The human minor histocompatibility antigen HA-1 as target for stem cell based immunotherapy of cancer : pre-clinical and clinical studies

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Chapter 10.

General discussion

Contents

10.1. Efficacy and limitations of SC based mHag specific immunotherapy of cancer

A series of measures such as dose-reduced conditioning (1), matching of patients and donors based on high-resolution HLA-typing (2,3) and improved supportive care have considerably reduced the treatment-related mortality of allogeneic SCT in the recent years (4). Additionally, the growing number of matched unrelated donors allowed an increasing number of patients to profit from the GvT effects of HLA-matched SCT (5). Nevertheless, relapse remains the major cause of mortality after allogeneic SCT (1). Thus, boosting the graft-versus-tumor (GvT) effect without risking the clinically linked graft-versus-host disease (GvHD) (6,7) is crucial for further improvements of the allogeneic SCT outcome. The rapidly growing number of molecularly identified mHags suitable as immunotherapeutic targets (Introduction, table 1) provides new options to combine allogeneic SCT with subsequent mHag specific immunotherapy. Nevertheless, the development of mHag specific immunotherapy is still at its beginning. The optimal prerequisites for the efficacy of immunotherapy, the optimal immunotherapeutic approaches and the optimal parameters for clinical guidance of immunotherapy are largely unknown. This thesis started with investigating the anti-cancer efficacy of HA-1 CTLs in newly established cancer models in vitro and in vivo.

10.1.1. General anti-cancer efficacy and limitations of HA-1 CTLs

Upon adoptive transfer, human CTLs need to survive in vivo, to circulate via the blood and lymph vessels to the cancer sites and to kill the malignant target cells. We tested the functionality of HA-1 CTLs against human leukemia (**chapter 2**) and solid tumors (**chapter 4**) both in models of minimal disease and of established malignancies in NOD/SCID mice in vivo. We made the following observations:

1) HA-1 CTLs circulate systemically into all organs (including the bone marrow, i.e. the main location for leukemia, multiple myeloma and bone metastases of solid tumors). This indicates that HA-1 CTLs are capable of attacking disseminated malignant cells probably in all organs.

2) HA-1 CTLs disappear rapidly from the circulation within 1 week after adoptive transfer. These results are in accordance with earlier reports in mice (8) and in men (9) showing rapid clearance of adoptively transferred mono- or polyclonal CTLs specific for a single epitope. Interestingly, allo-HLA specific T cell lines can expand upon antigen recognition in NOD/scid mice (10). The reason for the better maintenance of the allo-HLA T cell lines might be found in the different composition and way of generation of these T cell lines, i.e. co-transfer of cytokine producing CD4 T helper cells and in vitro stimulation against multiple (unknown) epitopes that are present in vivo (10). However, also the level of differentiation of the transferred CTLs may affect their in vivo persistence and transfusion of less differentiated mHag CTLs may result in an improved in vivo persistence (11). It remains to be determined whether limited in vivo persistence of donor-derived mHag CTLs is also problem in the context of mHag peptide vaccination.

3) HA-1 CTLs effectively suppress the progression of human leukemia and solid tumor metastases in vivo – given that the effector to target cell ratio in vivo is high. In contrast, progression of fully established malignancies could not be influenced by the infusion of HA-1 CTLs. These data suggest that minimal residual disease is – similar to the experiences in most cancer therapies - a crucial requirement for the anti-tumor efficacy of mHag CTLs. Consequently, mHag specific immunotherapy must be preceded by maximal tumor reduction to be successful. Conversely, mHag specific immunotherapy should not be applied as rescue approach in otherwise untreatable patients (4).

