70 expression was detected on the Ewing sarcoma cell lines CADO-ES, ET10, STA-ET2.1, VH64, WE68 and the neuroblastoma line CHP126.

Noteworthy, of one patient (patient (p) 404) with cultures from consecutive tumors, the culture derived from the local recurrent tumor exhibited equally high CD70 expression as the culture derived from the primary tumor of this patient. The level of CD70 expression on the primary cultures was similar to the CD70 levels on established osteosarcoma cell lines (Figure 1, panel C). In contrast to CD70, its receptor CD27 was not detected on any of the osteosarcoma cell lines (data not shown). Moreover, CD70 was detected on few established Ewing sarcoma cell lines (5/15) at lower intensities than on osteosarcoma cell lines, while CD70 was hardly detected on neuroblastoma (1/7) and rhabdomyosarcoma (0/5) cell lines (Figure 1, panel C).
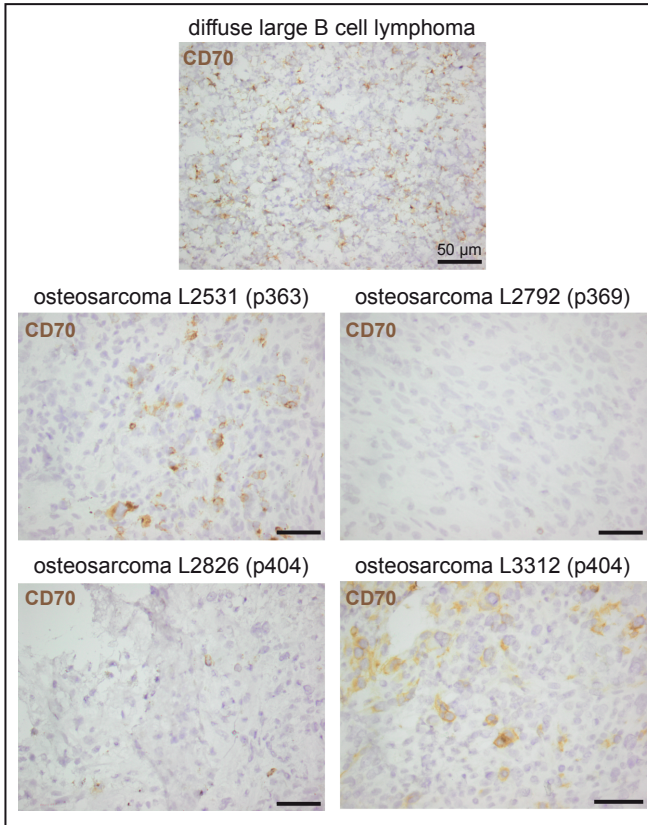
Figure 2: Heterogeneous CD70 protein expression in osteosarcoma lesions

Immunohistochemical CD70 protein staining in one specimen of diffuse large B cell lymphoma and four osteosarcoma specimens of three osteosarcoma patients (L2531 of p363; L2792 of p369; L2826 and L3312 of p404) (Table 1) Brown-colored CD70 staining patterns and counterstaining with Mayer's hematoxylin are displayed at a 40-fold magnification.

CD70^{+} primary osteosarcoma cultures are generated from parental tumors containing CD70^{+} cells

Since in particular most osteosarcoma cell lines and patient-derived primary osteosarcoma cultures exhibited high CD70 protein expression, it was examined whether CD70 was also expressed in (corresponding) osteosarcoma tumors. Detection of CD70 by immunohistochemistry in large tissue microarrays is hampered by the lack of commercially available anti-CD70 antibodies suitable for paraffin-embedded tissue [39]. Instead, CD70 detection in individual frozen specimens is feasible as tested and described for large diffuse B cell lymphoma tissue (Figure 2) [31]. Therefore, we analyzed and compared CD70 expression in available frozen specimens of our collection of parental tumor specimens (n=4) and corresponding primary cultures (Table 1). In addition, we compared CD70 expression between recurrent/metastatic lesions and primary tumors of the same patients (n=5) (Table 1).

Overall, CD70 expression tended to be more frequently detected in recurrent (2/4) and metastatic (1/3) tumors than in primary osteosarcoma tumors (1/5) (Table 1). CD70 was heterogeneously expressed in the tumors in a membranous pattern (Figure 2). Of the tumor-primary culture combinations, parental tissue of L2531 (p363) exhibited CD70-expressing cells in focal regions. The high CD70 intensity of these cells was consistent with the strong CD70 expression of the corresponding primary culture (Figure 1, panel B). Of L2792 (p369), CD70
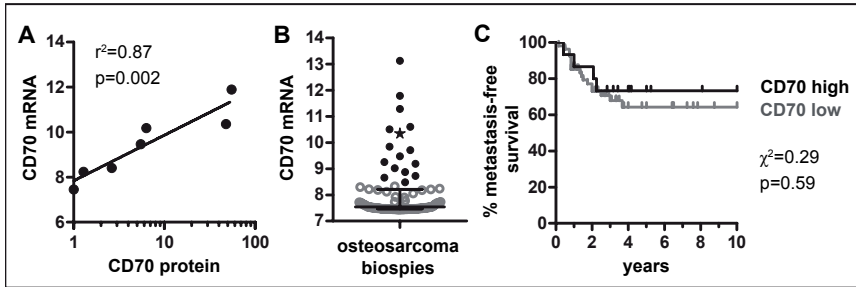
Figure 3: CD70 gene expression in osteosarcoma lesions does not influence survival
**(A)** CD70 protein expression (fold change geoMFI) on the surface of established osteosarcoma cell lines, as analyzed by flow cytometry (Figure 1), was correlated with relative CD70 gene (mRNA) expression of these cell lines, as analyzed by microarray. The regression coefficient (r^2) between CD70 protein and CD70 gene expression was 0.87 (*P*=0.002) as calculated by linear regression analysis. **(B)** CD70 relative gene (mRNA) expression in 83 biopsies of the primary tumor of 83 osteosarcoma patients depicted with the median and interquartile range. Elevated CD70 gene expression was evaluated as above the upper 25 percentile (16 patients, as indicated by the closed symbols). Asterix: biopsy L1372 of osteosarcoma patient 88 with high CD70 gene expression and available frozen tissue for immunohistochemical and immunofluorescent protein stainings as depicted in Figure 4. **(C)** Metastasis-free survival of osteosarcoma patients during ten years of follow-up comparing survival with high and low CD70 gene expression. Of the 83 patients (n= 16: CD70^{high} gene expression and n=67: CD70^{low} gene expression) depicted in panel B, 13 patients with metastasis at time of diagnosis were excluded, resulting in the inclusion of 70 patients (n=15: CD70^{high}, and n=55: CD70^{low}) for this analysis. Mantel-Cox univariate survival analysis resulted in a log-rank χ^2 score of 0.29 (*P*=0.59).

was neither detected in the parental tissue nor on the primary culture. Notably, of the one patient (p404) with tissue and primary cultures from consecutive tumors, a majority of cells of the local recurrent tumor (L3312) showed strong CD70 expression, whereas only a few cells were weakly positive in the primary tumor (L2826). Of this patient, the primary cultures of both tumors were strongly positive for CD70. In the residual four patients with combinations of consecutive tumor specimens, one metastatic tumor (L437, p47) contained CD70-expressing cells, while there were no CD70-expressing cells detectable in the primary tumor (L1046) of the same patient (Table 1).

