Innate immune responses of natural killer cells and macrophages against bone sarcomas : towards cellular immunotherapy

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CONCLUDING CHAPTER 7

Discussion and Future Directions

Towards Harnessing NK cells and Macrophages for Immunotherapy against Bone Sarcomas

CONTENTS

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.

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.

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

(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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