10.1.2. Specific considerations regarding HA-1 CTLs in the treatment of human solid tumors

As described in chapter 3, we established a novel assay to determine the in situ efficacy of mHag specific immunotherapy against 3D tumors. These 3D tumors cultured from cancer cell lines in collagen type I closely resembled clinical cancers by many histological features, their immunohistochemical staining patterns, the heterogeneous protein expression and the distinct tumor growth compartments of clinical cancers. Thereby, our 3D culture system is an excellent model for avascular tumor stages and micrometastases. **As described in chapter 3 and 4**, we applied this in vitro tumor model to show the potency and mHag specificity of mHag HA-1 CTLs to destroy human 3D microtumors in vitro. Our data are pointing towards crucial differences in the functional expression of the mHag HA-1 on solid tumors compared to tumor associated antigens (TAAs), which are commonly used as targets for cancer immunotherapy. While 3D tumor growth results in loss of TAA presentation (12,13) and in "multicellular apoptosis resistance" (14,15) within 24h, the functional mHag HA-1 expression and tumor susceptibility to mHag CTL lysis were not hampered by the 3D tumor growth in our study. The complete eradication of microtumors by HA-1 CTLs provides a first indication that HA-1 is expressed on the vast majority of tumor cells in our model. Overall, the capability of HA-1 CTLs to infiltrate microtumors, to inhibit tumor growth and, ultimately, to eradicate human microtumors in a highly mHag specific manner, qualifies HA-1 as powerful allogeneic target on solid tumors. When translating these findings into our NOD/scid mouse solid tumor model **(chapter 4)** we made two important observations. First, i.v. infused HA-1 CTLs were capable to infiltrate human lung metastasis fully established as 3D tumor structures in vivo and to inhibit the progression of these pulmonary metastases. Second, transfused HA-1 CTLs did – although detectable in all organs upon adoptive transfer – not infiltrate into s.c. macrotumors. This finding is very much comparable to the still unexplained observation that TAA CTLs upon adoptive transfer in man only infiltrate few cancer metastases within individual patients (16-18). Importantly, also explanted s.c. macrotumors were not infiltrated by co-incubated HA-1 CTLs. This finding may point towards differences in the composition of s.c. macrotumors, pulmonary metastases and in vitro microtumors. In fact, the compactness of the tumor histology decreased from s.c. macrotumors over pulmonary metastases to vitro microtumors. Moreover, the higher compactness of the larger s.c. macrotumors compared to the smaller pulmonary metastases was associated with a much higher content of connective tissue (i.e. desmoplasia) in these tumors in vivo. Thus, the tumor architecture might – as already suggested by earlier studies on tumor infiltration by tumor specific T-cells (19,20) - represent a physical barrier for HA-1 CTL infiltration (4). Since desmoplasia in man correlates with the progression of solid tumors (21), our data suggest that there are at least two interrelated factors associated with a high tumor load hampering the success of allogeneic immunotherapy of solid tumors, namely the unfavorable in vivo effector to target cell ratio and the tumor composition.

10.1.3. Consequences for HA-1 specific cancer immunotherapy

We have shown the first proof of concept that tumor mHag CTLs can induce strong effects against hematological malignancies and metastatic cancer in vivo. Thus, mismatched mHags with hematopoiesis- and solid tumor-restricted expression are excellent tools to boost the allo-immune response against cancer after HLA-matched allogeneic SCT. However, the limited in vivo persistence of transfused HA-1 CTLs, the effector to target ratio-dependent efficacy of HA-1 CTLs in vivo and the

limited solid tumor infiltration by HA-1 CTLs indicates that mHag specific immunotherapy will be most effective in minimal residual disease (4).

10.2. Immune escape mechanisms of cancers against HA-1 specific immunotherapy

A major problem of the various approaches of cancer immunotherapy is the escape of the malignant cells from the immune attack by tumor antigen specific T-cells. These immune escape mechanisms are extremely diverse and accumulate with tumor progression. They involve the loss of HLA expression (22), peptide processing defects (23), release of immune suppressive cytokines, tumor counter attack and other mechanisms (4,24). During the work on this thesis, we found two novel mechanisms suppressing the immune recognition of mHags on leukemia and solid tumor cells, namely chromosomal aberrations and promoter hypermethylation.

10.2.1. Deletion of mHags by chromosomal aberrations in cancer cells

Genetic instability due to increased DNA damage or ineffective DNA repair has a major impact on the pathogenesis both of inherited and sporadic human leukemias as disruption or fusion of genes causes initiation and progression of hematological neoplasias (25). Chromosomal aberrations are a common feature of this genetic instability and strongly affect the overall disease prognosis (26). Little is known about how leukemia-related chromosomal aberrations specifically affect antigen recognition by T-cells. **As described in chapter 5**, we have identified chromosomal deletion of the mHag genes as a novel mechanism which leads to the immune escape of human leukemia cells from mHag specific immunotherapy. We found in a patient heterozygous for HA-1 (H/R) and HA-2 (V/M) deletion of both the non-immunogenic HA-1R allele and the immunogenic HA-2V allele in purified leukemia cells. In contrast, the HA-1R and HA-2V alleles were present in PBMCs and in the non-leukemic bone marrow fraction. Loss of HA-1R in purified leukemia cells was caused by an unbalanced translocation affecting chromosome 19 (localizing the HA-1 gene) and loss of HA-2V was caused by an isochromosome 7 (localizing the HA-2 gene). The loss of HA-2V was associated with loss of functional recognition of leukemia cells with HA-2 CTLs. These data showed the proof of principle that common karyotypic abnormalities in leukemia cells can knock out mHag encoding genes and, thereby, abrogate their recognition by mHag CTLs. This mechanism of antigen loss is mainly relevant in mHag heterozygous patients, where loss of the single immunogenic mHag allele may completely abrogate leukemia cell recognition by mHag CTLs. Consequently, the observed mechanism of mHag loss requires particular attention when targeting specific mHags with immunotherapy. The described mechanism of mHag loss in leukemia cells occurred without the impact of allogeneic SCT, i.e. solely due to the chromosomal aberrations characterizing the respective leukemia. Interestingly, recent data have also shown that the immunological pressure subsequent to allogeneic SCT can lead to the elimination of antigens on leukemia cells. First, loss of the H-Y chromosome in leukemia cells was found in a acute lymphoblastic leukemia patients relapsing after sex-mismatched allogeneic SCT (27). Moreover, after transplantation of haploidentical hematopoietic stem cells and infusion of donor T cells, leukemic cells can escape from the donor's antileukemic T cells through the loss of the mismatched HLA haplotype. These “immune-modified” leukemia cells can finally lead to leukemia relapse (28). It remains to be determined whether alleles of hematopoiesis-restricted mHags such as HA-1 or HA-2 can be lost on leukemia cells in