Altogether, these results indicate that CD70 protein can be expressed in primary, recurrent and metastatic osteosarcoma lesions. CD70 expression was demonstrated on the majority of primary cultures and cell lines and only detected on primary cultures if these were derived from a tumor specimen containing CD70^{+} cells, indicating that CD70 expression was not caused by in vitro cell culture. Thus, these results suggest that CD70^{+} cells in the tumor preferentially grow out to CD70^{+} patient-derived cultures.

CD70 expression in osteosarcoma lesions is confined to tumor cells and does not influence patient survival

To determine whether CD70 expression on tumor cells would be associated with clinical outcome of patients with osteosarcoma, we needed to investigate CD70 expression levels in a large cohort of patients with data on follow-up. For this purpose, we wanted to use a public dataset on gene (mRNA) expression of a large collection of osteosarcoma biopsies.

Therefore, it was first investigated whether CD70 mRNA expression correlated with protein expression in osteosarcoma cell lines. CD70 protein expression in osteosarcoma cell lines indeed correlated with CD70 mRNA expression in these cells lines (r^2=0.87, *P*<0.002) (Figure 3, panel A).

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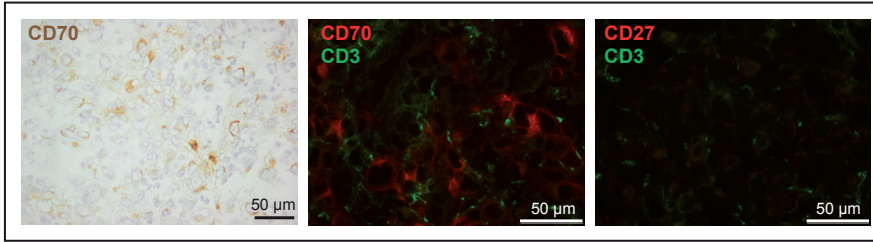
Figure 4: CD70 protein expression in osteosarcoma lesions is expressed on tumor cells and not on tumor-infiltrating T cells

Immunohistochemical stainings for CD70 and immunofluorescent double stainings for CD70 (red) and CD3 (green) as well as CD27 (red) and CD3 (green) (cell nuclei in blue) was assessed in sequential specimens of the osteosarcoma biopsy L1372 (p88) with high CD70 gene expression as indicated by the asterix in Figure 3, panel A.

Next, CD70 gene expression was evaluated in diagnostic biopsies of 83 osteosarcoma patients. A subset of patients (16/83) showed significantly higher CD70 gene expression (above the upper 25 percentile as indicated) (Figure 3, panel B). Of note, high CD70 gene expression corresponded to strong CD70 protein staining in the tumor biopsy as assessed for one patient (p88) with available frozen tissue (L1372) (Figure 4, panel A; patient (p88) indicated by * in Figure 3, panel A).

To investigate the association of CD70 expression by tumor cells with clinical outcome, we first assessed whether CD70 was expressed by tumor cells and not by tumor-infiltrating T cells in this biopsy with high CD70 gene/protein expression (L1372, p88), since activated T cells are known to express CD70 [13]. Strong immunofluorescent staining for CD70 was confined to tumor cells and did not co-localize with the T cell marker CD3 (Figure 4, panel B). In contrast to CD70, its receptor CD27 was detected neither in CD70+ nor CD70- osteosarcoma lesions and was not expressed by infiltrating T cells (Figure 4, panel C and data not shown).

Next, it was determined whether higher CD70 gene expression was correlated with the occurrence of metastasis, which is the most important parameter for survival of osteosarcoma patients. Thirteen patients presenting with metastasis at time of diagnosis were excluded from this analysis. In the remaining 70 patients, the occurrence of overt metastasis (metastasis-free survival) during ten years of follow-up was not statistically different between patients with high or low CD70 gene expression (log-rank χ^2=0.29, P=0.59) (Figure 3, panel C). As a reference, CD14 gene expression of the same patient cohort was substantially higher, and median CD14 expression was positively correlated with metastasis-free survival in this specific cohort (log-rank χ^2=7.48, P=0.006) as we have similarly previously reported (data not shown) [2]. There was no association between CD70 gene expression and a specific tumor location or histological subtype (data not shown).

In conclusion, high CD70 expression in osteosarcoma tumors is not associated with metastasis-free survival.

DISCUSSION

We demonstrate that CD70 is expressed on the majority of osteosarcoma cell lines as well as patient-derived osteosarcoma cultures which were derived from CD70^{+} tumor tissue. CD70 expression in tumor lesions was heterogeneous and restricted to tumor cells and not attributed to infiltrating T cells. CD70 gene expression in diagnostic biopsies was significantly higher in a subset of osteosarcoma patients but this difference was not correlated with metastasis-free survival.

Among pediatric bone and soft tissue cancers, we found that CD70 is preferentially expressed on osteosarcoma cells (9/13). CD70 was detected on few Ewing sarcoma cells (5/15) and was virtually absent from neuroblastoma and rhabdomyosarcoma cells, indicating that CD70 expression is restricted to certain cancer types like renal cell carcinoma and osteosarcoma and to a lesser extent brain cancers, larynx or pharynx cancer, melanoma, pancreatic cancer, ovarian carcinoma and Ewing sarcoma [7–9,33,34,39–41]. Among the osteosarcoma specimens we tested, CD70 expression was heterogeneous between patients as well as within the tumor. In a larger cohort of 83 osteosarcoma patients, a subset of tumor biopsies (19%) showed significantly higher CD70 gene expression.

This raises the question whether CD70 exerts a biological function in certain cancer types or certain cancer cells. In some tumor-transformed B cells, CD70 (downstream) signaling may affect proliferation and apoptosis, [31,34,42]. In spite of this, in most studies, biological effects of CD70 have been demonstrated to be mediated by interactions of CD70 with its receptor CD27, which can have a dual role in regulating anti-tumor adaptive immune responses. Acute challenge with CD70^{+} tumor cells was shown to induce anti-tumor T cell-mediated immunity [29,43]. Similarly, CD70–CD27 interactions promote anti-virus and anti-tumor T cell responses [44–47]. On the contrary, co-culture of CD70^{+} tumor cells and CD27^{+} immune cells was shown to inhibit alloreactive T cell proliferation and induce T cell apoptosis [34,43,48,49]. Similarly, continuous CD70–CD27 co-stimulation in CD70-transgenic mice or in mice chronically infected with mouse choriomeningitis virus exhausts the naïve T cell pool in favor of effector memory T cells that ultimately results in T cell dysfunction unless relieved from the CD70–CD27 brake [45,50,51]. In line with the latter observations, CD70^{+} tumor cells in renal cell carcinoma and B cell lymphoma may promote depletion of naïve CD27^{+} T cells and induce regulatory T cells in the tumor, respectively [52,53].

