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Novel immunotherapeutic strategies after stem cell transplantation

Boosting graft-versus-tumor immunity by interference with co-inhibitory signaling

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General introduction and outline of the thesis
General introduction

Allogeneic stem cell transplantation (SCT) can be a curative treatment option for patients suffering from hematological malignancies due to alloreactive T cell responses recognizing minor histocompatibility antigens (MiHAs) expressed on the malignant cells. Despite induction of robust MiHA-specific T cell responses and long-term persistence of alloreactive memory T cells specific for the tumor, often these T cells fail to respond efficiently to tumor recurrence. This lack of responsiveness may result from immunosuppressive mechanisms exploited by the tumor cells, including disrupted antigen presentation, secretion of immunosuppressive cytokines, recruitment of regulatory T cells and myeloid-derived suppressor cells (MDSCs), but also modulation of the expression levels of co-stimulatory and co-inhibitory molecules and their subsequent signaling. In this thesis, we investigated the involvement of the co-inhibitory molecules PD-1 and BTLA in the functional impairment of MiHA-specific T cell responses after allogeneic SCT. As in many patients the induction and/or reactivation of MiHA-specific T cell responses is inadequate, potent post-transplant immunotherapies are required to boost graft-versus-tumor (GVT) immunity. Dendritic cell (DC)-based vaccination boosting MiHA-specific T cell immunity is an appealing strategy to prevent or counteract tumor recurrence, but improvement of DC immunogenicity and interference with negative co-signaling pathways is essential to reactivate and boost GVT immunity, and improve clinical outcome post-transplantation. Therefore, we explored the pre-clinical potential of such immunotherapeutic strategies, including treatment with the combination of MiHA peptide-loaded DC vaccines and anti-PD-1 or anti-BTLA blocking antibodies, and vaccination with PD-1 ligand silenced DCs loaded with MiHA peptide or mRNA.

Hematological malignancies

Hematological malignancies comprise a group of heterogeneous neoplasms that account for approximately 7% of all newly diagnosed cancers in Europe. Based on clinical features these are generally categorized in four major groups: acute leukemias, chronic leukemias, malignant lymphomas and plasma cell malignancies. Among these groups etiology, incidence and prognosis greatly varies. Patient age, health status and genetic abnormalities of the tumor are important factors influencing disease prognosis, and consequently determine the choice of therapy. Standard treatment for hematological malignancies includes chemotherapy and radiotherapy, which reduce tumor burden and can induce long-term remission. Moreover, in the past years new therapeutic agents have been developed that more specifically target tumor cells. Currently, the first-line drug in chronic myeloid leukemia (CML) is imatinib, a tyrosine kinase activity blocker that inhibits the BCR-ABL fusion protein. Also treatment of B-cell lymphomas has greatly improved after the introduction of the anti-CD20 monoclonal antibody rituximab. Other promising drugs are bortezomib, a proteasome inhibitor, and lenalidomide, which is reported to
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Hematopoietic stem cell transplantation and donor lymphocyte infusions
Hematopoietic SCT is a well-established treatment modality for patients with a hematological malignancy. Patients are pretreated with chemotherapy with/without radiotherapy, which interferes with the cell division of fast-proliferating tumor cells. Thereby tumor burden is reduced and a situation of minimal residual disease (MRD) can be achieved. Unfortunately, also rapidly dividing normal cells in the bone marrow, gastrointestinal tract and hair follicles are affected, causing side-effects like pancytopenia, mucositis and hair loss. To restore hematopoiesis and provide the patient with a new and healthy immune system, hematopoietic SCT is performed. The hematopoietic stem cells can be isolated from bone marrow (BM), peripheral blood (PB) following mobilization with granulocyte colony-stimulating factor (G-CSF), or umbilical cord blood (UCB). Reconstitution of the various immune cell lines starts within 2-3 weeks after successful stem cell engraftment. In various randomized trials it has been demonstrated that engraftment and subsequent immune recovery is usually faster upon SCT with PB as compared to BM, which is probably due to the higher number of CD34+ hematopoietic stem cells in the mobilized PB stem cell grafts.

There are two types of hematopoietic SCT, namely autologous SCT that uses the patient’s own stem cells obtained prior to conditioning therapy, and allogeneic SCT where stem cells from another person (either a sibling or voluntary unrelated donor) are infused into the patient. Depending on the type of hematological malignancy, and certain patient characteristics the patient receives either an autologous or allogeneic stem cell graft. Although treatment related toxicity is low with autologous SCT, as compared to allogeneic SCT, the disadvantage is that the stem cell graft may contain residual tumor cells, contributing to eventual relapse of the disease. Allogeneic SCT on the other hand, not only provides the patient with a new healthy immune system, but can also be regarded as a highly potent cellular immunotherapy due to donor-derived GVT responses. These can competently eliminate residual tumor cells, resulting in long-term remission and possibly cure of the disease. Unfortunately, allogeneic SCT also causes high morbidity and mortality due to conditioning-related toxicity, infections and graft-versus-host-disease (GVHD). The last decade, reduced intensity chemotherapy regimens have been introduced that have greatly improved treatment related mortality (TRM), and allow older patients to benefit from allogeneic SCT as well. Furthermore, to decrease the incidence and severity of GVHD complete or partial T cell-depleted SCT can be performed, thereby limiting the induction of dangerous alloreactive T cell responses shortly after transplant. In addition, immunosuppressive drugs are administered during the first period after allogeneic SCT. After 6 months when the conditioning-related inflammation has resolved, pre-emptive donor lymphocyte infusions (DLI) can be given to boost or induce GVT immunity with limited risk of GVHD.

The immunogenic potential of hematological malignancies has been evidently demonstrated by the observation of increased tumor-reactive T cells and tumor regression following hematopoietic SCT. Especially, the implementation of allogeneic SCT has greatly enhanced the curative rate in leukemia and lymphoma. Furthermore, DLI has been shown to mediate clinical and molecular remissions in case of residual, refractory or relapsed disease. Nevertheless, hematological malignancies vary in their sensitivity to this treatment. In particular chronic phase CML is highly sensitive to DLI, which is likely due to the recipient DCs being part of the malignant clone. In addition, also acute myeloid leukemia (AML), MM and lymphoma can respond relatively well to DLI. In contrast, acute lymphoblastic leukemia (ALL) hardly responds to DLI, indicating that there are intrinsic differences between these cancers.

Immunobiology of GVT responses
Both in solid organ transplantation, as well as in hematopoietic SCT the human leukocyte antigen (HLA) molecules are known to play a critical role in donor graft acceptance, rejection and GVHD. There are two types of HLA molecules, class I (HLA-A, -B, -C) that are involved in antigen presentation to CD8+ T cells, and class II (HLA-DR, -DQ, -DP) which present antigens to CD4+ T cells. Without HLA-matching in allogeneic SCT, severe alloreactive T cell responses will be elicited towards normal tissues and cells expressing the foreign recipient-specific HLA molecules, causing the detrimental complication GVHD. Therefore, it is essential to transplant these patients with a donor stem cell graft that is matched for 8-10 out of 10 HLA molecules, not considering HLA-DP.

The most important immune effectors involved in GVT immunity are the donor-derived T cells and natural killer (NK) cells. NK cells recognize their target cells independent of HLA-antigen complexes as their activation is dependent on the balance of signals delivered by activating and inhibitory receptors. Normally NK cells receive inhibitory signals via HLA class I molecules, however on virus-infected cells and malignant cells HLA class I expression can be down-regulated, thereby mediating NK cell activation. In contrast, T cells can only recognize specific antigens presented by HLA molecules via their T cell receptor (TCR). Due to a wide variability in rearrangements of TCR gene segments, the specificity of the naive T cell repertoire is extremely diverse. Upon encounter...
with their cognate antigen and appropriate co-stimulation, the naive T cells become activated and respond. Important antigens in post-transplant anti-tumor immunity are tumor-associated antigens (TAAs) and alloantigens. TAAs are antigens which are highly expressed by tumor cells, but generally also at low levels by normal cells. The immunogenicity of TAAs varies, and immunotolerance could occur as these antigens are derived from self proteins. In contrast, highly potent T cell-based immunity can be elicited against recipient-specific alloantigens independent of HLA-matching. These T cell responses have been demonstrated to be directed against MiHAs, which are polymorphic peptides derived from cellular proteins that are presented in HLA molecules. Due to genetic variations amongst individuals, the patient and donor can be disparate for various MiHAs. Especially in case of transplantation with a graft from an unrelated donor the likelihood of polymorphic differences, and thus numerous MiHA disparities, is high. Often single nucleotide polymorphisms (SNPs) lead to an amino acid change, however frame-shifts, early stop codons and alternative splicing can also occur, resulting in a new immunogenic peptide. As the presentation of a MiHA peptide is restricted to a certain HLA molecule, both the MiHA allele frequency rate and HLA allele frequency determine the MiHA disparity rate within the SCT population. Upon processing and presentation of the MiHA peptide in the HLA molecules, the donor immune system can recognize the antigen as foreign and elicit powerful CD8+, but also CD4+. T cell responses resulting in attack of the MiHA-expressing target cells. While several autosomal-encoded MiHAs are exclusively expressed by hematopoietic cells and their malignant counterparts, others show an ubiquitous expression pattern and can cause GVHD, especially in the skin, liver and gastrointestinal tract.

Since the discovery of the first MiHAs and the awareness of the importance of these polymorphic antigens in GVT immunity, increasing numbers of MiHAs have been identified using different approaches like peptide elution, cDNA expression cloning and genetic linkage analysis. Although the conventional methods are time-consuming and laborious, with the introduction of new and revisited genetic techniques, like whole genome association scanning, this process is accelerated and multiple novel MiHAs can be identified simultaneously. In several studies an association between mismatches in MiHAs and clinical outcome after HLA-matched allogeneic SCT has been reported. On the molecular level, disparities in individual MiHAs, including HA-1, HA-2 and HA-8, were associated with an increased occurrence of GVHD and lower relapse rate, although other reports could not confirm this. In addition mismatches in the ubiquitously expressed HY antigens, which are the result of transplantation of a male recipient with a female donor graft, were associated with a higher incidence of severe acute and chronic GVHD and a reduced relapse rate. However, more important are post-transplantation MiHA-specific T cell responses and their association with clinical outcome. In various malignancies, the emergence of MiHA-specific T cell responses was shown to precede clinical remission in patients treated with allogeneic SCT and DLI. These observations indicate the attractiveness of MiHAs as potent target antigens in post-transplantation immunotherapy to prevent or treat tumor recurrences. Furthermore, by selection of hematopoietic-restricted MiHAs, that are solely expressed by the redundant patient hematopoietic system and residual tumor cells, GVT immunity can be selectively augmented, without causing GVHD. Interesting therapeutic MiHA-candidates in this respect are HA-1, HA-2, LRH-1, ARHGDIIB, BCL2A1 and UTA2-A1.

Dynamic of graft-versus-tumor T cell responses

In order to eradicate tumor cells, efficient activation of tumor-reactive T cells by highly competent antigen presenting cells (APCs) is crucial. DCs are the most potent professional APCs of the immune system that can effectively initiate and reactivates T cell-based immune responses. They originate from myeloid bone marrow precursors that differentiate into immature DCs, which are highly competent in scanning the environment for antigens, engulfment of the antigen and subsequent processing. Upon antigen uptake, maturation of the DCs is initiated resulting in increased expression of peptide-HLA complexes and acquisition of many accessory molecules involved in cell adhesion, cell migration, and co-stimulation. Subsequently, the mature DCs home to the lymph nodes where they present the processed antigen to passing T cells (Figure 1). To activate the T cells with the corresponding TCR two signals are required. Firstly, the TCR-CD3 complex needs to interact with the cognate peptide presented in HLA molecules on the DCs. Secondly, a co-stimulatory signal should be delivered, which is generally provided by CD28, expressed on the T cell, interacting with its ligands CD80 and CD86 on the DCs. In the absence of co-stimulation the T cell will become functionally anergic, and thereby tolerant to the antigen, which is one of the physiological mechanisms involved in elimination of self-reactive T cells. In addition to these two essential signals, inflammatory cytokines can also contribute to T cell activation. Furthermore, cytokines such as interleukin (IL)-2 and IL-15 are important for the proliferation and survival of the antigen-activated T cells.

As the MiHAs are foreign to the donor immune cells, presentation of the cognate MiHA peptide by recipient APCs in combination with correct co-stimulation results in activation of the MiHA-specific CD8+ T cells. These T cells then clonally expand and differentiate into effector cells, that produce inflammatory cytokines like interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and have strong cytolytic potential. Thereby, they can competitively eliminate MiHA-expressing malignant cells. After the initial immune response, most of the MiHA-reactive CD8+ T cells die through apoptosis during the contraction phase. Nevertheless, a small but substantial pool of long-lived memory cells survives, that is capable of quickly responding to recurrence of the antigen. These memory T cells play an important role in protective immunity in SCT patients. However, even though alloreactive CD8+ memory T cells are present for a long time after transplantation, these T cells do not always respond efficiently to recurring tumor cells, suggesting that their functionality is affected and this failure may contribute to the occurrence of tumor relapses.
Chapter 1

General introduction and outline of the thesis

Secondly, defects in components of the antigen presentation machinery can occur, including the transporter associated with antigen processing 1 (TAP-1) and subunits of the immunoproteasome (LMP-2, LMP-7), resulting in improper processing and loading of tumor antigens in the HLA class I molecules. In addition, tumor cells can down-regulate or even lose the expression of HLA class I proteins. The complete or partial loss of HLA-I can contribute to immune escape by the tumor cells.

Defects in antigen processing and presentation

At the tumor level, antigen presentation can be disrupted by for instance down-regulation of antigen expression or selection of tumor cells lacking the immunogenic antigen.

Immune escape by the tumor cells

Despite the powerful aspects of immune reactions, tumor cells exploit various mechanisms to evade immune recognition and destruction. These include both tumor intrinsic alterations, as well as the establishment of an immunosuppressive milieu (Figure 2).

Secondly, defects in components of the antigen presentation machinery can occur, including the transporter associated with antigen processing (TAP) and subunits of the immunoproteasome (LMP-2, LMP-7), resulting in improper processing and loading of tumor antigens in the HLA class I molecules. In addition, tumor cells can down-regulate or even lose the expression of HLA class I proteins. The complete or partial loss of HLA-I can contribute to immune escape by the tumor cells.
can be caused by deletions in chromosome 6, where the HLA alleles are located, or due to mutations in the β2-microglobulin, an essential molecule for stable HLA expression on the cell surface. All these alterations contribute to invisibility of the tumor cells, thereby hampering efficient recognition by tumor-reactive T cells.

**Resistance to immune-mediated cytotoxicity**
Tumor cells can also become resistant to attack by cytotoxic immune cells via interference with signaling of death receptors, like Fas and TRAIL, or by directly impairing the perforin/ granzyme cytotoxic pathways. Increased expression of anti-apoptotic molecules like Bcl-2, Bcl-xL, Mcl-1 or XIAP, which can be accompanied by reduced levels of pro-apoptotic molecules like Bak and Smac, render the cancer cells less sensitive to immune cell mediated apoptosis via inhibition of caspase activity. Furthermore, oncogenic transcription factors like STAT3 can be constitutively activated in both the cancer cells and the immune cells present in the tumor milieu. STAT3 regulates various different processes, including tumor cell proliferation and survival, but also immunosuppression by inhibiting mediators required for immune activation. These changes within the tumor cells can impede proper anti-tumor immune responses.

**Secretion of immunosuppressive factors**
Besides intrinsic alterations, the cancer cells can also create an immunosuppressive microenvironment. For instance by secretion of immunosuppressive cytokines and soluble factors the function of surrounding immune cells can be modulated. Depending on the tumor type different factors are secreted, amongst which IL-10, transforming growth factor (TGF)-β, prostaglandin (PGE)2 and MICA. IL-10 can suppress DC function and skew the T cell response towards the Th2 type, which is less effective in supporting tumor-reactive CTL formation and function than the Th1 counterparts. TGF-β inhibits DC activation as well, but also impairs T cell and NK cell function by diminishing T cell proliferation, down-regulating effector molecules such as perforin and IFN-γ, and blocking expression of the activating NK receptors Nkgp30 and NKG2D. In addition, soluble MICA acts as a decoy factor mediating NK cell inhibition via occupation of the NKG2D receptor. Another important factor secreted by tumor cells is indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the metabolization of the essential amino acid tryptophan via the kynurenine pathway. This results in tryptophan depletion within the tumor microenvironment, and subsequently induces cell cycle arrest and apoptosis of effector T cells.

**Recruitment of suppressive immune cells**
In addition to the direct immunosuppressive actions of the secreted soluble factors, these factors are also involved in recruitment of and differentiation into immunosuppressive immune cells, like regulatory T cells (T_{reg}) and suppressive or tolerogenic APCs. T_{reg} are characterized by the expression of CD25 and the transcription factor FOXP3, and are recruited toward the tumor cells via amongst others the CCR4 chemokines CCL17 and CCL22. Upon arrival in the tumor milieu, T_{reg} can exert their suppressive function via different mechanisms, including direct inhibition of T cell activation and suppression of APC function via cell-cell contact, and secretion of IL-10 and TGF-β. Furthermore, they can also induce IDO production by APCs and thereby indirectly suppress effector T cell function. Moreover, T_{reg} are postulated to possess cytotoxic potential upon activation and may directly kill target cells via perforin- and granzyme-dependent pathways. Notably, TGF-β in the tumor milieu can promote the conversion of effector T cells into T_{reg}. In addition, factors released in the tumor micro-environment attract monocytes and immature DCs, and mediate their differentiation into M2 macrophages, MDSCs and tolerogenic DCs. These suppressive APCs secrete a variety of cytokines and growth factors, like TGF-β and IL-10, that facilitate tumor growth and motility, but also promote Th2 responses or induce immune tolerance. In addition, T cell proliferation and function is inhibited due to arginine deprivation and NO generation resulting from arginine catabolism by arginase-1 and iNOS, two enzymes highly expressed in M2 macrophages and MDSCs. Furthermore, high levels of reactive oxygen species produced by these suppressive APCs can also contribute to T cell suppression and tumorigenesis.

**Signaling via co-inhibitory molecules**
In the last decade, another powerful immunosuppressive mechanism exploited by cancer cells gained much attention: the repressive action of co-inhibitory molecules, which is described in more detail in Chapter 3. Normally, expression levels of co-inhibitory molecules like CTLA-4 and PD-1 are up-regulated upon T cell activation. Ligation of these receptors to their corresponding ligands on APCs results in T cell inhibition, and via this natural feedback loop sustained T cell activation is prevented and the effector T cell response resolves. The balance in positive and negative co-signals determines the activation state of the T cells during immunity and tolerance. By down-regulating co-stimulatory molecules like CD80 and CD86, and up-regulating various co-inhibitory molecules tumor cells acquire an immune inhibitory phenotype. Furthermore, tumor-reactive T cells can express high levels of the co-inhibitory receptors such as CTLA-4, PD-1 and BTLA, as is described in Chapters 3, 4 and 5. It has been reported in chronic viral infections, that due to chronic antigen exposure these co-inhibitory molecules are up-regulated on the viral-specific T cells, and that sustained downstream signaling leads to T cell exhaustion. This phenomenon may also occur in cancer, especially in case of refractory or relapsed disease, and by exploiting such negative regulatory pathways tumor cells effectively hamper T cell-mediated immunity.

**Boosting anti-tumor immune responses with immunotherapy**
To cure hematological malignancies it is essential to explore therapeutic strategies that potently initiate and boost anti-tumor immune responses and induce tumor-specific
immune memory formation in order to prevent tumor relapse. Yet, severe treatment-related systemic toxicities should be avoided. Importantly, hematopoietic-restricted MiHAs, which are solely expressed by the redundant patient hematopoietic system and the hematological malignancy, hold the key to separate GVT and GVHD immunity after allogeneic SCT. Currently, various immunotherapeutic strategies are being investigated to boost anti-tumor immunity, including systemic antibody treatment and cellular therapies like adoptive transfer of T cells and NK cells, and vaccination-based approaches using various antigen formulations or DCs. In addition to the exploration of these strategies as monotherapy, various combinations of antibody treatment, cellular therapy, chemotheraphy and immunomodulatory drugs such as lenalidomide hold promise for future therapy of patients suffering from hematological malignancies.

**Antibody treatment**

Increasing numbers of clinical grade antibodies are being developed and investigated in the treatment of cancers. These antibodies can be applied as a monotherapy, but also in combination with other treatment modalities such as chemotherapy and cellular immunotherapy. Antibodies can mediate tumor clearance via various mechanisms. Upon binding to the target molecule, the Fc-tail of the antibody can be recognized by Fc-receptors on NK cells resulting in subsequent NK degranulation and lysis of the target cell. Besides this antibody-dependent cell-mediated cytotoxicity (ADCC), cytolyis can also be mediated via activation of the complement system upon binding of complement components to the Fc-tail of the antibody. In addition, the antibody itself may directly affect signal transduction of the targeted molecule, or induce apoptosis. Furthermore, cytotoxic drugs or radionucleides can be coupled to the antibodies, thereby the target cells can be specifically damaged and patients will encounter less side effects than with regular treatment. Notably, this last approach requires internalization of the targeted molecule upon antibody crosslinking.

For the treatment of hematological malignancies various monoclonal antibodies have been developed that target molecules highly expressed by the tumor cells. Amongst these are gemtuzumab (anti-CD33) and rituximab (anti-CD20). Notably, the expression of these target molecules is not restricted to malignant cells, and therefore also normal hematopoietic cells will be affected by antibody treatment. The gemtuzumab antibody was coupled to ozogamicin, a potent cytotoxic antibiotic, and has been used in the treatment of AML as in more than 80% of the patients CD33 is expressed by the leukemic blasts. Although, good overall response rates were observed in AML patients with relapsed disease, this immunotoxin was withdrawn from the market in 2010 due to lack of benefit compared to standard chemotherapy, and treatment related toxicity. Nevertheless, in certain patient subgroups treatment with adjusted doses of gemtuzumab ozogamicin had beneficial effects, and reassessment of this drug might be warranted. In contrast, the anti-CD20 antibody rituximab is already part of standard treatment in patients with B cell lymphoma. Rituximab was approved in 1997, and both monotherapy and combination treatment with chemotherapy resulted in significantly increased progression-free survival and overall survival. In chronic lymphoid leukemia (CLL) monotherapy with rituximab had low clinical activity, however in combination with fludarabine and cyclophosphamide prolonged progression-free survival was observed. Importantly, the side effects of rituximab treatment are usually limited and reversible.

Antibody treatment can also be explored to improve immune effector expansion and function in vivo. Treg present in the tumor micro-environment are known to suppress tumor-reactive T cell function. Therefore, anti-tumor immunity might be improved by depletion of these Treg. In order to do so different strategies have been developed that target the IL-2Rα chain, including denileukin diftitox (fusion protein of IL-2 and diphtheria toxin), LMB-2 (anti-CD25 immunotoxin) and daclizumab (anti-CD25 antibody). Recently, daclizumab was reported to reduce Treg numbers and improve anti-tumor T cell responses following infusion with a cancer vaccine in patients with metastatic breast cancer. However, in another study no improvement of anti-melanoma immunity was observed following daclizumab treatment, despite efficient depletion of Treg. As activated effector T cells also express CD25, the protective anti-tumor responses are restrained as well. Hence, it is essential to transiently target the Treg and limit the in vivo availability of daclizumab. Another exciting way to improve anti-tumor immune effector function is by favorably modulating the balance in co-stimulatory and co-inhibitory signals towards T cell stimulation. By administration of agonistic antibodies targeting co-stimulatory molecules, like CD28, 4-1BB and OX40, T cell activation and proliferation can be enhanced. In addition, co-inhibitory interactions can be blocked using antagonistic antibodies targeting CTLA-4, PD-1 and BTLA to release the brake on T cell functionality. Hence, it is essential to transiently target the Treg and limit the in vivo availability of daclizumab. The latter therapeutic strategy is described in more detail in Chapter 3, 4 and 5 of this thesis. Although, interference with these immune checkpoints seems a promising approach to increase anti-tumor immunity, one has to be careful not to elicit autoimmunity (i.e. GVHD in the allogeneic SCT setting).

**Adoptive transfer of immune effector cells**

The immune effector cells that play an important role in anti-tumor immunity are the T cells and NK cells. Therefore, adoptive transfer of these potent effector cells is an appealing means to prevent or treat relapse of the tumor cells, and so far various strategies have been exploited. Nevertheless, specificity is crucial to avoid systemic toxicity. The major breakthrough for successful adoptive transfer was the implementation of an immunodepleting conditioning regimen prior to infusion. Due to the resulting lymphopenia the infused cells are less easily rejected and can thus exert their effector function more efficiently. In addition, soluble factors, including IL-15, are released that promote survival of the transferred T and NK cells. One method to obtain sufficient numbers of T cells reactive against a TAA or MiHA is via isolation of these cells from the
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effect effector repertoire of patients, followed by a fast expansion protocol\(^{95,96}\). However, the cytotoxic potential of these T cells might be suboptimal due to in vivo pre-exposure to immunosuppressive drugs and inhibitory factors in the tumor milieu. Another technique is the isolation and expansion of naïve tumor-reactive T cells from a healthy donor by stimulation with peptide-presenting DCs\(^{12-14}\). However, this is a time-consuming and laborious process. The feasibility of both approaches has been demonstrated by several groups\(^{15-19}\). Importantly, one phase I trial reported in 5 out of 7 patients with relapsed leukemia a complete, but transient, remission upon adoptive transfer of MiHA-specific T cells expanded from post-transplant recipient PBMCs\(^{20}\). Unfortunately, the infused T cells failed to persist in vivo, which might be due to the terminal differentiation stage and, consequently, rapid exhaustion of these cells as a result of the extensive culture protocol.

A third way to efficiently generate high numbers of tumor-reactive T cells with high-affinity TCRs is by gene transfer of the antigen-TCR α and β chains into donor T cells\(^{21,22}\). To prevent the induction of GVHD in patients treated with allogeneic SCT, the TCR genes should preferably be transferred into donor T cells with a known specificity that cannot recognize and target GVHD-tissues, such as virus-specific T cells\(^{23}\). Another potential complication might be mispairing of the introduced and native TCR chains, thereby generating a new potentially harmful specificity\(^{24}\). Efforts are being made to prevent this mispairing, amongst which is the transfer of TCR α and β chains into γδ T cells. Successful TCR gene transfer and resultant cytolytic competence has been demonstrated for both TAAAs and MiHAAs\(^{25,26}\). Importantly, in the setting of allogeneic SCT matching of the HLA-restriction allele between recipient and donor is no longer required, as with gene transfer the complete MiHA-TCR is introduced into the donor T cells.

In contrast to T cells, NK cell activation is only dependent on the balance of signals delivered by activating and inhibitory receptors. Since HLA class I molecules normally inhibit NK cell function, they will be activated and exert their cytolytic function in case of low HLA expression levels on the tumor cells. However, tumor cells can down-regulate surface expression of activating NK ligands, and secrete soluble decoy molecules that occupy the activating receptor NKG2D, and thereby interfere with the efficient NK-mediated recognition and elimination\(^{27}\). Importantly, anti-tumor responses have been observed in AML patients who received NK cell infusions, both in the allogeneic SCT and the non-transplant setting, without causing severe toxicity\(^{28,29}\). In addition, the infused NK cells even alleviated GVHD after allogeneic SCT. These characteristics designate NK cells as a promising immunotherapeutic strategy in the treatment of hematological malignancies\(^{30}\). Nevertheless, similar to the T cell infusions, long expansion and differentiation culture protocols are required to obtain sufficient numbers of NK cells, and these might affect their in vivo functionality. Notably, in our group, a high log-scale expansion protocol was developed to culture large numbers of pure NK cells from umbilical cord blood CD34+ stem cells\(^{31}\). Upon differentiation, these NK cells expressed high levels of activating NK receptors and efficiently killed myeloid leukemia cells \textit{ex vivo}\(^{32}\). The in vivo potential of this NK cell product is currently being explored in a phase I clinical trial in elderly AML patients who are not eligible for SCT.

Vaccination strategies

Patients can also be treated with tumor antigen presenting cell vaccines, either as monotherapy or in combination with T cell infusions and blockade of co-inhibitory signaling pathways, as these have the capacity to stimulate the tumor-reactive T cells in vivo. Another strategy is to infuse long peptides, which are engulfed, processed and cross-presented by the patient’s DC subsets\(^{33}\). As these approaches allow in vivo antigen presentation, the natural cycle of T cell activation, expansion, contraction and memory formation is followed, ensuring long-term persistence of tumor-reactive T cell immunity.

In the recent years, different vaccination strategies have been explored including vaccines of \textit{ex vivo} generated DCs, DC fusions with tumor cells, and tumor cell lysates.