response to mHag specific immune reactivities after allogeneic SCT and whether such an antigen loss is also associated with leukemia relapse.

10.2.2. Epigenetic regulation of HA-1 expression in malignancies

However, not only genetic aberrations may lead to loss of cancer cell recognition by mHag CTLs. **As described in chapter 6**, we found that also epigenetic mechanisms, i.e. heritable changes in the gene expression that are not accompanied by changes in DNA sequences, may play an important role in silencing the HA-1 mRNA expression in cancer cells. Particularly DNA hypermethylation in promoter regions is a frequent phenomenon in cancerogenesis and mostly associated with silencing of genes (29,30). We found that the HA-1 promoter region is localized within a CpG island and, thus, within a potential target for cancer-related promoter-hypermethylation (29,30). Furthermore, the HA-1 promoter region was frequently 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. Interestingly, HA-1 promoter methylation was detectable also in some primary breast cancers. Moreover, we found that HA-1 gene expression was inducible in all HA-1^{neg} cancer cell lines by treatment with 5-AZA-CdR. Our data also showed that HA-1 promoter hypermethylation is not the only mechanism silencing the HA-1 gene expression. Namely, treatment with 5-AZA-CdR induced HA-1 gene expression in two HA-1^{neg} cancer cell lines despite the HA-1 promoter being unmethylated. Moreover, also the HDAC inhibitor TSA induced de novo HA-1 expression in some HA-1^{neg} tumor cell lines, which indicates that also histone deacetylation may contribute to the regulation of the HA-1 gene expression in solid tumors. It remains unclear which mechanism dominantly regulates HA-1 expression in HA-1^{neg} primary cancers and whether this might vary in different stages of tumor progression or different tumor entities. Overall, these data suggest that different epigenetic mechanisms are involved in silencing the HA-1 gene expression in solid tumor cells facilitating their escape from immunological anti-HA-1 responses. Of note, DNA hypermethylation is also an important feature of leukemias and – particularly – of myelodysplastic syndrome (MDS) (31). It remains to be determined whether HA-1 promoter methylation is also present particularly in MDS and secondary leukemia subsequent to MDS and whether this phenomenon is – equal to solid tumors - also associated with silencing of HA-1 mRNA expression (4).

10.2.3. Consequences for HA-1 specific cancer immunotherapy

Although we have identified chromosomal aberrations as potential immune escape mechanism only in leukemia so far, also solid tumors frequently have complex chromosomal aberrations due to chromosomal instability (32). Consequently, since some mHags e.g. HA-1 and BCL2A1 are also expressed on solid tumors, deletion of the relevant mHag genes in the tumor cells may also lead to a loss of mHag recognition of solid tumors. Overall, our data on mHag related immune escape mechanisms indicate that it is crucial for successful mHag specific cancer immunotherapy to know the mHag karyotype, the HA-1 mRNA/protein expression status and – if applicable – the functional HA-1 expression on isolated malignant cells. Thus, appropriate selection of patients for mHag specific immunotherapy should include HA-1 expression analysis on the malignancy – both in leukemia and in solid tumors. While functional analysis of HA-1 expression on leukemia cells is rather feasible using e.g. cytotoxicity assays on sorted leukemia cells, HA-1 expression analysis on solid tumors can be difficult due to the absence of fresh tumor material at the time point of decision for mHag specific immunotherapy, high percentages of necrotic tumor cells and contaminating HA-1 positive