Hence, during a persistent tumor–immune cell interaction, CD70 expression on tumor cells may support tumor escape from immunosurveillance and thus be disadvantageous for patient outcome. However, to our knowledge, a significant association of CD70 expression on tumor cells with patient survival has not been described in these human tumors [41]. In our cohort of osteosarcoma patients, CD70 gene expression at the time of diagnosis did not correlate with metastasis-free survival, suggesting that at least in osteosarcoma, CD70 expression does not promote tumor progression. Moreover, CD27 was neither detected on tumor cells nor on infiltrating T cells in osteosarcoma lesions, suggesting that CD70 on tumor cells is not involved in CD27-dependent tumor–immune cell interactions. In spite of the ambiguous role of CD70 in cancer development and progression, its substantial expression in certain cancers would make CD70 an attractive immunotherapeutic target. This is supported by the highly restricted

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expression of CD70 on immune cells and its virtual absence in normal non-hematopoietic tissue, which may limit side-effects of CD70 targeted treatment [7,9,39]. In this perspective, chimeric antigen receptor T cells (CD27-CD3ζ) have been reported to kill CD70Ztumor cells and mediate tumor regression in mice [54]. Moreover, therapeutic chimeric and humanized anti-CD70 antibodies are currently developed. In preclinical studies, anti-CD70 antibodies demonstrated indirect anti-tumor efficacy mediated by NK cells and macrophages involving antibody-dependent cellular cytotoxicity and tumor cell phagocytosis, respectively, as we have similarly recently reported using the clinically-approved anti-epidermal growth factor receptor antibody cetuximab [55-58] (this thesis, chapter 3, 4 and 5). In addition, if conjugated to cytotoxic drugs anti-CD70 antibodies can mediate direct anti-tumor effects [7,39,59]. Since we had no access to chimeric or humanized anti-CD70 antibodies, we could not test direct or indirect cytotoxicity against CD70-expressing osteosarcoma cells. Altogether, in osteosarcoma patients with CD70-postive tumors, CD70 may constitute an attractive candidate for novel targeted immunotherapy.

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CONCLUDING CHAPTER 7

Discussion and Future Directions

Towards Harnessing NK cells and Macrophages for Immunotherapy against Bone Sarcomas

CONTENTS

7

Immune cells play a key role in the control of cancer development, referred to as cancer immunosurveillance. Cancer cells are rapidly dividing cells with inherent genomic instability which enables their potential to produce more or less immunogenic variants. In association with the pressure of immunosurveillance, this process favors the selection of cancer cells which are able to evade immune responses. Cancer escape is supported by the immunosuppressive milieu of the cancer microenvironment, restricting anti-cancer innate and adaptive immune responses [1;2].

As documented in this thesis and in addition to T cell-mediated anti-cancer responses, NK cells and macrophages have the potential to kill cancer cells and interfere with cancer cell growth, respectively. In view of counteracting tumor escape and immunosuppression and optimizing the anti-cancer potential of NK cells and macrophages against osteosarcoma and Ewing sarcoma, the following points are discussed below. **(1)** The cytolytic potential of NK cells may need to be improved and directed to cancer cells in such a way that it can resist inhibitory influences. **(2)** The anti-cancer potential of macrophages may need to be activated and improved. **(3)** It would be favorable to convert the immunosuppressive cancer microenvironment to a pro-inflammatory, immune cell-attracting milieu.

1. How to Enhance and Direct NK cell Cytotoxicity to Cancer Cells and Circumvent Immunosuppression?

In **chapter 3** it is demonstrated that the cytotoxic activity of NK cells can be effectively enhanced and directed to osteosarcoma and Ewing sarcoma cell lines if the cancer cells are coated with a therapeutic antibody like cetuximab, targeting the epidermal growth factor receptor (EGFR) on the cancer cell surface. In the presence of cetuximab, FcγRIII/CD16–mediated NK cell cytotoxicity (ADCC) against autologous osteosarcoma cells was substantially enhanced and as effective as by cytokine (IL-15)–activated NK cells. Low EGFR expression levels on the cancer cell surface were sufficient to trigger ADCC by NK cells.

However, despite their susceptibility to NK cell cytotoxicity in short-term NK cell-tumor cell interactions (in particular at high NK–tumor cell ratios), bone sarcoma cells may be able to inhibit NK cell activation and functionality during prolonged interactions (at low NK–tumor cell ratios). In **chapter 4** it is illustrated that prolonged physical contact with certain sarcoma cell lines can trigger down-regulation of NK cell activating receptors, even in the presence of the NK cell activator IL-15. This inhibiting effect resulted in the impairment of NK cell cytolytic activity mediated through these receptors, and inhibited lysis of osteosarcoma and Ewing sarcoma cells. Notably, NK cells pre-activated with IL-15 for five days prior to sarcoma cell interaction were resistant to the immunosuppressive effects of sarcoma cells. Moreover, the FcγRIII/ CD16–mediated antibody-dependent cytotoxic potential of NK cells remained functional after prolonged sarcoma cell interactions. The membrane-bound factors expressed by some but not all sarcoma cells, conferring selective inhibition of NK cell functionality and leaving ADCC intact, remain to be further investigated. This might be achieved by manipulating the expression of known/unknown inhibitory genes/proteins using for instance RNA silencing techniques and subsequent screening for effects on NK cell function in response to gene-modified target cells. The identification of these inhibitory genes and proteins might result in the design of small molecules or antibodies targeting these genes/proteins to block the inhibitory effects on NK cell functionality.

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The results of these two chapters imply that NK cell cytotoxicity may need to be **(a)** enhanced by cytokine stimulation prior to cancer cell encounter and/or **(b)** triggered by therapeutic antibodies to circumvent immunosuppressive influences of the cancer microenvironment.

➔ **(a)** Cytokine treatment alone may be insufficient to enhance NK cell function in vivo and potentially toxic [3]. NK cells with improved anti-cancer reactivity could be introduced by adoptive transfer, as previously demonstrated using ex vivo IL-2–activated, haploidentical NK cells in patient with hematological malignancies [4;5]. This approach has not been reported to achieve objective responses in patients with solid cancers [3]. In fact, in the case of autologous NK cells which are more prone to be inhibited by KIR-ligand interactions, adoptively-transferred IL-2–pre-activated NK cells have been shown to lose cytolytic activity in recipients with melanoma [6]. Thus, to prolong functional NK cell persistence in the cancer microenvironment, NK cells need to be sufficiently pre-activated. It remains to be determined whether this could be achieved by IL-15 which rendered NK cells resistant to sarcoma-mediated inhibition in vitro as noted above. In this perspective, IL-15 is known to be an important factor for NK cell activation and NK cell survival and IL-15–activated NK cells have been shown to mediate tumor ablation in murine studies [7;8]. Other studies have recently suggested that functional NK cell persistence after adoptive transfer in mice can be further improved by combination of IL-15 with IL12+IL18 +/- IL-2 [9;10].

➔ **(b)** Application of cancer-reactive antibodies like cetuximab might sustain NK cell cytotoxicity since ADCC remained functional after contact with sarcoma cells. Induction of ADCC has recently been proposed to trigger NK cell cytotoxicity in an otherwise immunosuppressive cancer microenvironment in melanoma patients [6]. Also, induction of ADCC may improve lysis of cancer cell variants poorly sensitive to antibody-independent NK cell cytotoxicity [11]. Notably, induction of ADCC by NK cells against sarcoma cells increased the activation status of NK cells, which may mobilize and improve NK cell functionality *in vivo* as suggested in previous studies [12-14]. Furthermore, NK cell activation by cetuximab-coated cancer cells can induce IFN-γ production which has been shown to stimulate dendritic cell maturation and priming of tumor antigen-restricted (EGFR and MAGE-3) CD8 T cell responses *in vitro*, associated with EGFR-specific CD8 T cell expansion after cetuximab therapy in patients with head-neck cancer [15]. Thus, in response to antibody therapy, NK cells not only have the potential to directly kill cancer cells but may also contribute to the generation of systemic anti-cancer Th1 responses which could potentially be directed against a broad repertoire of naturally-selected cancer antigens. As compared to T cells, NK cells have limited capacity for proliferation and survival, restricting eradication of large tumors by NK cells. Thus, the contribution of NK cells in the initiation of potent anti-cancer adaptive immunity may be critical for tumor control and the clinical success of antibody therapy as recently highlighted in preclinical studies [16-18].