Nowadays, for immunotherapeutic purposes DCs are generally cultured from PB monocytes as high numbers of monocytes can be easily obtained from either patient or donor, and differentiated into mature DCs\(^{41-43}\). Depending on the tumor antigen, e.g. TAA or MiHA, against which T cell immunity should be elicited and its expression profile, the DCs might have to be loaded with the target antigen. In the setting of allogeneic SCT, recipient-derived DCs express the MiHA endogenously and they can be infused without further modifications\(^{44}\). The advantage of this approach is that also currently unknown MiHAs are expressed by the DC vaccine, however these DCs can be compromised by the malignancy and treatment regimen. Another approach is the use of donor-derived DCs, which might be more potent and easier to obtain than those of the patient. Upon culture, donor-derived DCs have to be pulsed with the MiHA to be capable of eliciting MiHA-specific T cell responses. The advantage of using donor-derived DCs is that by exploiting hematopoietic-restricted MiHAs GV T immunity can be selectively boosted, without evoking severe GVHD\(^{44,45}\). Various antigen loading approaches have been developed, including co-culture of the DCs with tumor lysate, exogenous loading of the HLA molecules with a short peptide, and target antigen mRNA electroproporation\(^{46,47}\). The limitation of peptide loading is that only one specific CD4+ or CD8+ T cell response can be induced, whereas DC pulsing with either tumor lysates or antigen mRNA allows natural processing and long-lasting presentation of a variety of different epitopes presented by both HLA class I and II molecules. In that way, the breadth of the elicited immune response could be strongly enlarged.

The feasibility and safety of DC-based vaccination in cancer patients has been shown for a number of malignant diseases, including melanoma, non-Hodgkin lymphoma and MM\(^{48-50,132}\). However, despite the induction of anti-tumor T cell responses and tumor regression in these DC-vaccinated patients, overall response rates and durable responses are not yet optimal. The magnitude of the anti-tumor immune responses, the functionality of the boosted T cells and the induction of long-term memory needs to be enhanced.
Aims and outline of this thesis

Treatment of hematological malignancies with T cell-depleted allogeneic SCT followed by delayed add back of donor lymphocytes, has reduced the incidence and severity of GVHD and in that way has greatly improved the quality of life post-transplantation. Unfortunately, still too many patients relapse and even though in some patients MiHA-specific memory T cells are present, these T cells do not always efficiently respond to the recurring tumor cells. These observations suggest that the tumor cells suppress anti-tumor immune responses. For that reason, it is crucial to gain more knowledge on these immunosuppressive mechanisms exploited by the tumor cells in order to develop potent adjuvant immunotherapeutic approaches that can be applied after allogeneic SCT. The aim of this thesis is to explore the role of co-inhibitory molecules in the functional suppression of GVT immunity post-transplantation, and to investigate and explore different immunotherapeutic strategies that interfere with these negative signals to competently boost MiHA-specific T cell responses in order to prevent or treat recurrences of the tumor cells.

In Chapter 2, we sought to investigate further proof that MiHAs are crucial antigens in the beneficial GVT responses after allogeneic SCT. In a retrospective analysis, we assessed whether DNA disparity in either autosomal-encoded MiHAs or the ubiquitously-expressed HY antigens is associated with less GVHD and improved relapse-free survival. Moreover, we zoom in on the importance of MiHA mismatching on post-transplant outcome of MM patients transplanted with a sibling graft. Finally, we study the immunogenic potential of MiHAs to induce specific T cell responses in mismatched patients, and analyze whether detection of these T cell responses correlates with better clinical outcome post-transplantation. Importantly, we observed that disparity in autosomal MiHAs and detection of MiHA-specific T cell responses was associated with improved relapse-free survival, while the incidence and severity of GVHD was not affected.

In Chapter 3 the role of co-inhibitory receptors in T cell immunity in hematological malignancies is reviewed. Numerous studies indicate that co-inhibitory pathways impair tumor-reactive T cell function in hematological and solid cancers, and contribute to tumor immune escape. Here, we discuss promising pre-clinical and clinical data of immunotherapeutic approaches interfering with these co-inhibitory networks. In Chapter 4 we investigate whether the PD-1/PD-L pathway is involved in the functional impairment of GVT immune responses. We examine the expression levels of various co-stimulatory and co-inhibitory ligands on leukemic progenitor cells using flow cytometrical analysis. In addition, PD-1 expression on MiHA-specific T cells is analyzed. Finally, we stimulate these MiHA-specific T cells with peptide-loaded DCs in the presence of PD-1 blocking antibody. This resulted in enhanced MiHA-specific T cell expansion and cytokine production, especially in patients that developed relapsed disease. Beside PD-1, more co-inhibitory receptors may be involved in tumor immune escape. In Chapter 5 we study the role of the co-inhibitory receptor BTLA in the suppression of MiHA-specific T cell function after allogeneic SCT. First, we determine BTLA expression levels on MiHA-specific memory T cells using flow cytometry. Next, the expression of the BTLA ligand HVEM by various hematological malignancies is assessed. To investigate whether BTLA-HVEM signaling contributes to the functional impairment of MiHA-specific T cells we stimulate these T cells with DCs in combination with anti-BTLA and/or anti-PD-1 blocking antibody. Interestingly, some patients responded better to BTLA blockade, while others showed more pronounced effects following PD-1 blockade. The data in these chapters indicate that PD-1 and BTLA interactions contribute to the functional impairment of GVT immune responses, providing a rationale for incorporating co-signaling blockade against these receptors in post-transplant immunotherapy.

However upon allogeneic SCT, the patient is in a highly activated immunological state due to chemotherapy-induced tissue damage and inflammation, especially in GVHD-prone target tissues such as skin and gut. Releasing immune checkpoints at this time could be dangerous, as T cells would home to the inflamed alloantigen-expressing GVHD sites and destroy healthy cells. Therefore, we developed another immunotherapeutic strategy in Chapter 6. Here, we investigate whether we could augment MiHA-specific T cell responses by stimulation with PD-L1 and PD-L2 silenced monocyte-derived DCs. DCs are electroporated with PD-L1 and PD-L2 siRNA at the immature state, followed by 2 days of maturation. Subsequently, we analyze DC phenotype and their capacity to stimulate either allogeneic or recall antigen T cell responses. Importantly, these PD-L knockdown DCs showed superior potential to expand ex vivo MiHA-specific CD8+ effector and memory T cells from leukemia patients. To generate a clinical-grade applicable DC vaccine with further improved immunogenic potential, we explore in Chapter 7 the...
combination of PD-1 ligand siRNA and target antigen mRNA delivery. While PD-L1 siRNA electroporation must be performed at the immature DC stage to efficiently prevent the up-regulation of PD-L1 and PD-L2 during maturation, mRNA electroporation is most optimal at the mature DC stage. Hence, we investigated a non-viral clinical-grade applicable siRNA transfection method based on lipid nanoparticles (LNPs). First, we examine the siRNA delivery efficacy of various LNP formulations. Subsequently, we analyze the combination of siRNA-LNP transfection and target antigen mRNA electroporation. Finally, we investigate the potential of these PD-L1 silenced DCs pulsed with antigen mRNA to boost MiHA-specific T cell immunity. Importantly, these DCs potently augmented antigen-specific CD8+ memory T cell responses from transplanted cancer patients. Together, these findings indicate that our PD-L silenced DCs are attractive cells for clinical-grade production and adjuvant immunotherapy to competitively induce and boost GVT immunity post-transplantation.

Finally, in Chapter 8 the findings reported in this manuscript are summarized and discussed, and future perspectives are outlined.

References

Chapter 1

General introduction and outline of the thesis


Association of disparities in known minor histocompatibility antigens with relapse-free survival and graft-versus-host-disease after allogeneic stem cell transplantation
Abstract

Allogeneic stem cell transplantation (allo-SCT) can induce remission in patients with hematological malignancies due to graft-versus-tumor (GVT) responses. This immune-mediated anti-tumor effect, however, is often accompanied by detrimental graft-versus-host disease (GVHD). Both GVT and GVHD are mediated by minor histocompatibility antigen (MiHA)-specific T cells recognizing peptide products from polymorphic genes that differ between recipient and donor. In this study, we evaluated whether mismatches in a panel of seventeen MiHAs are associated with clinical outcome after partial T cell-depleted allo-SCT. Comprehensive statistical analysis revealed that DNA mismatches for one or more autosomal-encoded MiHAs was associated with increased relapse-free survival in sibling transplants ($P=0.04$), particularly in patients suffering from multiple myeloma ($P=0.02$). Moreover, mismatches for the ubiquitous Y chromosome-derived MiHAs resulted in a higher incidence of acute GVHD (grade 3-4; $P=0.004$), while autosomal MiHA mismatches, ubiquitous or restricted to hematopoietic cells, were not associated with severe GVHD. Finally, we demonstrated considerable differences between MiHAs in their capability to induce in vivo T cell responses using dual-color tetramer analysis of peripheral blood samples collected post-SCT. Importantly, detection of MiHA-specific T cell responses was associated with improved relapse-free survival in recipients of sibling transplants ($P=0.01$). Our findings provide a rationale to further boost GVT immunity towards autosomal MiHAs with a hematopoietic restriction to improve outcome after human leukocyte antigen-matched allo-SCT.

Introduction

Allogeneic stem cell transplantation (allo-SCT) combined with donor lymphocyte infusion (DLI) is a potent treatment for patients with hematological malignancies\(^{15}\). Many clinical and experimental studies in human leukocyte antigen (HLA)-identical allo-SCT provide evidence that both the potentially curative graft-versus-tumor (GVT) effect and graft-versus-host disease (GVHD) develop as a result of donor T cell responses directed against disparate minor histocompatibility antigens (MiHAs)\(^{3,4}\). These MiHAs are polymorphic HLA-bound peptides derived from cellular proteins that can induce powerful alloreactive T cell responses. It has been demonstrated that emergence of MiHA-specific T cells precedes clinical remission in patients treated with DLI\(^{4,6,7}\). While various MiHAs, including Y chromosome-encoded MiHAs, are expressed ubiquitously, increasing numbers of autosomal-encoded MiHAs are being identified that are exclusively expressed by hematopoietic cells and their malignant counterparts\(^{8-10}\). The molecular identification of these GVHD- and GVT-associated MiHAs has made it possible to study the clinical impact of MiHA mismatches and their specific T cell responses post-transplantation.

Several studies in HLA-matched allo-SCT have reported an association between mismatches in MiHAs and clinical outcome. Mismatches in individual MiHAs, including HA-1, HA-2 and HA-8, have been associated with increased GVHD occurrence and lower relapse rates\(^{11-13}\), but other studies could not confirm these results\(^{14-16}\). Furthermore, previous studies have predominantly investigated cohorts of HLA-matched non-T cell-depleted transplants, and found only an increased rate of chronic GVHD (cGVHD) and reduced relapse rate upon HY MiHA disparity\(^{17,18}\). Moreover, investigation of the role of MiHA incompatibility in transplant outcome is hampered by the requirement to restrict studies to specific HLA types and low frequencies of particular MiHA alleles. Recently however, it was reported that HLA-A2+ chronic myeloid leukemia (CML) patients who developed acute GVHD (aGVHD) showed an improved overall survival (OS) and relapse-free survival (RFS) when receiving a transplant from a HA-1-mismatched donor\(^{19}\).

In this study, we performed a retrospective analysis on the impact of a panel of seventeen immunogenic MiHA mismatches in a relatively large cohort of patients who received partial T cell-depleted allo-SCT. In sibling transplants, mismatches in one or more of the studied autosomal-encoded MiHAs resulted in an improved RFS ($P = 0.04$), especially in multiple myeloma (MM) patients ($P = 0.02$). In contrast, no significant association between autosomal MiHA mismatches and acute or chronic GVHD was observed, whereas the occurrence of a HY disparity led to more grade 3-4 aGVHD ($P = 0.004$). Finally, this report describes for the first time the potential of disparate MiHAs to induce productive T cell responses post-transplantation. Tetramer analysis revealed that the ability of different MiHAs to mount specific CD8+ T cell responses post-transplant varies strongly (0% - 60%). More importantly, presence of MiHA-specific T cell immunity was associated with improved RFS ($P = 0.01$), without inducing severe aGVHD.
or cGVHD. Together, these data provide rationale for further boosting of GVT immunity towards autosomal hematopoietic-restricted MiHAs to improve relapse-free survival after HLA-matched allo-SCT.

Patients, materials and methods

Patients and donors

Three hundred and twenty-seven (N=327) adult SCT recipients and their donors were included in this study. They were selected from the total transplant cohort of our centre, treated between 1995 and 2010 with HLA-matched partial T cell-depleted allo-SCT for a hematological malignancy. HLA was typed using sequence-specific PCR. In sibling transplants, patients were transplanted with a HLA-identical sibling donor, and in matched unrelated transplants (MUD), patients were transplanted with an 8 to 10 out of 10 HLA-matched voluntary donor, not considering HLA-OP. Only patient-donor couples with the HLA types HLA-A1, -A2, -A3, -A24, -B7, -B8 or -B44 were included, because the selected set of MiHAs was restricted to these HLA types. Furthermore, couples were selected based on the availability of both patient and donor material. The characteristics of patients, donors and SCT procedures are shown in Table 1. Patients and donors had given their informed consent to the prospective collection of data and samples for investigational use, which was approved by the Radboud University Nijmegen Medical Centre’s Institutional Review Board.

Treatment protocol

All patients were treated according to protocols described previously20,22. Myeloablative conditioning regimens consisted of cyclophosphamide (60 mg/kg for 2 days) in combination with either total body irradiation (TBI; 4.5 Gy for 2 days) or busulfan (4 mg/kg for 4 days). Idarubicin (42 mg/m² in 48 hours intravenously) was often added to reduce the risk of relapse in the setting of partial T cell-depleted SCT.25 Non-myeloablative conditioning regimens consisted mainly of cyclophosphamide (1200 mg/m² for 4 days) in combination with fludarabine (30 mg/m² for 4 days), sometimes only TBI (2 Gy). Patients receiving a MUD-graft received anti-thymocyte globulin (ATG) (2 mg/kg for 4 days) in combination with fludarabine (30 mg/m² for 4 days), sometimes only TBI (2 Gy). Patients receiving a MUD-graft received anti-thymocyte globulin (ATG) (2 mg/kg for 4 days). After conditioning, patients received a partial T cell-depleted graft derived either from bone marrow (BM) or mobilized peripheral blood stem cells (PBSCs). The median number of CD34+ stem cells in the graft was 1.9 x 10⁶ cells/kg (range 0.5-6.5) for BM and 5.6 x 10⁶ cells/kg (range 1.3-13.8) for PBSCs, and the median number of CD3+ T cells in BM grafts was 0.7 x 10⁶ T cells/kg and in PBSC grafts 0.5 x 10⁶ T cells (Table 1). GVHD prophylaxis consisted of cyclosporine A (CsA) only in almost all patients, and was dosed 1.5 mg/kg bid daily (bid) intravenously for the first two weeks, and thereafter 1 mg/kg bid intravenously or 2.5-3 mg/kg bid orally. Tapering of CsA was started in the absence of GVHD after two months, and stopped at three months. Several patients received prophylactic or therapeutic DLI following transplantation. Prophylactic DLI was restricted to patients who had stopped CsA for at least three months, and had not developed aGVHD grade ≥2 or cGVHD.

Definition of outcome variables

Acute GVHD was graded according to the criteria of Przepiorka et al.26 and cGVHD was classified according to the revised Seattle criteria of Lee et al.27. Patient risk scores for outcome were determined according to the Gratwohl score28. OS, RFS, and non-relapse mortality (NRM) were defined according to the standard criteria proposed by the EBMT.

MiHA genotyping using the KASPar system

HLA-matched SCT donor-recipient pairs were genotyped for a panel of 17 MiHAs with the KASPar assay system (KBioscience, Hoddesdon, UK), which is a fluorescence-based competitive allele-specific PCR using non-labeled primers. Details of this method can be found at http://www.kbioscience.co.uk.

Tetramer staining and validation by T cell culture

PE- and APC-labeled MiHA tetramers were produced as described previously22. Tetramer stainings were performed directly on cryopreserved peripheral blood mononuclear cells (PBMCs) after thawing, and 7 days after ex vivo re-stimulation with the appropriate MiHA peptide29. For this, PBMCs were stimulated once with MiHA peptide-pulsed (10 μM) Epstein-Barr virus lymphoblastoid cell lines (EBV-LCLs) on day 0. MiHA peptides were loaded on a corresponding EBV-LCLs stably transduced with either HLA-A2, -A3, or -B7. For additional HLA types (i.e. HLA-A24 and HLA-B8) matching healthy donor EBV-LCLs were used. After initial ex vivo re-stimulation, 100 IU/ml IL-2 (Chiron, Emeryville, CA) and 10 ng/ml IL-15 (Immunotools, Friesoythe, Germany) was added at days 2 and 5. For tetramer staining, ~1 x 10⁶ cells were stained with 0.2 μg tetramer for 15 min at room temperature. The stability of all tetramers was verified using HPLC analysis in combination with a HLA-binding assay (MHC-ELISA or MHC-bead assay)29. In addition, the functional reactivity of all tetramers, except HEATR, was confirmed by staining cytotoxic T cell clones specific for the corresponding epitope, and subsequent flow cytometrical analysis. After tetramer staining, cells were washed with PBS/0.5% bovine serum albumin (BSA, Sigma, St Louis, MO, USA) and labeled with AlexaFluor700-conjugated CD8 (Invitrogen, Carlsbad, CA, USA) in combination with FITC-conjugated CD4, CD14, CD16 and CD19 (Beckman Coulter) for 30 min at 4°C. Finally, cells were washed and resuspended in PBS/0.5% BSA containing 0.2 μM Sytox Blue (Invitrogen) to allow dead-cell exclusion. Data acquisition was performed on a Cya-ADP analyzer (Beckman Coulter) and analyzed using Kaluza 1.1 software (Beckman Coulter). CD8+ T cells were defined as viable Sytox Blue-negative, single-cell lymphocytes, CD8-positive and FITC (CD4, CD14, CD16, CD19) negative cells. Within the CD8+ T cell population, cells positive for both
tetramers (APC and PE) were quantified (Supplementary Figure 1). Patients were classified as having a positive tetramer response, when MiHA-specific CD8+ T cells (≥0.01% tetramer+ cells within the CD8+ T cell population) were found either directly after thawing, and/or 7 days after ex vivo peptide re-stimulation using peripheral blood samples obtained during the effector or memory phase of the immune response.

Statistical analysis

The outcome variables aGVHD and cGVHD, relapse, RFS, OS, and NRM after allo-SCT were analyzed in relation to MiHA disparity. Associations with RFS and OS were analyzed using Kaplan Meier curves and log-rank tests. Statistical differences in the RFS incidences at certain time points were analyzed using Kaplan Meier point estimates and their associated errors. Associations with cumulative incidences of aGVHD, cGVHD, relapse, and NRM were estimated respecting the presence of competing risks using the Gray test. As competing risks we considered death within 100 days from other toxicities or relapse for aGVHD, death after 100 days from other toxicities or relapse for cGVHD, NRM for relapse, and death from relapse for NRM.

Furthermore, in case a P-value was ≤0.20 in univariable analyses, Cox regression analyses (for the endpoints RFS and OS) and Fine and Gray regression analyses (for the endpoints aGVHD, cGVHD, relapse, and NRM) were used to adjust for the following confounding risk factors: patient age, stem cell source, year of transplant, conditioning regimen, diagnosis-subgroup, CMV seropositivity of either recipient and/or donor, and MiHA disparity associates with RFS and GVHD after allo-SCT

Table 1  Recipient, donor, and SCT characteristics (N=327)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Recipient age, years, mean (range)</th>
<th>Donor age, years, mean (range)</th>
<th>Recipient gender male, no (%)</th>
<th>Donor gender male, no (%)</th>
<th>Gender combination, no (%)</th>
<th>Donor relation, no (%)</th>
<th>Disease category, no (%)</th>
<th>Stem cell source</th>
<th>SCT date, no (%)</th>
<th>Conditioning regimens, no (%)</th>
<th>GVHD prophylaxis, no (%)</th>
<th>Disease status, no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>46 (18-67)</td>
<td>46 (11-71)</td>
<td>195 (60%)</td>
<td>191 (68%)</td>
<td>Male patient/female donor</td>
<td>Matched sibling donor</td>
<td>AML/MDS</td>
<td>Mobilized peripheral blood, no (%)</td>
<td>1995-1999</td>
<td>Myeloablative:</td>
<td>Cyclosporine alone</td>
<td>Early</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Other</td>
<td>Matched unrelated donor</td>
<td>- CD34 x10^6/kg, median (range)</td>
<td>175 (54%)</td>
<td>(Ida-Cy-Bus)</td>
<td>- Flu-Cy</td>
<td>305 (93%)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.6 (1.1-13.8)</td>
<td>18 (5.5%)</td>
<td>(Ida-Cy-TBI)</td>
<td>- Flu-Cy-ATG</td>
<td>13 (4%)</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 (0.04-1.7)</td>
<td>182 (55.5%)</td>
<td>Cy-ATG-Bus</td>
<td>- TBI alone</td>
<td>5 (4%)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52 (46%)</td>
<td>8 (2.5%)</td>
<td>Cy-ATG-TBI</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9 (0.5-6.5)</td>
<td>39 (12%)</td>
<td>Non-myeloablative:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7 (0.5-1.1)</td>
<td>50 (15.5%)</td>
<td>Flu-Cy</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16 (5%)</td>
<td>14 (4%)</td>
<td>Flu-Cy-ATG</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (3%)</td>
<td>19 (59%)</td>
<td>TBI alone</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13 (4%)</td>
<td>81 (25%)</td>
<td>Cyclosporine alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (3%)</td>
<td>53 (16%)</td>
<td>Cyclosporine/MMF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 (3%)</td>
<td></td>
<td>None</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; CLL, chronic lymphoid leukemia; MM, multiple myeloma; SCT, stem cell transplantation; Ida, idarubicin; Cy, cyclophosphamide; Bus, busulfan; TBI, total body irradiation; ATG, anti-thymocyte globulin; Flu, fludarabine; MMF, mycophenolate mofetil; GVHD, graft-versus-host disease; CMV, cytomegalovirus.
Chapter 2

MiHA disparity associates with RFS and GVHD after allo-SCT

Autosomal MiHA disparity is associated with improved relapse-free survival in sibling transplants

Clinical outcome parameters after HLA-matched allo-SCT

We analyzed the clinical outcome after partial T cell-depleted allo-SCT in patients (N=327) suffering from a hematological malignancy. The median follow-up of patients alive at last follow-up was 71 years (range 0.5-17), and 1.1 years (range 0.05-12.6) in those who died. In Figure 1A-B, the survival curves for OS (5 year: 68%) and RFS (5 year: 44%) are depicted for the complete cohort. NRM after 5 years was 15.6% (i.e. 43.4% of all deaths, Figure 1C) and 40.4% of the patients experienced relapse. Acute GVHD occurred in 19.6% of patients and was severe (grade 3-4) in only 6.5%. Of all evaluable patients, 22.7% developed limited cGVHD and 14.2% extensive cGVHD.

MiHA allele frequency and phenotype disparity rate

To determine the MiHA allele frequency and disparity rates, recipient and donor DNA was typed. Paired couples were only genotyped when the appropriate HLA molecule was expressed to which the MiHA is restricted, leading to actual immunogenic disparity rates. HY.A2, HY.B7 or HY.B8 disparity was scored when a female-donor-male-recipient (FDMR) transplantation was performed. The highest disparity rates, within the restricting HLA type, were observed for all Y chromosome-derived MiHAs (20.9%-26.5%), as well as for ACC-2 (25.0%), LRH-1 (19.6%) and HA-1 (16.4%). Overall, 60 (18.2%) and 137 (41.6%) of the 327 pairs were disparate for Y-chromosome or autosomal chromosome encoded MiHAs, respectively (Table 2).

HY disparity is associated with increased aGVHD

In HY mismatched patients a significantly higher grade 3–4 aGVHD incidence of 19% at day 100 after SCT was observed than in HY matched patients (4%; univariable analysis \( P < 0.001 \)) when respecting for the presence of competing risks. Multivariable analysis confirmed this association for HY incompatibility with the increased risk to develop aGVHD grade 3–4 (hazard ratio (HR) 4.1, 95% confidence interval (CI): 1.6-10.3, \( P < 0.001 \) Figure 2A). Notably, none of the non-sex linked MiHA mismatched categories (HA-1 or autosomal MiHAs mismatched) showed an association with acute or chronic GVHD (Figure 2A-B), possibly due to the hematopoietic-restricted tissue expression pattern of the majority of the autosomal MiHAs tested.

Autosomal MiHA disparity is associated with improved relapse-free survival in sibling transplants

Statistical analysis revealed that mismatches in the studied MiHAs did not have an impact on relapse-free survival in the complete transplant cohort (Figure 3A). Because recipients transplanted with a MUD graft might have a HLA-DP mismatch, as well as a higher rate of unknown MiHA mismatches, we separately analyzed the effect of disparities in known MiHAs on clinical outcome in the sibling cohort (N=264). Interestingly, recipients transplanted with an autosomal MiHA-mismatched sibling graft showed significantly better RFS in multivariable analysis (HR 0.68, 95%CI: 0.48-0.98, \( P =0.04 \); Figure 3B, Table 3). This beneficial effect of autosomal MiHA disparity on RFS could be
MM patients show improved RFS upon transplantation with a MiHA-mismatched sibling graft

Interestingly, our multivariable analysis including the correction for diagnosis subgroups revealed an association between autosomal MiHA disparity and improved RFS in HLA-matched sibling transplantation, which indicates a diagnosis independent effect. To study which patients benefit the most from a disparity in the studied MiHAs, we separately analyzed the influence of MiHA mismatches on clinical outcome in different diagnosis subgroups. Notably, we were only able to confirm the significant correlation between the occurrence of a disparate autosomal MiHA and clinical outcome in the MM subgroup. Of the 22 mismatched MM patients, 15 patients were mismatched for 1 MiHA, 6 patients for 2 MiHAs and 1 patient for 4 MiHAs. Importantly, transplanted MM patients showed improved RFS when at least one autosomal MiHA mismatch was present (HR 0.41, 95%CI: 0.19-0.89, \(P=0.02\), Figure 4A). In addition, patients with an autosomal MiHA mismatch developed significantly less relapse as observed in univariable Fine and Gray competing risk analysis (HR 0.46, 95%CI: 0.21-1.00, \(P=0.049\), Figure 4B). The occurrence attributed to both a trend in improved NRM (HR 0.59, 95%CI: 0.29-1.25, \(P=0.19\)) and less relapse (HR 0.77, 95%CI: 0.50-1.11, \(P=0.14\)) as assessed in multivariable Fine and Gray competing risk analyses. Furthermore, also in sibling allo-SCT HY disparity was associated with a higher incidence of grade 3-4 aGVHD (HR 4.2, 95%CI: 1.6-10.9, \(P=0.004\), Table 3).

Table 2 MiHA disparity rate

<table>
<thead>
<tr>
<th>MiHA</th>
<th>HLA restriction</th>
<th>Peptide sequence</th>
<th>Reference</th>
<th>N(^a)</th>
<th>Number of disparate pairs (rates)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-3</td>
<td>A1</td>
<td>VTEPGTAQY</td>
<td>41</td>
<td>82/87</td>
<td>3 (3.7%)</td>
</tr>
<tr>
<td>HA-1</td>
<td>A2</td>
<td>VLHDDLEA</td>
<td>41</td>
<td>195/197</td>
<td>32 (16.4%)</td>
</tr>
<tr>
<td>HA-2</td>
<td>A2</td>
<td>YGEVVSV</td>
<td>42</td>
<td>195/197</td>
<td>6 (3.1%)</td>
</tr>
<tr>
<td>HA-8</td>
<td>A2</td>
<td>RTLDKIVLEV</td>
<td>44</td>
<td>194/197</td>
<td>22 (11.3%)</td>
</tr>
<tr>
<td>HY</td>
<td>A2</td>
<td>FIDSYCOQ</td>
<td>3</td>
<td>190/197</td>
<td>42 (21.3%)</td>
</tr>
<tr>
<td>ADIR</td>
<td>A2</td>
<td>SVAPALALAFPA</td>
<td>41</td>
<td>195/197</td>
<td>30 (15.4%)</td>
</tr>
<tr>
<td>HwA1</td>
<td>A2</td>
<td>CIPPSLLFPA</td>
<td>41</td>
<td>195/197</td>
<td>6 (3.1%)</td>
</tr>
<tr>
<td>SP110</td>
<td>A3</td>
<td>SLPRGSTTPK</td>
<td>47</td>
<td>92/93</td>
<td>7 (7.6%)</td>
</tr>
<tr>
<td>PANE1</td>
<td>A3</td>
<td>RWVDPGVVLK</td>
<td>48</td>
<td>92/93</td>
<td>5 (5.4%)</td>
</tr>
<tr>
<td>ACC-1</td>
<td>A24</td>
<td>DVLOQVLOQ</td>
<td>43</td>
<td>54/54</td>
<td>6 (11.1%)</td>
</tr>
<tr>
<td>ACC-2</td>
<td>B44</td>
<td>KEFEDINWW</td>
<td>49</td>
<td>64/65</td>
<td>16 (25.0%)</td>
</tr>
<tr>
<td>LRH-1</td>
<td>B7</td>
<td>TPQVRQVNC</td>
<td>35</td>
<td>97/98</td>
<td>19 (19.6%)</td>
</tr>
<tr>
<td>HY</td>
<td>B7</td>
<td>SPVDDKARAEL</td>
<td>0</td>
<td>98/98</td>
<td>26 (26.5%)</td>
</tr>
<tr>
<td>ECGF</td>
<td>B7</td>
<td>RPHAIRPLAL</td>
<td>53</td>
<td>97/98</td>
<td>5 (5.2%)</td>
</tr>
<tr>
<td>ZAPHR</td>
<td>B7</td>
<td>IPRDSWVWEL</td>
<td>51</td>
<td>97/98</td>
<td>12 (12.4%)</td>
</tr>
<tr>
<td>HY</td>
<td>B8</td>
<td>LPHNMIDL</td>
<td>52</td>
<td>67/67</td>
<td>14 (20.9%)</td>
</tr>
<tr>
<td>HEATR</td>
<td>B8</td>
<td>ISKERAEL</td>
<td>53</td>
<td>64/67</td>
<td>8 (12.5%)</td>
</tr>
</tbody>
</table>

\(N\), number of patient donor couples typed, within the total number of couples presenting the correct HLA molecule. \(N\)\(^a\) The number of disparate pairs and disparity rates of a particular MiHA were determined within all typed couples presenting the correct HLA molecule.

