Cancer immunotherapies

Summary

Natural killer (NK) cells are part of the first line immune defense against malignant tumor cells and can readily kill damaged cells. NK cells can distinguish damaged cells from healthy cells through an extensive array of activating and inhibitory receptors and they react to target cells when the net balance of all integrated signals is shifted towards more activation than inhibition. This way, NK cell responses are regulated to allow killing of damaged cells without overactivity against healthy cells. An important group of inhibitory receptors are Human Leukocyte Antigen (HLA) class I molecules, whereas one of the most potent activating receptors is CD16A (FcγRIIIA). The latter can induce strong NK cell activation and subsequent tumor killing by binding to IgG antibodies and mediating antibody-dependent cellular cytotoxicity (ADCC).

As NK cells are potent tumor killers without major off-target toxicity against healthy cells, NK cell-based immunotherapies are considered a promising approach to improve cancer treatments. Clinical studies demonstrated that NK cell infusions, derived either from the patient or from a healthy donor, are well tolerated by the patients and can achieve promising anti-tumor results. Despite being equipped with powerful cytotoxic mechanisms, the NK cells' anti-tumor functions are, however, often inhibited by the tumor or the tumor microenvironments (TME). Immunosuppressive TME impose a challenge for many ongoing treatment approaches, including but not limited to immunotherapies, and the NK cell dysfunction inside tumor areas remains a challenge to be solved. In our research group, we focus on developing effective NK cell-based therapies by using NK cells derived from healthy donors and by utilizing two cancer types as models, namely the hematological malignancy multiple myeloma (MM) and breast cancer.

This thesis describes several strategies how to potentiate the NK cell responses against multiple myeloma and breast cancer cells with the aim to develop NK cells that retain their anti-tumor effector functions in an immunosuppressive TME. In **chapter 2**, we summarized the functional relevance of the inhibitory receptors KIR and NKG2A for NK cells in multiple myeloma, a tumor type that largely expresses the HLA class I molecules HLA-ABC and HLA-E, which are the corresponding ligands to the KIR and NKG2A receptors, respectively. Moreover, we described strategies that could interfere with inhibitory NK cell signals to improve the NK cell efficacy against multiple myeloma (**chapter 2**). In the following chapters, we tested the relevance of interfering with HLA-mediated NK cell inhibition in multiple myeloma and breast cancer to lower the NK cell activation threshold.

In most of the following experimental chapters, we isolated NK cells from peripheral blood of healthy donors and activated them with a high dose of the cytokine IL-2. After overnight activation with IL-2, NK cells were co-cultured with tumor cells to assess the NK cell anti-tumor functions, namely NK cell activation status (degranulation by CD107a), cytokine secretion (IFN-γ), and tumor killing efficacy (cytotoxicity assays). Following up on previous research by our group about the role of KIR receptors on NK cells to target multiple myeloma cells, we investigated in **chapter 3** to which extent the NKG2A receptor influenced the anti-tumor responses of cytokine-activated NK cells against multiple myeloma. Next to NK cell inhibition, KIR and NKG2A receptors are also involved in NK cell licensing, a maturation process that results in more potent NK cells. Therefore, KIR and NKG2A play a dual role in NK cell responses; first by creating more potent NK cells through licensing, and second by inhibiting NK cell responses when the cognate ligand HLA class I is expressed. In **chapter 3**, we found that expression of NKG2A inhibited NK cell degranulation of NKG2A^{+} NK cells only against HLA-E^{high} target cells, but it did not impair NK cell responses against target cells that expressed low levels of HLA-E. NKG2A expression even slightly enhanced NK cell activity against HLA-E^{low} target cells. The ADCC-mediating antibody Daratumumab enhanced NK cell degranulation of all NK cell subsets. These findings imply that NKG2A expression can have a beneficial effect on NK cells, presumably due to licensing, and that interfering with NKG2A, to create less inhibition, can be important for targeting HLA-E^{high} tumors, but might not be necessary when HLA-E expression is low or absent.