In **chapter 6** it is documented that the immunoregulatory protein CD70 is expressed on some (primary) osteosarcoma cell lines and in (corresponding) tumor lesions of osteosarcoma patients. CD27, the receptor for CD70, was not detected on cancer cells or infiltrating T cells in osteosarcoma lesions, suggesting that CD70-CD27 interactions are not involved in interactions between CD70+ tumor cells and CD27+ immune cells in osteosarcoma. Overall, since CD70 expression on normal cells is very restricted and transient, molecules like CD70 may act as

additional but more specific target antigens for direct or indirect immunotherapeutic approaches, for instance via anti-CD70 antibodies, with a reduced risk for off-target adverse effects.

2. How to Improve Anti-Cancer Activity of Macrophages?

Macrophages frequently infiltrate solid cancers, and in osteosarcoma macrophage infiltration is correlated with favorable patient outcome [19], suggesting anti-cancer activity of macrophages in osteosarcoma. In **chapter 5** it is demonstrated that M1 macrophages are able to inhibit osteosarcoma cell growth if activated with a bacterial stimulus like liposomal muramyl tripeptide (L-MTP-PE). Importantly, induction of anti-cancer activity by L-MTP-PE required the presence of IFN-γ, a notion which is relevant for improving the outcome of currently applied L-MTP-PE therapy in osteosarcoma patients [20]. In addition, it is described that IL-10–polarized M2 macrophages, which are considered to support cancer development, have the capacity to inhibit growth of some osteosarcoma cells if directed to antibody (cetuximab)-coated cancer cells.

Thus, different types of macrophages, which are already present in osteosarcoma lesions, can be manipulated to exert anti-cancer responses if activated through factors reminiscent of an acute infection in the cancer microenvironment and/or in combination with therapeutic antibodies. It is noteworthy that the critical macrophage-priming stimulus IFN-γ could be provided by NK cells, since it has been shown that NK cells can produce significant IFN-γ amounts in response to cytokine activation and presumably also in response to therapeutic antibodies [9;15]. Hence, antibody therapy can mobilize multiple (innate) immune cells such as NK cells and macrophages which may cooperate in anti-cancer responses.

3. How to Disrupt Immunosuppression and Improve Tumor-Infiltration by NK cells?

To harness the cytotoxic potential of NK cells for immunotherapy, NK cell functionality needs to be modulated to withstand inhibitory influences of the cancer microenvironment. Alternatively, the immunosuppressive character of the cancer microenvironment needs to be abolished. An acute infection or infection-mimicking immunostimulators may facilitate the conversion of an immunosuppressive cancer microenvironment to a more pro-inflammatory milieu that supports recruitment and anti-cancer activity of immune cells [21;22]. In **chapter 2** it is described that NK cells can become activated in response to an adenoviral infection, in particular adenovirus type 35. Phenotypic and functional activation of NK cells was reciprocally mediated by toll-like receptor 9–dependent IFN-α production by plasmacytoid dendritic cells upon adenovirus exposure. Thus infections or constituents of infectious agents may not only induce anti-cancer activity of macrophages (chapter 5) but also rescue and enhance NK cell activation in interplay with other (innate) immune cells. Oncolytic viruses selectively infect and kill cancer cells, especially when shielded from neutralizing antibodies and delivered by tumor-homing cells; this can trigger recruitment and activation of NK cells and other immune cells, inducing anti-cancer (but also non-tumor–directed anti-virus) innate and adaptive immune responses with the potential for objective responses in patients [23-29]. Thus, establishing an infection in the cancer microenvironment by oncolytic viruses which are able to infect and kill bone sarcoma cells may facilitate tumor elimination, increase the immunogenicity of the cancer microenvironment and enable cancer immunosurveillance. Finally, if able to resist inhibition, NK cell cytotoxicity itself could initiate tumor elimination which may counteract immunosuppression.

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For the success of NK cell-based immunotherapy it will be necessary that NK cells can migrate to and accumulate in the cancer microenvironment. However, since NK cell infiltration in solid cancers including osteosarcoma is generally poor [30-34] (and unpublished observation), future studies need to address how the infiltration of NK cells into the cancer microenvironment can be improved. Oncolytic viruses could be modified to over-express chemokines such as CCL5, CX3CL1, and CXCR3 ligands, which have been shown to regulate tumor infiltration and anti-cancer responses of NK cells in murine studies [35-37]. In particular, chemerin has recently been reported to be down-regulated in several human solid cancers in association with dismal prognosis, while over-expression of chemerin in murine tumors can improve NK cell infiltration and NK cell-dependent tumor ablation [38]. Moreover, it has been shown that antibody-based immunotherapy in patients with breast cancer (trastuzumab) and B cell malignancies (rituximab) and tyrosine kinase inhibitor-based immunotherapy in patients with gastrointestinal stromal tumors can mediate mobilization and tumor infiltration of NK cells *in vivo* [13;32;39]. In murine studies it has recently been demonstrated that novel anti-EGFR antibodies can induce tumor-infiltration of NK cells (and macrophages) with superior efficacy than cetuximab [40].

4. Future Directions

To better understand the biological relevance of NK cells and ADCC for anti-tumor effects in response to antibody (e.g., cetuximab) therapy, the effect of NK cell depletion (and adoptive transfer) may be elucidated in combination with analysis of the activation status of tumor-infiltrating and peripheral NK cells as well as NK cell accumulation in (metastatic) tumor lesions. For these purposes murine xenograft or syngeneic mesenchymal stem cell–induced osteosarcoma models, the latter more closely resembling human osteosarcoma [41], could be employed. These models may allow to explore the effect of an established (immunosuppressive), bone-associated cancer microenvironment, as encountered by NK cells in patients, on NK cell functionality and the ability of NK cells to eliminate large solid tumor masses as compared to treatment-ablated minimal tumor numbers (minimal residual disease).

In addition, a contribution of macrophages to tumor elimination in response to antibody therapy and/or activation by L-MTP-PE (+/- IFN-γ) could be clarified in these osteosarcoma models. It may be investigated whether these activating stimuli can influence macrophage polarization and macrophage recruitment to the tumor. In future clinical studies, it could be determined whether treatment with L-MTP-PE results in more pronounced clinical responses in osteosarcoma patients with higher macrophage infiltration in tumor lesions. It should be addressed whether L-MTP-PE therapy can be improved by the presence of endogenously-induced or exogenously-delivered IFN-γ (e.g., by integration in MTP-PE–containing liposomes) and how this treatment affects the phenotype and infiltration of macrophages in (consecutive metastatic) tumor lesions.

In view of harnessing the antibody-dependent cytotoxic function of NK cells, it will be of interest to determine whether NK cells penetrating bone sarcomas retain functional FcγRIII/CD16 expression as observed for NK cells from peripheral blood of osteosarcoma patients. To delineate NK cell responses to antibody therapy in patients, it would be of interest to explore the distribution as well as phenotypic and functional activation of NK cells in blood and (consecutive metastatic) tumor lesions of patients with osteosarcoma or other solid cancers. Since tumor infiltration of NK cells needs to be improved, it will be of interest to investigate whether

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(a) therapeutic antibodies, inducing ADCC and NK cell activation like anti-EGFR cetuximab, (b) cytokine-induced NK cell activation or (c) even the application of oncolytic viruses can modulate NK cell trafficking to bone sarcoma lesions using murine osteosarcoma models and by analyzing patient tumor specimens.