\(N\)\(^b\), the number of disparate pairs and disparity rates were determined within all typed couples presenting the correct HLA molecule.

Figure 2 Occurrence of GVHD following MiHA-mismatched transplantation

Within the complete cohort, the incidence of (A) aGVHD grade 3-4 and (B) limited/extensive cGVHD was analyzed using Fine and Gray competing risk regression models. Groups were categorized based on MiHA disparity in mismatched and matched at the DNA level. GVHD, graft-versus-host-disease; MiHA, minor histocompatibility antigen.
Chapter 2: MiHA disparity associates with RFS and GVHD after allo-SCT

MiHA disparity associates with RFS and GVHD after allo-SCT

of a disparate MiHA was also correlated with an increase in limited cGVHD (univariable analysis $P = 0.03$, Figure 4C). Notably, the effects on clinical outcome parameters were not a result of differences in disease status between MiHA matched and mismatched patients (Figure 4D). Interestingly, 22.6% of the MiHA mismatched MM patients had a HA-1 disparity (Figure 4E). HA-1 disparity was previously reported to be associated with improved RFS in CML patients who developed aGVHD. Altogether, these data suggest that mismatches in the studied MiHAs induce improved graft-versus-myeloma immunity following partial T cell-depleted sibling allo-SCT.

Table 3: Analysis of patient characteristics and clinical outcome within the SIB cohort

<table>
<thead>
<tr>
<th>Outcome parameter</th>
<th>MiHA disparity</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>$P$-value</td>
</tr>
<tr>
<td>aGVHD gr 3-4</td>
<td>HY</td>
<td>4.80 (2.00-11.20)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>1.10 (0.50-2.50)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGVHD lim/ext</td>
<td>HY</td>
<td>1.30 (0.70-2.20)</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>1.01 (0.63-1.67)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to relapse</td>
<td>HY</td>
<td>0.56 (0.35-0.98)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>0.77 (0.53-1.11)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFS</td>
<td>HY</td>
<td>0.75 (0.49-1.16)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>0.73 (0.52-1.03)</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td>HY</td>
<td>0.90 (0.52-1.55)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>0.88 (0.56-1.35)</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRM</td>
<td>HY</td>
<td>1.30 (0.70-2.70)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>MiHA</td>
<td>0.59 (0.29-1.25)</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

Abbreviations: aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; RFS, relapse-free survival; OS, overall survival; NRM, non-relapse mortality; CMV, cytomegalovirus; CI, confidence interval; MiHA, minor histocompatibility antigen; NA, not applicable. Associations with OS and RFS were analyzed using Kaplan Meier curves and log-rank tests. Associations with cumulative incidences of aGVHD, cGVHD, relapse and NRM were estimated respecting the presence of competing risks using the Gray test. Furthermore, in case a $P$-value was $≤ 0.20$ in univariable analyses, Cox regression analyses (for the endpoints OS and RFS) and Fine and Gray regression analyses (for the endpoints aGVHD, cGVHD, relapse and NRM) were used to adjust for known confounding risk factors.
Multiple myeloma patients transplanted with a related MiHA-mismatched graft show improved relapse-free survival

Multiple myeloma patients transplanted with a sibling graft were grouped based on autosomal MiHA disparity in mismatched and matched at the DNA level. Between these subgroups differences in relapse-free survival and relapse were analyzed using the log-rank test and Fine and Gray competing risk regression model, respectively. N, number of patients within the group. P-values of univariable analyses are given. C. The incidence of cGVHD and D. disease status was analyzed using the Fisher Exact test. E. Within the total of mismatched MiHAs, the relative contribution of each MiHA is depicted. Allo-SCT, allogeneic stem cell transplantation; cGVHD, chronic graft-versus-host-disease; Lim, limited; Ext, extensive; Int, intermediate disease; Adv, advanced disease; n.s., not significant; MiHA, minor histocompatibility antigen.

Ability to induce tetramer+ MiHA-specific T cell responses varies among MiHAs

Although genetic MiHA incompatibility shows significant differences on clinical outcome post-SCT, this does not necessarily mean that corresponding T cell responses actually occur. Therefore, we investigated the potential of 15 of the 17 studied MiHAs to induce productive MiHA-specific T cell responses in vivo. For this, we analyzed recipient PBMC material obtained at a median of 9 months (range 2-70 months) after allo-SCT using a dual-color MiHA-multimer approach. Patients were classified as having a positive tetramer response, when MiHA-specific CD8+ T cells were detected directly after thawing and/or after one ex vivo peptide re-stimulation. Although we were unable to analyze all patients at the same time interval, we believe that a positive detection of MiHA tetramer+ CD8+ T cells at variable time points reflects either an ongoing effector immune response, or a sustained effector-memory response after immune contraction. In Figure 5A, representative tetramer screenings of three different allo-SCT recipients are depicted. Already in freshly thawed samples low numbers of MiHA-specific CD8+ T cells were observed in 27 out of 40 (67.5%) MiHA T cell responsive patients. In addition, in 13 out of 40 (32.5%) responsive patients, tetramer+ T cells were detectable after 1 week stimulation with MiHA peptide-pulsed EBV-LCLs. Notably, when examining the whole cohort of MiHA-mismatched recipients, certain MiHA-specific T cell responses were observed more frequently than others (Figure 5B). Especially, disparity for HA-1, HA-2, PANE1, LRH-1, ACC-1 and the HY-chromosome encoded antigens HY.A2 and HY.B7 resulted in MiHA-specific CD8+ T cell responses in 25-60% of the MiHA-mismatched patients. HA-8, SP110- and ZAPHIR-specific CD8+ T cells were found in 10-20%. In contrast, no productive CD8+ T cell responses against ADIR, HwA11, ECGF, HEATR and HY.B8 were observed, despite genetic disparity. These results indicate that certain MiHAs appear relatively more productive in inducing MiHA-specific CD8+ T cell responses after partial T cell-depleted allo-SCT than others.

Finally, we examined whether presence of a MiHA-specific T cell response, including those targeting autosomal MiHAs (N=25 in the complete cohort, and N=20 in the SIB cohort) or HY (N=15 in both the complete cohort and the SIB cohort), was associated with improved outcome post-transplantation. When analyzing the whole RFS curves no significant differences were observed (complete cohort: HR 0.82, 95%CI: 0.52-1.30, P =0.39; SIB cohort: HR 0.73, 95%CI: 0.44-1.22, P =0.23). Nevertheless in the first years after allo-SCT, the RFS of patients with and without a MiHA-specific T cell response clearly differs. Therefore, the RFS incidences at 3 years post-transplant were compared and we found that detection of MiHA-specific T cell responses was associated with...
Chapter 2

MiHA disparity associates with RFS and GVHD after allo-SCT

Recipient PBMC samples obtained after allo-SCT were analyzed for presence of MiHA-specific CD8\(^+\) T cells using the dual-color MiHA-multimer flow cytometry assay. Patients were classified as having a positive tetramer response when MiHA-specific CD8\(^+\) T cells were found directly after thawing, or 7 days after stimulation with peptide-loaded EBV-LCLs. (A) The number in the dot plots indicates the percentage of MiHA-specific cells positive for both tetramers (PE and APC) within the CD8\(^+\), CD4\(^+\), CD14\(^-\), CD16\(^-\), CD19\(^-\) T cell population. Three representative examples are shown. (B) For each disparate MiHA, the number of tetramer\(^+\) responses (white bars) within the total number of screenings (grey bars) is depicted for the complete cohort of MiHA-mismatched recipients. Percentages indicate the relative number of productive responses. (C,D) Relapse-free survival was analyzed for (C) the complete cohort of recipients versus (D) patients transplanted with a sibling graft using the log-rank test. Groups were categorized based on detection of MiHA-specific T cell responses (black line) vs. no MiHA-specific T cell responses or no mismatching for any of the studied MIs (grey line) after allo-SCT. Allo-SCT, allogeneic stem cell transplantation; N, number of patients within the group; MiHA, minor histocompatibility antigen. Statistical differences in RFS incidences were analyzed using Kaplan Meier point estimates and their associated errors, univariable analysis: \(P = 0.03\), Figure SC), as well as in the sibling transplants (RFS incidence of 73% vs. 52%, univariable analysis: \(P = 0.01\), Figure SD). Notably, this association with improved RFS at 3 years after allo-SCT can be mainly attributed to less relapse in patients with a MiHA-specific T cell response (complete cohort: relapse incidence of 26% vs. 39%, \(P = 0.09\); SIB cohort: relapse incidence of 24% vs. 40%, \(P = 0.047\), and to a smaller extent to improved NRM (complete cohort: NRM incidence of 5% vs. 10%, \(P = 0.26\); SIB cohort: NRM incidence of 3% vs. 9%, \(P = 0.10\)) as assessed in univariable Fine and Gray competing risk analyses. Despite inclusion of T cell responses against ubiquitously expressed MiHAs, including HA-8 and HY (i.e. 17 out of 40 or 35 in the complete and the SIB cohort respectively), the incidence of neither grade 3-4 aGVHD (\(P > 0.7\)) nor limited/extensive cGVHD was affected (\(P > 0.2\), both univariable Fine and Grey competing risk analysis). To conclude, these results indicate that productive MiHA-specific T cell responses contribute to the beneficial GVT immunity following partial T cell-depleted allo-SCT.

**Discussion**

As the dominant target antigens in HLA-matched allo-SCT, MiHAs play a pivotal role in both GVT responses, as well as GVHD. Precise understanding of involved MiHA-specific T cell responses may not only lead to a better prediction of clinical outcome in allo-SCT recipients, but also provide a rationale for the selection of the most potent MiHAs in post-transplant immunotherapy. Interestingly, our statistical analyses of immunogenic MiHA disparity rates in sibling transplants revealed that DNA mismatches in autosomal-

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**Figure 5** Detection of tetramer\(^+\) MiHA-specific CD8\(^+\) T cell responses is associated with improved RFS after allo-SCT

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**Table 1**

<table>
<thead>
<tr>
<th>MiHA</th>
<th>Tetramer Responses</th>
<th>Number of Patients</th>
<th>NRM Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-1</td>
<td>50%</td>
<td>250</td>
<td>3%</td>
</tr>
<tr>
<td>HA-2</td>
<td>60%</td>
<td>150</td>
<td>4%</td>
</tr>
<tr>
<td>HA-3</td>
<td>70%</td>
<td>100</td>
<td>5%</td>
</tr>
</tbody>
</table>

---

**Figure 6**

Relapse-free survival was analyzed for (A) the complete cohort of recipients versus (B) patients transplanted with a sibling graft using the log-rank test. Groups were categorized based on detection of MiHA-specific T cell responses (black line) vs. no MiHA-specific T cell responses or no mismatching for any of the studied MIs (grey line) after allo-SCT. Allo-SCT, allogeneic stem cell transplantation; N, number of patients within the group; MiHA, minor histocompatibility antigen. Statistical differences in RFS incidences were analyzed using Kaplan Meier point estimates and their associated errors, univariable analysis: \(P = 0.03\), Figure SC), as well as in the sibling transplants (RFS incidence of 73% vs. 52%, univariable analysis: \(P = 0.01\), Figure SD). Notably, this association with improved RFS at 3 years after allo-SCT can be mainly attributed to less relapse in patients with a MiHA-specific T cell response (complete cohort: relapse incidence of 26% vs. 39%, \(P = 0.09\); SIB cohort: relapse incidence of 24% vs. 40%, \(P = 0.047\), and to a smaller extent to improved NRM (complete cohort: NRM incidence of 5% vs. 10%, \(P = 0.26\); SIB cohort: NRM incidence of 3% vs. 9%, \(P = 0.10\)) as assessed in univariable Fine and Gray competing risk analyses. Despite inclusion of T cell responses against ubiquitously expressed MiHAs, including HA-8 and HY (i.e. 17 out of 40 or 35 in the complete and the SIB cohort respectively), the incidence of neither grade 3-4 aGVHD (\(P > 0.7\)) nor limited/extensive cGVHD was affected (\(P > 0.2\), both univariable Fine and Grey competing risk analysis). To conclude, these results indicate that productive MiHA-specific T cell responses contribute to the beneficial GVT immunity following partial T cell-depleted allo-SCT.

**Discussion**

As the dominant target antigens in HLA-matched allo-SCT, MiHAs play a pivotal role in both GVT responses, as well as GVHD. Precise understanding of involved MiHA-specific T cell responses may not only lead to a better prediction of clinical outcome in allo-SCT recipients, but also provide a rationale for the selection of the most potent MiHAs in post-transplant immunotherapy. Interestingly, our statistical analyses of immunogenic MiHA disparity rates in sibling transplants revealed that DNA mismatches in autosomal-
encoded MiHAs are associated with improved clinical outcome. In particular, MM patients showed a lower incidence of relapse and increased RFS when transplanted with a MiHA-mismatched sibling transplant. In addition, we demonstrated considerable variance in the relative immunogenicity of different MiHAs in inducing productive T cell responses post-transplantation. Most importantly, presence of these MiHA-specific T cell responses was associated with improved GVT immunity after partial T-cell depleted allo-SCT.

Characteristics of all recipients and their corresponding donors were analyzed for clinical outcome parameters (Table 3). Importantly, we observed that mismatched autosomal MiHAs, including those with a ubiquitous expression pattern, were not correlated with higher incidences of severe acute or chronic GvHD after partial T cell-depleted SCT. In accordance with previous reports, only the HY MiHA was associated with increased frequency of grade 3-4 aGvHD. Similar findings were previously reported by Gratwohl et al. and Stern et al. who used female-to-male alloreactivity as a model for MiHA HY mismatches. However, not only MiHAs play an important role in the FDMR transplant, also non-inherited maternal/paternal antigens (NIMA and NIPA) are involved. In non-T cell-depleted haploidentical sibling NIMA-mismatched allo-SCT, lower aGvHD rates were observed than in NIPA-mismatched recipients. Our current study, we have no information on the NIMA or NIPA status of the transplant couples and cannot exclude the influence of these antigens on clinical outcome after partial T cell-depleted allo-SCT. Moreover, unknown MiHAs not present in our panel and other general genetic disparities might be important as well. Therefore, we analyzed patients transplanted with a sibling donor separately, thereby circumventing the higher likelihood of unknown polymorphic differences between recipients and donors. Analysis showed again a role for a disparate HY MiHA, and although more cases of severe aGvHD (grade 3-4) were observed, this gender mismatch showed a trend towards a lower incidence of relapse (P = 0.095, Table 3), which was previously reported by Gratwohl et al.

Our findings indicate that a disparity in at least one known autosomal MiHA was associated with higher RFS, without increasing aGvHD incidence or severity after partial T cell-depleted allo-SCT. This contribution of autosomal MiHA mismatches to improved RFS in the sibling transplant setting becomes even more apparent when compared with patients transplanted with a MUD graft. In the MUD cohort, effects of mismatched MiHAs on RFS could not be observed, which might be attributed to the smaller size of this cohort. Furthermore, these MUD patients receive ATG treatment, which results in an additional in vivo T cell depletion, thereby reducing the chance of inducing tumor-reactive MiHA-specific T cell responses.

Besides the role of alloreactive T cells in the GVT response, the underlying malignancy of the recipients might also be important. We found that autosomal MiHA disparity in sibling transplants was associated with improved RFS in multivariable analysis, which included the correction for diagnosis subgroups, suggesting a diagnosis independent effect. However, we could only confirm the observed significant correlation between the occurrence of disparate autosomal MiHAs and improved RFS in the MM subgroup. The reason why we did not observe similar effects in the other diagnosis-subgroups could be related to differences in the immune susceptibility of the various malignancies and the number of patients included in this study. Furthermore, partial T cell-depleted SCT results in a low incidence of acute and chronic GVHD, which might have downgraded the clinical impact of MiHA mismatches. When focusing on patients suffering from MM within the sibling cohort, an evident role for disparate MiHAs on both relapse incidence, as well as RFS was observed independent of disease status. Moreover, the occurrence of disparate MiHAs was also associated with a higher incidence of limited cGvHD. These results show that especially MM patients can benefit from a MiHA-driven GVT effect in the presence of an acceptable degree of cGvHD. It is well known that MM is an immunogenic tumor and that patients can respond well to DLI. This phenomenon could be the result of multiple factors, likely including high antigen presentation, good susceptibility to killing, and possibly a good window for immune recognition and killing due to the relatively slow tumor growth. Furthermore, post-transplant treatment with immunomodulatory drugs such as lenalidomide has a promoting impact on GVt immunity.

Overall, we observed low incidence of aGvHD (6.5% grade 3-4) in our partial T cell-depleted setting, even when ubiquitous expressed MiHA mismatches were present. This indicates that there is a potential safe clinical application for transplant mismatching of certain strongly immunogenic MiHAs in allo-SCT. Previously, the MiHAs HA-1, HA-2, LRH-1 and ACC-1 have been implicated to selectively induce a GVT effect without GvHD. We show that they are also potent in inducing MiHA-specific T cell responses in vivo, adding to the promise of these MiHAs not only in transplant mismatching but also in vaccination or adoptive T cell strategies. However, timing of the tetramer-based analysis of post-transplant PBMC samples is crucial in detecting MiHA-specific T cell responses, and our study had a rather wide time frame of sampling after allo-SCT, which probably resulted in underestimating of the frequency of positive responses. In addition, some of the MiHA-specific T cell responses could be masked (subdominant) by other (unknown) MiHAs. Moreover, the underlying malignancy might skew the MiHA-specific T cell repertoire towards a particular hematopoietic compartment such as the BM, which may prevent the detection of MiHA-specific T cells in peripheral blood. Therefore, absence of tetramer T cells during analysis does not necessarily mean a complete lack of MiHA-specific T cells in vivo. Nevertheless, we observed that presence of MiHA-specific T cell responses resulted in improved RFS at 3 years after allo-SCT despite heterogeneity of the cohort. However, after 5 years the RFS curves of patients with and without MiHA-specific immunity no longer differ. As the group size is relatively limited at start and becomes even smaller in time due to positive events or censoring of patients, the data are likely less reliable at late time-points. Furthermore, due to the heterogeneity of
the cohort, over time the patient group is likely skewed toward late-relapsing malignancies with low immunogenicity. It could also well be that the patients that develop late relapses have impaired MiHA-specific T cell immunity due to immune escape mechanisms exploited by surviving tumor cells, like the PD-1/PD-L1 and BTLA/HVEM co-inhibitory pathways as we have reported previously. \cite{10,11}. Due to this negative signaling T cells can become exhausted in time, and patients may lose the advantage of having MiHA-specific immunity post-transplantation.

Importantly, occurrence of these in vivo MiHA-specific T cell responses, including those recognizing ubiquitously expressed MiHAs, amongst which the HY antigens, was not associated with an increased incidence of severe acute or chronic GvHD. Unfortunately, the group of patients having MiHA-specific T cell immunity was too small to focus on diagnosis subgroups or perform multivariable analyses. Therefore, these observations should be confirmed in a larger and more homogenous cohort of allo-SCT recipients with longer follow-up.

In conclusion, this study shows that the occurrence of MiHA mismatches is associated with improved clinical outcome after partial T cell-depleted HLA-matched allo-SCT, particularly in MM patients but likely also in other hematological malignancies. The observed positive effects might be attributed to MiHA-specific CD8+ T cells inducing GVT responses. However, not all MiHAs seem to have the same potential to induce MiHA-specific T cells as was shown by tetramer analysis of PBMC samples collected post-transplant. By further studying the MiHAs that are most productive in this respect, we can select hematopoietic-restricted MiHAs as safe and potent target antigens in allo-SCT in order to prevent or treat tumor recurrences with post-transplant immunotherapeutic strategies.

Acknowledgements

We would like to thank dr. Arnold van der Meer for providing us with DNA samples of transplant couples, Rob Woestenenk for assistance in flow cytometry, and all hematologists of our transplant center for providing patient material. Theo de Witte is a member of the advisory board of Novartis, Celgene and Clavis. The remaining authors have no competing financial interests to declare. This work was supported by a NIH grant (RO1CA18880) and a grant from the Dutch Cancer Society (KWF 2008-4018).

References


Supplementary Figure 1
Gating strategy for detection of tetramer+ MiHA-specific T cell responses

Recipient PBMC samples, obtained after allo-SCT or DLI were stained with MiHA-tetramers in PE and APC, CD8-AlexaFluor 700, CD4-, CD14-, CD16- and CD19-FITC and Sytox Blue. Subsequently, cell populations were analyzed by flow cytometry. Sytox blue– single cells were selected and gated on CD8+ FITC lymphocytes. Within this population the percentage of cells positive for both tetramers was determined.
Co-inhibitory molecules in hematological malignancies: targets for therapeutic intervention

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Abstract

The adaptive immune system can be a potent defense mechanism against cancer, however it is often hampered by immune suppressive mechanisms in the tumor microenvironment. Co-inhibitory molecules expressed by tumor cells, immune cells and stromal cells in the tumor milieu can dominantly attenuate T cell responses against cancer cells. Today, a variety of co-inhibitory molecules, including CTLA-4, PD-1, BTLA, LAG3, TIM-3 and CD200R, have been implicated in immune escape of cancer cells. Sustained signaling via these co-inhibitory molecules results in functional exhaustion of T cells, during which the ability to proliferate, secrete cytokines and mediate lysis of tumor cells is sequentially lost. In this review, we discuss the influence of co-inhibitory pathways in suppressing autologous and allogeneic T cell-mediated immunity against hematological malignancies. In addition, promising pre-clinical and clinical data of immunotherapeutic approaches interfering with negative co-signaling, either as monotherapy or in conjunction with vaccination strategies, are reviewed. Numerous studies indicate that co-inhibitory signaling hampers the clinical benefit of current immunotherapies. Therefore, manipulation of co-inhibitory networks is an attractive adjuvant immunotherapeutic intervention for hematological cancers after standard treatment with chemotherapy and hematopoietic stem cell transplantation.

Introduction

Despite the powerful aspects of immune reactions, most often tumor cells are able to evade immune recognition and destruction. Mechanisms exploited by tumor cells to escape T cell-mediated immunity include disruption of antigen presentation, down-regulation of HLA molecules, secretion of immune suppressive cytokines, as well as recruitment of regulatory T cells (T_{reg}) and myeloid-derived suppressor cells. In the last decade, another powerful immune suppressive mechanism gained much attention: the repressive action of co-inhibitory molecules. Activation of T cells is predominantly dependent on both co-stimulatory and co-inhibitory molecules, including members of the B7/CD28 family. The balance between positive and negative co-signals determines the functionality of T cells during immunity and tolerance. In addition to the native role of co-signaling, tumor cells can evade immune control by down-regulating co-stimulatory molecules such as CD80 and CD86, and up-regulating various co-inhibitory ligands, thereby limiting the therapeutic potential of current immunotherapy against cancer.

Standard treatment for hematological cancers includes chemotherapy and radiotherapy, which reduce tumor burden and can induce long-term remission. Moreover, in the past years new therapeutics, including imatinib, dasatinib, rituximab, bortezomib and lenalidomide, have been developed that target tumor cells. However, drug resistance and relapse remain major problems. In addition, cellular immunotherapy is an attractive treatment option to cure hematological malignancies. Such cell-based immunotherapies include allogeneic stem cell transplantation (allo-SCT), T cell and NK cell adoptive transfer, and vaccination-based approaches using various antigen formulations or dendritic cells (DCs). Allo-SCT can be regarded as the most powerful cell-based immunotherapy, due to the graft-versus-tumor (GVT) responses constituted by alloreactive T cells. These alloreactive T cell responses eradicate the malignant cells upon recognition of polymorphic HLA-presented peptides, known as minor histocompatibility antigens (MHA). Allo-SCT greatly enhanced the cure rate for aggressive hematological cancers, although many patients fail to launch productive immune responses and develop relapses. Moreover, a major drawback of allo-SCT is the occurrence of graft-versus-host disease (GVHD), a potentially life-threatening side effect predominantly caused by alloreactive T cells recognizing healthy tissues, notably the skin, liver and gastrointestinal tract. Since hematopoietic MHA are solely expressed by the redundant patient hematopoietic system and the hematological malignancy, they hold the key to separate GVT from GVHD. Studies by us and others demonstrated that the cellular immunotherapies described above are often hampered by the action of co-inhibitory molecules that attenuate tumor-reactive T cell responses, resulting in sub-optimal clinical results. This review will address the role of co-inhibitory molecules in immune evasion by hematological malignancies, and discuss options to circumvent T cell inhibition without severe adverse effects. In addition, we address whether a differential effect of co-inhibitory molecules...
exists in GVT and GVHD, creating an opportunity to limit GVHD toxicity without dampening anti-tumor immunity.

**Co-inhibitory molecules in hematological malignancies**

Today a variety of co-inhibitory molecules have been implicated in immune escape of cancer. Here, we will discuss the co-inhibitory molecules involved in suppressing anti-tumor immunity against hematological malignancies (summarized in Table 1).

**CTLA-4**

**Expression and function of CTLA-4**

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4; CD152) was the first co-inhibitory molecule identified, and is partly similar to the co-signaling molecule CD28. However, while CD28 is constitutively expressed on the membrane of naive T cells, CTLA-4 is primarily localized in intracellular compartments and rapidly translocates to the cell membrane upon T cell activation. The inhibitory function of CTLA-4 was revealed in knockout mice, which developed lethal lymphoproliferative disease with multiorgan T cell infiltration. Like CD28, CTLA-4 has an extracellular domain containing the MYPPPY binding motif, enabling both receptors to interact with CD80 (B7-1) and CD86 (B7-2) expressed by antigen-presenting cells (APCs). However, the binding affinity of CTLA-4 for these ligands is 10-100 fold higher, thereby outcompeting CD28 and promoting immune inhibition.