hematopoietic cells. Therefore, simple read outs assessing HA-1 expression in tumors (e.g. by antibodies) are urgently needed to determine at least the HA-1 positivity on protein level in solid tumors. However, not only negativity for HA-1 expression in all cancer cells of an individual malignancy might be a problem for targeting of cancers by HA-1 specific immunotherapy. Previous data on HA-1 mRNA expression in primary tumors indicated that HA-1 can also be only heterogeneously expressed in HA-1^{pos} cancers – a phenomenon also observed for autologous TAAs (33). A solution for this problem might be our finding that the hypomethylating agent 5-AZA-CdR restored HA-1 expression only in HA-1^{neg} solid tumor cells without affecting normal non-hematopoietic cells. Thus, hypomethylating agents might ensure the expression of HA-1 in a maximal number of cancer cells before HA-1 specific immunotherapy. In addition to the induction of HA-1 mRNA expression in HA-1^{neg} cancers, hypomethylating drugs are capable of reversing drug resistance of cancer cells in clinical studies (34) and of reinducing a broad spectrum of genes associated with an effective CTL-tumor interaction. 5-AZA-CdR can improve tumor cell killing by the induction of cell adhesion molecules, e.g. ICAM-1 and LFA-3 (35) and by the restoration of down-regulated HLA class I molecules frequently occurring in cancer cells as shown for melanoma cells (36,37). Moreover, 5-AZA-CdR can induce a broad spectrum of CTAs, e.g. MAGE-A3 and NY-ESO-1 in solid tumor cells in vitro (33,38-40) and in vivo (41). Thus, hypomethylating drugs may facilitate immunotherapeutic targeting of multiple antigens (4), potentially including mHags other than HA-1 which are otherwise not expressed by solid tumors.

10.3. Optimal design of HA-1 specific immunotherapy

Our in vivo data in the humanized animal models **in chapters 2 and 4** demonstrate that HA-1 CTLs are therapeutically highly effective against leukemia and solid tumors in minimal disease. However, particularly the short in vivo persistence of transferred CTLs, the necessity of high numbers of HA-1 CTLs in vivo and the low solid tumor infiltration remain important limitations of HA-1 CTLs in vivo. To date, there are two main options to exploit mHag differences between donor and recipient in adoptive immunotherapy, namely cellular adoptive immunotherapy with in vitro generated mHag CTLs and mHag peptide vaccination.

10.3.1. Optimal design of cellular adoptive immunotherapy

Adoptive transfer of in vitro generated SC donor derived mHag CTLs is possible since mHag CTLs can be generated by stimulation of PBMCs with mHag peptide loaded dendritic cells in vitro (42,43). However, this approach is extremely laborious and expensive. Moreover, the success of in vitro generation of HA-1 CTLs from individual donors is not always certain (42,44). Additionally, our in vivo data showed that adoptive CTL transfer can only be successful, if mHag CTLs are provided in large quantities. mHag CTLs can be generated not only by in vitro stimulation of T-cells with mHag peptide loaded DCs (42) but also by transfer of mHag specific T cell receptor (TCR) genes into donor peripheral blood mononuclear cells which has been successfully shown for the mHags HA-1 (45) and HA-2 (46). However, the generation of mHag CTLs both by using mHag peptide loaded donor DCs and by retroviral TCR transfer is hampered since antigen specific stimulation results in progressive transition from T_{CM} to T_{EM} cells associated with acquisition of effector function, loss of proliferative activity and reduced T-cell survival in vivo (i.e. “linear model of T-cell memory”) (47). As shown in **chapter 7,** application of IL-7 and IL-15 during the genetic modification of T-cells with a suicide gene resulted - despite subsequent

repetitive T-cell stimulation with mHag peptide loaded dendritic cells (DCs) - in gene-modified T-cells with high proliferative capacity and the capability of differentiation into highly cytotoxic CTLs. Most importantly, these gene-modified T-cells were capable of eliminating human leukemia in immunodeficient mice in vivo. At present, the reason why these gene-modified human T lymphocytes generated after CD28 costimulation with beads and culturing with IL-7/IL-15, but not with the other combinations tested, retain mHag CTL alloreactivity is unknown. The different cytokines belonging to the γ-chain family apparently have distinct roles in T-cell responses. IL-2 and IL-15, for example, are potent T-cell growth factors that share the IL-2/IL-15 receptor β. As opposed to IL-2, IL-15 does not prime T cells for activation-induced cell death (48) and strongly contrasts regulatory T cells (49). The preservation of mHag-reactivity may be explained by a combined action of the two cytokines, which could specifically promote the proliferation and the survival of suicide gene-modified precursors for mHag CTLs. Rather unexpectedly, despite their predominant T_{EM} phenotype, gene-modified mHag CTLs could undergo multiple rounds of expansion in vitro without losing anti-leukemia efficacy. The persistence of the proliferative potential in subpopulations of mHag T-cells despite repetitive stimulation may point towards the existence of T-cells with stem cells features in our experiments. The "stem T-cell model" proposes that antigen encounter determines the early generation of a long-lived putative memory stem T-cell characterized by the ability to self-renew and differentiate into memory T-cells and effectors (11). In fact, in a mouse model of GvHD, $CD8^+$ T cells primed in vitro for a short period of time with host antigens in the presence of IL-15, were able to persist after adoptive transfer throughout the disease course and to give rise to different T-cell types, including effectors (50). In analogy, after antigenic stimulation, we found that a subpopulation of suicide gene positive mHag HA-1 CTLs, identified by the expression of IL-7Ra, was characterized by the highest proliferative potential and by the capability to self-renew and to differentiate into potent anti-leukemia effectors. Overall, these characteristics match with those of memory stem T-cells (4).