Overall, to exploit the cytotoxic potential of NK cells against bone sarcomas, antibody therapy should be scheduled in the presence of endogenous or pre-activated adoptively-transferred NK cells to sustain NK cell functionality, which might contribute to the generation of potent and enduring anti-cancer adaptive immunity. Finally, NK cell anti-cancer function may be more effective in a less or non-immunosuppressive cancer microenvironment. Thus, NK cells may be harnessed to eliminate residual cancer cells which have survived after conventional anti-cancer therapies.

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SUMMARY OF THIS THESIS

Introduction

Osteosarcoma and Ewing sarcoma are the most common bone cancers in children and young adults. Despite advanced surgical techniques and multi-drug chemotherapy, one-third of the patients still succumb to recurrent disease with poor prognosis. Likewise, patients with metastatic and chemotherapy-resistant disease have a poor outcome. Thus, novel targeted therapies are needed that combine potent and specific anti-cancer activity with limited toxicity toward normal tissues.

Previous research lines have provided evidence that natural killer (NK) cells and macrophages, both cell types of the innate immune system, are able to contribute to anti-cancer responses against osteosarcoma and Ewing sarcoma cells. NK cells are able to eliminate virus-infected and tumor-transformed cells and can support adaptive immune responses. The cytotoxic function of NK cells is not MHC class I-restricted; thus, while being able to evade cytotoxic T lymphocyte responses, tumor cells with reduced MHC class I expression can be recognized by NK cells. NK cells are additionally attractive for the design of anti-tumor immunotherapies, since NK cells have not been associated with severe adverse effects such as graft-versus-host disease if therapeutically introduced into patients. During cancer development, cancer cells are presumed to evolve immune escape variants, a process which is supported by the immunosuppressive milieu of the cancer microenvironment, inhibiting anti-cancer immune responses. Thus, to harness NK cells and macrophages for immunotherapeutic approaches, their anti-cancer potential may need to be induced in such a way that it can resist inhibitory influences.

In the **introductory chapter 1**, clinical and biological properties of osteosarcoma and Ewing sarcoma are discussed followed by an overview of cancer immunology and immunotherapy. The results of the preclinical studies described in the **chapters 2–6** are further discussed in the **concluding chapter 7**, addressing implications for anti-cancer immunotherapeutic strategies involving NK cells and macrophages.

Results

In **chapter 2,** an experimental model for measuring NK cell activation is explored in response to a prototypical viral infection by adenovirus type 5 (HAdV5) and HAdV35. It is described that increased expression of the NK cell activation marker CD69 and enhanced NK cell cytotoxic activity in response to HAdV5 relied on the contribution of T cells and IL-2. In contrast, NK cell activation in response to HAdV35 occurred in the absence of T cells and was mediated by the significantly higher production of interferon-α by pDC. Interferon-α production by pDC was dependent on toll-like receptor-9 signaling and was enhanced by the reciprocal interaction of NK cells and pDC.

In the following research chapters it is explored how the anti-cancer potential of NK cells and macrophages can be enhanced and directed to osteosarcoma and Ewing sarcoma cells. In **chapter 3** it is shown that the cytotoxic activity of NK cells can be more specifically directed to sarcoma cell lines if the cancer cells are coated with a therapeutic antibody like cetuximab, targeting the epidermal growth factor receptor (EGFR) on the cancer cell surface. In the presence

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of cetuximab, FcγRIII/CD16-mediated, antibody-dependent NK cell cytotoxicity (ADCC) against autologous osteosarcoma cells was substantially enhanced and as effective as by cytokine (IL-15)–activated NK cells. ADCC by NK cells required only minimal EGFR expression on the cancer cell surface. Thus, NK cells are able to exert substantial cytolytic activity against osteosarcoma and Ewing sarcoma cell lines as demonstrated in short-time 4-hour cytotoxicity assays.

However, as illustrated in **chapter 4**, prolonged two-day sarcoma–NK cell interactions, potentially occurring in the cancer microenvironment *in vivo*, can alter NK cell functionality. It is shown that certain sarcoma cell lines can trigger down-regulation of NK cell-activating receptors, such as NKG2D, DNAM-1 and NKp30, even in the presence of the NK cell activating cytokine IL-15. This inhibiting effect was dependent on physical contact and resulted in impaired NK cell cytotoxicity mediated through these receptors, inhibiting lysis of osteosarcoma and Ewing sarcoma cells. Notably, when activated with IL-15 for five days prior to sarcoma cell interaction, these pre-activated NK cells were resistant to the immunosuppressive effects of sarcoma cells. Moreover, the FcγRIII/CD16-mediated antibody (cetuximab)-dependent cytotoxic potential of NK cells remained functional after prolonged sarcoma cell interactions. Hence, a combination of cytokine activation with therapeutic antibodies may improve and sustain the capacity of NK cells to contribute to cancer cell elimination in a potentially immunosuppressive cancer microenvironment.

Macrophages frequently infiltrate solid cancers. In osteosarcoma, macrophage infiltration is correlated with favorable patient outcome, suggesting anti-cancer activity of macrophages in osteosarcoma. In **chapter 5** it is demonstrated that human M1-type macrophages are able to inhibit osteosarcoma cell growth if activated with a bacterial stimulus like liposomal muramyl tripeptide (L-MTP-PE). Importantly, induction of anti-cancer activity by L-MTP-PE required the presence of IFN-γ, a notion which is relevant for improving the outcome of currently applied L-MTP-PE therapy in osteosarcoma patients. IL-10–polarized M2 macrophages, which are considered to support cancer development, were able to inhibit growth of some osteosarcoma cells if directed to antibody (cetuximab)-coated cancer cells. Hence, tumor-infiltrating macrophages may be manipulated to exert anti-cancer responses if activated through factors reminiscent of an infection in the cancer microenvironment and/or by the application of cancer cell-reactive therapeutic antibodies.

In **chapter 6**, it is documented that the immunoregulatory protein CD70 is expressed on the majority of osteosarcoma cell lines and patient-derived osteosarcoma cultures, whereas only few Ewing sarcoma cell lines expressed CD70. CD70 expression in primary and recurrent osteosarcoma lesions was heterogeneous and restricted to tumor cells and not attributed to tumor-infiltrating T cells. CD27, the receptor for CD70, was expressed neither on tumor cells nor on tumor-infiltrating T cells in CD70-positive or CD70-negative osteosarcoma lesions. CD70 gene expression did not correlate with the occurrence of metastasis and survival of osteosarcoma patients. Since CD70 expression in normal tissue is restricted and transient, CD70 may represent a novel, more cancer-specific target for anti-cancer immunotherapy in patients with CD70 positive tumors.

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Conclusion

The research performed in this thesis demonstrates that human NK cells and macrophages have the potential to exert anti-cancer responses if sufficiently activated by immunostimulators, and directed to osteosarcoma and Ewing sarcoma by therapeutic antibodies. These findings may help to improve currently applied therapies and design novel immunotherapeutic strategies which counteract potential cancer escape and immunosuppression and contribute to cancer cell elimination. For an effective contribution of NK cell-based immunotherapy to cancer cell elimination, it will be important to further investigate how tumor cell escape from immune control can be overcome and how the migration and penetration of NK cells into the cancer microenvironment can be enhanced.