As CTLA-4 is up-regulated upon T cell receptor (TCR) ligation, it plays an important role in dampening effector T cell activation and regulating immune homeostasis. Additionally, CTLA-4 signaling in immunosuppressive T<sub>REG</sub> mediates the control of auto-reactive T cells, as in vivo interference with CTLA-4 on these cells elicited pathological autoimmunity. The effect of CTLA-4 interference could either be due to depletion and/or inhibition of T<sub>REG</sub>. Wing et al. showed that T<sub>REG</sub>-specific CTLA-4 deficiency resulted in impaired suppressive T<sub>REG</sub> function, since CTLA-4 enables the down-regulation of CD80/CD86 on APCs, which can be partly due to endocytosis of CD80 and CD86 by T<sub>REG</sub>. This renders a less stimulatory APC, resulting in a lasting cell-extrinsic inhibitory effect. CTLA-4 signaling can attenuate adaptive immune responses in chronic viral infections and cancer. CTLA-4 as such is not a marker of exhausted cells, but elevated levels on viral antigen-specific T cells correlated with their dysfunction in patients with chronic viral infections, which could be restored by CTLA-4 blockade. Also in cancer, high expression of CTLA-4 was correlated with antigen-specific T cell dysfunction in metastatic melanoma. In various CD80- and CD86-positive solid tumor models, monotherapy with CTLA-4 blocking antibody resulted in elimination of established tumors and long-lasting anti-tumor immunity. Several clinical trials have been performed with anti-CTLA-4 antibodies, mostly with ipilimumab in melanoma. Interestingly, an increase in overall survival of melanoma patients has been observed. However, not all patients gain clinical benefit and individual responses are hard to predict. Furthermore, occurrence of adverse toxic

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**Table 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Expression on normal cells</th>
<th>Expression on malignant cells</th>
<th>Binding partners</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>activated T&lt;sub&gt;+&lt;/sub&gt;, T&lt;sub&gt;REG&lt;/sub&gt;, DCs, macrophages</td>
<td>AML, NHL, MM&lt;sup&gt;18;20&lt;/sup&gt;</td>
<td>CD80, CD86</td>
</tr>
<tr>
<td>PD-1</td>
<td>activated T, B, NK, monocytes, myeloid cells</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>PD-L1, PD-L2</td>
</tr>
<tr>
<td>PD-L1</td>
<td>activated B, DCs, macrophages, monocytes, non-lymphoid tissues</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>PD-L2</td>
</tr>
<tr>
<td>PD-L2</td>
<td>activated T, DCs, macrophages, monocytes, non-lymphoid tissues</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>PD-L1</td>
</tr>
<tr>
<td>BTLA</td>
<td>T, B, DCs, myeloid cells, DCs</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>HVEM</td>
</tr>
<tr>
<td>LAG-3</td>
<td>activated T, T&lt;sub&gt;REG&lt;/sub&gt;, NK, pDCs, DCs, NK, monocytes, myeloid cells, fibroblasts, endothelium</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>MHC-1 II</td>
</tr>
<tr>
<td>TIM-3</td>
<td>Th1 CD4&lt;sup&gt;+&lt;/sup&gt; T, CD8&lt;sup&gt;+&lt;/sup&gt; T, DCs, NK, monocytes, epithelium</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Galectin-9</td>
</tr>
<tr>
<td>CD200R</td>
<td>activated T, NK, DCs, fibroblasts, epithelium, monocytes, granulocytes</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Galactin-9, CTS, TIGG</td>
</tr>
<tr>
<td>CD200</td>
<td>activated T, NK, DCs, mast cells, monocytes, myeloid cells, neurons, epithelium</td>
<td>AML, NHL, MM&lt;sup&gt;18&lt;/sup&gt;</td>
<td>CD200R</td>
</tr>
</tbody>
</table>

1. Cells: T<sub>REG</sub> (regulatory T cells), B cells; DCs, dendritic cells; pDC, plasmacytoid dendritic cell; NK, natural killer (T) cells; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; NHL, (B-cell) non-Hodgkin lymphoma; MM, multiple myeloma.
effects remains a problem. Interestingly, in one trial patients responding to ipilimumab were reported to have high titers of anti-MICA antibodies, probably because of enhanced CD4+ T cell function resulting in increased antibody responses. These antibodies may revert the functional inhibition of natural killer (NK) and CD8+ T cells induced by tumor-secreted MICA. In 2011, the FDA and EMA approved ipilimumab treatment for advanced melanoma, paving the way for further exploration of therapies targeting co-inhibitory molecules in cancer. Although anti-CTLA-4 treatment works in vivo, either alone or in combination with vaccines, in vitro CTLA-4 blockade has not been very successful in reversing T cell dysfunction. This might be due to limitations of the in vitro models, as CTLA-4 blockade probably exerts its in vivo action via multiple immune mediators (e.g., effector T cells, Tregs, antibody responses). 

CTLA-4 in hematological malignancies

Numerous experimental and clinical studies have demonstrated that co-inhibitory molecules hamper T cell immunity against hematological cancers in both the autologous and allogeneic setting (Table 2 and 3). For instance, a causal relationship between CTLA-4 and Tregs has demonstrated in lymphoma patients. A large proportion of the lymphoma-infiltrating lymphocytes was identified as CTLA-4+ Tregs, and Tregs-mediated T cell suppression could be abrogated by CTLA-4 blockade. Additionally, CTLA-4+CD80/CD86 interactions also take place between T cells and tumor cells. In multiple myeloma (MM) patients, CD86 but not CD80 was expressed by tumor cells, while CTLA-4 was up-regulated on T cells, resulting in anergy of tumor-specific T cells. In concordance with these results, T cells from chronic lymphoid leukemia (CLL) patients responded to anti-CD3 activation by a decrease in CD28 and an increase in CTLA-4 expression, resulting in an inhibitory phenotype. Similar to MM, we and others showed that acute myeloid leukemia (AML) cells heterogeneously express CD86, but CD80 levels are generally low or absent. As CD80 and CD86 can mediate either T cell stimulation via CD28 or T cell inhibition via CTLA-4, their role in the induction of tumor-specific T cell immunity was investigated in an AML model. Expression of CD86 on AML resulted in tumor rejection, whereas CD80- AML tumors grew progressively. The latter observation was shown to be CTLA-4 dependent, as blockade with anti-CTLA-4 resulted in clearance of CD80- AML cells.

Because of their potent suppressive function, co-inhibitory molecules became major targets of preclinical and clinical blocking studies. For example, in a murine thymoma model, CTLA-4 blockade after DC vaccination improved survival and resulted in a sustained increase in the number of antigen-specific T cells. In a phase I study that included four non-Hodgkin lymphoma (NHL) patients, two subjects developed a clinical response upon ipilimumab treatment. No enhanced T cell-mediated anti-tumor reactivity could be observed, although Tregs levels decreased, suggesting that CTLA-4’s effectiveness may be attributed to Tregs depletion via antibody-dependent cell-mediated cytotoxicity (ADCC). In a follow-up study with 18 NHL patients, ipilimumab administration resulted in clinical responses in 2 patients, and in a number of patients enhanced T cell responses against KLH and tetanus toxoid were observed. Overall toxic effects were limited in these studies, and although durable responses were rare, the response rates resembled those of the first clinical trials in solid cancers. Since only small numbers of patients with hematological malignancies have been treated so far, more research is warranted to draw conclusions.

Allogeneic T cell function after allo-SCT is also strongly influenced by co-inhibitory molecules. The importance of CTLA-4 in modulating allogeneic immune responses has been confirmed by association of certain CTLA-4 genotypes with the incidence of leukemia relapse and overall survival after allo-SCT. Although not all functional consequences of reported polymorphisms have been elucidated, the CD80 single nucleotide polymorphism is postulated to influence the transcription of the sCTLA-4 variant, hampering normal CTLA-4 function. Interestingly, it was demonstrated that CTLA-4 blockade shortly after allo-SCT increased GVHD in a CD28-dependent manner. However, when anti-CTLA-4 was administered at later time points after allo-SCT, the GV/T effect was boosted without signs of GVHD. Shortly after allo-SCT, conditioning-related mucosal barrier injury, leading to a pro-inflammatory cytokine storm, tissue damage and inflammation, may induce major T cell activation in GVHD tissues. However, at later time points these inflammatory events have diminished, and there is no general T cell activation. In patients, ipilimumab administration at late time points after allo-SCT has been explored in one phase I trial. Following a single infusion of ipilimumab in 29 allo-SCT patients with a recurrent or progressive hematological malignancy, 3 clinical responses were observed. Importantly, no induction or exacerbation of clinical GVHD was reported, although similar to other CTLA-4 blockade trials 14% of the patients showed organ-specific immune adverse events. The lack of GVHD induction is likely attributed to the median interval of 1 year between last donor cell infusion and ipilimumab administration. This provides a window for anti-tumor immunotherapy in the post-transplantation setting and emphasizes the importance of appropriate timing.

PD-1 Expression and function of PD-1

Programmed death-1 (PD-1, CD279) is another immunoreceptor belonging to the B7/ CD28 family. In 1992, PD-1 was identified on hybridoma T cells undergoing apoptosis and was believed to be a programmed cell death-induced gene. Further characterization demonstrated that PD-1 is inducibly expressed on stimulated CD4+ T cells, CD8+ T cells, B cells and monocytes. PD-1 binds two B7 family ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273). Their interaction with PD-1 differs in affinity and type, due to a conformational transition in PD-L1, but not PD-L2, upon binding. While PD-L2 expression is mainly restricted to APCs, like DCs and macrophages, PD-L1 is expressed on many non-lymphoid tissues as well. Furthermore, multiple tumor types express PD-L1 and its expression is elevated following IFN-γ exposure. PD-L1 molecules on tumor cells can
Chapter 3 Co-inhibitory molecules in hematological malignancies

It has been well demonstrated that PD-1 plays a crucial role in T cell regulation in various immune responses such as peripheral tolerance, autoimmunity, infection and anti-tumor immunity. Indeed, PD-L1 expression has been associated with poor prognosis in solid tumors. Interestingly, PD-L1 is also able to bind CD80, mediating T cell inhibition. In addition to downstream signaling of PD-L1, also engagement of PD-L2 resulted in T cell inhibition, further illustrating the complexity of these interactions.

Table 2 Outcome of interference with murine co-inhibitory molecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Therapy</th>
<th>Tumor</th>
<th>Outcome</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4 + ova-DC vaccination</td>
<td>Thymoma</td>
<td>Improved tumor rejection, enhanced antigen-specific T cell responses</td>
<td>23</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4; CTLA-4 deletional knockout</td>
<td>AML</td>
<td>Increased survival and improved tumor rejection</td>
<td>22</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4</td>
<td>AML</td>
<td>Enhanced T cell response, prolonged survival</td>
<td>54</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1; PD-1 knockout</td>
<td>MM</td>
<td>Delayed tumor growth; complete tumor rejection in PD-1 knockout</td>
<td>43</td>
</tr>
<tr>
<td>PD-1</td>
<td>HSCT + whole cell vaccination + anti-PD-L1</td>
<td>MM</td>
<td>Increased survival</td>
<td>52</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1; PD-1 knockout</td>
<td>AML</td>
<td>Enhanced T cell response, improved tumor rejection, increased survival</td>
<td>53</td>
</tr>
<tr>
<td>PD-1/TIM-3</td>
<td>Anti-PD-L1 and/or mTim-3 hFc</td>
<td>AML</td>
<td>Delayed tumor growth upon monotherapy, improved tumor rejection upon combined blockade</td>
<td>61</td>
</tr>
<tr>
<td>CD200</td>
<td>Anti-CD200</td>
<td>AML</td>
<td>Increased survival</td>
<td>86</td>
</tr>
<tr>
<td>CD200</td>
<td>Anti-CD200</td>
<td>B-CLL</td>
<td>Improved tumor rejection</td>
<td>87</td>
</tr>
<tr>
<td>CD200</td>
<td>Anti-CD200</td>
<td>B-cell lymphoma</td>
<td>Delayed tumor growth</td>
<td>88</td>
</tr>
<tr>
<td>Allogeneic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4</td>
<td>AML</td>
<td>Enhanced T cell response and GVHD early after BMT; enhanced tumor-specific T cell response later after BMT with low GVHD</td>
<td>28</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1</td>
<td>Lymphoma</td>
<td>Enhanced T cell response</td>
<td>62</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1</td>
<td>None</td>
<td>Enhanced alloreactive T cell response, no GVHD</td>
<td>64</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1; PD-1 knockout</td>
<td>CML</td>
<td>Increased survival</td>
<td>65</td>
</tr>
<tr>
<td>BTLA</td>
<td>BTLA agonist</td>
<td>B-cell lymphoma</td>
<td>Early after BMT; prevention of GVHD, later after BMT; no effect on GVHD; effective GVT response</td>
<td>78</td>
</tr>
<tr>
<td>BTLA</td>
<td>BTLA agonist</td>
<td>Mastocytoma/T-cell lymphoma</td>
<td>Inhibition of alloreactive T cell response, prevention from GVHD</td>
<td>79</td>
</tr>
<tr>
<td>HVEM</td>
<td>Specific blockade of BTLA binding</td>
<td>None</td>
<td>Prevention from acute GVHD</td>
<td>80</td>
</tr>
<tr>
<td>PD-1H/VISTA</td>
<td>PD-1H/VISTA agonist</td>
<td>None</td>
<td>Prevention from acute GVHD</td>
<td>100</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; B-CLL, B-cell chronic lymphoid leukemia; CML, chronic myeloid leukemia; MM, multiple myeloma; BMT, bone marrow transplantation; HSCT, hematopoietic stem cell transplantation; GVHD, graft-versus-host disease; GVT, graft-versus-tumor.

deliver negative signals towards PD-1-expressing tumor-reactive T cells, thereby inhibiting anti-tumor immunity. Indeed, PD-L1 expression has been associated with poor prognosis in solid tumors. Interestingly, PD-L1 is also able to bind CD80, mediating T cell inhibition. In addition to downstream signaling of PD-L1, also engagement of PD-L2 resulted in T cell inhibition, further illustrating the complexity of these interactions.
to these signaling effects, the PD-1 gene itself is subject to epigenetic regulation, as increased PD-1 expression on activated CD8\(^+\) T cells results from demethylation of the \(\text{Pdcd1}\) locus\(^{48}\). During conversion to functional memory T cells remethylation of \(\text{Pdcd1}\) occurs, whereas in exhausted T cells the \(\text{Pdcd1}\) regulatory region remains demethylated.

### PD-1 in hematological malignancies

In addition to CTLA-4, PD-1/PD-L interactions were shown to be of importance in hematological malignancies. For instance, PD-L1 overexpression enhanced MM invasiveness and rendered tumor cells less susceptible to cytotoxic T lymphocytes (CTLs)\(^{49}\). This effect was

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**Table 3** Outcome of interference with human co-inhibitory molecules

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Therapy</th>
<th>Tumor</th>
<th>Outcome</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>Ipilimumab, in vivo</td>
<td>NHL</td>
<td>2/4 tumor regression, no increase in vaccine-specific T cell responses, reduction in T(\text{REG}) number early after treatment. Toxicity: mainly grade 1-2, 1x grade 3</td>
<td>24</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Ipilimumab, in vivo</td>
<td>Relapsed/refractory B cell NHL</td>
<td>2/18 clinical response, 5/16 enhanced T cell response to recall antigen. Toxicity: mainly grade 1-2, 6/18 grade 3</td>
<td>25</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4, ex vivo</td>
<td>HL</td>
<td>Abrogated T(\text{REG}) suppression</td>
<td>17</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Anti-CTLA-4, ex vivo</td>
<td>CLL</td>
<td>Enhanced tumor-specific T cell response</td>
<td>108</td>
</tr>
<tr>
<td>PD-1</td>
<td>BMS-936,558, ex vivo</td>
<td>ALL, AML, CML, NHL, MM</td>
<td>Enhanced alloreactive T cell response</td>
<td>20</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1, ex vivo</td>
<td>NHL</td>
<td>Enhanced T cell response</td>
<td>52</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1 and anti-PD-L2, ex vivo</td>
<td>HL</td>
<td>Restored T cell response</td>
<td>53</td>
</tr>
<tr>
<td>PD-1</td>
<td>Anti-PD-L1, ex vivo</td>
<td>HCV-lymphoma</td>
<td>Abrogated T(\text{REG}) suppression, reduction in T(\text{REG}) number</td>
<td>55</td>
</tr>
<tr>
<td>PD-1</td>
<td>CT-011, in vivo</td>
<td>AML, CML, HL, NHL, MDS, MM</td>
<td>6/17 clinical response, 1 complete remission, no toxicity</td>
<td>57</td>
</tr>
<tr>
<td>PD-1</td>
<td>CT-011 w/wo lenalidomide, ex vivo</td>
<td>MM</td>
<td>Enhanced NK cytotoxicity, additive effect of lenalidomide</td>
<td>58</td>
</tr>
<tr>
<td>PD-1</td>
<td>CT-011 + tumor/DC vaccination, ex vivo</td>
<td>MM</td>
<td>Reduction in T(\text{REG}) number, enhanced T cell response</td>
<td>59</td>
</tr>
<tr>
<td>PD-1</td>
<td>DC vaccination with PD-L silencing, ex vivo</td>
<td>AML, CML</td>
<td>Enhanced alloreactive T cell response</td>
<td>106</td>
</tr>
<tr>
<td>BTLA</td>
<td>Anti-BTLA, ex vivo</td>
<td>ALL, AML, CML, NHL, MM</td>
<td>Enhanced alloreactive T cell response</td>
<td>62</td>
</tr>
<tr>
<td>CD200</td>
<td>Anti-CD200, ex vivo</td>
<td>AML</td>
<td>Enhanced NK cytotoxicity</td>
<td>64</td>
</tr>
<tr>
<td>CD200</td>
<td>Anti-CD200, ex vivo</td>
<td>CLL</td>
<td>Enhanced antigen-specific T cell responses, reduction in T(\text{REG}) number</td>
<td>65</td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; HCV, hepatitis C virus; T\(\text{REG}\), regulatory T cell; NK, natural killer cell; MiHA, minor histocompatibility antigen; GVHD, graft-versus-host disease; GI, gastro-intestinal tract.
alleviated in PD-1 knockout mice or by anti-PD-L1 antibody treatment, demonstrating the importance of the PD-1/PD-L1 pathway in this process. This role of PD-1 was also confirmed in an AML model, and interestingly, PD-L1 expression was elevated on tumor cells in vivo compared to in vitro. In another report, an increased level of PD-L1 on MM cells together with enhanced PD-1 expression on exhausted T cells was demonstrated. As expected, in mice PD-L1 blockade improved survival after autologous SCT and whole cell vaccination from 0% to 40%. In humans, PD-L1 expression was observed on NHL tumor cells, and blockade greatly enhanced cytokine production of autologous tumor-reactive T cells.

Furthermore, it was shown that tumor cells of Hodgkin lymphoma (HL) patients can express both PD-L1 and PD-L2, and PD-1 expression was elevated on HL-infiltrating T cells. Also in this case, blockade of PD-L1 mediated increased cytokine secretion by the infiltrated T cells. Additionally, long-term persistent murine leukemia cells were shown to sequentially up-regulate PD-L1 and CD80, thereby conferring protection against immune destruction. Upon PD-L1 or CTLA-4 blockade, CTL-mediated lysis of these persistent AML cells was improved. Similar to the link of CTLA-4 and Tregs, an elevated number of Tregs exhibiting high PD-1 expression was described in HCV-associated lymphoma. In addition to PD-1 expression on CD8 T cells and Tregs, PD-L1 expression on APCs was important for tumor persistence of murine AML. Combining PD-L1 blockade with Treg depletion showed superior efficacy in clearance of AML, due to alleviation of PD-1-dependent Treg-mediated suppression.

Although clinical PD-1 blockade has not been as extensively tested as ipilimumab for CTLA-4, multiple clinical grade antagonistic anti-PD-1 antibodies have been developed, i.e. CT-011, BMS-936,558 and MK-3475 (NCT01295827). Furthermore, two anti-PD-L1 antibodies, BMS-936,559 (NCT00729664) and MPDL2180A (NCT01375842), one anti-PD-L2 antibody (NCT00658892) and a PD-L2 fusion protein AMP-224 (NCT01352884) are being tested in phase I clinical trials. Three studies involving hematological cancers were performed with CT-011. One phase I clinical trial was conducted in patients with varying hematological malignancies, and showed a clinical response in 6 out of 17 patients, with few adverse events. Although the CD4+ T cell count was elevated in the treated patients, no additional evidence of T cell activation was found. In a pre-clinical study, ex vivo treatment with CT-011 enhanced the functionality of NK cells against autologous primary MM cells. In addition, the drug lenalidomide down-regulated PD-L1 levels, and an additive effect was shown by combining lenalidomide with CT-011, rendering this combination a promising therapy for MM patients. Another study examined whether PD-1 blockade improves the effectiveness of myeloma/DC vaccination therapy, since it is known that both myeloma cells and myeloma/DC hybridomas highly express PD-L1. Indeed, ex vivo addition of CT-011 resulted in enhanced myeloma lysis by T cells, as well as a reduction in the number of Tregs. However, until now the most promising effects have been obtained with the monoclonal human anti-PD-1 antibody BMS-936,558 (MDX-1106, ONO-4538). Administration to patients with solid tumors was well tolerated and only one serious adverse event, inflammatory colitis, was reported. Follow-up reports presented at ASCO 2010 and GU ASCO 2011 showed that persistent clinical responses were observed in approximately 30% of patients with renal cell carcinoma, prostate cancer, melanoma and lung cancer upon treatment with repetitive doses of anti-PD-1 antibodies. The lack of strong toxic effects in this study holds promise that PD-1 blockade might have a more subtle effect than CTLA-4 blockade, thereby highlighting anti-PD-1 antibodies as interesting candidates for cancer therapy.

The role of PD-1 in allo-SCT has been investigated both in mice and men. In two similar murine studies dissecting the role of alloantigens in GVT and GVHD reactivity, it was found that alloreactive T cells recognizing antigens on GVHD-prone tissues are driven into dysfunction and apoptosis. Furthermore, the interaction of non-hematopoietic cells with alloreactive T cells prevented the formation of proper alloreactive memory cells by exploiting the PD-1/PD-L1 pathway. This means that in addition to the detrimental effect of GVHD as such, also the beneficial GVT effect is hampered as alloreactive T cells become functionally impaired. Notably, PD-L1 blockade late after allo-SCT may partly restore the GVT reactivity without inducing GVHD. Moreover, these results support the importance of targeting hematopoietic-restricted MiHAs, since these are solely expressed by hematopoietic tumor cells and residual healthy immune cells of the recipient, but not by GVHD-prone tissues. In one of the few studies investigating chronic myeloid leukemia (CML), using a retrovirus-induced CML model, it was demonstrated that tumor-specific T cells can become exhausted. In this model, consisting of PD-1+ tumor-specific T cells and PD-L1+ CML cells, exhaustion was overcome by either using PD-1 deficient cells or anti-PD-L1 administration. We and others have investigated the role of PD-1 in GVT immunity in allo-SCT patients. High PD-1 expression was observed on alloreactive CD8+ Treg cells that specifically recognize hematopoietic-restricted MiHAs in myeloid leukemia patients. In agreement, Mumpercht et al. showed that the total T cell population from CML patients had elevated levels of PD-1. Also CD117+ progenitor AML cells displayed low levels of CD80 and CD86, whereas PD-L1 was highly expressed, especially under inflammatory conditions. Since these observations were made in allo-SCT patients who relapsed after initial powerful MiHA-specific T cell responses, we postulated that PD-1 expression is involved in T cell exhaustion. By stimulation with MiHA-loaded DCs ex vivo, we aimed at activating these PD-1+ MiHA-specific Treg cells, however results were suboptimal, suggesting an impaired state. Importantly, upon treatment with anti-PD-1 or anti-PD-L1 blocking antibodies we were able to reinvigorate MiHA-specific Treg proliferation. Notably, the effect of PD-1 blockade on MiHA-specific Treg cells from relapsed patients compared to patients in long-term remission was significantly stronger, indicating the function of PD-1 in T cell exhaustion and subsequent tumor immune evasion.
BTLA

Expression and function of BTLA

B and T lymphocyte attenuator (BTLA), i.e. CD272, was identified in 2003 as an inhibitory receptor with structural similarities to CTLA-4 and PD-1. BTLA is mainly expressed by immune cells, including T and B cells, DCs and myeloid cells. In contrast to other B7/CD28 family members, BTLA binds a member of the tumor necrosis factor receptor (TNFR) superfamily, namely Herpes virus entry mediator (HVEM). Though, HVEM is part of an intricate signaling network as it has at least four additional binding partners that distinctively mediate T cell responses: i.e. CD160, LIGHT (for lymphotxin-a, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes), Lymphotxin-a, a (LTα) and Herpes simplex virus glycoprotein D (gD). BTLA or CD160 signaling upon HVEM binding result in T cell inhibition. However, HVEM present on T cells acts as a co-stimulatory receptor, thereby constituting a bidirectional pathway. Interestingly, naive T cells express both HVEM and BTLA, and these molecules form a T cell intrinsic heterodimer complex. Due to formation of this complex, HVEM is unavailable for extrinsic ligands, and no co-stimulatory signal is transduced. Studies in BTLA-deficient mice revealed a predisposition to experimentally induced autoimmune encephalomyelitis. Furthermore, these mice develop late-onset spontaneous autoimmune hepatitis-like disease and multi-organ lymphocyte infiltration, implying the involvement of BTLA in maintaining self tolerance. In humans, persistent expression of BTLA was observed on EBV- and CMV-specific CD8+ T cells, negatively affecting T cell function. Furthermore, in melanoma patients high BTLA expression correlated with impaired tumor-specific T cell function. These tumor-specific T cell responses could be restored in vitro by interference with the BTLA/HVEM pathway in combination with vaccination therapy. In addition, co-expression of PD-1, BTLA and T-cell immunoglobulin and mucin-domain-3 (TIM-3) rendered melanoma-specific CD8+ T cells highly dysfunctional, which could be restored by combined blockade of all three co-inhibitory molecules.

BTLA in hematological malignancies

A single administration of agonistic anti-BTLA antibody directly after allo-SCT in mice completely prevented GvHD, while this treatment did not hamper GvT responses. Moreover, when using an antibody that specifically blocked the interaction of HVEM with BTLA, but not LIGHT, GvHD was attenuated. This suggests that in this model the co-stimulatory function of HVEM was dominant over its co-inhibitory activity. Since both HVEM and BTLA can be expressed by T cells, bidirectional signaling can occur. Therefore, combining specific blocking antibodies with cell-specific knockout models for these molecules will be necessary to further unravel the intricate interactions between HVEM, BTLA, CD160 and LIGHT. In addition, we investigated the effect of a BTLA blocking antibody on MiHA-specific T cell function in allo-SCT patients. As shown for PD-1, we observed that BTLA was also highly expressed on MiHA-specific T cell. Moreover, in 7 out of 11 patients BTLA blockade resulted in increased outgrowth of MiHA-specific T cells of transplanted patients. Interestingly, in 3 patients BTLA blockade effects were more prominent than those of PD-1, indicating that BTLA has a non-redundant function to PD-1, and therefore it holds promise in post-SCT therapies.

New co-inhibitory players

In addition to the afore discussed molecules, CD200R, TIM-3, LAG-3 and PD-1/HV/1STA were recently shown to contribute to T cell inhibition and/or exhaustion in hematological cancers. CD200 receptor (CD200R) is an inhibitory receptor previously thought to be most important on myeloid cells, but it is also expressed in the lymphoid lineage such as NK, CD4+ and CD8+ T cells, especially upon stimulation. Its ligand, CD200 (OX2) is a glycoprotein expressed on a broad number of cell types, including solid tumors and hematological malignancies. In addition to the previously discussed co-inhibitory molecules, CD200R inhibits both T and NK cell functionality. Furthermore, CD200/CD200R interactions are involved in tumor immune evasion, as CD200 expression on AML cells promoted tumor growth in mice. Interestingly, patients with CD200+ AML cells displayed a lower number of activated NK cells, and the effect on NK functionality was correlated to CD200 expression on the leukemia cells. In concordance with this, blockade of CD200 enhanced Fnd α release and cytotoxicity by NK cells. Moreover, CD200 blockade restored T cell proliferation and tumor control by immune cells for human CD200+ CLL both in vitro and in a humanized mouse model. However, in a follow-up report treatment with anti-CD200 antibody caused loss of T cell-mediated tumor control due to clearance of the T cells. This was attributed to ADCC of CD200+ T cells caused by the IgG1 variant of anti-CD200, which was not observed for the IgG4 isotype. Therefore, blocking strategies should be carefully designed before proceeding to the clinic.

In addition, the co-signaling receptor TIM-3 is expressed on Th1, CD4+ and CD8+ T cells, and is involved in co-inhibition. In mice, the interaction of TIM-3 with its ligand Galectin-9 was demonstrated to be inhibitory in autoimmune diseases and malignancies. Furthermore, in HIV+ and melanoma patients, dysfunctional T cells have been shown to co-express TIM-3. In this regard interference with TIM-3 signaling is an interesting treatment option, and enhanced tumor vaccine efficacy has been observed by TIM-3 blockade. Interestingly, both TIM-3 and PD-1 were expressed on a subset of exhausted CD8+ T cells in a murine AML model and expression increased during tumor progression. While either TIM-3 or PD-L1 blockade alone was not sufficient to improve survival, a combination of the two antibodies decreased tumor burden and enhanced survival. Furthermore, in human lymphoma an interesting role for TIM-3 has been described on tumor endothelium. TIM-3 expressed on these endothelial cells mediated impaired CD4+ T cell responses, and thereby promoted lymphoma onset, growth, and dissemination. In contrast, a stimulatory role for TIM-3 and Galectin-9 has been reported in the interaction.
of CD8+ T cells and DCs10. This discrepancy is reflected in research investigating its mechanism of action, where T cell receptor stimulation is enhanced upon TIM-3 signaling18. This might be explained by the fact that T cell exhaustion could be caused by prolonged TCR signaling, and TIM-3 accelerates this process. Another explanation is that depending on which ligand binds to TIM-3, different modes of signaling are initiated. Therefore, TIM-3 may act as either a co-stimulatory or a co-inhibitory factor, similar to BTLA.

Lymphocyte-activation gene 3 (LAG3, CD223) is a co-inhibitory receptor highly similar to CD4 and binds HLA class II molecules21,22. Importantly, LAG3 was implicated to inhibit T cell function in HL patients23,24. LAG3 seems to be non-redundant from PD-1, as both are expressed on distinct populations of CD8+ T cells. Recently, it was shown that in mice PD-1 and LAG3 act synergistically in the onset of autoimmune diseases and tumor escape25,26. Furthermore, in HL, both Treg and LAG3+ CD4+ T cells were shown to be involved in tumor immune evasion, since the expression of FoxP3 and LAG3 coincided with the impairment of tumor-specific T cell responses27. Therefore, LAG3 is an interesting candidate to combine with therapies that utilize Treg depletion or PD-1 blockade.