10.3.2. Optimal design of HA-1 peptide vaccines

Another more practical and potentially efficient strategy of mHag specific immunotherapy is 'vaccination'. In this strategy, mHag peptides are administered to the patient where donor derived antigen presenting cells (APCs) will boost the donor-derived mHag specific immune response emerging after mHag mismatched SCT (51). However, the optimal design of mHag peptide vaccines is unclear. **In chapter 8,** we investigated "peptide length extension", i.e. a novel vaccination concept that had previously shown superior immune responses in animal studies (52). Namely, peptides exactly fitting the binding grooves of MHC molecules (so called short peptides, SPs) have been described to induce tolerance in some animal models due to systemic peptide spreading associated with peptide presentation by non-professional APCs. In contrast, length extended peptides (long peptides, LPs), i.e. peptides containing the immunogenic epitope sequence but incapable to fit directly fit into MHC molecules require peptide processing which is optimally performed by professional APCs (53,54). This ensures that LP presentation is restricted to cells with optimal co-stimulating capacities leading to immune responses with a lower tolerance risk compared to SPs. Our human in vitro studies confirmed that presentation of HA-1 LPs is, indeed, more restricted to activated DCs than the HA-1 SP, since hematopoietic cells such as T-cells, B-cells and non-adherent monocytes hardly presented the HA-1 LPs. However, also fibroblasts presented HA-1 LPs very well. Thus, HA-1 LPs can also be presented in the absence of optimal co-stimulation. This finding is important, since systemic spreading of peptides and subsequent

presentation in a low co-stimulatory context e.g. in the lungs was linked to the tolerance induction in the Ad5E1 murine system in vivo (55-57). Another finding associated with the superior immune responses induced by LPs in animal studies was prolonged antigen presentation in vivo. However, our study revealed that the decays of HA-1 LP and SP presentation on cellular level are comparable. Remarkably, the HA-1 SP was still detectable up to 7 days after DC loading with 100 uM peptide. This extremely long presentation might result from the high HLA binding affinity of HA-1 and its low dissociation rate from HLA (58,59) and might be of considerable importance for the success of vaccination with HA-1 SP loaded DCs (4) (see below "10.5. Future directions"). Finally, the presentation efficiency of HA-1 LPs was much lower than of the HA-1 SP. This might have important consequences for HA-1 peptide vaccination after allogeneic SCT, since it is unknown, when after allogeneic SCT DCs have reached full functionality enabling processing and presentation of HA-1 LPs (60). Overall, vaccination with length extended HA-1 peptides may work in vivo. However, our in vitro data do not provide convincing evidence that current HA-1 LPs will provide better results than SPs.

10.3.3. Consequences for HA-1 specific cancer immunotherapy

Our data provide guidance for the optimal generation of mHag CTLs for adoptive transfer and for the development of mHag peptide vaccines. Our data indicate that provision of homeostatic cytokines during in vitro culture of mHag CTLs is crucial for the maintenance of their proliferative capacity. Moreover, the identification of IL-7Ra as stem T-cell marker provides for the first time the option to select optimally expanding CTLs based on a single cell surface marker. The possibility to quantify stem T-cells in vitro based on this marker may also help to further improve and to compare new in vitro CTL expansion protocols. Protocols considering these principles may facilitate the emergence of large enough quantities sufficient for adoptive CTL transfer – both with and without genetic modification (4). Conversely, our studies on HA-1 LP vaccines do not provide convincing evidence that current HA-1 LPs can provide better results than the well established HA-1 SPs in clinical studies. The major problem of HA-1 LPs was the low presentation also by professional APCs. However, the current drawbacks of HA-1 LPs might be overcome in future studies by linkage of HA-1 LPs to TLR agonists (61) or antibodies (62) to facilitate receptor-mediated antigen uptake and to increase extent and duration of HA-1 LP presentation. Alternatively to the direct application of peptides to patients, HA-1 SPs can be loaded ex vivo on DCs which also ensures that the antigen presentation is long-lasting and restricted to optimal antigen presenting cells (4) (this concept is discussed below in "10.5. Future directions").