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SAMENVATTING VAN HET PROEFSCHRIFT

Inleiding

Osteosarcoom en Ewing-sarcoom zijn de meest voorkomende kwaadaardige bottumoren bij kinderen en jong volwassenen. Ondanks chirurgische verwijdering van de tumor in combinatie met hoge dosis combinatie chemotherapie, zal een derde van de patiënten recidiveren waarna de prognose slecht is. Ook patiënten met gemetastaseerde tumoren of tumoren die niet reageren op chemotherapie, hebben slechte overlevingskansen. Nieuwe behandelmethoden, die specifiek tegen de tumorcellen maar niet tegen gezonde weefsels gericht zijn, dienen ontwikkeld te worden.

In eerder onderzoek was aangetoond, dat Natural Killer (NK) cellen en macrofagen, beide behorende tot het aspecifieke immuunsysteem, anti-tumor activiteit bezitten tegen osteosarcoom en Ewing-sarcoom cellen. NK-cellen zijn in staat virus-geïnfecteerde cellen en kankercellen te lyseren. Daarnaast kunnen NK-cellen de activatie van het specifieke immuunsysteem ondersteunen. Tumorcellen ontsnappen soms aan herkenning door cytotoxische T-lymfocyten door de expressie van MHC klasse I moleculen te verlagen. NK-cellen daarentegen herkennen juist cellen die geen of weinig MHC klasse I tot expressie brengen. Omdat er tot op heden nog geen relatie tussen NK-cel therapie en klinisch ongunstige effecten zoals graft-versus-host ziekte en toxiciteit is gerapporteerd, zijn NK-cellen een aantrekkelijke optie om immunotherapie op te baseren.

Voor een effectieve immunotherapie is het verder nog noodzakelijk dat de effector cellen ongevoelig zijn voor de immuun-onderdrukkende eigenschappen van de tumor. Zowel de tumor cellen zelf als het stromale weefsel rondom de tumor kunnen de effector cellen onderdrukken of ontsnappen aan herkenning.

In het **inleidende hoofdstuk 1** worden de klinische en biologische kenmerken van het osteosarcoom en Ewing-sarcoom besproken. Aansluitend volgt er een overzicht waarin het veld van de tumorimmunologie en de verschillende immuuntherapieën wordt beschreven. De resultaten van de preklinische studies, beschreven in de **hoofdstukken 2–6**, worden in het **afsluitende hoofdstuk 7** samengevat, met daarbij de implicaties voor immuuntherapieën die op NK-cellen en macrofagen gebaseerd zijn.

Resultaten

In **hoofdstuk 2** wordt een experimenteel model behandeld om de activiteit van NK-cellen te meten, gestimuleerd door twee verschillende serotypen van het adenovirus. Wanneer bloedcellen geïnfecteerd werden met humaan adenovirus type 5 (HAdV5), was zowel de verhoogde expressie van de NK-cel activatie marker CD69 als de hogere cytotoxische activiteit van NK-cellen, afhankelijk van T-cellen en met name hun interleukine-2 productie. NK-cellen gestimuleerd door infectie van bloedcellen door een ander serotype, namelijk HAdV35, werden daarentegen onafhankelijk van T-cellen geactiveerd. In dit geval werd NK-cel activatie geïnduceerd door verhoogde productie van interferon-α door plasmacytoïde dendritische cellen (pDC). De pDC herkenden het virus-DNA via hun DNA-sensor, toll-like receptor 9. Bovendien werd de interferon-α productie versterkt door de wederzijds interactie tussen pDC en NK-cellen.

In **hoofdstuk 3** is uitgezocht hoe het cytotoxische potentieel van NK-cellen tegen osteosarcoom en Ewing-sarcoom cellen versterkt en specifieker gericht kan worden. Hier wordt beschreven dat

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sarcoomcellen beter herkend en gelyseerd kunnen worden door NK-cellen als de tumorcellen met therapeutische antilichamen zoals cetuximab (gericht tegen de receptor voor epidermale groei factor (EGFR)) beladen zijn. In aanwezigheid van cetuximab was de cytotoxische activiteit van NK-cellen, ook die van patiënten die een osteosarcoom hebben, significant verhoogd tegen autologe EGFR-postieve osteosarcoom cellen, dat wil zeggen tumorcelkweken die uit dezelfde patiënten gegenereerd zijn. De cytotoxiciteit van NK-cellen, gemedieerd door cetuximab, was even effectief als die van NK-cellen, die gestimuleerd waren met het sterk activerende cytokine interleukine-15 (IL-15). Zelfs bij lage hoeveelheden EGFR op de tumorcellen, was de lyserende werking van NK-cellen reeds maximaal; er werd geen dosis-effect relatie gevonden.

Op deze manier zijn NK-cellen in staat om substantiële cytotoxische activiteit tegen osteosarcoom en Ewing-sarcoom cellijnen uit te oefenen, zoals aangetoond in de korte vier uur durende chroom-release experimenten. Maar, zoals beschreven in **hoofdstuk 4**, kunnen langdurige interacties (zoals twee dagen) tussen sarcoomcellen en NK-cellen, die mogelijk bij de patiënt in het omliggende tumorweefsel plaatsvinden, de activiteit van NK-cellen tegen sarcomen verminderen. Sommige sarcoom cellijnen kunnen down regulatie van activerende receptoren zoals NKG2D, DNAM-1 en NKp30 op NK-cellen veroorzaken, zelfs in aanwezigheid van het sterk activerende cytokine IL-15. Deze lagere expressie van receptoren werd gemedieerd door fysiek contact tussen de tumorcel en de NK-cel, en had als gevolg dat de cytotoxische activiteit van NK-cellen verminderde, dus een remmend effect had op de functie van NK-cellen. Opvallend was dat de antilichaam-gemedieerde cytotoxische capaciteit van NK-cellen ongevoelig was voor de remming door sarcoomcellen. Wanneer de NK-cellen vóórdat ze in contact kwamen met de sarcoomcellen met IL-15 geactiveerd waren, was hun cytotoxische activiteit bestand tegen de remmende effecten van de tumorcellen. Een combinatie van NK-cel activatie door middel van cytokinen en toevoeging van therapeutische antilichamen zou de functionaliteit van NK cellen in patiënten zodanig kunnen versterken dat eliminatie van tumorcellen, ondanks inhiberende factoren van de tumor(omgeving), effectief blijft.

In solide tumoren is vaak infiltratie te zien van macrofagen en hoewel bij vele tumoren dit een negatieve prognostische factor is, is deze infiltratie in osteosarcoom patiënten gecorreleerd met een betere prognose. Deze observatie suggereert dat macrofagen anti-tumor activiteit tegen het osteosarcoom bezitten. In **hoofdstuk 5** wordt beschreven dat M1-type macrofagen in staat zijn de groei van osteosarcoom cellen te remmen, als deze macrofagen geactiveerd zijn met een bacteriële stimulus, zoals liposomal muramyl tripeptide (L-MTP-PE). De inductie van anti-tumor activiteit van M1-type macrofagen door L-MTP-PE was wel afhankelijk van co-stimulatie met het cytokine interferon-γ. Inductie van interferon-γ tijdens macrofaag activatie door L-MTP-PE zou dus belangrijk kunnen zijn om het effect van L-MTP-PE therapie in patiënten met osteosarcoom te verbeteren. Bovendien waren IL-10–gepolariseerde macrofagen, waarvan over het algemeen beschreven wordt dat die de tumorontwikkeling ondersteunen, in staat osteosarcoom celgroei te remmen, als het antilichaam cetuximab aanwezigheid was. De anti-tumor activiteit van tumor-infiltrerende macrofagen zou dus gestimuleerd kunnen worden door factoren die op een infectie in de kanker omgeving lijken, namelijk een bacterieel bestanddeel in een interferon-γ rijke omgeving, of door toediening van een therapeutische antilichaam.