Recently another immunoregulatory molecule with similarities to PD-1, as well as to PD-L1 was simultaneously discovered by two groups: PD-1H (PD-1Homolog)28 or VISTA (V-domain Ig suppressor of T cell activation)29,30. This molecule is broadly expressed on hematopoietic cells and is up-regulated on APCs and T cells upon activation. Mice treated with a single dose of PD-1H/VISTA-antibody did not develop GVHD after allo-SCT, however the mechanism of action was not elucidated29. Another study identified PD-1H/VISTA as an inhibitory ligand on APCs and tumor cells31,32. Here, PD-1H/VISTA Ig-fusion protein conveyed a lasting negative signal to T cells, and expression of the protein on APCs suppressed T cell proliferation. Importantly, PD-1H/VISTA expression on tumor cells resulted in diminished anti-tumor immunity. The human ortholog was determined on the genomic level, and due to the important role of this immunoregulatory molecule in GVHD and tumor escape, PD-1H/VISTA is anticipated to be a potential therapeutic target.

Future prospects

Several therapeutic strategies to interfere with the function of co-inhibitory molecules are being explored in order to enhance anti-tumor T cell immunity. The challenge of interference with immune checkpoints is to boost anti-tumor reactivity while avoiding systemic toxicity. This could potentially be achieved by 1) combining the alleviation of co-inhibition with other therapeutic options, 2) blocking co-inhibitory molecules that are intrinsically skewed towards anti-tumor responses rather than GVHD or autoimmune effects and 3) optimal dosage and timing of antibody administration. Appealing combinations are the simultaneous targeting of multiple co-inhibitory receptors, or incorporation in existing cellular therapies. For example, DC vaccination may be applied together with blocking antibodies or siRNA knockdown of co-inhibitory molecules to boost anti-tumor immunity, or by administration of agonistic antibodies against co-inhibitory molecules implicated in GVHD, adverse effects may be reduced (Figure 1).

Although anti-CTLA-4 and anti-PD-1 monotherapy have shown promising results, combination therapy with other treatment modalities such as immunomodulatory anti-cancer agents, vaccines or T cell depletion is potentially necessary to effectively cure hematological cancers. At the moment, several clinical trials are underway that target co-inhibitory receptors in hematologic cancers. The effectiveness of CTLA-4 blockade by ipilimumab is investigated in lymphoma patients (NCT00047164), and the anti-PD-1
knowledge of a growing number of co-inhibitory molecules, novel mono- and combinatorial treatment options become available. In the end, this could lead to optimized immunotherapy against hematological cancers, with limited risk of adverse events like the induction of autoimmune diseases and GVHD.

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monoclonal antibody CT-011 is combined with three different therapies. In lymphoma patients, CT-011 is administered following autologous SCT (NCT00532259) and combined with rituximab (NCT00904722). Furthermore, the combination of CT-011 with a DC/AML fusion vaccine is being investigated as a therapy for AML patients (NCT01096602). Recently, we explored another treatment option in which an antigen-specific stimulation is combined with interference of co-inhibition. We demonstrated that stimulation with PD-L1/L2 silenced MiHA-loaded DCs boosted the expansion of MiHA-specific T cells ex vivo. Following these promising results, we will start a clinical trial shortly combining DLI with vaccination of PD-L1/L2 silenced donor DCs loaded with hematopoietic-restricted MiHA (CCMO-trial no. NL37318). We believe that these clinical studies provide a platform for incorporating blockade of co-inhibitory molecules in adjuvant therapy of hematological malignancies, with numerous options for combination therapies. Importantly, the risk of breaking tolerance systemically by blockade of one co-inhibitory molecule could be prevented by using lower levels of multiple blocking antibodies targeting different inhibitory molecules simultaneously, since together these may boost immune responses in a non-redundant manner. This is stressed by the fact that exhausted T cells are known to display multiple different co-inhibitory receptors. By analyzing the downstream pathways of different co-inhibitory receptors one could limit these options and exclude combinations of receptors that have redundant effects, and focus on synergistic combinations. Notably, a clinical trial in solid tumors has started which combines blocking antibodies against PD-1 and CTLA-4 (NCT01024231), harnessing the power of these two non-redundant immune checkpoints.

The crux in allo-SCT is the separation of GVT and GVHD reactivity. Although CTLA-4 and PD-1 clearly contribute to T cell exhaustion, their activation might have too broad consequences early after allo-SCT, and interfering with their signaling might deteriorate GVHD. Interestingly, anti-BTLA was recently reported to impair GVHD while allowing GVT reactions. Whether or not this important distinction in alloreactive responses also exists in humans needs to be evaluated, but it renders BTLA an important candidate for post-transplantation immunotherapy. Furthermore, the timing of co-receptor blockade seems to be essential to boost GVT without causing GVHD. Early after allo-SCT, the patient is in a highly activated immunological state, due to chemotherapy-induced tissue damage and subsequent inflammation, especially in GVHD-target tissues such as skin and gut. To release immune checkpoints at this time would be dangerous, since T cells would home to these inflamed alloantigen-expressing GVHD sites and destroy healthy cells. However, a delayed treatment window following allo-SCT is possible, when there is no systemic ‘inflammatory’ state. However, at the tumor site where the inflammation is sustained anti-tumor MiHA-specific T cells may be specifically boosted, resulting in renewed GVT effects.

Altogether, co-inhibitory molecules play a pivotal role in natural and immunotherapy-induced T cell-mediated immunity against hematological cancers. With increasing
References


Chapter 3 Co-inhibitory molecules in hematological malignancies


PD-1/PD-L1 interactions contribute to functional impairment of minor histocompatibility antigen-specific CD8\(^+\) T cells in patients with relapsed cancer after allogeneic stem cell transplantation

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Abstract

Tumor relapses remain a serious problem after allogeneic stem cell transplantation (allo-SCT), despite the long-term persistence of minor histocompatibility antigen (MiHA)-specific memory CD8+ T cells specific for the tumor. We hypothesized that these memory T cells may lose their function over time in transplanted patients. Here, we offer functional and mechanistic support for this hypothesis, based on immune inhibition by programmed death-1 (PD-1) expressed on MiHA-specific CD8+ T cells and the associated role of the PD-1 ligand PD-L1 on myeloid leukemia cells, especially under inflammatory conditions. PD-L1 was highly up-regulated on immature human leukemic progenitor cells, whereas co-stimulatory molecules such as CD80 and CD86 were not expressed. Thus, immature leukemic progenitor cells seemed to evade the immune system by inhibiting T-cell function via the PD-1/PD-L1 pathway. Blocking PD-1 signaling using human antibodies led to elevated proliferation and IFN-γ production of MiHA-specific T cells co-cultured with PD-L1-expressing leukemia cells. Moreover, patients with relapsed leukemia after initial MiHA-specific T-cell responses displayed high PD-L1 expression on CD34+ leukemia cells and increased PD-1 levels on MiHA-specific CD8+ T cells. Importantly, blocking PD-1/PD-L1 interactions augments proliferation of MiHA-specific CD8+ memory T cells from relapsed patients. Taken together, our findings indicate that the PD-1/PD-L1 pathway can be hijacked as an immune escape mechanism in hematological malignancies. Furthermore, they suggest that blocking the PD-1 immune checkpoint offers an appealing immunotherapeutic strategy following allo-SCT in patients with persisting or relapsed disease.

Introduction

Alloreactive CD8+ T cells play a crucial role in the graft-versus-tumor (GVT) response following allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI). In human leukocyte antigen (HLA)-matched allo-SCT, these alloreactive CD8+ T cell responses are directed against minor histocompatibility antigens (MiHAs). Previously, we have characterized CD8+ T cell immunity toward a hematopoietic-restricted MiHA, designated LRH-1, which is presented by HLA-B7 and encoded by the P2X5 purinergic receptor gene. LRH-1-specific CD8+ T cell responses can be frequently detected in myeloid leukemia patients following DLI, and has been associated with leukemic remission. Moreover, we showed that CD34+ myeloid leukemia progenitor cells can be efficiently targeted in vitro by LRH-1-specific CD8+ cytotoxic T lymphocytes (CTLs), indicating that these CTLs play a significant role in GVT-specific immunity against myeloid leukemia. However, we have observed that despite the presence of LRH-1-specific CD8+ memory T (Tmem) cells for many years, late relapses do occur in patients with advanced myeloid leukemia. Furthermore, we noted that LRH-1-specific CD8+ Tmem cells do not always efficiently expand with recurrence of leukemia cells, suggesting that these T cells become functionally impaired.

Mechanisms exploited by tumor cells to inhibit CD8+ T cell-mediated immunity include disruption of antigen presentation, down-regulation of HLA molecules, and induction of immune suppressive components such as Programmed death-1 (PD-1) signaling. PD-1 plays a crucial role in T cell regulation in various immune responses and is involved in peripheral tolerance, autoimmunity, infection and anti-tumor immunity. Elevated PD-1 expression on antigen-specific CD8+ T cells in chronic viral infections has been recognized as a hallmark for T cell exhaustion resulting in diminished cytokine production, proliferation and cytolytic activity upon antigen restimulation. PD-1 binds two ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). Although PD-L2 expression is mainly restricted to antigen presenting cells (APCs) like dendritic cells (DCs) and macrophages, PD-L1 is also expressed by many non-hematological cell types. Furthermore, PD-L1 can be expressed on multiple tumor types and its expression is elevated following IFN-γ exposure. PD-L1 molecules on tumor cells can deliver negative signals through PD-1 to tumor-reactive CTLs, thereby inhibiting anti-tumor immunity. In agreement, PD-L1 expression has been associated with poor prognosis in various cancers including hepatocellular carcinoma and melanoma. Notably, studies in models of murine myeloid leukemia have also demonstrated that PD-1/PD-L1 interactions play an important role in immune evasion. Interestingly, PD-L1 is also able to bind CD80, resulting in T cell inhibition. Therefore, PD-L1 molecules on human leukemia cells may restrain CD8+ T cell responses involved in GVT immunity after allo-SCT.
In this study, we examined the role of PD-1/PD-L1 interactions in functional impairment of MiHA-specific CD8+ T cells. We observed that PD-L1 is induced following IFN-γ and TNF-α exposure. Furthermore, we observed that activated LRH-1-specific CD8+ T cells express PD-1 during the course of the immune response. Importantly, we showed that blockade of PD-1/PD-L1 interactions using clinical grade human antibodies increases the proliferation and IFN-γ production of MiHA-specific CD8+ T cells when stimulated with PD-L1-expressing acute myeloid leukemia (AML) cells and DCs. Together, these findings indicate that the PD-1 signaling pathway suppresses MiHA-specific CD8+ T cell responses and PD-1 blockade may be an attractive approach to boost GVT immunity in patients with persistent or relapsed disease.

Materials and Methods

Patient and donor material

Peripheral blood (PB) and bone marrow (BM) samples of leukemia patients have been collected after written informed consent in ongoing clinical stem cell transplantation protocols approved by the RUNMC Institutional Review Board. We used peripheral blood mononuclear cells (PBMCs) obtained from patients with hematological malignancies who developed MiHA-specific CD8+ T cell responses after DLI. Patient 1 (Pt 1) suffered from accelerated-phase (AP) chronic myeloid leukemia (CML) and was successfully treated with therapeutic DLI after allo-SCT.15 The patient relapsed 4 years after DLI. Patient 2 (Pt 2) suffered from AML and developed a strong LRH-1-specific CD8+ T cell response upon pre-emptive DLI, but developed extramedullary relapses without leukemic involvement in the bone marrow16. Characteristics of these patients and other transplanted patients investigated in this study are included in Table 2.

DCs were generated from monocytes isolated from PBMCs of healthy donors by plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Immature DCs (iDCs) were generated by culturing adherent monocytes in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 2% HS, 500 U/ml IL-4 and 800 U/ml GM-CSF (both Immunotools, Friesoythe, Germany). After 3 days, cells were harvested, counted, and the percentage of MiHA-tetramer+ cells was determined.

In this study, we examined the role of PD-1/PD-L1 interactions in functional impairment of MiHA-specific CD8+ T cells. We observed that PD-L1 is induced following IFN-γ and TNF-α exposure. Furthermore, we observed that activated LRH-1-specific CD8+ T cells express PD-1 during the course of the immune response. Importantly, we showed that blockade of PD-1/PD-L1 interactions using clinical grade human antibodies increases the proliferation and IFN-γ production of MiHA-specific CD8+ T cells when stimulated with PD-L1-expressing acute myeloid leukemia (AML) cells and DCs. Together, these findings indicate that the PD-1 signaling pathway suppresses MiHA-specific CD8+ T cell responses and PD-1 blockade may be an attractive approach to boost GVT immunity in patients with persistent or relapsed disease.

Mixed lymphocyte-AML reactions and CTL stimulation assays

After culturing and preincubation, AML cells and DCs were washed, counted, and seeded in 96-well round bottom plates (Corning Costar, New York, NY, USA). In lymphocyte-AML reaction assays, allogeneic CD8+ T cells were isolated from non-adherent or complete PBMC fractions from healthy donors by direct magnetic labeling with the appropriate MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Subsequently, 1015 CD8+ T cells were added to AML cells and mDCs at different E/T ratios. In MiHA-specific T cell expansion assays, PD-L1+ iDCs were loaded with 10 μM LRH-1 peptide TPNNQRQNVLC for 30 minutes at room temperature and co-cultured with LRH-1-specific CTL RP1 at a stimulation ratio of 10:1.

PD-1 and PD-L1 blocking antibodies were added at a final concentration of 10 μg/ml. Human antibodies to human PD-1 (BMS-936,558; MDX-1106; ONO-4538) and PD-L1 (BMS-936,559; MDX-1105) and a matching IgG4 isotype control were kindly provided by dr. Alan Korman (Bristol-Myers Squibb, Biologics Discovery, Millipitas, CA, USA). BMS-936,558 and BMS-936,559 are genetically engineered, fully human IgG4 antibodies currently evaluated in clinical trials for selected tumor treatments14. All co-cultures were performed in a total volume of 200 μl IMDM/10% HS. After 5 days of co-culture, supernatant was harvested for cytokine analysis. At day 5, 0.5 μCi [3H]-thymidine (Perkin Elmer, Groningen, the Netherlands) was added to each well. After overnight incubation, [3H]-thymidine incorporation was measured using a 1205 Wallac Betaplate counter (Perkin Elmer).

MiHA-specific T+ cell proliferation assays

MiHA-specific CD8+ T cells present in PBMCs from patients Pt 1, 2, 15-21 (Table 2) were stimulated for one to three consecutive weeks ex vivo with either MiHA peptide alone or with MiHA peptide-loaded PD-L1+ iDCs or PD-L1-PD-L2+ mDCs, as described previously15. PD-1 and PD-L1 blocking antibodies were added at a final concentration of 10 μg/ml. After 5 days, 500 μl supernatant was removed and fresh IMDM/10% HS containing 50 U/ml IL-2 (Chiron, Emeryville, CA, USA) and 5 ng/ml IL-15 (ImmunoTools) was added. At day 7, cells were harvested, counted, and the percentage of MiHA-tetramer+ CD8+ T cells was determined.

Flowcytometry

Expression of co-signaling ligands on myeloid leukemia cells and DCs was analyzed by staining with the following fluorochrome-conjugated antibodies: CD14 (clone TU4K4, Dako, Glostrup, Denmark), CD33 (clone D3H6.L60.251), CD34 (clone 5B1), CD117 (clone 104D21), CD54 (clone 84H10), CD80 (clone MAB104), CD83 (clone H1B5a), CD86 (clone HAS.287, all from Beckman Coulter, Fullerton, CA, USA), anti-PD-L1 (clone MIH1), anti-PD-L2 (clone MIH18, both from Becton Dickinson, Franklin Lakes, NJ, USA) and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2b PE (Beckman Coulter). PD-1 expression on and percentage of MiHA-specific CD8+ T cells was determined as described previously15 using anti-PD-1 (clone MIH4, Becton Dickinson). Cells were analyzed using the Coulter FC500 flowcytometer (Beckman Coulter).
Immunohistochemistry staining
Paraffin-embedded chloroma tissues were stained as previously described\(^1 \). Briefly, antigen retrieval was performed using 10 minutes of boiling in 0.01 M of sodium citrate pH 6.0 followed by incubation with primary antibodies anti-PD-L1 (clone MIH, eBioscience, San Diego, CA, USA), anti-PD-L2 (clone MIH18, eBioscience), anti-CD8 (clone C8/144B, Dako), anti-CD34 (clone QBEnd/10, Klinpath, Duiven, the Netherlands) and anti-FOXp3 (clone 236A/ E7, ITK Diagnostics, Uithoorn, the Netherlands). Staining was visualized either by 3,3-diaminobenzidine (DAB) or aminoethyl carbazole (AEC) staining.

IFN-γ and granzyme B ELISA
Production of IFN-γ and granzyme B by stimulated T cells was determined by enzyme-linked immunosorbent assay (ELISA; IFN-γ: Pierce Endogen, Rockford, IL, USA; granzyme B: Mabtech, Nacka Strand, Sweden) according to manufacturer’s protocol.

Real-time quantitative reverse transcription PCR and microarray analysis
Total RNA was isolated from cell samples using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis and PCR amplification were performed as described\(^2 \). The hydroxymethylbilane synthase (HMBS) housekeeping gene was used to normalize PD-L1 and PD-L2 expression. PD-L1 and PD-L2 mRNA expression is shown in \( \Delta \Delta CT \) values and was quantified relative to cell line U266, which was set at 1. \( \Delta \Delta CT \) was calculated as follows: 2\(^{[-\Delta CT_{sample} - \Delta CT_{U266}]} \), in which \( \Delta CT \) was normalized for HMBS by calculating \( \Delta CT = CT_{gene} - CT_{HMBS} \) per sample.

For microarray analysis, LRH-1-tetrameric human CD8\(^{+} \) T cells were sorted (median sorted cell number: 1,300; range: 200-15,000 cells) on an Epics Elite sorter (Beckman Coulter, Naka Strand, Sweden) according to manufacturer’s protocol.

Statistical analysis
Paired one-tailed student-t test or one-way ANOVA followed by a Bonferroni post-hoc test were used when appropriate.

### Results

**Myeloid leukemia progenitor cells differentially express PD-L1 compared with CD80 and CD86**
To investigate whether myeloid leukemia cells in relapsed patients after allo-SCT express PD-L1 under inflammatory conditions, we analyzed leukemia samples from two patients who relapsed despite circulating LRH-1-specific CD8\(^{+} \) T cells (Table 1). We found that CD34\(^{+} \) CML-AP cells from a relapsed patient (Pt 1) at four years after DLI expressed PD-L1 upon stimulation with IFN-γ (Figure 1A), while expression of co-stimulatory molecules CD80 and CD86 on these CD34\(^{+} \) CML-AP cells was low. Furthermore, we observed high PD-L1 expression on CD34\(^{+} \) leukemia cells in a chloroma biopsy of an AML patient (Pt 2) who relapsed three years after DLI (Figure 1B). Again, we found that these extramedullary AML cells expressed low levels of CD80 and CD86 (data not shown). Staining of specific T cell markers revealed that CD8\(^{+} \) T cells extensively infiltrated the chloroma, while FOXP3\(^{+} \) regulatory T cells were hardly detectable (Supplementary Figure 1). These data suggest that selective expression of PD-L1 on these relapsed leukemia cells could have been involved in evading LRH-1-specific CD8\(^{+} \) T cell immunity.

### Table 1 Myeloid leukemia patient characteristics and PD-L1 expression

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<th>Patient</th>
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Characteristics of myeloid leukemia patients which have been analyzed for PD-L1 expression. Patient samples at diagnosis, except indicated by ‘relapse’ CML-CP, chronic myeloid leukemia-chronic phase; CML-AP, chronic myeloid leukemia-accelerated phase; PB, peripheral blood; BM, bone marrow; unstim, unstimulated; MFI, mean fluorescence intensity; ND, not determined; WBC, white blood cell count in 10\(^{9}\)/L; peripheral blood. Determined by immunohistochemistry.
Leukemia cells were exposed to 100 U/ml IFN-γ and 1.25 ng/ml TNF-α 16 hours before analysis. (B) Expression of PD-L1 and PD-L2 by CD34+ AML cells in a chloroma biopsy of Pt 2 at time of relapse, 3 years after DLI, was determined by immunohistochemistry. Staining was visualized using DAB (CD34) or AEC (PD-L1, PD-L2 and isotype control). Magnification 1000x. (C) CD33+ AML cells from 9-12 different patients were incubated with 100 U/ml IFN-γ and 1.25 ng/ml TNF-α for 16 hours, after which PD-L1 and PD-L2 mRNA expression with (+) and without (-) IFN-γ and TNF-α was measured. (D) Expression of co-stimulatory ligands on CD33+ AML cells, in the absence (-) or presence (+) of IFN-γ and TNF-α, was determined by flowcytometry. (E) Expression of PD-L1, PD-L2, CD80, CD86 and CD54 on CD33+CD117+CD14+ progenitor AML cells from 8 patients. Expression is depicted as mean ± SD. Paired one-tailed student t-test was performed to identify significant differences. *P<0.05; **P<0.01; ***P<0.001.

To determine whether selective induction of PD-L1 expression under inflammatory conditions is a general phenomenon in leukemia, we analyzed a panel of 12 primary AML samples for expression of co-signaling ligands following treatment with IFN-γ and TNF-α (Table 1). Indeed, these cytokines induced an 137-fold and 31-fold up-regulation of PD-L1 and PD-L2 mRNA, respectively (Figure 1C). Furthermore, consistent with the findings in the two relapsed leukemia patients, PD-L1 cell surface expression was significantly up-regulated (> 20% PD-L1+ cells) on AML cells of 7 out of 10 newly diagnosed patients, while expression of PD-L2 was only slightly induced (Figure 1D). Notably, PD-L1-expressing AML cells displayed very low expression of CD80 and a variable expression of CD86, which was not influenced by IFN-γ/TNF-α treatment.

Because AML clones comprise heterogeneous populations of malignant cells, we studied whether different AML populations exhibited differential expression of co-signaling molecules. Using multi-color flowcytometry, we defined three distinct AML populations defined as CD33+CD117+CD14+ AML progenitor cells, CD33+CD117+CD14+ AML myelo/monoblasts and CD33+CD117+CD14+ AML promonocytes (Supplementary Figure 2A-B). A panel of nine AML patients with different FAB classifications was used for analyzing expression of co-signaling ligands upon IFN-γ ± TNF-α stimulation. Interestingly, the most immature CD33+CD117+CD14+ AML cells exhibited high PD-L1 expression (range: 46-94% PD-L1+ cells) in combination with almost absent or very low expression of PD-L2, CD80 and CD86 under inflammatory conditions (Figure 1E). The CD33+CD117+CD14+ AML myelo/monoblasts showed slightly more up-regulation of PD-L1 and CD80 expression as well as higher CD86 expression (Supplementary Figure 2C). Mature CD33+CD117+CD14+ AML promonocytes displayed combined up-regulation of PD-L1, PD-L2, CD80 and CD86 expression (Supplementary Figure 2D).

Collectively, these data demonstrate that immature AML cells which contain the putative leukemic stem cells selectively up-regulate PD-L1 expression following short-term exposure to IFN-γ and TNF-α, enabling these leukemia progenitor cells to inhibit T cell-mediated attack via the PD-1/PD-L1 pathway.
MiHA-specific CTL expansion and function is enhanced by PD-1 blockade

To elucidate the role of PD-L1 on AML cells in inhibiting the recognition by MiHA-specific CD8+ T cells, we performed antigen restimulation experiments using CTL clone RP1 that recognizes the hematopoietic-restricted MiHA LRH-1 on AML progenitor cells. RP1, as well as CTLs against other MiHAs, up-regulated expression of PD-1 upon co-culture with MiHA AML (Supplementary Figure 3 and data not shown). Antibody blockade of PD-1 signaling using human antibodies resulted in improved proliferation and IFN-γ production by CTL RP1 upon engagement of PD-L1-expressing primary AML cells from Pt 11 loaded with MiHA peptide (Figure 2E-G). As hypothesized, we found that PD-1 blockade strongly elevated the proliferation and IFN-γ production by CTL RP1 when stimulated with peptide-loaded PD-L1+ iDCs (Figure 2F-H) and PD-L1+ iDCs (Figure 2H). Cytotoxicity of CTLs versus AML cells was also enhanced after PD-1 and PD-L1 blockade (Figure 2I), whereas no cytotoxicity was observed versus iDCs (Figure 2J). These data indicate that MiHA-specific CD8+ effector T cells can be inhibited via the PD-1/PD-L1 pathway either by AML or resident APC populations that selectively express PD-L1 in the leukemia microenvironment.

Effect of PD-L1-expressing AML cells on allogeneic T cells

To investigate whether PD-L1 expression on AML cells can dampen allogeneic T cell responses, we performed mixed lymphocyte reactions with PD-L1-expressing AML cells and allogeneic CD3+ T cells in the absence or presence of anti-PD-1/BMS-936,558 and anti-PD-L1/BMS-936,559 blocking antibodies. Blocking with either anti-PD-1 or anti-PD-L1 antibody significantly increased proliferation of CD3+ T cells upon stimulation with allogeneic PD-L1+ AML cells from AML-M4 Pt 9 (Figure 2A), whereas allogeneic T cell proliferation stimulated with PD-L1+ and PD-L2+ mDCs could only be boosted with anti-PD-1 (Figure 2B). This difference can be explained by the high PD-L2 expression on mDCs resulting in insufficient interference of PD-1 signaling by the PD-L1 antibody. In agreement with the T cell proliferation data, IFN-γ production was also increased by blocking of PD-1 interactions between T cells and PD-L1+ AML cells (Figure 2C) or PD-L1+ iDCs (Figure 2D). These results demonstrate that PD-L1 expression on AML cells decreases T cell proliferation and cytokine production.

Figure 2. PD-1 blockade enhances T cell responses to stimulation with primary AML cells and DCs

Allogeneic CD3+ T cells were co-cultured with PD-L1-expressing leukemia cells of AML Pt 9 (A and C) or PD-L1/PD-L2+ mDCs (B and D) with addition of blocking anti-PD-1 or anti-PD-L1 antibodies. Proliferation was measured on day 5 (A and B) and IFN-γ production was evaluated (C and D). LRH-1-specific CTL RP1 was co-cultured with peptide-loaded primary leukemic cells of AML Pt 11 (E and G) or peptide-loaded PD-L1+ iDCs (F and H). IFN-γ production was measured (E and F) in addition to IFN-γ production (G and H). Cytotoxicity was measured by granzyme B secretion (I and J). One representative experiment out of 3 is shown. One-way ANOVA was performed with isotype antibody as control. *P<0.05, **P<0.01, ***P<0.001.

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PD-1 signaling impairs MiHA-specific CD8+ T cells
PD-1 signaling impairs MiHA-specific CD8+ T cells

Next, we investigated whether PD-1 is expressed by LRH-1-specific T cells in CML-AP Pt 1 and AML-M0 Pt 2 who relapsed four and three years, respectively, after the initial DLI that induced long-lasting LRH-1-specific CD8+ T cell responses in these patients. PD-1 expression could be detected on LRH-1-specific CD8+ T cells during the complete course of the immune response after DLI which peaked at week 28 for CML-AP Pt 1 and at week 10 for AML-M0 Pt 2 (Figure 3A). We observed relatively elevated expression of PD-1 on LRH-1 tetramer-positive T cells compared with tetramer-negative CD8+ T cells in the same patient (Figure 3A-B). After DLI, PD-1 levels at the cell surface of LRH-1-specific CD8+ T cells gradually declined, but >95% of the tetramer-positive T cells remained PD-1 positive during the contraction phase. To determine PD-1 expression on the apparently impaired LRH-1-specific CD8+ Tmem cells, we sorted LRH-1-specific CD8+ Tmem cells (Pt 1: 1.8% at week 28; Pt 2: 2.9% at week 15) and low frequencies of LRH-1-specific CD8+ Tmem cells (Pt 1: 0.08% at week 225; Pt 2: 0.05% at week 151) and performed microarray analysis using amplified cDNA. PD-1 mRNA levels of LRH-1-specific CD8+ Tmem cells at the time of relapse were elevated or similar compared with LRH-1-specific CD8+ T mem cells at the peak of the response for Pt 1 and Pt 2, respectively (Figure 3C). These data indicate that LRH-1-specific CD8+ T cells express elevated levels of PD-1 on the cell surface, which remain present during the contraction and late memory phase of the immune response following DLI.