In **chapter 4**, patient-derived primary breast cancer cells were found to be relative resistant to NK cells, which seemed to be at least partially driven by HLA class I inhibition. Moreover, we presented that reducing HLA class I inhibition through selection of NK cell donors with a genetic mismatch between KIR on NK cells and HLA on tumor cells (KIR-HLA ligand mismatched NK cells) strongly enhanced NK cell degranulation. Additionally, all NK cells were more potent effector cells when combined with the antibody Trastuzumab. Trastuzumab is a clinically-approved

anti-HER2 antibody that is known to mediate ADCC, which can provide very potent NK cell activation. Importantly, the combination of KIR-HLA ligand mismatched NK cell donors and ADCC-inducing antibody Trastuzumab remained effective in a hypoxic environment, which is frequently observed in breast cancer.

To achieve successful NK cell-based therapies, the complex and often immunosuppressive TME must be considered. Particularly for solid tumors, NK cell infiltration into tumor tissues is a crucial first step. In **chapter 5**, we assessed NK cell density, phenotype, and cellular distribution in two breast cancer cohorts by a multiplexed imaging technique to gain more in-depth knowledge about NK cells in situ. We reported that NK cells were the least abundant cell type of all evaluated leukocyte subsets and appeared not highly cytotoxic based on low granzyme B expression, supporting that NK cell infiltration must be improved (**chapter 5**).

Another factor to examine in the TME are low levels of nutrients including glucose, which can occur as a consequence of tumor growth. Since glucose is an important fuel for NK cells, we measured the glucose concentration in the bone marrow of multiple myeloma patients in **chapter 6**. Compared to the average glucose levels in blood, glucose levels in the bone marrow of most patients were reduced. Our study further demonstrated that such reduced glucose concentrations (500 mg/L) did not impair the anti-tumor responses neither of cytokine-activated NK cells nor of expanded NK cells (**chapter 6**), suggesting that activated NK cells can withstand a low glucose TME.

Within the TME, tumor-associated cells such as immunosuppressive myeloid cells have been reported in literature to limit the anti-tumor potential of NK cells. To study the interaction between activated donor NK cells and tumor-associated cells, we generated two in vitro-polarized macrophage cell types, M1 and tumor-associated macrophages (TAM), and co-cultured these with NK cells (**chapter 7**). We found that NK cells responded to these macrophage target cells, measured by degranulation and IFN-γ secretion. With the aim to boost NK cells against the immunosuppressive TME, we tested whether an ADCC-triggering antibody could promote NK cell responses against the M1 and TAM macrophages and we found that NK cells degranulated stronger and produced more IFN-γ in combination with Avelumab directed against PD-L1^{high} macrophages. By secreting pro-inflammatory cytokines such as IFN-γ in response to tumor-associated cells, NK cells may serve as adjuvants by triggering anti-tumor responses in other immune cell types. This effect would be an important additional function next to killing tumor directly.

To escape immune responses, tumor cells can alter their ligand expression for example by shedding NK cell activating ligands or by altering HLA class I expression. In **chapter 8**, we described a role for genetic polymorphism in the occurrence of alternatively spliced RNA variants of HLA-C. If translated to a functional protein, the HLA-C variants might occur as soluble forms as they precisely lacked the transmembrane region, encoded by exon 5. Since all HLA-C molecules are recognized by NK cell receptors, it will be interesting to further determine whether the observed role of polymorphism results in altered HLA-C expression levels and thus influences NK cell responses, like it has previously been described for HLA-E and HLA-G.

In **chapter 9**, the findings of this thesis are discussed in the context of current challenges and advancements for NK cell-based cancer therapies. In summary, the strategies presented in this thesis provide small buildings blocks towards developing effective NK cell-based immunotherapies that retain their potency in an immunosuppressive TME.