In **hoofdstuk 6** wordt beschreven dat het immuunregulerende eiwit CD70 tot expressie gebracht wordt op een meerderheid van de osteosarcoom cellijnen en primaire osteosarcoom

celkweken. Op Ewing-sarcoom cellijnen was daarentegen nauwelijks CD70 aantoonbaar. In zowel diagnostisch als in recidief osteosarcoom weefsel is CD70-expressie heterogeen en beperkt zich tot de tumorcellen. CD27, de receptor voor CD70, werd niet gedetecteerd op tumorcellen. Infiltrerende T-cellen in tumorweefsel waren negatief voor zowel CD70 als CD27-expressie, onafhankelijk van CD70 expressie in het tumorweefsel. CD70-gen expressie was niet gecorreleerd met het ontstaan van metastasen of de overlevingskansen van osteosarcoom patiënten. Gezien het feit dat expressie van CD70 in gezond weefsel heel beperkt en transient is, zou CD70 een nieuw en tumor specifieke target kunnen zijn voor immuuntherapieën in patiënten met CD70-positieve tumoren.

Conclusies

Het onderzoek beschreven in dit proefschrift illustreert dat humane NK-cellen en macrofagen in staat zijn anti-tumor activiteit ten toon te spreiden als deze immuuncellen geactiveerd zijn door de juiste stimuli of in aanwezigheid van therapeutische antilichamen. Deze resultaten kunnen helpen om de huidige therapieën te verbeteren en de ontwikkeling van nieuwe immuuntherapieën te stimuleren. Daarbij zal in het bijzonder aandacht gegeven dienen te worden aan de gevoeligheid van deze therapieën voor immuun-onderdrukkende effecten van de tumor of tumor omgeving. Om eliminatie van tumorcellen door een NK cel immunotherapie effectief te laten zijn zal in de toekomst enerzijds onderzocht dienen te worden hoe de ontsnapping van tumorcellen aan het immuunsysteem kan worden voorkomen en anderzijds hoe de migratie van NK cellen in de tumor micro-omgeving geoptimaliseerd kan worden.

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ZUSAMMENFASSUNG DIESER DISSERTATION

Einleitung

Das Osteosarkom und das Ewing-Sarkom sind Knochenkrebsarten, die am häufigsten bei Kindern und jungen Erwachsenen auftreten. Trotz fortschrittlicher Operationstechniken zur Entfernung von primärem Tumorgewebe und Tumormetastasen, sowie eines breiten Spektrums an chemotherapeutischen Substanzen bricht die Krebserkrankung bei einem Drittel der Patienten nach vielversprechender erster Behandlung erneut aus, was eine deutliche Verschlechterung der weiteren Prognose dieser Patienten nach sich zieht. Gleichermaßen ist die Lebenserwartung von Patienten, die bereits in einem frühen Stadium Tumormetastasen oder Therapie-resistente Tumore aufweisen, sehr eingeschränkt. Um die Perspektive dieser Patienten zu verbessern, werden daher neue Therapiemethoden benötigt, die Krebszellen gezielt angreifen, dabei jedoch gesundes Gewebe möglichst wenig beinträchtigen.

In früheren Forschungsansätzen wurde gezeigt, dass Natürliche-Killerzellen (NK-Zellen) und Makrophagen, beides Zelltypen des sogenannten angeborenen Immunsystems, anti-Tumor–Aktivität gegenüber Sarkomzellen besitzen bzw. begünstigen. NK-Zellen sind in der Lage infizierte Zellen sowie Krebszellen zu lysieren und können Immunantworten des erworbenen Immunsystems unterstützen und modulieren. NK-Zellen erkennen Zielzellen, die im Vergleich zu gesunden Körperzellen meist eine verminderte Anzahl an deaktivierenden MHC-Klasse-I–Molekülen und zudem eine erhöhte Anzahl an aktivierenden Signalmolekülen exprimieren: dies trifft vor allem auf gestresste und transformierte Zellen zu. Daher können NK-Zellen auch die Tumorzellen bekämpfen, die sich der Kontrolle des erworbenen Immunsystem und somit T-Lymphozyten entziehen. Der Einsatz von NK-Zellen für immuntherapeutische Ansätze ist auch deshalb vielversprechend, da NK-Zellen keine gefährlichen Nebenwirkungen wie Graft-versus-Host Reaktionen hervorrufen, wenn NK-Zellen zu therapeutischen Zwecken in Patienten infundiert werden.

Es wird angenommen, dass im Laufe der Krebsentstehung, unter dem Selektionsdruck des Immunsystems, Tumorzellvarianten hervorgebracht werden, die in der Lage sind anti-Tumor–Immunantworten zu unterlaufen oder auch entgegenzuwirken. Dieser Prozess wird durch die immununterdrückenden Eigenschaften der Tumorzellen selbst aber auch durch das umliegende Gewebe der Tumorumgebung begünstigt, wodurch Immunantworten gegenüber Krebszellen gehemmt werden. Um folglich NK-Zellen und Makrophagen für immuntherapeutische Ansätze zu verwenden, muss das anti-Tumor–Potential dieser Immunzellen derart verstärkt werden, dass dieses in der Tumorumgebung nicht mehr gehemmt wird.

Im **einleitenden Kapitel 1** dieser Dissertation werden die klinischen und biologischen Eigenschaften des Osteosarkoms und Ewing-Sarkoms vorgestellt. Auf diesen Abschnitt folgt ein Überblick über das Feld der Tumorimmunologie sowie verschiedener Immuntherapien. Die vorklinischen Forschungsergebnisse dieser Arbeit werden in den **Kapiteln 2–6** beschrieben und im **abschließenden Kapitel 7** übergreifend zusammengefasst; Schlussfolgerungen und Implikationen für die Verwendung von NK-Zellen und Makrophagen für Immuntherapien werden diskutiert.

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Ergebnisse

In **Kapitel 2** wird ein experimentelles Modell zum Messen der Aktivität von NK-Zellen als Antwort auf eine Virusinfektion durch den Adenovirus Subtyp 5 (HAdV5) und HAdV35 aufgestellt. Die Ergebnisse dieser Experimenten zeigen, dass eine Aktivierung von NK-Zellen durch Stimulierung mit HAdV5 – ermittelt anhand einer erhöhten Expression des Aktivierungsmarkers CD69 auf der Oberfläche von NK-Zellen als auch einer erhöhten zytotoxische Aktivität der NK-Zellen – nur in Gegenwart von T-Zellen und von dem durch T-Zellen produzierten Zytokin (Botenstoff) Interleukin-2 erfolgen konnte. Im Gegensatz hierzu konnten NK-Zellen durch HAdV35 ohne den Einfluss von T-Zellen aktiviert werden. Dies wurde durch die vermehrte Ausschüttung des Zytokins Interferon-α (IFN-α) durch Plasmazytoide Dendritische Zellen (pDC) vermittelt. Die vermehrte Ausschüttung von IFN-α durch mit HAdV35 infizierte pDC war abhängig von der Aktivierung des Toll-like Rezeptor-9 (Detektor für Virus-DNA) und wurde darüber hinaus durch die wechselseitige Interaktion zwischen NK-Zellen und pDC verstärkt.