10.4. Suitable parameters for the clinical guidance of mHag specific immunotherapy

The broad applicability of HA-1 as immunotherapeutic target in nearly all hematological cancers challenges the definition of uniform endpoints in clinical studies. Namely, the large variety of disease specific markers (e.g. various molecular markers in leukemia, paraprotein in myeloma or computer tomography in lymphoma) complicates the comparison of clinical responses to immunotherapy in different diseases. Moreover, absence of such markers particularly in cytogenetically normal hematopoietic malignancies hamper a disease specific response monitoring (63,64). The HA-1 expression in all hematopoietic cells indicated that HA-1 CTLs eliminate both malignant and non-malignant host hematopoietic cells (65-68). Thus, the suppression of the residual host hematopoiesis determined by chimerism analysis could serve as a universal marker for the clinical efficacy of HA-1

specific immunotherapy. Additionally, it was unknown whether the HA-1 specific immune response can eradicate the residual host hematopoiesis, including host stem cells, or whether a persistent immunological pressure of donor HA-1 CTLs is needed to control the residual host hematopoiesis after allogeneic SCT.

10.4.1. Complete cancer eradication versus continuous cancer suppression by donor CTLs

As described in chapter 9, we investigated 1) which parameters might be suitable to determine the immunological and clinical response to HA-1 peptide vaccination and 2) which therapeutic effects can be maximally achieved by HA-1 specific immunotherapy. We found HA-1 specific immune responses in around half of the patients transplanted with an HA-1 mismatched donor. These immune responses were first measurable either with onset of GvHD or subsequent to the termination of immune suppression and mostly persisted after emergence for many months at lower levels. HA-1 CTLs could be – in contrast to CTLs against the autologous tumor associated antigen WT-1 in earlier post-transplant studies (69) - easily isolated, expanded and functionally tested from almost all HA-1 tetramer$^+$ PBMC samples. This analysis revealed that HA-1 CTLs emerging after allogeneic SCT largely consist of high avidity CTLs. Interestingly, around half of the patients, though transplanted with HA-1 mismatched donors, did not show HA-1 CTLs in the entire post-transplant period. As yet it is unclear, which factors determine whether HA-1 specific responses occur after allogeneic SCT. Pre-immunizations of healthy donors may have an impact (70), since both mHag specific CTLs and regulatory T-cells can be induced through pregnancy in mothers and in children (71,72). These immunizations can occur in mothers against paternal mHags expressed by their children and in the children, both females and males, against the non-inherited maternal mHags (72). Additional studies are required to determine the predictive value of the donor immunization for HA-1 CTL emergence in allo-transplanted patients.

The initial hypothesis of this study was that donor HA-1 CTLs do not discriminate between normal and malignant host hematopoietic cells. Thus, HA-1 CTLs may suppress the levels of host chimerism. In accordance with this hypothesis, HA-1 CTL$^+$ patients showed low levels of host chimerism during the presence of HA-1 CTLs. Moreover, increase of HA-1 CTLs in the peripheral blood was mostly accompanied with a decline of host chimeric cells and vice versa. Finally, HA-1 CTL$^-$ patients showed persistent host chimerism at high levels throughout the post SCT period. These data suggest for the first time that HA-1 CTLs are important mediators of the conversion to and maintenance of full donor chimerism after allogeneic HLA-matched, HA-1 mismatched SCT. The observation that rapid increase of host chimerism and relapse were associated with disappearance of functional HA-1 CTLs underlines that persistence of HA-1 CTLs is required to keep the host hematopoiesis including the malignant hematopoietic cells in check. The reasons for the disappearance of HA-1 CTLs remain unclear since HA-1 tetramer$^+$ cells had predominantly a memory phenotype showing only seldom signs of terminal differentiation. Emergence of mHag specific or unspecific regulatory T-cells (72,73), progressive telomere shortening (74) in the HA-1 tetramer$^+$ cells or emergence of a host-anti-graft immune response (75) need to be considered and require further studies. The persistence of host chimeric cells in most of the patients throughout the post transplant period raised the question whether HA-1 CTLs would be ultimately capable of definitely eradicating the host hematopoiesis or whether a continuous immunological pressure is needed to keep the host hematopoiesis in check. This question becomes increasingly relevant with the application of dose-reduced conditioning regimens. Namely, dose reduced conditioning frequently results in a slower replacement of the host- by donor-hematopoiesis compared to

