# English summary

This thesis describes studies that help to design optimal clinical trials aiming at eradicating leukemia and solid tumors by immunotherapy targeting so called minor histcompatibility antigens (mHags). This new therapy approach will be applied in cancer patients subsequent to allogeneic stem cell transplantation (SCT).

Human leukocyte antigen (HLA) matched allogeneic stem cell transplantation (SCT) is an established curative treatment for hematopoietic malignancies and an investigative immunotherapeutic approach for solid tumors. The curative effect of allogeneic SCT is based on so called graft versus-tumor responses (GvT) mediated by the transplanted donor immune system. This means that killer cells, particularly T-cells, co-transplanted with the donor stem cells are capable of recognizing leukemia and solid tumor cells of the patient leading to an elimination of the malignant cells. These curative immune responses typically co-incide with a severe side effect of allogeneic SCT, namely graft-versus-host disease (GvHD). GvHD is the result of an overwhelming immune response of the donor T-cells particularly leading to killing of epithelial cells in the entire body. This condition is associated with a considerable morbidity and mortality. In order to prevent severe GvHD, patients and donors are commonly matched based on so called major histocompatibility antigens, which are called “human leukocyte antigens (HLA)” in the human system. However, HLA-matching is not sufficient to prevent GvHD entirely. Consequently, antigens other than HLA differing between patient and donor must be responsible for the remaining immune responses. These antigens are called minor histocompatibility antigens (mHags). Importantly, not only GvHD, but also the GvT effects are mainly driven by mHags mismatched between patient and donor in the HLA-matched setting.

mHags are polymorphic “peptides”, i.e. protein fragments presented in the context of HLA-molecules on the cell surface. Polymorphic means that they exist in different variants (so called alleles) which can differ between patient and donor. The mHag alleles present in a patient or donor can be easily tested before SCT based on new typing technologies in order to predict possible mHag specific immune responses after allogeneic SCT. Another important feature of mHags is that they have a so called “differential tissue distribution”. This means that some of the mHags are expressed ubiquitously, i.e. by all cells in the body, while others are only expressed by hematopoietic (including leukemia) or solid tumor cells. Those mHags ubiquitously expressed in the body have been identified as the prime in situ targets of GvHD. Therefore, hematopoiesis- and solid tumor-restricted mHags, e.g. HA-1, provide the unique option to boost the GvT effect without inducing severe GvHD.

In general, there are two approaches to apply mHag specific immunotherapy of cancer, namely adoptive transfer of in vitro generated mHag specific CTLs (mHag CTLs) and vaccination with tumor mHag peptides to boost the mHag CTLs emerging after allogeneic SCT. However, until the begin of this thesis, the proof of concept for the anti-cancer efficacy of mHag specific immunotherapy in vivo was missing. Moreover, the optimal clinical parameters for mHag specific immunotherapy were unknown and mHag specific immune escape mechanisms of cancer were not identified. Additionally, new approaches for the in vitro expansion of mHag CTLs and the design of mHag peptide vaccines were required. Finally, the optimal parameters for the clinical guidance of mHags specific immunotherapy of cancer were undefined.

**This thesis studied** 1) the proof of concept for the efficacy of mHag specific immunotherapy in vitro and in vivo, 2) the optimal circumstances under which mHag CTLs are most effective against established leukemia and solid tumors, 3) new immune escape mechanisms in cancer that need to be considered when targeting mHags, 4) the optimal protocols for the in vitro expansion of mHags CTLs, 5) the optimal design of mHag peptide vaccines and 6) the optimal parameters for the clinical guidance of mHags specific immunotherapy of cancer.

**Chapter 2-4** are providing the proof of concept that HA-1 CTLs are capable of eliminating human leukemia and solid tumors in vitro and in vivo. In detail, **chapter 2** studied the anti-tumor efficacy of HA-1 CTLs against human leukemia in models of minimal and established disease in immunodeficient mice. HA-1 CTLs circulated systemically into all organs and effectively suppressed the progression of human leukemia in a minimal disease setting in vivo. However, this study also showed that HA-1 CTLs disappear rapidly from the circulation after adoptive transfer and that progression of fully established leukemia could not be influenced by the infusion of HA-1 CTLs. **Chapter 3** describes the establishment of a novel assay that enables studying the in situ interaction of mHag CTLs with human solid tumors in vitro. The solid tumors in this model are generated from established tumor cell lines in a collagen type I matrix vitro and closely resemble many histological features of the corresponding clinical cancers. The 3D feature of the tumors allows to determine e.g. the level of tumor infiltration necessary to eliminate human tumors. **Chapter 4** describes the potency and mHag specificity of human mHag HA-1 CTLs to destroy human 3D microtumors in vitro and the anti-tumor efficacy of HA-1 CTLs in vivo. Infusion of HA-1 CTLs into immunodeficient mice previously engrafted with human breast cancer cells resulted in infiltration of human 3D lung metastasis and inhibition of cancer progression. However, infused HA-1 CTLs did not infiltrate established s.c. macrotumors. Although the exact reasons for the low HA-1 CTL infiltration of large established solid tumors remain unknown, the summary of the findings of **chapter 2-4** indicate that mHag specific immunotherapy of cancer will be most effective after maximal tumor reduction.