In den folgenden Forschungsprojekten wird untersucht, auf welche Weise das anti-Tumor–Potential von NK-Zellen und auch Makrophagen gesteigert und zielgenauer auf Osteosarkom- und Ewing-Sarkomzellen gerichtet werden kann. In **Kapitel 3** wird gezeigt, dass die zytotoxische Aktivität von NK-Zellen spezifischer auf Sarkomzelllinien gelenkt werden kann, wenn die Krebszellen mit bestimmten therapeutischen Antikörpern wie zum Beispiel Cetuximab bedeckt sind, welche in diesem Fall den Rezeptor des epidermalen Wachstumsfaktors (EGFR) auf der Oberfläche der Krebszellen binden. Cetuximab induziert die sogenannte FcγRIII/CD16-vermittelte, Antikörper-abhängige Zytotoxität von NK-Zellen. Dadurch wurde in Gegenwart von Cetuximab die Lyse von aus Osteosarkom-Patienten kultivierten Krebszellen durch autologe NK-Zellen vervielfacht: d.h. durch NK-Zellen, die ebenfalls aus diesen Patienten isoliert worden waren. Die durch Antikörper (Cetuximab) induzierte zytotoxische Aktivität der NK-Zellen war vergleichbar mit jener von IL-15–aktivierten NK-Zellen und war selbst bei geringer Expression von EGFR auf der Krebszelloberfläche wirkungsvoll. Diese Experimente legen dar, dass NK-Zellen in kurzzeitigen, vierstündigen Experimenten substantielle zytotoxische Aktivität gegenüber Osteosarkom- und Ewing-Sarkomzellen besitzen.

Wie jedoch in **Kapitel 4** veranschaulicht wird, sind längere, zwei Tage andauernde Interaktionen zwischen Sarkomzellen und NK-Zellen, wie sie potentiell eher den Bedingungen in der Tumorumgebung von Patienten entsprechen, in der Lage die Funtionalität von NK-Zellen zu beinflussen. In diesem Abschnitt wird gezeigt, dass bestimmte Sarkomzelllinien nach Kultivierung mit NK-Zellen eine verminderte Expression von aktivierenden NK-Zell–Rezeptoren wie z.B. von NKG2D, DNAM-1 und NKp30 bewirken können, und dies sogar wenn die NK-Zellen gleichzeitig mit dem aktivierenden Zytokin IL-15 behandelt wurden. Weiterhin wurde durch diesen negativen Effekt auch die zytotoxischen Aktivität der NK-Zellen und somit die Lyse von Sarkomzellen vermindert, da diese von der Funktionalität der oben genannten NK-Zellrezeptoren abhängt. Dieser hemmende Effekt beruhte auf dem physischen Kontakt zwischen Tumorzellen und NK-Zellen. Wenn die NK-Zellen allerdings vor der Interaktion mit Sarkomzellen über einen Zeitraum von fünf Tagen mit IL-15 aktiviert worden waren, wurden diese voraktivierten NK-Zellen nicht mehr durch die immununterdrückenden Eigenschaften der Sarkomzellen beeinträchtigt. Außerdem wurde die FcγRIII/CD16-vermittelte, Antikörper (Cetuximab)-

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abhängige Zytotoxität von NK-Zellen durch die anhaltende Interaktion mit Sarkomzellen nicht gestört. Diese Ergebnisse lassen vermuten, dass eine Kombination von aktivierenden Zytokinen und therapeutischen Antikörpern die zytotoxische Kapazität von NK-Zellen aufrechterhalten und soweit verstärken könnte, dass NK-Zellen auch unter Einfluss von immununterdrückenden Faktoren in der Tumorumgebung die Eliminierung von Krebszellen unterstützen könnten.

Es ist bekannt, dass Makrophagen häufig solide Tumore infiltrieren. In einer früheren Studie an Osteosarkom-Patienten wurde festgestellt, dass Patienten mit einer höheren Anzahl an Makrophagen im Tumorgewebe eine bessere Prognose aufweisen. Diese Ergebnisse legen nahe, dass Makrophagen im Osteosarkom an anti-Tumor–Reaktionen beteiligt sind. In **Kapitel 5** wird dargestellt, dass humane M1-Typ Makrophagen in der Lage sind das Zellwachstum von Osteosarkomzellen zu hemmen, sofern diese Makrophagen zuvor mit bakteriellen Substanzen wie z.B. liposomalen Muramyltripeptiden (L-MTP-PE) aktiviert wurden. Die Induktion der anti-Tumor–Aktivität durch L-MTP-PE erforderte allerdings die gleichzeitige Zugabe von IFN-γ, eine Beobachtung, die für die Optimierung der gegenwärtig angewendeten L-MTP-PE-Therapie bei Osteosarkom-Patienten relevant ist. Auch IL-10–polarisierte M2-Typ Makrophagen, welche die Entstehung und das Wachstum von Tumoren vermutlich begünstigen, konnten in die Lage versetzt werden, das Wachstum von Osteosarkomzellen zu hemmen, jedoch nur wenn die Krebszellen mit Antikörpern (Cetuximab) behandelt waren. Diese Ergebnisse deuten darauf hin, dass Tumor-infiltrierende Makrophagen möglicherweise auch in Patienten für anti-Tumor–Antworten mobilisiert werden könnten, durch Zugabe von Tumorzell-markierenden therapeutischen Antikörpern und/oder von aktivierenden Faktoren, die eine Infektion in der Tumorumgebung simulieren.

In **Kapitel 6** wird dokumentiert, dass das immunregulatorische Protein CD70 auf einer Mehrzahl von Osteosarkomzelllinien und aus Patienten gewonnenen Osteosarkomzellkulturen exprimiert wird, während CD70 nur auf wenigen Ewing-Sarkomzelllinien nachweisbar ist. Untersuchungen verschiedener Gewebeproben von primären und erneut auftretenden Osteosarkomtumoren ergaben, dass die Expression von CD70 in diesen Tumoren heterogen ist, auf Tumorzellen begrenzt und nicht assoziiert ist mit Tumor-infiltrierenden T-Zellen. CD27, der natürliche Rezeptor für CD70, wurde weder auf Krebszellen noch auf Tumor-infiltrierenden T-Zellen in CD70-positiven oder CD70-negativen Osteosarkomproben nachgewiesen. Die Genexpression von CD70 im Tumorgewebe hatte keinen Einfluss auf das Auftreten von Metasasen und somit auf die Überlebensrate dieser Patienten. Da die Expression von CD70 im gesunden Gewebe sehr eingeschränkt bzw. kurzlebig ist, könnte CD70 daher in Patienten mit CD70-positiven Tumoren ein neues, Krebszell-spezifischeres Zielprotein für Immuntherapien darstellen.

Fazit

In dieser Arbeit wird dargelegt, dass humane NK-Zellen und auch Makrophagen das Potential für anti-Tumor–Antworten besitzen, wenn diese Immunzellen hinreichend mit immunstimulierenden Faktoren aktiviert und/oder mit therapeutischen Antikörpern auf Osteosarkom- und Ewing-Sarkomzellen gelenkt werden. Diese Ergebnisse mögen zur Verfeinerung derzeit angewendeter Therapien sowie zur Entwicklung neuer immuntherapeutischer Behandlungsansätze beitragen, die den immununterdrückenden Faktoren der Tumorumgebung entgegenwirken und die Eliminierung von Tumorzellen unterstützen.

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CURRICULUM VITAE

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