myeloablative regimens (76). In our study, we found that HA-1 CTLs are not only capable of eliminating hematopoietic progenitor cells in vitro (66,77,78), but more importantly also stem cells. Namely, co-incubation of HA-1 CTLs with UCB and BM derived $CD34^+$ or $CD133^+$ stem cells largely abrogated HPC and CAFC colony formation in vitro. Moreover, HA-1 CTLs eliminated NOD/SCID mouse repopulating stem cells for at least 16 weeks post transplant. These data suggest that HA-1 CTLs can ultimately eliminate human hematopoietic stem cells in vivo and, thereby, boost the conversion to full donor chimerism after allogeneic SCT. Of note, apart from the normal hematopoiesis, also the malignant hematopoiesis considerably contributes to the host chimerism in leukemia (but less in lymphomas). Although HA-1 is expressed on virtually all hematological malignancies, the formal proof for the functional HA-1 expression on malignant stem cells is very cumbersome due to the controversial or still absent characterizations of these cells in the various malignancies (79-85). Nevertheless, our data indicate that an HA-1 CTL to stem cell ratio of at least 0.3-1:1 within in the stem cell niche would be necessary for the eradication of the host hematopoiesis. It remains to be determined whether HA-1 specific immunotherapy is capable of enriching HA-1 CTLs in the BM compartment at these quantities.

10.4.2. Consequences for clinical studies

Our data provide guidance for the design and definition of suitable clinical and immunological endpoints of HA-1 specific immunotherapy studies after HLA-matched, HA-1 mismatched SCT. First, suppression of host chimerism appears to be a universal marker for the clinical response to HA-1 specific immunotherapy studies since it correlates with the anti-HA-1 response and because it is – in contrast to disease related markers (such as molecular markers, paraprotein or computer tomography) - independent of the disease for which the patient had received allogeneic SCT. Nevertheless, disease related indicators remain necessary to provide a complete picture of clinical responses. Second, the inverse correlation between the suppression of the host hematopoiesis and the extent of the anti-HA-1 response indicates that HA-1 specific immunotherapy should aim at inducing an anti-HA-1 response in all patients after HLA-matched, HA-1 mismatched SCT. This also implies that HA-1 specific immunotherapy should start preemptively to eradicate the residual host cells before relapse can occur. Third, HA-1 specific immunotherapy should aim at generating not only peaks but also a long-lasting immune response against HA-1, because HA-1 CTL disappearance in some patients was associated with relapse and because the efficacy of immunotherapy to generate high HA-1 CTL numbers in the stem cell niche is still unknown.

10.5. Future directions

Allogeneic HLA-matched SCT is increasingly applied in patients with high relapse risk after allogeneic SCT (5). Thus, enhancement of the GvT effect with low GvHD risk is vital for further improvements of the SCT outcome. Tumor mHags are the ideal target antigens after allogeneic HLA-matched SCT to prevent relapse without risking severe GvHD. Therefore, enormous efforts have been made in the recent years to implement mHag specific immunotherapy as additional procedure subsequent to allogeneic HLA-matched SCT. The data presented in this thesis have considerably influenced the design of these clinical studies.

Two approaches of mHag vaccination have recently entered the clinic:

1) The first approach of mHag peptide vaccination is the application of HA-1 SPs for the treatment of hematological malignancies (Koen van Besien and Arnold Ganser, personal communication) and renal

cell carcinoma (Niederwieser, personal communication). Aim of these studies is to investigate after non-myeloablative SCT the potency of mHag HA-1 (and HA-2 for hematological malignancies) peptide vaccination with or without donor lymphocyte infusions (DLI) to induce mHag CTL responses. As exemplified in Figure 1, both studies apply HA-1 SPs every 50 days starting 1 month after stop of immune suppression. Goal of these studies is to induce an anti HA-1 response in all vaccinated patients, to boost the number of HA-1 CTLs in the blood (and bone marrow of patients), to maintain the anti-HA-1 response over a long period of time and to eradicate the host hematopoiesis, including the residual leukemia and solid tumor cells of patients after allogeneic SCT (4).