PD-1 blockade augments proliferation of MiHA-specific CD8+ T cells

To further elucidate the role of PD-1 in impairment of LRH-1-specific CD8+ Tmem cells, we performed functional assays using PBMCs from CML-AP Pt 1 containing 0.05-0.10% LRH-1-specific CD8+ Tmem cells several years after the initial stimulus. Stimulation of PBMCs of Pt 1 with peptide alone in the presence of IL-2 and IL-15 did not result in an increase of LRH-1-specific T cells (data not shown). Notably, PD-1 or PD-L1 blockade resulted in a 2- to 4-fold increase in the number of LRH-1-specific T cells (Figure 4A). However, peptide stimulation in the presence of PD-1 blockade, but in the absence of professional APCs, resulted in insufficient T cell outgrowth. Therefore, we stimulated PBMCs containing LRH-1-specific Tmem cells with peptide-loaded PD-L1-expressing iDCs in the presence of PD-1 blockade. Blockade with anti-PD-1 antibody resulted in a 20-times higher number of LRH-1-specific CD8+ T cells after three stimulations with peptide-loaded PD-L1+ iDCs (Figure 4B). Consistently, we observed a specific increase to 4.4% LRH-1-specific CD8+ T cells compared to 0.6% with the isotype control after repeated DC stimulations using blockade with anti-PD-1 (Figure 4C).

Similar assays were performed with PBMCs obtained 7 and 36 months post-DLI containing low numbers of LRH-1-specific Tmem cells from AML-M0 Pt 2. In these assays, we used mDCs in order to prevent repetitive T cell stimulation. At 7 months post-DLI, blocking with anti-PD-1 and anti-PD-L1 antibody resulted in increased outgrowth of LRH-1-specific CD8+ T cells up to 8.1% and 6.4%, respectively, compared to 2.0% in the presence of an isotype control (Figure 4D). In addition, during relapse at 36 months post-DLI upon PD-1 and PD-L1 blockade LRH-1-specific CD8+ T cells increased to 11.6% and 8.86%, respectively, compared to 0.59% for isotype control (Table 2). To further confirm the effect of PD-1/PD-L1 blockade on the proliferative capacity of other MiHA-specific T cells, we stimulated PBMCs from a relapsed MM patient (Pt 16) containing HA-1-specific T cells, we observed a specific increase to 4.4% LRH-1-specific CD8+ T cells (data not shown). Notably, PD-1 or PD-L1 blockade resulted in a 2- to 4-fold increase in the number of LRH-1-specific T cells (Figure 4A). However, peptide stimulation in the presence of PD-1 blockade, but in the absence of professional APCs, resulted in insufficient T cell outgrowth. Therefore, we stimulated PBMCs containing LRH-1-specific Tmem cells with peptide-loaded PD-L1-expressing iDCs in the presence of PD-1 blockade. Blockade with anti-PD-1 antibody resulted in a 20-times higher number of LRH-1-specific CD8+ T cells after three stimulations with peptide-loaded PD-L1+ iDCs (Figure 4B). Consistently, we observed a specific increase to 4.4% LRH-1-specific CD8+ T cells compared to 0.6% with the isotype control after repeated DC stimulations using blockade with anti-PD-1 (Figure 4C).

PD-1 is highly expressed by circulating MiHA-specific CD8+ T cells in vivo

Figure 3 MiHA-specific T cells express elevated levels of PD-1 in vivo
(A) PD-1 expression of LRH-1-specific T cells in CML-AP Pt 1 and AML-M0 Pt 2 was determined by flowcytometry. Time post-DLI is indicated in weeks, percentage of LRH-1-specific T cells of total CD8+ T cells in brackets and MFI of LRH-1-specific T cells is displayed. (B) PD-1 expression of LRH-1-specific T cells compared to total CD8+ T cells in the same patient. (C) LRH-1-specific T cells were isolated by flowcytometry-assisted cell sorting. Subsequently, RNA was isolated and PD-1 mRNA levels were determined at the peak of the response, and in LRH-1-specific Tmem, cells 4.5 or 3 years after the initial response. Raw intensity values measured from microarray analysis are depicted.
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(A) PBMCs of CML-AP Pt 1 containing low levels of LRH-1-specific T\textsubscript{Mem} cells years after the initial response were stimulated by addition of LRH-1 peptide in the presence of blocking antibodies against PD-1 or PD-L1. Numbers of LRH-1-specific cells were enumerated by flow cytometry, and numbers at isotype control were set to 1. Results are from three independent experiments from a sample 225 weeks after DLI (\(0, 175, 195\)) and one sample taken 275 weeks after DLI (\(3, 5, 7\)).

(B) iDCs were loaded with LRH-1 peptide and added weekly to PBMCs, containing LRH-1-specific T\textsubscript{Mem} cells, for 3 weeks, combined with blocking antibodies. (C) LRH-1-specific T cell percentages of total CD8+ were identified by flow cytometry.

(D) mDCs loaded with LRH-1 peptide were used to stimulate LRH-1-specific T\textsubscript{Mem} cells of AML-M0 Pt 2. Subsequently, the percentage of LRH-1-specific T cells after one week was determined by flow cytometry.

(E) mDCs loaded with HA-1 peptide were used to stimulate HA-1-specific T\textsubscript{Mem} cells of relapsed MM Pt 16. Subsequently, the percentage of HA-1-specific T cells after one week was determined by flow cytometry.

Table 2 Characteristics of transplanted patients and effect of PD-1-blockade on MiHA-specific T cell proliferation

<table>
<thead>
<tr>
<th>Pt</th>
<th>Disease</th>
<th>MiHA T cell response</th>
<th>Sample date (months post-SCT or post-DLI)</th>
<th>Effect PD-1 Blockade(a)</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CML-AP</td>
<td>LRH-1</td>
<td>52 post-tDLI</td>
<td>19.8</td>
<td>Hematologic relapse at 47 mo post-tDLI</td>
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<td>AML</td>
<td>LRH-1</td>
<td>7 post-pDLI</td>
<td>15.4</td>
<td>Chloroma relapses at 33, 50, 78 post-pDLI</td>
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<tr>
<td>2</td>
<td>AML</td>
<td>LRH-1</td>
<td>36 post-pDLI</td>
<td>2.8</td>
<td>Chloroma relapses at 33, 50, 78 post-pDLI</td>
</tr>
<tr>
<td>15</td>
<td>CML-BC</td>
<td>HA-1</td>
<td>9 post-pDLI</td>
<td>1.5</td>
<td>Remission; death due to GVHD at 11 mo post-pDLI</td>
</tr>
<tr>
<td>16</td>
<td>MM</td>
<td>HA-1</td>
<td>6 post-SCT</td>
<td>5.5</td>
<td>Active disease at 6 mo post-SCT. Plasma cells 5%; M-protein 7 gr/L</td>
</tr>
<tr>
<td>17</td>
<td>Pre-T ALL</td>
<td>HA-2</td>
<td>12 post-SCT</td>
<td>2.5</td>
<td>Remission; alive at 174 mo post-SCT</td>
</tr>
<tr>
<td>18</td>
<td>CML</td>
<td>SP110</td>
<td>20 post-SCT</td>
<td>8.7</td>
<td>Cytogenetic relapse at 14 mo post-SCT</td>
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<tr>
<td>19</td>
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<td>0.7</td>
<td>Remission; alive at 213 mo post-SCT</td>
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<td>6 post-SCT</td>
<td>3.3</td>
<td>Remission; alive at 31 mo post-SCT</td>
</tr>
</tbody>
</table>

Characteristics of patients with hematological malignancies displaying MiHA-specific T cell responses. Pt, patient; CML-AP, chronic myeloid leukemia accelerated phase; CML-BC, chronic myeloid leukemia blast crisis; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; T-ALL, T cell acute lymphoblastic leukemia; pDLI, pre-emptive donor lymphocyte infusion; tDLI, therapeutic donor lymphocyte infusion; SCT, stem cell transplantation; MiHA-specific response: MiHA for which a response was observed; Mo, months; GVHD, graft-versus-host disease.

\(a\) Effect PD-1 blockade: ratio absolute number of tetramer+ cells DC + peptide + anti-PD-1/absolute number of tetramer+ cells DC + peptide + isotype control.
addition, we investigated whether PD-1 blockade increased the absolute amount of MiHA-specific T cells. For AML Pt 2 and MM Pt 16, we observed a robust absolute increase of MiHA-specific T cells upon PD-1 and PD-L1 blockade (Figure 5A).

Next, we investigated whether the effect of PD-1/PD-L1 is exclusive for dysfunctional MiHA-specific T cells in relapsed patients, or that it also affects potential non-impaired T cells in patients with remission after allo-SCT. Therefore, we investigated the effect of PD-1/PD-L1 blocking in CML-BC Pt 15 and pre-T ALL Pt 17 (Table 2). PD-1 and PD-L1 blockade enhanced the absolute number of MiHA-specific T cells, but the effect was moderate (Figure 5B). Finally, we compared the effect of PD-1 blockade on MiHA-specific T cells from relapsed patients to those from patients in remission. Importantly, we showed that PD-1 blockade had a significantly superior effect on dysfunctional MiHA-specific T cells from relapsed patients (Figure 5C). Collectively, these results demonstrate that PD-1 signaling impairs the proliferative capacity of MiHA-specific CD8+ T cells upon antigen stimulation prior to or during relapse, and this functional impairment can be abrogated by PD-1/PD-L1 immune checkpoint blockade.

Discussion

Allo-SCT is a potentially curative treatment for advanced myeloid leukemia. The effect largely depends on alloreactive CD8+ T cells targeting MiHAs on leukemic blasts and progenitor cells. However, MiHA-specific CD8+ T cell responses induced after transplantation are in many patients not sufficient to sustain complete remission. Distinct mechanisms are involved in reducing anti-tumor T cell responses, allowing malignant cells to escape immune destruction. Among these mechanisms, T cell inhibition or even exhaustion due to signaling of the PD-1/PD-L pathway may diminish immune responses by limiting the expansion and functionality of CD8+ T cells. Recently, we showed that LRH-1+ leukemia can relapse without inducing secondary LRH-1-specific CD8+ Tmem cell expansion, suggesting that these Tmem cells are either suppressed or not activated. In this study, we examined the role of PD-1/PD-L1 interactions in functional impairment of LRH-1-specific CD8+ T cells reactive to myeloid leukemia. Interestingly, we showed that PD-L1 and to some extent PD-L2 was expressed by CD34+ progenitor myeloid leukemia cells of two patients with relapses after initial efficient T cell responses. Furthermore, we confirmed expression of PD-L1 on a broader panel of AML samples at diagnosis. Previously, it has been shown that PD-L1 expression is elevated on relapse AML cells compared to diagnosis material. We investigated this in one CML patient, and indeed, PD-L1 expression was higher on relapse tumor cells than cells at diagnosis (Table 1). Especially CD117-CD14 early progenitor myeloid leukemia cells, which contain the leukemic stem cells, highly expressed PD-L1. PD-L1 expression increased upon exposure to inflammatory cytokines, whereas expression of CD80 and CD86 remained low. Consequently, prolonged PD-1/
PD-L1 interactions may lead to functional exhaustion of LRH-1-specific T\textsubscript{rem} cells, and relapse of the leukemia may occur without induction of a secondary immune response. To investigate whether LRH-1-specific T cells display an impaired phenotype, we analyzed T cells of two patients with LRH-1-specific responses. It is known that PD-1 is elevated on T cells specific for viral epitopes in chronic viral infections\(^ {13}\). Also during chronic CML disease it has been shown that PD-1 levels on the total population of CD8\(^ +\) T cells are elevated\(^ {14}\). Here, we showed for the first time that MiHA-specific T\textsubscript{rem} cells can have an elevated level of PD-1. Both patients with non-responding T\textsubscript{rem} cells had leukemia relapses following a robust initial LRH-1-specific T cell response. Whether or not elevated PD-1 expression on MiHA-specific T cells correlates with immune escape and subsequent relapse of myeloid leukemia needs to be determined in a larger cohort of patients. However, we found that the PD-1/PD-L1 pathway negatively influences the function of PD-1-expressing LRH-1-specific CTLs. Most importantly, we demonstrated that blocking PD-1/PD-L1 interactions with human blocking antibodies resulted in increased outgrowth of MiHA-specific T\textsubscript{rem} cells from allo-SCT recipients. We also observed a stimulatory effect of PD-1 blockade on MiHA-specific T cells from patients in remission, which is not unexpected due to the role of PD-1 in regulation of T cell activation. However, the abrogation of PD-1 signaling had a significant stronger effect on the proliferation of MiHA-specific T cells in relapsed patients compared to patients in remission.

Besides PD-1, several other inhibitory receptors are known to play a role in functional T cell exhaustion, such as CTLA-4, LAG3, BTLA, TIM-3, CD160 and CD244\(^ {14}\). In future years, the influence of this array of co-inhibitory receptors will be further elucidated and perhaps combinations of blocking antibodies against targets such as PD-1 and LAG3 will result in highly re-activated MiHA-specific T cell responses\(^ {15}\). But, as PD-1 is involved in peripheral tolerance, autoimmune events following PD-1 blockade therapy may occur\(^ {16}\). In a recent phase I study, the clinical grade anti-PD-1 antibody BMS-936,558, also used in our study, was administered to patients with solid tumors. Anti-PD-1 was well tolerated and only one serious adverse event, inflammatory colitis, was observed in a melanoma patient. Remarkably, one durable complete response and two partial responses were observed\(^ {16}\). Our current in vitro data illustrate that PD-1 blockade is an attractive approach to reinvigorate impaired MiHA-specific T cells in patients with persisting or relapsed leukemia. However, in the setting of allogeneic SCT, PD-1 blockade could aggravate graft-versus-host-disease. For optimal boosting selective GVT immunity in the post-SCT setting, we would like to combine active immunotherapy by DC vaccination using hematopoietic-restricted MiHAs with PD-1 blockade. By inducing a time-limited alleviation of PD-1 signaling combined with an antigen-specific stimulus, we aim to resuscitate the impaired MiHA-specific T cells, without causing autoimmune effects or GVHD. Another strategy is to specifically knock-down PD-L1 and/or PD-L2 on MiHA-loaded DC vaccines by siRNA. In a recent article, we showed that stimulation with PD-L1/L2 knockdown DCs resulted in specific outgrowth of initially unresponsive MiHA-specific T cells\(^ {17}\). This strategy would

minimize off-target stimulatory effects, since the hyperstimulatory DCs are loaded with hematopoietic-restricted MiHAs. Results of clinical trials being performed with BMS-936,558 and BMS-936,559 in parallel with preclinical mouse models using blocking antibodies in a post-SCT setting will determine the ideal therapy combination.

In conclusion, we demonstrated PD-1 expression on myeloid leukemia cells, especially under inflammatory conditions. Interestingly, CD117\(^ +\) early progenitor myeloid leukemia cells express high levels of PD-L1, but low CD80 and CD86 expression. Furthermore, we showed that blockade by human anti-PD-1 or anti-PD-L1 results in increased proliferation of and IFN-γ and granzyme B production by LRH-1-specific CTLs stimulated with PD-L1\(+\) leukemia cells. In addition, LRH-1-specific CD8\(^ +\) T cells exhibited elevated PD-1 expression in vivo. Most importantly, we could specifically resuscitate initially unresponsive MiHA-specific T\textsubscript{rem} cells by PD-1/PD-L1 blockade. Therefore, we postulate that PD-1 blockade could be a powerful addition to post-SCT therapy. Combining MiHA-specific DC vaccination with PD-1 blockade may reinvigorate impaired MiHA-specific T\textsubscript{rem} cells and restore immune control, thereby preventing or attacking leukemia relapses.

Acknowledgements

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References


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Supplementary Figure 1
High numbers of CD8+ cells have infiltrated the extramedullary AML, whereas only minimal numbers of FOXP3+ regulatory T cells are present.

Staining was visualized using DAB. Magnification CD8 100x, FOXP3 400x.

Supplementary Figure 2
Subpopulation distribution and expression of co-signaling molecules within AML

(A) The total AML population was identified by CD33 expression, and AML subsets were further defined by CD14 and CD117 expression. Example of the gating strategy of different AML subpopulations using a bone marrow sample of AML-M2 patient 5.

(B) Distribution of leukemic subpopulations within 9 AML samples of morphologically diagnosed FAB classification M0, M1, M2, M4 and M5. Expression of co-stimulatory molecules after overnight incubation with IFN-γ and TNF-α on (C) CD117-CD14- intermediate AML cells or (D) CD117 CD14+ AML promonocytes.

Supplementary Figure 3
LRH-1-specific CTL line expresses and up-regulates PD-1 during co-culture with primary AML cells

(A) During 6 days of co-culture of CTL with CD33+ AML cells of Pt 11, expression of PD-1 on LRH-1-specific CTL was monitored by flowcytometry. (B) Expression of PD-1 on LRH-1-specific CTLs on day 6.
B and T lymphocyte attenuator mediates inhibition of tumor-reactive CD8+ T cells in patients after allogeneic stem cell transplantation


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Chapter 5

Abstract

Allogeneic stem cell transplantation (allo-SCT) can cure hematological malignancies by inducing alloreactive T cell responses targeting minor histocompatibility antigens (MiHAs) expressed on malignant cells. Despite induction of robust MiHA-specific T cell responses and long-term persistence of alloreactive memory T cells specific for the tumor, often these T cells fail to respond efficiently to tumor relapse. Previously, we demonstrated the involvement of the co-inhibitory receptor Programmed death-1 (PD-1) in suppressing MiHA-specific CD8+ T cell immunity. Here, we investigated whether B and T lymphocyte attenuator (BTLA) plays a similar role in functional impairment of MiHA-specific T cells after allo-SCT. In addition to PD-1, we observed higher BTLA expression on MiHA-specific CD8+ T cells compared to the total population of CD8+ effector-memory T cells. In addition, BTLA’s ligand Herpes virus entry mediator (HVEM) was found constitutively expressed by myeloid leukemia, B-cell lymphoma and multiple myeloma cells. Interference with the BTLA/HVEM pathway, using a BTLA blocking antibody, augmented proliferation of MiHA-specific CD8+ T cells by HVEM-expressing dendritic cells. Interestingly, we demonstrated that blocking BTLA or PD-1 enhanced ex vivo proliferation of MiHA-specific CD8+ T cells in respectively 7 and 9 out of 11 allo-SCT patients. Notably, in 3 out of 11 patients the effect of BTLA blockade was more prominent than that of PD-1. Furthermore, these expanded MiHA-specific CD8+ T cells competently produced effector cytokines and degranulated upon antigen reencounter. Together, these results demonstrate that BTLA/HVEM interactions impair MiHA-specific T cell functionality, providing a rationale for interfering with BTLA signaling in post-SCT therapies.

Introduction

Allogeneic stem cell transplantation (allo-SCT) can be a curative treatment for hematological malignancies\(^1\). The therapeutic effectiveness is attributed to the graft-versus-tumor (GVT) effect, which is constituted by donor T cells targeting minor histocompatibility antigens (MiHAs) expressed by tumor cells\(^2\). Recipient dendritic cells (DCs) present MiHAs to donor-derived MiHA-specific CD8+ T cells, whereupon these cells get activated and expand. Subsequently, they migrate to the tumor sites and lyse the cancer cells\(^3\). Adaptive immune responses, including alloreactive responses, terminate in a contraction phase, leaving a small pool of long-lived MiHA-specific memory cells\(^4\). Unfortunately, despite the presence of these memory cells many patients with a hematological malignancy relapse post-transplantation\(^5;6\), suggesting that MiHA-specific T cell functionality is affected. Tumor cells are known to evade immune attack via multiple mechanisms\(^7\), amongst which is interference with T cell activation. This is a tightly coordinated event, involving T cell receptor-mediated recognition of the cognate peptide, ligation of co-signaling receptors, and cytokine signalling\(^8\). In recent years, the distinct role of co-inhibitory receptors in this process has been extensively investigated\(^9\). Expression of these receptors has been linked to functional impairment of T cells in various diseases, such as chronic viral infections\(^10;11\) and cancer\(^12\).

We recently investigated whether MiHA-specific T cells in patients after allo-SCT are functionally impaired by signaling of co-inhibitory receptors, and demonstrated that blockade of the Programmed death-1 (PD-1; CD279) pathway augmented proliferation and cytokine-production of MiHA-specific T cells\(^13\). PD-1 is involved in T cell exhaustion, a phenomenon characterized by the gradual loss of T cell functionality, i.e. diminished cytokine production, proliferative capacity and cytolytic activity\(^14\). In addition, it was observed that concurrent expression of multiple co-inhibitory receptors determines the functional state of T cells\(^15\). One of these co-inhibitory receptors is B- and T-lymphocyte attenuator (BTLA; CD272)\(^16\). This receptor is a member of the CD28/B7 family, but in contrast to PD-1 and Cytotoxic T-lymphocyte antigen 4 (CTLA-4), BTLA binds to a member of the Tumor necrosis factor receptor superfamily, Herpes virus entry mediator (HVEM)\(^17\). Other binding partners of HVEM are CD160 and LIGHT (for Lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM), a receptor expressed by T lymphocytes\(^18\). HVEM ligation to the receptors BTLA and CD160 was shown to be inhibitory, while the interaction of HVEM with LIGHT mediates T cell stimulation\(^19\). Recently, a role for BTLA was implicated in the escape of melanoma cells from T cell immunity\(^20\). The authors demonstrated that high BTLA expression on Melan-A+ tumor cells correlated to inhibition of antigen-specific cytokine production in melanoma patients. Therefore, we investigated the role of BTLA in the functional impairment of MiHA-specific T cells in allo-SCT patients. Here, we demonstrated that these alloreactive cells had high expression of both BTLA and PD-1. Moreover, we showed that HVEM and BTLA were
constitutively expressed by hematological cancer cells, suggesting a role of the BTLA/ HVEM pathway in tumor immune evasion in allo-SCT patients. Most importantly, we showed augmented proliferation of MiHA-specific T cells upon ex vivo stimulation following BTLA blockade. Together, these results demonstrate that BTLA interactions contribute to the functional impairment of MiHA-specific T cells, providing a rationale for incorporating BTLA interference in post-SCT therapy.

Materials and methods

Patient and donor material
Mononuclear cells were isolated from healthy donor buffy coats (Sanquin, Nijmegen, the Netherlands), or blood and bone marrow samples of patients with hematological malignancies. To examine primary tumor cells, bone marrow samples of 8 acute myeloid leukemia (AML) patients and 10 multiple myeloma (MM) patients obtained at diagnosis were used. Furthermore, we used PBMCs from transplanted patients who developed MiHA- or cytomegalovirus (CMV)-specific CD8+ T cell responses. These were obtained 3-13 months after allo-SCT. Characteristics of these patients are described in Table 1. Because no apheresis material of the corresponding donors was available, dendritic cells were cultured from apheresis material of allogeneic HLA-A*2- or HLA-B7+ donors. All cells of healthy donors and patients were obtained after written informed consent.

Cell culture
Hematopoietic tumor cell lines were cultured in IMDM (Invitrogen, Carlsbad, CA, USA) supplemented with 10-20% FCS (Integro, Zaandam, the Netherlands). U937 cells were cultured in IMDM/10% FCS with 22.5 ng/ml IL-6 (Immunotools, Friesoythe, Germany). AML-193 cells were cultured in IMDM/20% human serum (HS; PAA laboratories, Pasching, Austria) containing 2 ng/ml IL-3 (Cellgenix, Freiburg, Germany) and 2 ng/ml GM-CSF (Immunotools). Hematopoietic tumor cell lines were cultured in IMDM (Invitrogen, Carlsbad, CA, USA) supplemented with 10-20% FCS (Integro, Zaandam, the Netherlands). U937 cells were cultured in IMDM/10% FCS with 22.5 ng/ml IL-6 (Immunotools, Friesoythe, Germany). AML-193 cells were cultured in IMDM/20% human serum (HS; PAA laboratories, Pasching, Austria) containing 2 ng/ml IL-3 (Cellgenix, Freiburg, Germany) and 2 ng/ml GM-CSF (Immunotools).

Generation of monocyte-derived DCs
Healthy donor monocytes were isolated from peripheral blood mononuclear cells (PBMCs) via plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Immature DCs were generated by culturing monocytes in X-VIVO-15 medium (Lonza, Verviers, Belgium) supplemented with 2% HS, 500 U/ml IL-4 (Immunotools) and 800 U/ml GM-CSF (PAA laboratories). After 3 days, cells were harvested and cultured in 6-well plates (Corning Costar, Lowell, MA, USA) at 1 x 10^6 DCs/well in X-VIVO-15/2% HS containing 500 U/ml IL-4 and 800 U/ml GM-CSF. Maturation of DCs was induced at day 6 by culturing in X-VIVO-15/2% HS containing 500 U/ml IL-4, 800 U/ml GM-CSF, 5 ng/ml IL-1β, 15 ng/ml IL-6, 20 ng/ml TNF-α (all Immunotools) and 1 µg/ml PGE2 (Pharmacia & Upjohn, Bridgewater, NJ, USA). At day 8, mature DCs were harvested and used in T cell stimulation assays.

Flow cytometry
BTLA and PD-1 expression on CD8+ T cell subsets of healthy donors and allo-SCT patients was analyzed by staining PBMCs with antibodies against CCR7 (clone 150503, R&D systems, Abingdon, United Kingdom), CD45RA (clone H100, Biolegend, Antwerp, Belgium), CD3 (clone UCHT1, Beckman Coulter, Fullerton, CA, USA) and CD8 (clone 3B5, Invitrogen) in combination with BTLA (clone J168-340), PD-1 (clone MIH4, both Becton Dickinson, Franklin Lakes, NJ, USA) or IgG1 isotype control (Dako, Glostrup, Denmark). In case of PBMCs containing MiHA+, CMV-pp65- or influenza virus (FLU)-specific CD8+ T cells, cells were first labeled with 1.5-2 µg APC-conjugated tetramers containing the corresponding peptide. Tetramers were kindly provided by prof. dr. Frederik Falkenburg (Department of Hematology, LUMC, Leiden, the Netherlands).

Human AML and MM cell lines, primary patient samples and healthy donor leukocytes were phenotyped by staining the cells with antibodies recognizing HVEM (clone 122, Biolegend), BTLA, CD160 (clone BY55, Beckman Coulter), LIGHT (clone 7-3(7), eBioscience, Vienna, Austria) or IgG1 FITC/PE dual-color isotype control (Dako). Primary AML cells were gated on CD33+ cells using a CD33 antibody (clone D3HLE60.251, Beckman-Coulter). Primary MM cells were gated on CD38+CD138+ cells using antibodies against CD38 (clone HIT2, Becton Dickinson) and CD138 (clone B-A38, Beckman Coulter). Healthy donor leukocytes were labeled with antibodies recognizing CD3 (clone UCHT1), CD8 (clone SFC11/Thy2D3, Beckman Coulter), CD14 (clone HCD14, Biolegend) and CD19 (clone HD37, Dako).

MiHA-specific T cell cultures were incubated with PE-labeled tetramer and, subsequently, labeled with FITC-conjugated CD8 (clone LT8, Prolimmune, Oxford, United Kingdom) and PE-Cy7-conjugated CD25. After washing with PBS/0.5% bovine serum albumin (BSA; Sigma, St Louis, MO, USA), cells were resuspended in washing buffer containing 0.1% 7-amino-actinomycin D (7-AAD; Sigma). Cells were analyzed using the Coulter FC500, Navios, or the Cyan-ADP flow cytometer (all Beckman Coulter).

BTLA and PD-1 blocking antibodies
Mouse anti-human BTLA IgG1 blocking antibody, BTLA-8.2, was kindly provided by prof. dr. Daniel Olive (INSERM UMR 891, Institut Paoli Calmettes, Marseille, France). Mouse anti-human PD-1 blocking antibody (clone MIH4, both Becton Dickinson, Abingdon, United Kingdom), CD45RA (clone H100, Biolegend, Antwerp, Belgium), CD3 (clone UCHT1, Beckman Coulter, Fullerton, CA, USA) and CD8 (clone 3B5, Invitrogen) in combination with BTLA (clone J168-340), PD-1 (clone MIH4, both Becton Dickinson, Franklin Lakes, NJ, USA) or IgG1 isotype control (Dako, Glostrup, Denmark). In case of PBMCs containing MiHA+, CMV-pp65- or influenza virus (FLU)-specific CD8+ T cells, cells were first labeled with 1.5-2 µg APC-conjugated tetramers containing the corresponding peptide. Tetramers were kindly provided by prof. dr. Frederik Falkenburg (Department of Hematology, LUMC, Leiden, the Netherlands). Human AML and MM cell lines, primary patient samples and healthy donor leukocytes were phenotyped by staining the cells with antibodies recognizing HVEM (clone 122, Biolegend), BTLA, CD160 (clone BY55, Beckman Coulter), LIGHT (clone 7-3(7), eBioscience, Vienna, Austria) or IgG1 FITC/PE dual-color isotype control (Dako). Primary AML cells were gated on CD33+ cells using a CD33 antibody (clone D3HLE60.251, Beckman-Coulter). Primary MM cells were gated on CD38+CD138+ cells using antibodies against CD38 (clone HIT2, Becton Dickinson) and CD138 (clone B-A38, Beckman Coulter). Healthy donor leukocytes were labeled with antibodies recognizing CD3 (clone UCHT1), CD8 (clone SFC11/Thy2D3, Beckman Coulter), CD14 (clone HCD14, Biolegend) and CD19 (clone HD37, Dako).