**Chapter 5 and 6** are investigating the question which mHag specific immune escape mechanisms in cancer need to be considered when targeting mHags. In detail, **chapter 5** shows that common chromosomal aberrations in leukemia cells can knock out mHag encoding genes and, thereby, abrogate their recognition by mHag CTLs. **Chapter 6** describes that promoter methylation, i.e. a frequent chemical DNA modification acquired during cancerogenesis and mostly associated with silencing of genes, plays a role in HA-1 gene silencing in solid tumor cell lines not expressing HA-1 mRNA. Namely, HA-1 promoter region is mostly methylated in HA-$1^{neg}$ solid tumor cell lines but unmethylated in HA-$1^{pos}$ solid tumor cell lines, normal non-hematopoietic and hematopoietic cell lines. Moreover, application of hypomethlating drugs induced HA-1 expression in HA-$1^{neg}$ tumor cells and sensitized them for recognition by HA-1 specific cytotoxic T-lymphocytes in vitro. Overall, **chapter 5 and 6** identify chromosomal aberrations and HA-1 promoter methylation as so far unknown mechanisms that enable leukemia and solid tumor cells to escape recognition by mHag CTLs. Thus, knowing the mHag karyotype, the HA-1 mRNA/protein expression status and the functional HA-1 expression on isolated malignant cells is necessary to optimally plan mHag specific immunotherapy in individual patients.

**Chapter 7** investigates how the in vitro expansion of mHag CTLs can be improved in order to generate sufficient numbers of in vitro generated CTLs for adoptive CTL transfer into patients. Namely, repetitive in vitro stimulation of mHag CTLs commonly results in progressive CTL differentiation, loss of proliferative activity and ultimately, CTL death. This problem is particularly evident during genetic modification of CTLs with retroviral vectors (i.e. "gene therapy"). Homeostatic cytokines like IL-7 and IL-15 have been described to maintain the proliferative activity of CTLs. In **chapter 7,** IL-7 and IL-15 were applied to the CTL cultures during the genetic modification of T-cells with a suicide gene. Repetitive stimulation of the T-cell lines with mHag peptide loaded dendritic cells (DCs) resulted in gene-modified T-cells with stem cell features, i.e. T-cells with high proliferative capacity and the capability of differentiation into highly cytotoxic CTLs. T-cells with these features were IL-7Ra positive. Overall, these data show that homeostatic cytokines during in vitro culture of mHag CTLs might help to expand larger quantities of mHag CTLs in vitro. Moreover, exploitation of IL-7Ra as marker might help to select mHag CTLs with optimal expansion potential.

**Chapter 8** investigates whether HA-1 peptide vaccines can be improved by extending the peptide length. This study was inspired by previous animal studies showing differences in the presentation of so called "long peptides" (LPs) (i.e. peptides containing the sequence of the actual HLA class I T-cell epitope, but being extended at both ends) and the conventional so called short peptides (SPs) (i.e. peptides representing exactly the HLA class I T-cell epitope of an antigen). Namely, LP presentation was described to be more restricted to professional APCs and to be longer-lasting compared to the SPs. In the study of **chapter 8,** HA-1 LP presentation but not SP presentation was largely restricted to activated DCs and almost absent on other hematopoietic cells. Importantly, DCs presented the HA-1 LP 2-3 log levels less efficiently than the SP. Finally, the decays of HA-1 LP and SP presentation on DCs were comparable. In conclusion, HA-1 LP and SPs differ in their in vitro properties. However, it remains questionable whether HA-1 LP vaccines will induce better immune responses compared to SPs in clinical studies.

**Chapter 9** investigates how clinical responses to HA-1 specific immunotherapy can be uniformely determined in different diseases. Namely, HA-1 is expressed in virtually all hematological malignancies which are characterized by different disease-specific markers. **Chapter 9** describes the relationship between HA-1 CTLs in the peripheral blood and the long-term kinetic of host hematopoietic chimerism after allogeneic HLA-matched, HA-1 mismatched SCT. Host chimerism was measured by highly sensitive mHag specific quantitative PCR. Functional HA-1 CTLs were detectable in 6/10 patients showing an inverse correlation between HA-1 CTL numbers and host hematopoietic chimerism levels. Conversely, HA-1 $CTL^{neg}$ patients had persistently high host chimerism levels. Co-incubation of $CD34^{+}$ cells with HA-1 CTLs largely prevented human hematopoietic engraftment in immunodeficient mice. Overall, these data indicate that therapeutic boosting of HA-1 CTLs may control chimerism by eliminating the host hematopoiesis on stem cell level. Thus, host chimerism is a suitable marker to determine clinical responses to HA-1 specific immunotherapy in different diseases.

**In conclusion**, this thesis provides the proof of concept for the anti-leukemia and –solid tumor efficacy of mHag specific immunotherapy in vitro and in vivo. HA-1 specific immunotherapy is best performed in a minimal disease setting. Consequently, maximum tumor reduction with conventional therapies is

required for successful mHag specific immunotherapy. Immune escape mechanisms in cancer need to be considered when targeting mHags. Thus, functional assays should determine the expression of HA-1 on the cancer cell surface before starting immunotherapy. In vitro expansion of mHags CTLs for adoptive CTL transfer is best performed in the presence of homeostatic cytokines. Short peptides might be optimal mHag peptide vaccines. Finally, chimerism is an optimal clinical parameter to determine the clinical response to HA-1 specific immunotherapy in different diseases. The data acquired in this thesis have provided relevant knowledge that is already partially implemented in the design of new HA-1 vaccination studies aiming at curing leukemia and solid tumors.