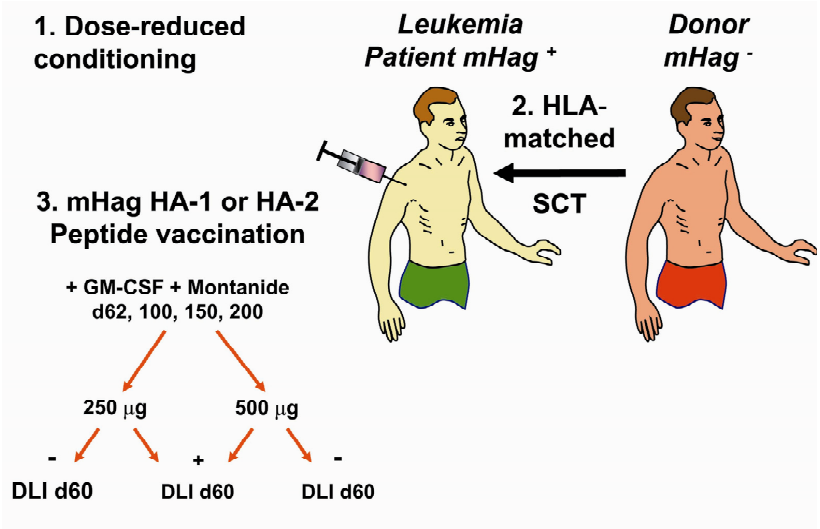

Figure 1. Schematic overview of a mHag peptide vaccination study. Patients with advanced hematological malignancies will receive after HLA-matched, mHag mismatched allogeneic SCT repetitive vaccinations with HA-1 or HA-2 peptides in combination with or without DLI.

2) The second currently tested approach is the vaccination with mHag SP ex-vivo loaded host DCs. This concept will be tested in patients with multiple myeloma, i.e. patients where long-lasting graft-versus-myeloma responses are usually lacking (86). mHag SP ex-vivo loaded host DCs combine the advantages of HA-1 SPs, i.e. the long lasting and highly efficient antigen presentation with the advantage of HA-1 LPs, i.e. antigen presentation restricted to professional APCs. Moreover, the use of host DCs will enable a donor immune response against multiple mismatched and mostly unknown mHags expressed on the host DCs locally at the vaccination site. This broad donor-anti-host immune response shall generate a pro-inflammatory environment which might be optimal for the induction of an immune response against the mHag peptides loaded on the DCs. This concept is based on the observation in animal studies that TAA specific CTLs show best efficacy in the pro-inflammatory environment of GvHD (87). Similarly, HA-1 specific CTLs particularly emerge during GvHD (88). Overall, a moderate proinflammatory environment might be optimal for the expansion and efficacy of tumor-reactive CTLs. This new approach of vaccination with mHag peptide loaded host DCs will be tested in multiple myeloma patients who will receive these DC vaccines in combination with DLI. The therapy schedule of this clinical trial is shown in Figure 2 (4).

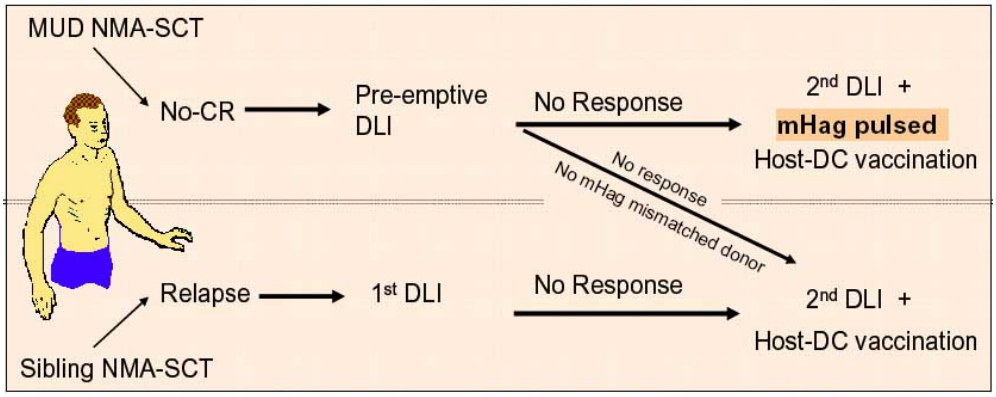

Figure 2. Schematic overview of two complementary DC vaccination protocols with or without loading mHag peptides on the host-DC vaccine. Multiple myeloma patients without a mHag mismatch receive host DCs and DLIs, while patients with a mHag mismatch receive host DCs pulsed with mHag peptides and DLIs.

A series of factors will support the broad application of mHags as immunotherapeutic targets in the future. First, the number of known mHags with high and exclusive expression on the malignant cells is currently rapidly increasing (see table 1, introduction). Already with six tumor mHags HA-1, HA.2, HB-1, BCL2A1, SP110 and PANE1 alone, there are sufficient immunologically relevant mHag mismatches present in 21% (sibling donor) or 33% (matched unrelated donor) of the HLA-matched donor / patient couples allowing mHag specific immunotherapy (89). Second, the growing availability of matched unrelated donors (MUD) facilitates the intended selection of donors based on known mHag mismatches with the patient suitable for mHag specific immunotherapy. Finally, the results of the first clinical phase I/II mHag based immunotherapeutic vaccination studies will provide crucial information how the clinical efficacy of mHag based therapies can be further improved (4).

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