MiHA-specific T cell cultures were incubated with PE-labeled tetramer and, subsequently, labeled with FITC-conjugated CD8 (clone LT8, Prolimmune, Oxford, United Kingdom) and PE-Cy7-conjugated CD25. After washing with PBS/0.5% bovine serum albumin (BSA; Sigma, St Louis, MO, USA), cells were resuspended in washing buffer containing 0.1% 7-amino-actinomycin D (7-AAD; Sigma). Cells were analyzed using the Coulter FC500, Navios, or the Cyan-ADP flow cytometer (all Beckman Coulter).

Ex vivo antigen-specific T cell expansion and degranulation assays
CMV-pp65- or MiHA-specific CD8+ memory T cells present in PBMCs from respectively healthy donors or patients (Patients 1-1, Table 1) were stimulate for one week ex vivo with peptide-loaded DCs in the presence of BTLA and/or PD-1 blocking antibody. Mature allogeneic DCs, cultured from apheresis material of HLA-A*2- or HLA-B7+ donors, were
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Results

MiHA-specific CD8\(^+\) effector memory T cells show high BTLA and PD-1 expression

Flow cytometric analysis was performed on CD8\(^+\) T cells from allo-SCT patients and healthy donors to determine BTLA and PD-1 expression levels on MiHA- and CMV- and FLU-specific CD8\(^+\) T cells in comparison to distinct CD8\(^+\) T cell differentiation stages. CD8\(^+\) T cell subset composition was analyzed by gating on CCR7 and CD69R (Figure 1A-B). In healthy donors the total CD8\(^+\) T cell pool was comprised of naive (N), effector-memory (EM) and central-memory (CM) cells. Allo-SCT patients on the contrary, had very low numbers of naive and CM CD8\(^+\) T cells, and their CD8\(^+\) T cells predominantly consisted of EM cells (82.4% ± 2.3%). Similar to CMV-pp65-specific CD8\(^+\) T cells in healthy donors (94.1% ± 3.0%), MiHA- and CMV-pp65-specific CD8\(^+\) T cells in allo-SCT patients were mainly of EM phenotype (95.7% ± 2.1% and 95.7% ± 1.3% respectively). However, the FLU-specific CD8\(^+\) T cell population in healthy donors comprised both CM (35.0% ± 9.8%) and EM (62.9% ± 10.3%) cells.

In healthy individuals, BTLA was highly expressed on naive CD8\(^+\) T cells and gradually decreased on more differentiated T cells, with EM CD8\(^+\) T cells showing markedly lower BTLA levels than the other T cell subsets (Figure 1C, Supplementary Figure 1A). When comparing CD8\(^+\) T cell subsets of healthy donors with those of allo-SCT patients, the number of naive BTLA\(^+\) T cells was significantly lower in allo-SCT patients. In contrast to BTLA, PD-1 expression by naive T cells was low and markedly increased upon T cell differentiation into effector and memory cells (Figure 1C, Supplementary Figure 1). In allo-SCT patients, the percentage of PD-1\(^+\) cells within each CD8\(^+\) T cell subset was significantly higher than in healthy individuals.

As most of the MiHA- and CMV-specific CD8\(^+\) T cells displayed an EM phenotype, we compared the expression of BTLA and PD-1 within the EM subset of tetramer\(^+\) and total CD8\(^+\) T cells (Figure 1D, Supplementary Figure 1B-C). Both in healthy donors and allo-SCT patients, the percentage of BTLA\(^+\) cells was significantly higher for CMV-specific CD8\(^+\) EM T cells than for total CD8\(^+\) EM T cells. The percentage of BTLA-positive FLU-specific T cells was similar to that in the total CD8\(^+\) EM T cell population of healthy donors, although BTLA levels on these T cells were slightly lower (Supplementary Figure 1C). Interestingly, MiHA-specific CD8\(^+\) T cells of allo-SCT patients highly expressed both BTLA and PD-1. Also CMV- and FLU-specific T cells in healthy donors showed increased PD-1 expression, however to a lesser extent than in allo-SCT patients. Collectively, these data demonstrate that MiHA-specific EM T cells of allo-SCT patients have elevated BTLA and PD-1 expression.

HVEM and BTLA are highly expressed by human hematopoietic tumor cells

To determine the expression of HVEM, BTLA, CD160 and LIGHT on hematopoietic malignancies, we analyzed various tumor cell lines, patient bone marrow samples and healthy donor leukocytes for cell surface expression using flow cytometry. First, we analyzed myeloid leukemia (both AML and chronic myeloid leukemia, CML), B-cell...
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Figure 1  BTLA and PD-1 are highly expressed by MiHA-specific CD8+ T cells

CD8+ T cell subsets of healthy donors and allo-SCT patients were examined for BTLA and PD-1 expression using flow cytometry. (A-B) T cell subset composition was analyzed by gating on CCR7 and CD45RA expression within the total or antigen-specific CD3+CD8+ T cell populations. Naive (N), CCR7+CD45RA+; Central memory (CM), CCR7+CD45RA−; Effector memory (EM), CCR7−. (A) Representative examples of the CD8 subset composition in 1 HD and 1 allo-SCT patient (Pt) are given. (B) Combined data on CD8 subset composition for HD and allo-SCT patients is shown (HD CD8: N=8, HD CMV and FLU: N=5, Patient CD8: N=6; Pt CMV and MiHA: N=5). Data are expressed as mean ±SEM. (C) The percentage BTLA and PD-1 positive cells was examined within the different CD8+ T cell subsets. (D) Within the EM subset of the total or antigen-specific CD3+CD8+ T cell population, the percentage of BTLA and PD-1 positive cells was examined within the different CD8+ T cell subsets. Statistical analysis was performed using a two-sample two-tailed t-test assuming independent samples. *P <0.05, **P <0.01, ***P <0.001. CD8+, CD8+ T cells; CMV, cytomegalovirus; FLU, influenza virus; MiHA, minor histocompatibility antigen. White bars, healthy donors; grey bars, allo-SCT patients.

Figure 2  Hematopoietic tumor cells highly express HVEM and BTLA

Surface expression of HVEM, BTLA, CD160 and LIGHT on hematopoietic tumor cells and healthy donor control cells was analyzed using flow cytometry. (A) For each protein the percentage positive cells was determined in various hematopoietic tumor cell lines. (B) HVEM, BTLA and LIGHT (black lines) protein expression by primary tumor cells as compared to isotype control (filled grey). Data of one representative AML and one MM patient are shown, respectively patient 19 and 24 of Supplementary Table 2. The numbers in the plots represent the mean fluorescence intensity (MFI). (C-D) The percentages of HVEM, BTLA, CD160 and LIGHT positive cells were determined in bone marrow samples of 8 AML patients and 10 MM patients, and PBMC samples of 5 healthy donors. (C) AML and MM cells were gated on the CD33+ and CD38+CD138− populations, respectively. (D) Healthy donor CD4+ T cells were gated as CD3+CD8−CD14− B cells as CD33+ and CD14+ and B cells as CD19+. Lines indicate the mean. Statistical analysis was performed using a two-sample two-tailed t-test assuming independent samples. *P <0.05, **P <0.01, ***P <0.001. AML, acute myeloid leukemia; MM, multiple myeloma.
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PD-1 blockade augments the proliferative capacity of fully functional CMV-specific CD8+ T cells

First, we investigated the effect of BTLA and/or PD-1 blockade on CMV-specific T cell responses, since in healthy donors a high level of PD-1 and BTLA on pp65-specific T cells was observed. Therefore, we stimulated PBMCs of 3 healthy donors with CMV-pp65 peptide loaded DCs in the presence or absence of blocking antibody. After one week, CMV-specific CD8+ T cell proliferation and function was analyzed. In Figure 3A, the percentage of tetramer+ CD8+ T cells is shown for 1 representative healthy donor. Blockade of BTLA resulted in a slightly higher percentage of CMV-specific T cells after 1 week. In contrast, PD-1 blockade clearly boosted the percentage and absolute number of CMV-specific CD8+ T cells (Figure 3B). The combination of BTLA and PD-1 blockade had no synergistic effects in this donor. To combine data of all healthy donors, the fold expansion was calculated as the absolute number of MiHA-specific CD8+ T cells in the presence of antibody blockade relative to isotype control treatment (Figure 3C). Interestingly, only PD-1 blockade and the combination with anti-BTLA treatment significantly enhanced the expansion of CMV-specific CD8+ T cells. To visualize the proliferation of the CMV-specific T cells, we labeled PBMCs of donor 2 with CFSE and performed a 1 week stimulation assay. After overnight restimulation with 5 µM CMV peptide in the presence of anti-CD107a antibody, the proliferation and degranulation capacity of the CMV-specific CD8+ T cells was analyzed. Upon stimulation in the presence of PD-1 blocking antibody, more cells had a CFSE-negative phenotype as compared to isotype control treated cells (Figure 3D). Furthermore, more than 66% of the tetramer+ CD8+ T cells showed high CD107a staining upon overnight restimulation with CMV peptide (Figure 3E). Notably, in this donor BTLA blockade did not further increase the degranulation capacity of the CMV-specific CD8+ T cells, while blocking PD-1 resulted in a higher percentage of CD107a+ antigen-specific T cells. Moreover, combined BTLA and PD-1 blockade further boosted CMV-specific T cell

Figure 3 Blockade of PD-1 results in enhanced proliferation of functional CMV-specific CD8+ T cells in healthy donors

PBMCs of healthy donors containing CMV-pp65-specific CD8+ T cells were stimulated for 1 week with mature DCs loaded with the CMV peptide at a ratio of 1:0.1. To block the inhibitory interactions, cells were pre-incubated with 10 µg/ml anti-BTLA and/or anti-PD-1. CMV-specific T cell expansion and function was subsequently analyzed using flow cytometry. (A) One week after stimulation, cells were analyzed for tetramer-positive CD8+ T cells using flow cytometry. Data of one representative healthy donor out of 3 donors is shown. (B) Total number of CMV-pp65-specific CD8+ T cells of donor 1 after 1 week of stimulation with CMV peptide loaded DCs in the presence or absence of blocking antibody. (C) The effect of BTLA blockade on CMV-specific T cell expansion was calculated by dividing the absolute number of tetramer-positive T cells following BTLA and/or PD-1 blockade with the absolute number of tetramer-positive T cells treated with isotype control antibody. Data of 3 different donors is shown. (D-F) To visualize T cell proliferation and function following 1 week stimulation in the presence of BTLA and/or PD-1 blockade, PBMCs labeled with or without 0.25 µM CFSE were re-stimulated overnight with 5 µM antigen peptide in the presence of CD107a antibody or Brefeldin A. The following day, CFSE dilution (D), CD107a staining (E), and intracellular IFN-γ and TNF-α levels (F) were analyzed within the CMV-specific CD8+ T cell population. Numbers in the plot indicate the percentage of cells in each quadrant. Data of one out of 2 donors is shown. Statistical analysis was performed using a Kruskal-Wallis analysis followed by a Dunn’s post-hoc test. *P < 0.05. MFI, mean fluorescence intensity.

these expression patterns in primary human tumor cells, we analyzed bone marrow samples of 8 AML and 10 MM patients obtained at time of diagnosis. AML and MM cells were gated on the CD32+SSlow and CD38+CD138+ populations, respectively. Similar expression patterns were found for HVEM and LIGHT in the primary tumor samples compared to the tumor cell lines (Figure 2B–C, Supplementary Table 2, Supplementary Figure 2B). While CD160 was again not expressed by the malignant cells, BTLA expression was substantially higher on the primary AML and MM cells than on the corresponding tumor cell lines. We also examined healthy donor control cells, and observed high HVEM and BTLA expression on T cells, monocytes and B cells (Figure 2D). However, expression levels were significantly higher on the monocytes and B cells (Supplementary Figure 2C). Furthermore, monocytes also displayed significantly higher LIGHT expression than the other cell types. Together, these data show that human AML and MM cells display high levels of HVEM, while BTLA is highly expressed on MM cells and moderately on AML cells.
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deprivation. Finally, we examined the production of effector cytokines IFN-γ and TNF-α of 1 week expanded CMV-specific T cells. Following overnight restimulation, most of the CMV-specific CD8⁺ T cells produced either IFN-γ alone or in combination with TNF-α (Figure 3F). Blockade of BTLA did not improve the percentage of antigen-specific T cells producing cytokines. On the contrary, blockade of PD-1 alone or in combination with BTLA, enhanced the percentage of IFN-γ⁺ and IFN-γ⁺TNFα⁺ cells within the CMV-specific CD8⁺ T cell population of this donor. These results indicate that blockade of PD-1, but not BTLA, results in increased proliferation of CMV-specific CD8⁺ T cells of healthy donors, and that these antigen-specific T cells are highly functional upon restimulation with the cognate peptide.

Both BTLA and PD-1 blockade augment expansion of MiHA-specific CD8⁺ T cells ex vivo

To investigate the role of BTLA in the inhibition of MiHA-specific CD8⁺ T cell responses, we performed functional assays using PBMCs from 11 patients with a hematological malignancy who developed MiHA-specific T cell responses after allo-SCT. Previously, we demonstrated that reinvigoration of MiHA-specific CD8⁺ T cell responses requires mature DCs loaded with the cognate MiHA^{HA-LR}. Therefore, we first analyzed expression levels of HVEM and its binding partners on mature monocyte-derived donor DCs (Figure 4A). Similarly to hematological tumor cells, mature DCs displayed high HVEM expression and moderate levels of BTLA and LIGHT. CD160, on the other hand, was not expressed. Subsequently, these DCs were pulsed with MiHA peptide and used for ex vivo stimulation of MiHA-specific memory T cells in the presence of BTLA and/or PD-1 blocking antibody. In Figure 4B, results of three representative patients are shown. At start, PBMCs of patients 4, 8 and 10 contained 0.09%, 0.76% and 0.11% CD8⁺ T cells recognizing either the MiHA LRH-1 or HA-1. In these patients, we observed augmented percentages of MiHA-specific CD8⁺ T cells after blockade of BTLA and/or PD-1 compared to isotype control treated cells. To combine data of all patients, the fold expansion was calculated as the absolute number of MiHA-specific CD8⁺ T cells after antibody blockade relative to isotype control treatment. In 7 out of 11 patients, BTLA blockade augmented MiHA-specific T cell expansion more than 2-fold (range 2.1-11.7 fold, Figure 4C, Table 1). Furthermore, PD-1 blockade enhanced MiHA-specific T cell numbers more than 2-fold in 9 out of 11 patients (range 2.5-12.5 fold.

**Figure 4** BTLA blockade increases ex vivo expansion of MiHA-specific CD8⁺ memory T cells

PBMCs of patients containing MiHA-specific CD8⁺ T cells were stimulated for 1 week with mature DCs loaded with the cognate peptide at a ratio of 1:10. To block the inhibitory interactions, cells were pre-incubated with 10 µg/ml anti-BTLA and/or anti-PD-1. DC phenotype and MiHA-specific T cell expansion was subsequently analyzed using flow cytometry. (A) Surface expression of HVEM, BTLA, CD160 and LIGHT (black lines) as compared to isotype control (filled grey) was measured on
Finally, we examined CFSE dilution, degranulation and cytokine production by the expanded MiHA-specific CD8+ T cells following overnight restimulation with the cognate peptide. After 1 week of stimulation with peptide-loaded DCs, >90% of the MiHA-specific CD8+ T cells were CFSE- (Figure 5A). However, no differential effect of BTLA or PD-1 blockade could be observed. Furthermore, these expanded MiHA-specific EM T cells efficiently degranulated upon antigen reencounter, as demonstrated by CD107a staining (Figure 5B). This in contrast to the tetramer-negative CD8+ T cell population, of which <5% of the cells stained positive for CD107a (data not shown). In some patients, single PD-1 blockade resulted in somewhat increased percentages of CD107a+ antigen-specific T cells, however this was not observed for the other treatments. In Figure 5C, intracellular IFN-γ and TNF-α expression levels by MiHA-specific T cells of one representative patient (no. 2) are shown. Following antigen restimulation more than 90% of the antigen-specific CD8+ T cells produced

Table 1 Characteristics of transplanted patients and effect of BTLA and/or PD-1 blockade on MiHA-specific CD8+ T cell proliferation

<table>
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<tr>
<th>Pt no.</th>
<th>Disease</th>
<th>MiHA T cell responsea</th>
<th>Sample date (months)</th>
<th>DLI prior to sample date</th>
<th>Disease status at sample date</th>
<th>GVHD at sample date</th>
<th>ISD at sample date</th>
<th>Clinical outcome</th>
<th>Effect αBTLAb</th>
<th>Effect αPD-1</th>
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<tr>
<td>1</td>
<td>pre-T-ALL</td>
<td>HA2</td>
<td>12 post-SCT</td>
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<td>Complete remission</td>
<td>Limited cGVHD</td>
<td>CSA, Prednisone</td>
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<td>Complete remission</td>
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<td>No</td>
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<td>No</td>
<td>Active disease at 6 mo post-SCT; Plasma cells 5%; M-protein 7 gr/L</td>
<td>1.0</td>
<td>8.7</td>
<td>9.4</td>
</tr>
<tr>
<td>10</td>
<td>NHL</td>
<td>HA1</td>
<td>12 post-SCT</td>
<td>No</td>
<td>Complete remission</td>
<td>No</td>
<td>No</td>
<td>Remission; alive at 165 mo post-SCT</td>
<td>11.7</td>
<td>5.3</td>
<td>8.4</td>
</tr>
<tr>
<td>11</td>
<td>NHL</td>
<td>HYA2</td>
<td>3 post-SCT</td>
<td>No</td>
<td>Relapse</td>
<td>No</td>
<td>Prednisone</td>
<td>Hematologic relapse at 3 mo post-SCT</td>
<td>2.6</td>
<td>1.6</td>
<td>5.8</td>
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Characteristics of patients with hematological malignancies displaying MiHA-specific CD8+ T cell responses. MiHA-specific response: MiHA for which a T cell response was observed. Effect αBTLA: ratio absolute number of tetramer+ CD8+ T cells after stimulation with DCs + peptide + αBTLA/absolute number of tetramer+ CD8+ T cells after stimulation with DCs + peptide + isotype control. AML, acute myeloid leukemia; CML-AP, chronic myeloid leukemia-accelerated phase; CML-BC, chronic myeloid leukemia-blast crisis; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; pre-T ALL, pre-T cell acute lymphoblastic leukemia; SCT, stem cell transplantation; DLI, donor lymphocyte infusion, given at 4-10 months after SCT; cGVHD, chronic graft-versus-host-disease; ISD, immunosuppressive drugs; CSA, Cyclosporine A; Mo, months.

Figure 4D. Interestingly, in 3 out of the 7 BTLA responders the effect of BTLA blockade was more prominent than that of PD-1 blockade. However, no significant difference was observed when comparing the effects of BTLA and PD-1 blockade for all patients. Moreover, combined blockade of BTLA and PD-1 boosted MiHA-specific CD8+ T cell expansion, though when compared to single receptor blockade no additive effect was observed.
the effector cytokines IFN-γ and/or TNF-α (Figure 5E). Although, the percentage of cytokine producing T cells did not further increase, the expression levels of IFN-γ and TNF-α within the MiHA-specific CD8+ T cells seemed to be higher following prior BTLA and/or PD-1 blockade. Especially, TNF-α seemed differentially up-regulated in MiHA-specific T cells treated with BTLA blocking antibody. In conclusion, we demonstrate that next to PD-1, BTLA is also involved in MiHA-specific T cell inhibition, and that the relative contribution of these two receptors to functional impairment differs between patients.

**Figure 5** Expanded MiHA-specific CD8+ memory T cells are highly functional

PBMCs of patients containing MiHA-specific CD8+ T cells were stimulated for 1 week with mature DCs loaded with the cognate peptide at a ratio of 10:1. To visualize the effect on T cell proliferation and function by BTLA and/or PD-1 blockade, PBMCs labeled with or without 1.25 µM CFSE were after one week restimulated overnight with 5 µM antigen peptide in the presence of CD107a. The following day, the percentage of CFSE after one week restimulated overnight with 5 µM antigen peptide in the presence of CD107a and function by BTLA and/or PD-1 blockade, PBMCs labeled with or without 1.25 µM CFSE were DCs loaded with the cognate peptide at a ratio of 1:0.1. To visualize the effect on T cell proliferation PBMCs of patients containing MiHA-specific CD8+ memory T cells were highly functional.

**Discussion**

Alloreactive MiHA-specific CD8+ T cells play a pivotal role in GVT responses after allo-SCT and DLI. Unfortunately, many patients develop relapses despite the long-term presence of MiHA-specific memory T cells, suggesting that these cells become functionally impaired. Tumor cells deploy distinct mechanisms to evade immune attack, including expression of ligands for co-inhibitory receptors on T cells. Previously, we demonstrated that the co-inhibitory PD-1/PD-L pathway is involved in the functional impairment of MiHA-memory responses after allo-SCT, and that these could be reinvigorated by DC stimulation in combination with PD-1 blockade. Another co-inhibitory receptor, BTLA, was recently shown to be involved in dysfunction of melanoma-specific T cells. This prompted us to investigate the potential inhibitory role of BTLA on MiHA-specific T cells in cancer patients after allo-SCT.

First, we investigated BTLA and PD-1 expression on the different CD8+ effector/memory subsets in healthy donors and allo-SCT patients. In concordance with the work from Derre et al., we found that BTLA expression in healthy donors is highest on naive CD8+ T cells, and is gradually reduced in CM and EM cells. This phenomenon was similar for CD8+ T cells of patients, although overall BTLA expression was lower. In contrast, PD-1 expression shows a reversed profile, with low expression on naive T cells and high presence on CM and EM, which was also reported by Duraiswamy et al. In allo-SCT patients PD-1 levels in the distinct subsets were evidently higher than those in corresponding healthy donor T cells. This is probably because of the presence of a long-lasting inflammatory milieu due to the pre-SCT conditioning regimen, development of alloreactive T cell responses, occurrence of GVHD and reactivation of viruses and subsequent immune responses. The differential expression profiles of BTLA and PD-1 indicate that there is a distinct role for these molecules to exert their physiological function. Since peripheral blood of allo-SCT patients predominantly contains EM CD8+ T cells with very low numbers of naive and CM T cells, analysis of BTLA and PD-1 expression was limited to the EM subset. Skewing of the CD8+ T cell population to the EM subset is a common phenomenon following allo-SCT since many T cells become activated, as mentioned before. When focusing on the MiHA-specific T cells these are also mainly of EM type, as expected due to in vivo allogeneic activation.

Subsequently, we investigated BTLA and PD-1 expression specifically on CMV-, FLU- and MiHA-specific T cells of healthy donors and allo-SCT patients, respectively. In accordance with findings by others, healthy donor CMV-specific T cells showed relative increased...
BTLA and PD-1 expression, which is probably due to frequent activation by latent CMV. On the contrary, lower BTLA expression was observed in case of cleared viral infections, like influenza. In general, BTLA levels on total CD8+ T cells in patients were slightly lower compared to those in healthy individuals. Interestingly, CMV- and MiHA-specific T cells of allo-SCT patients were highly BTLA positive despite their EM phenotype. PD-1 levels, on the contrary, were already high on the total CD8+ T cell pool, as well as, on CMV- and MiHA-specific EM T cells. These data confirm our recent observations and can be attributed to the high numbers of activated alloreactive and microbial-reactive T cells as mentioned before. Notably, MiHA-specific T cells of allo-SCT patients showed high expression levels of both PD-1 and BTLA.

To investigate whether BTLA ligation could be a potential evasion mechanism, we next examined ligand expression levels on tumor cells. Therefore, we analyzed hematopoietic tumor cells for the presence of BTLA, HVEM, CD160 and LIGHT. Interestingly, both HVEM and its binding partner BTLA were expressed on primary leukemia and myeloma cells, whereas LIGHT and CD160 were less present. Our data correspond with literature, describing HVEM to be constitutively expressed on primary myeloma cells. Furthermore, we report here for the first time that primary AML cells highly express HVEM. Notably, in contrast to the ligands for PD-1, HVEM is constitutively expressed and further up-regulation under inflammatory conditions was not observed (data not shown). Since BTLA can also be expressed on the tumor cells, we also investigated whether T cells were positive for HVEM. Indeed, HVEM was observed on human T cells, indicating that a bidirectional HVEM-BTLA signaling route between tumor cell and T cells can occur. However, the effect of BTLA signal transduction into the tumor cell is unknown. Furthermore besides BTLA, HVEM itself has been reported to function as a receptor as well.

Altogether, this results in a complex network of interactions for BTLA, HVEM and their binding partners, which needs to be elucidated in future experiments.

The concurrent expression of BTLA on MiHA-specific T cells and HVEM by malignant cells suggested that this co-inhibitory pathway contributed to the functional inhibition of MiHA-specific T cells in vivo. Therefore, we performed ex vivo blockade experiments using DCs as antigen-presenting cells. Based on their strong stimulatory potential, DCs are attractive vaccines for ex vivo and in vivo stimulation of MiHA-specific T cell responses. Importantly, DCs also express HVEM, BTLA and to some extent LIGHT, providing a rationale for combined DC stimulation and BTLA blockade. In addition, by interfering with the ligation of HVEM to BTLA an additive effect could be realized by increased interaction of HVEM with LIGHT, yielding a more co-stimulatory signal. After blockade of BTLA interactions, we observed enhanced proliferation of MiHA-specific T cells in 64% of the patients, indicating that BTLA ligation also contributes to impairment of alloreactive T cells in transplanted cancer patients. Based on our previous findings, where we identified the involvement of PD-1 in MiHA-specific T cell dysfunction, we next compared the relative contribution of both co-inhibitory receptors in this process. Compared to BTLA blockade, PD-1 blockade exerted evident boosting of antigen-specific T cell expansion in 82% of the patients. Interestingly, some patients responded better to BTLA blockade, while others showed more pronounced effects following PD-1 blockade. Whether the increase in number of cells is due to enhanced cell cycling was investigated in a CFSE dilution assay. For CMV-specific CD8+ T cells we observed a higher percentage of CFSE-diluted dividing cells following PD-1 blockade. However, we did not observe these effects in MiHA-specific CD8+ memory T cells. The increased number of antigen-specific T cells may here be attributed to less apoptosis following BTLA and/or PD-1 blockade. In addition, we examined whether the boosted antigen-specific T cells exhibited competent functionality. Upon antigen re-encounter, both CMV- and MiHA-specific CD8+ T cells efficiently degranulated and produced effector cytokines. Interestingly, blocking of BTLA seemed to mediate increased TNF-α production in MiHA-specific T cells.

Possibly, the differences observed between PD-1 and BTLA blocking can be attributed to inherent differences between the antibodies. The anti-PD-1 antibody is fully humanized and has proven its functionality in phase I clinical trials, whereas the anti-BTLA antibody is murine and still in the pre-clinical stage. Although few immune-related adverse events have been reported for PD-1 blockade, dosing and timing of the BTLA blocking antibody has to be carefully examined in clinical trials, as both molecules are involved in the maintenance of self tolerance. Especially, in the allo-SCT setting interference with co-inhibitory molecules could deteriorate GVHD. In mouse models, however, BTLA has been reported to have a differential role in GVT responses, and not in GVHD. Although we found high expression of both BTLA and PD-1 on MiHA-specific CD8+ T cells, we could not correlate their expression levels to the effect on T cell proliferation upon blocking either of these pathways (data not shown). Perhaps the impairment via these receptors depends more on the amount of signaling it has experienced, than the expression of the receptor as such, as has been suggested previously for PD-1. Furthermore, no evident correlation in BTLA effect and disease status could be observed, though patients in long-term remission after allo-SCT seemed to respond better. This is in contrast to PD-1, which was shown to be more involved in the impairment of MiHA T cell responses in relapsed patients. It could be that the inhibitory effect of BTLA and PD-1 on MiHA-specific T cells has been influenced by the treatment with immunosuppressive drugs, however in our study only few patients received ISD at time of analysis and no firm conclusions could be drawn. As the reversed expression profiles of BTLA and PD-1 on normal T cells already demonstrate, these molecules are likely to have distinct roles in hampering MiHA-specific T cell responses. Finally, we examined whether an additive effect could be observed by combined blockade of BTLA and PD-1. However, we did not observe an unambiguous additive effect on MiHA-specific T cell proliferation. This might suggest that the two receptors share common intracellular signaling pathways. It has been reported that both PD-1 and BTLA relay their co-inhibitory signal via SHP-1 and SHP-2. Nonetheless, a third interactor with the intracellular domain of BTLA has been described, Grb-2.
Altogether, we have demonstrated that BTLA and PD-1 levels are elevated on MiHA-specific T cells post-transplantation, and that BTLA’s ligand HVEM is present on hematological tumors as well as DCs. Importantly, blocking BTLA results in enhanced outgrowth of MiHA-specific T cells upon DC stimulation. Moreover, others reported a differential role of BTLA in the induction of GVHD and GvT responses\(^1\), making it an interesting target to prevent adverse alloreactive effects. This prompts BTLA as a novel target in combination with DC vaccination. We did not observe additive effects of simultaneous PD-1 and BTLA blockade and could not predict effects of either blocking antibody. Therefore, further research into the distinct roles of BTLA, PD-1 and other co-inhibitory molecules is important to understand the functional state of impaired T cells. This will result in more targeted therapy by applying blocking antibodies to the appropriate receptors. In conclusion, we showed that BTLA interactions impair functionality of MiHA-specific T cells, providing a rationale for the incorporation of BTLA blockade in post-SCT therapies.

Acknowledgements
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Chapter 5

BTLA blockade enhances MiHA-specific T cell function


Chapter 5

BTLA blockade enhances MiHA-specific T cell function

Supplementary Table 1
HVEM, BTLA, CD160 and LIGHT expression levels on hematopoietic tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>% HVEM</th>
<th>% BTLA</th>
<th>% CD160</th>
<th>% LIGHT</th>
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<tbody>
<tr>
<td>KG1a</td>
<td>AML-M0</td>
<td>85.0</td>
<td>8.9</td>
<td>3.6</td>
<td>6.8</td>
</tr>
<tr>
<td>KG1</td>
<td>AML-M1</td>
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<td>10.0</td>
<td>2.1</td>
<td>5.3</td>
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<tr>
<td>Kasumi</td>
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<td>8.0</td>
<td>0.8</td>
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<tr>
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<td>9.2</td>
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<td>Mono-Mac-6</td>
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Various hematopoietic tumor cell lines were analyzed for the percentage of HVEM, BTLA, CD160 and LIGHT positive cells. AML, acute myeloid leukemia; CML, chronic myeloid leukemia; B-NHL, B-cell non-Hodgkin lymphoma; MM, multiple myeloma; ND, not determined.

Supplementary Table 2
HVEM, BTLA, CD160 and LIGHT expression levels on primary AML and MM cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type</th>
<th>Malignant cells (%)</th>
<th>% HVEM</th>
<th>% BTLA</th>
<th>% CD160</th>
<th>% LIGHT</th>
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Peripheral blood samples of AML and MM patients, obtained at time of diagnosis, were analyzed for percentage of HVEM, BTLA, CD160 and LIGHT positive cells. AML cells were gated as CD33<sup>+</sup>SS<sup>low</sup> cells. MM cells were gated as CD38<sup>+</sup>CD138<sup>+</sup> cells. *The percentage blasts was defined by morphological analysis. AML, acute myeloid leukemia; MM, multiple myeloma; ND, not determined.
Chapter 5

BTLA blockade enhances MiHA-specific T cell function

Supplementary Figure 1

BTLA and PD-1 are highly expressed by MiHA-specific CD8* T cells

CD8* T cell subsets of healthy donors (HD) and allo-SCT patients (pt) were examined for BTLA and PD-1 expression using flow cytometry. T cell subsets were analyzed by gating on CCR7 and CD45RA expression within the total or antigen-specific CD3*CD8* T cell populations. Naive (N), CCR7*CD45RA*; Central memory (CM), CCR7*CD45RA-; Effector memory (EM), CCR7-. (A) Median fluorescence intensity (FI) of BTLA and PD-1 was examined within the different T cell subsets of the total CD8* T cell population. (B) Surface expression of BTLA and PD-1 (filled black) as compared to isotype control (filled grey) on total CD8* and antigen-specific EM T cells is depicted for the same donors and patients as in Figure 1A. The numbers in the plots represent the median FI. (C) BTLA and PD-1 surface expression levels were analyzed within the EM subset of the total or antigen-specific CD3*CD8* T cell populations for all donors and patients. HD CD8: N=8, HD CMV and FLU: N=5, Pt CD8: N=6, Pt CMV and MiHA: N=5. Statistical analysis was performed using a two-sample two-tailed t-test assuming independent samples, or One-way ANOVA followed by a Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001.

Supplementary Figure 2

Hematopoietic tumor cells highly express HVEM and BTLA

Surface expression of HVEM, BTLA, CD160 and LIGHT on hematopoietic tumor cells and healthy donor control cells was analyzed using flow cytometry. For each protein the mean fluorescence intensity (MFI) was determined on (A) various hematopoietic tumor cell lines, (B) primary tumor cells, and (C) healthy donor control cells. (A) AML (N=8) and MM (N=10) cells were gated on the CD33* and CD38*CD138* populations, respectively. (C) Healthy donor CD4* T cells were gated as CD3*CD8-, CD8* T cells as CD3*CD8-, monocytes as CD14* and B cells as CD19* (N=5). Statistical analysis was performed using a two-sample two-tailed t-test assuming independent samples, or One-way ANOVA followed by a Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001.
siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8+ T cells
Chapter 6

Knockdown of PD-L1 on DC augments T cell function

Abstract

Tumor relapse after HLA-matched allogeneic stem cell transplantation (SCT) remains a serious problem, despite long-term presence of minor histocompatibility antigen (MiHA)-specific memory T cells. Dendritic cell (DC)-based vaccination boosting MiHA-specific T cell immunity is an appealing strategy to prevent or counteract tumor recurrence, but improvement is necessary to increase the clinical benefit. Here, we investigated whether knockdown of Programmed death ligand 1 (PD-L1) and PD-L2 on monocyte-derived DCs results in improved T cell activation. Electroporation of single siRNA sequences into immature DCs resulted in efficient, specific and long-lasting knockdown of PD-L1 and PD-L2 expression. PD-L knockdown DCs strongly augmented interferon-γ and interleukin-2 production by stimulated T cells in an allogeneic MLR, while no effect was observed on T cell proliferation. Moreover, we demonstrated that PD-L gene-silencing, especially combined PD-L1 and PD-L2 knockdown, resulted in improved proliferation and cytokine production of KLH-specific CD4+ T cells. Most importantly, PD-L knockdown DCs showed superior potential to expand MiHA-specific CD8+ effector and memory T cells from leukemia patients early after donor lymphocyte infusion and later during relapse. These data demonstrate that PD-L siRNA electroporated DCs are highly effective in enhancing T cell proliferation and cytokine production, and therefore attractive for improving the efficacy of DC vaccines in cancer patients.

Introduction

Alloreactive CD8+ T cells play a crucial role in graft-versus-tumor (GVT) responses following allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI)1. In human leukocyte antigen (HLA)-matched allo-SCT, these alloreactive CD8+ T cell responses are directed against minor histocompatibility antigens (MHAs), which are HLA-bound peptides derived from polymorphic genes that differ between donor and recipient12. Upon infusion into the pre-conditioned patient, donor-derived MiHA-specific CD8+ T cells clonally expand and become effector cells, which subsequently have the capacity to target MiHA-expressing malignant cells1. After the primary immune response, most MiHA-reactive CD8+ T cells die through apoptosis and only a small pool of long-lived memory cells survives that is able to respond quickly upon reencounter of the antigen1. Importantly, we and others have shown that following DLI the emergence of MiHA-specific CD8+ effector T cells coincides with a decrease in number of malignant cells4-6. Moreover, years after the initial response MiHA-specific CD8+ memory T cells can still be detected, playing an important role in protective immunity in transplanted patients. However, these alloreactive CD8+ memory T cells do not always respond efficiently to recurring tumor cells and this failure may contribute to the occurrence of tumor relapses6. This lack of responsiveness may result from inefficient memory T cell activation due to absence of effective antigen presentation, impaired co-stimulation and/or enhanced signaling by co-inhibitory receptors. Interestingly, we recently found that the Programmed death-1 (PD-1)/PD-Ligand axis is involved in inhibiting the function of MiHA-specific CD8+ T cells upon their engagement with PD-L1 expressing myeloid leukemia cells and dendritic cells (DCs)9.

PD-L is a co-inhibitory receptor that is inducibly expressed by T and B cells upon activation. Engagement of this receptor with either of its ligands, i.e. PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), results in phosphorylation of the cytoplasmic tail and negatively regulates T cell receptor (TCR)-induced cytokine production and proliferation via the phosphatidylinositol-3 kinase pathway10-11. While PD-L2 expression is mainly restricted to activated lymphocytes and antigen presenting cells (APCs) like DCs and macrophages, PD-L1 is also expressed by many non-lymphoid tissues and tumor cells12-13. In the past few years, a crucial role for the PD-1/PD-L1 pathway has been demonstrated in chronic viral infections like HIV and hepatitis C (HCV)14-16. It has been shown that HIV- and HCV-specific T cells have increased PD-1 expression resulting in functional exhaustion of these cells. Moreover, PD-1/PD-L interactions have been implicated in functional impairment of tumor antigen-reactive CD8+ T cells in solid tumors and chronic myeloid leukemia17-19. Therefore, tumor cells may exploit the same pathway to escape eradication by MiHA-specific CD8+ memory T cells after allo-SCT.

DC vaccination therapy would be an appealing strategy to revive functionally inactive MiHA-specific CD8+ memory T cells and, by this means restore an effective immune response against recurring or persistent MiHA-expressing tumor cells. DCs have been
widely exploited as adjuvants in vaccination therapy because of their capability to effectively initiate and reactivate T cell-based immune responses. In GvT and graft-versus-host-disease (GVHD) responses the process of alloreactive T cell stimulation and activation is also orchestrated by MHA-presenting DCs. Nowadays, the most commonly used cell source for DC generation is peripheral blood monocytes, since high numbers of monocytes can be easily obtained and differentiated into mature DCs (mDC). Using IL-4 and GM-CSF, immature DCs (iDCs) are obtained after 5-7 days of culture, followed by 2 days of maturation in the presence of a cytokine cocktail containing IL-1β, IL-6, TNF-α and PGE2. Upon maturation, expression of peptide-HLA complexes is increased and many accessory molecules involved in cell adhesion, cell migration and co-stimulation are acquired. For instance, CCR7 plays an important role in DC migration to draining lymph nodes, while adhesion molecules CD54 and CD58 are involved in T cell activation. Furthermore, mDCs have high expression of co-stimulatory molecules CD80, CD86 and Inducible T-cell co-stimulator ligand (ICOS-L). However, co-inhibitory molecules such as PD-L1, PD-L2, Herpes virus entry mediator (HVEM) and B7-H3 are also highly up-regulated. Expression levels and interactions of these co-stimulatory and co-inhibitory ligands with their counter-receptors on T cells ultimately determine the turn of the balance towards antigen-specific T cell activation or inhibition.

Here, we investigated whether knockdown of PD-1 ligands in monocyte-derived DCs results in improved T cell function. We identified small interfering RNAs (siRNAs) that efficiently silenced PD-L1 and PD-L2 expression following electroporation of iDCs. We demonstrated that PD-L1 gene-silencing resulted in significant improvement of IFN-γ and IL-2 production, and proliferation of keyhole limpet hemocyanin (KLH)-experienced T cells. Moreover, PD-L1 knockdown DCs showed superior potential to expand PD-1-expressing MiHA-specific CD8+ T cells. Interestingly, PD-L1 knockdown DCs showed superior capacity to expand PD-1-expressing MiHA-specific CD8+ effector memory T cells from leukemia patients shortly after DLI and later during relapse. Together, these findings indicate that PD-L silenced DCs are attractive for improving the efficacy of DC vaccines in cancer patients.

Materials and methods

Patient and donor material
Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats (Sanquin blood bank, Nijmegen, the Netherlands). Blood samples were collected after written informed consent was obtained in ongoing clinical SCT and DC vaccination protocols, approved by the Radboud University Nijmegen Medical Centre Institutional Review Board. PBMCs containing KLH-specific T cells were obtained from multiple myeloma (MM) patients vaccinated with autologous DCs pulsed with KLH, as an adjuvant and immune monitoring tool, and various tumor-associated antigens in an ongoing clinical trial. These patients received three DC vaccines intravenously and intradermally with biweekly intervals. PBMCs obtained two weeks after the second vaccination were used for functional studies and DCs were cultured from autologous apheresis material collected before start of vaccination therapy. To examine the stimulatory capacity of PD-L knockdown DCs on MiHA-specific T cell responses, we used PBMCs obtained from 3 leukemia patients who developed a MiHA-specific CD8+ T cell response after DLI. Patient UPN643 suffered from acute myeloid leukemia (AML) and developed a strong LRH-1-specific CD8+ T cell response upon preemptive DLI. Then, the patient remained in complete clinical and cytogenetic remission until 3 years after DLI, whereupon extramedullary relapses developed without leukemic involvement in the bone marrow. Patient UPN640 had a blast crisis (BC) chronic myeloid leukemia (CML) and was treated with preemptive DLI after allo-SCT. This resulted in a HA-1-specific CD8+ effector T cell response. The third patient (UPN389) suffered from accelerated phase (AP) chronic myeloid leukemia (CML) and was successfully treated with therapeutic DLI after allo-SCT. Following DLI a LRH-1-specific CD8+ T cell response was observed, which coincided with remission of CML-AP.

Here, we investigated whether knockdown of PD-1 ligands in monocyte-derived DCs results in improved T cell function. We identified small interfering RNAs (siRNAs) that efficiently silenced PD-L1 and PD-L2 expression following electroporation of iDCs. We demonstrated that PD-L1 gene-silencing resulted in significant improvement of IFN-γ and IL-2 production, and proliferation of keyhole limpet hemocyanin (KLH)-experienced T cells. Moreover, PD-L1 knockdown DCs showed superior potential to expand PD-1-expressing MiHA-specific CD8+ effector memory T cells from leukemia patients shortly after DLI and later during relapse. Together, these findings indicate that PD-L silenced DCs are attractive for improving the efficacy of DC vaccines in cancer patients.

Cell culture
LRH-1-specific CD8+ cytotoxic T lymphocyte (CTL) culture RP1 was isolated from CML-AP patient UPN389 and recognizes the 9-mer epitope TPNQRQNVC in the context of HLA-B*0702. CTL RP1 was cultured as described previously. Recipient and donor Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCL) of patient UPN389 were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS; Integro, Zaandam, the Netherlands).

Generation of monocyte-derived DCs
Monocytes were isolated from PBMCs via plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Immature DCs were generated by culturing monocytes in X-VIVO-15 medium (Lonza, Verviers, Belgium) supplemented with 2% human serum (HS; PAA laboratories, Pasching, Austria), 500 U/ml IL-4 (Immunotools, Friesoythe, Germany) and 800 U/ml GM-CSF (Immunotools). After 2-3 days, half of the medium was replaced with fresh X-VIVO-15/2% HS medium containing 1000 U/ml IL-4 and 1600 U/ml GM-CSF. Maturation of DCs was induced at day 6-7 by culturing 0.5 x 10^6 immature (electroporated) DC/ml in 6-well plates (Corning Costar) in X-VIVO-15/2% HS containing 500 U/ml IL-4, 800 U/ml GM-CSF, 5 ng/ml IL-1β, 15 ng/ml IL-6, 20 ng/ml TNF-α (all Immunotools) and 1 µg/ml PGE2 (Pharmacia & Upjohn, Bridgewater, NJ, USA). At day 8, mDCs were harvested and used for T cell stimulation, unless stated otherwise.
Stealth™ siRNA duplexes

Three different PD-L1 and PD-L2 Stealth™ siRNA duplexes and two recommended Stealth™ negative control siRNA duplexes for either low or medium GC content were obtained from Invitrogen. The PD-L1 siRNA and PD-L2 siRNA target sequences are listed in Table 1. All siRNAs were dissolved or diluted to a concentration of 25 μM in DEPC-water (Invitrogen) and subsequently stored at -20°C.

Table 1 Target sequences of StealthTM PD-L siRNAs

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense sequence (5' → 3')</th>
<th>Anti-sense sequence (5' → 3')</th>
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<tr>
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<td>UAUGCUGAACCUCUAGGCUUCCUC</td>
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<tr>
<td>PD-L1 no. 2</td>
<td>CCUCUCCUGCAUUCUGCGAAGC AU</td>
<td>AAUUGGUCAGCAUAUGCAGUGG</td>
</tr>
<tr>
<td>PD-L1 no. 3</td>
<td>UGAUCACCAUULCGAGGAGAGGAUA</td>
<td>UUAUGCAGGGCUUCCUUCA</td>
</tr>
<tr>
<td>PD-L2 no. 1</td>
<td>GGGAGGAUAUGCCUGACUCGCAAU</td>
<td>AUCUGGUGAAGCGCAUUAGGC</td>
</tr>
<tr>
<td>PD-L2 no. 2</td>
<td>GGGACUCAUGCCUCAGCGUUA</td>
<td>UUACUGAGAGCAGCAGUGU</td>
</tr>
<tr>
<td>PD-L2 no. 3</td>
<td>CCUCUCCUGCAUUCUGCGAAGC AU</td>
<td>AAUUGAAGGCAUUAGCAGGG</td>
</tr>
</tbody>
</table>

DC electroporation

Immature DCs were harvested at day 6-7, washed once with Hank’s balanced salt solution (HBSS; Lonza) and twice with phenol red free Optimem buffer (Gibco Invitrogen, Carlsbad, CA, USA). Cells were resuspended in Optimem buffer and ≤7.5 x 10⁶ cells were added to a 0.4 cm gene pulser cuvette (Biorad, Hercules, CA, USA). Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

RNA isolation and real-time quantitative reverse transcription PCR

Total RNA was isolated from mature PD-L knockdown DCs using the Quick-RNA-miniprep (Zymo Research Corporation, Orange, CA, USA). cDNA synthesis and PCR amplification were performed as described[28,29] The Hydroxymethylbilane synthase (HMBS) housekeeping gene was used to normalize gene expression. One μl of cDNA was amplified in a 50 μl reaction mixture containing 1.25 U AmpliTaq Gold (Applied Biosystems, Forster City, CA, USA), 300 nM gene-specific forward and reverse primer, 100-300 nM gene-specific Taqman probe (100 nM for PD-L2, and 300 nM for PD-L1 and HMBS), 250 μM of each dNTP, 5 mM MgCl₂, and 1x Taqman PCR buffer (Applied Biosystems). The following gene-specific primers and Taqman probes were used: PD-L1 FW 5'-CATCTTATATGCGCTTTG-GTAGCA-3', PD-L1 RV 5'-GGATTACGTCTCCTACAAATGTG-3' and PD-L1 MGB probe 5'-(TET)-ACATTACATCTCCGTGGTAAAG-3'; PD-L2 FW 5'-CACTTGCGTCCTCACTATTTT-3', PD-L2 RV 5'-TGGGATGACAGGCTTTTCTTTTG-3' and PD-L2 probe 5'-(TET)-TTCATAGGCCAGT-GATACGCTTAAGAACAACACTCT(TAMRA)-3'; HMBS, HMBS-FW 5'-GGCAATGCGCCTGCAAA; HMBS-RV 5'-GGTGACCCACGCGAATCACG-3' and HMBS-probe 5'-(VIC)-CTCATCTTGGCGCTTTCTCCGCC(TAMRA)-3'. PCR amplification was performed using an ABI Prism 7700 (Applied Biosystems) with the following PCR conditions: enzyme activation for 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PD-L1 and PD-L2 ΔCt values were normalized for HMBS by calculating ΔCt = Ct(Gene) - Ct(HMBS) per sample. Finally, PD-L1 and PD-L2 mRNA expression levels were quantified relative to DCs electroporated without siRNA as follows: 2^(-ΔΔCt)electroporated with siRNA/2^(-ΔΔCt)electroporated without siRNA.

Flow cytometry

Phenotype and maturation state of DCs was analyzed by staining with the following antibodies: anti-CD14 (clone TUK4, Dako, Glostrup, Denmark), anti-CD80, clone MA104, anti-CD83 (clone HI122, FabGen, Burlington, MA, USA), anti-CD-L1 (clone M142), anti-CD-L2 (clone M1H8, both from Becton Dickinson, Rhenania-Lakes, NL, USA) and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2c PE (Beckman Coulter). LRH-1- and HA-1-specific T cells were detected by staining cell suspensions with PE-labeled LRH-1/HLA-B7 tetramers containing peptide TPNQQNCV (Dako) and PE-labeled HA-1/HLA-A2 tetramers containing peptide VLHDDELQA (Becton Dickinson). T cells were incubated with 1.5-2 μg/ml tetramer for 15 min at room temperature. Subsequently, cells were labeled with the appropriate concentrations of anti-CD8 (ProImmune, Oxford, United Kingdom) and anti-CD3 (Beckman Coulter) (30 min at 4°C. After washing with PBS/0.5% bovine serum albumin (BSA; Sigma, St Louis, MO, USA), cells were resuspended in washing buffer containing 0.1% 7-amino-actinomycin D (7-AAD; Becton Dickinson, Franklin Lakes, NJ, USA) and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2c PE (Beckman Coulter). T cells were incubated with 1.5-2 μg/ml tetramer for 15 min at room temperature. Subsequently, T cells were labeled with the appropriate concentrations of anti-CD8 (ProImmune, Oxford, United Kingdom) and anti-CD3 (Beckman Coulter) for 30 min at 4°C. After washing with PBS/0.5% bovine serum albumin (BSA; Sigma, St Louis, MO, USA), cells were resuspended in washing buffer containing 0.1% 7-amino-actinomycin D (7-AAD; Sigma). Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

Allogeneic mixed lymphocyte reactions

Allogeneic CD4⁺ and CD8⁺ T cells were isolated from non-adherent or complete PBMC fractions by direct magnetic labeling with the appropriate MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Cells were resuspended in IMDM/10% HS to a concentration of 0.5 x 10⁶ cells/ml and stimulated with mature PD-L1 knockdown DCs at T cell:DC ratios ranging from 1:0.1 to 1:0.0125. These co-cultures were performed in 48-wells plates (Cellstar; Greiner Bio-one) in a total volume of 1 ml IMDM/10% HS. After 1-5 days of co-culture, supernatant was harvested for cytokine analysis. At day 5, cells were resuspended and transferred to 96-wells round bottom plates (Corning Costar). Subsequently, 0.5 μCi [³H]-thymidine (Perkin Elmer, Groningen, the
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Netherlands) was added to each well. After overnight incubation, [3H]-thymidine incorporation was measured using a 1205 Wallac Betaplate counter (Perkin Elmer).

**KLH-specific T cell activation assays**

Autologous monocyte-derived DCs were cultured from apheresis material of MM patients as described above. At day 3, DCs were pulsed with or without 50 µg/ml KLH (Vacume®, Biosyn Corporation, Fellbach, Germany). Patient PBMCs containing KLH-specific T cells were thawed, resuspended in IMDM/10% HS to a concentration of 0.5 x 10^6 cells/ml and mixed with autologous 2 day mature PD-L knockdown DCs at a T cell:DC ratio of 1:0.05. These co-cultures were performed in 6-fold in 96-wells round bottom plates (Corning Costar) in a total volume of 200 µl. After 1 and 5 days of co-culture, supernatant was harvested and was determined by flow cytometry. At day 5, proliferation was analyzed using [3H]-thymidine, as described before.

**MiHA-specific T cell expansion assays**

LRH-1-specific CTL RP1 was resuspended in IMDM/10% HS to a concentration of 0.05 x 10^6 cells/ml and plated in 96-wells round bottom plates (Corning Costar). HLA-B7* PD-L knockdown DCs were loaded with or without 10 µM LRH-1 peptide TPQQRQNC for 30 minutes at room temperature and, subsequently, co-cultured with CTLs at a stimulation ratio of 1:1 in a total volume of 200 µl. After 1 day, 100 µl supernatant was harvested and fresh IMDM/10% HS containing 100 U/ml IL-2 (Chiron, Emeryville, CA, USA) was added. At day 4, CTL proliferation was analyzed using [3H]-thymidine, as described before, and supernatant was analyzed for cytokine levels.

MiHA-specific CD8+ memory T cells present in PBMCs from patients UPN543, UPN640 and UPN389 were stimulated for one to three consecutive weeks ex vivo with un loaded or MiHA peptide-loaded PD-L1 knock down DCs. Mature allogeneic PD-L1 knockdown or control DCs, cultured from apheresis material of a HLA-B7* LRH-1- or HLA-A2* HA-1 donor, were loaded with or without 5 µM MiHA peptide (LRH-1: TPQQRQNC, HA-1: VHLDDPLEA) for 30 minutes at room temperature. PBMCs and DCs were subsequently co-cultured at a ratio of 1:1 in 2 ml IMDM/10% HS in K-24 wells plates (Corning-Costar). After 5 days, CD4+ T cells were depleted from the cultures by direct magnetic labeling with CD4 IMag beads (Becton Dickinson). CD4-depleted fractions were resuspended in IMDM/10% HS supplemented with 50 U/ml IL-2 and 5 ng/ml IL-15 (Immucyte). At day 6 or 7, cells were harvested, counted and the percentage of LRH-1- or HA-1-specific CD8+ T cells was determined by flow cytometry.

**CD107a degranulation assay**

PBMC cultures stimulated for 2 consecutive weeks with LRH-1 peptide-loaded PD-L1 knockdown DCs were used in a CD107a secretion assay to determine functional recognition of the LRH-1 antigen by measuring CTL degranulation. As target cells we used the corresponding recipient EBV-LCLs and donor EBV-LCLs loaded with or without 5 µM LRH-1 peptide. Briefly, 2 x 10^4 cells of each T cell culture were stimulated with target cells at an effector:target ratio of 1:1 in a total volume of 500 µl IMDM/10% HS supplemented with 25 U/ml IL-2 and 6.5 µl anti-CD107a (Becton Dickinson). After overnight co-culture, the percentage of CD107a+ cells was determined within the LRH-1-tetramer+ CD8+ T cell population using flow cytometry.

**Cytokine analyses**

IFN-γ and granzyme B levels in culture supernatants were analyzed using enzyme-linked immunosorbent assays (ELISA; IFN-γ: Pierce Endogen, Rockford, IL, USA; Granzyme B: MabTech, Nacka Strand, Sweden). Release of IL-2, IL-4, IL-5, TNF-α and IFN-γ by the T cells was simultaneously determined in pooled supernatant using a Th1/Th2 BD™ cytokine bead array (Becton Dickinson) following the manufacturer’s protocol and measured using flow cytometry.

**Statistical analysis**

Statistical significance of differences between PD-L knockdown DCs and medium GC siRNA control DCs was analyzed using One-way ANOVA followed by a Bonferroni post-hoc test. P-values <0.05 were considered significant.

**Results**

Efficient, specific and long-lasting siRNA-mediated knockdown of PD-L1 and PD-L2 on DCs

To determine the efficacy and specificity of three different siRNAs in silencing PD-L1 and PD-L2 expression on monocyte-derived DCs, we studied the magnitude, off-target effects and duration of knockdown on mRNA and protein level. Immature DCs were electroporated with or without 0.25 nmol siRNA and subsequently cultured in maturation medium containing IL-1ß, IL-6, TNF-α and PGE2. All three PD-L1 siRNAs appreciably reduced PD-L1 expression to the same extent. To validate knockdown on the protein level, we analyzed PD-L1 mRNA levels at two days after electroporation, while the negative control siRNAs had no effect (Figure 1A). Similarly, all three PD-L2 siRNAs efficiently knocked down PD-L2 mRNA expression to the same extent. To validate knockdown on the protein level, we analyzed PD-L1 and PD-L2 surface expression on siRNA-electroporated DCs two days after maturation. PD-L1 siRNA number 2 induced the most pronounced reduction of PD-L1 cell surface expression, resulting in an average of 21% PD-L1+ DCs (N=6, Figure 1B-C). In case of PD-L2 all three siRNAs were equally effective, resulting in <10% PD-L2+ DCs. Therefore, we decided to continue with PD-L1 and PD-L2 siRNA number 2 and the accompanying medium GC negative control siRNA. To verify that our siRNAs of interest did not affect DC maturation or have other off-target effects, protein expression levels of various maturation
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Efficacy, specificity and duration of siRNA-mediated PD-L1 and PD-L2 silencing on DCs

Immature DCs were electroporated with 0.25 nmol PD-L1, PD-L2 or negative control (MED GC vs. LOW GC) siRNA duplexes and subsequently cultured in maturation medium containing IL-18, IL-6, TNF-α and PGE2 for 2 days or 1-5 days. (A) PD-L1 and PD-L2 expression was measured and subsequently normalized for HMBS expression using RT Q-PCR. PD-L1 mRNA expression in DCs electroporated without siRNA was set to 1. (B) PD-L1 and PD-L2 protein expression (black lines) as compared to isotype control (grey histograms) was analyzed using flow cytometry. The numbers in the plots represent the mean fluorescence intensity (MFI). Data of 1 representative donor out of 6 are shown. (C) Percentage of PD-L1+ or PD-L2+ DCs was determined for 3-6 donors by flow cytometry. (D) Expression of maturation and co-stimulatory molecules by electroporated DCs was analyzed using flow cytometry. The bars represent DCs electroporated without siRNA (black), with MED GC control siRNA (white), PD-L1 siRNA (dark grey) and PD-L2 siRNA (light grey). Data are expressed as mean ± SEM of 3-6 donors. (E) Percentage of PD-L1+ or PD-L2+ DCs over time was determined by flow cytometry. The percentage of PD-L1+ DCs electroporated without siRNA was set to 100%. The lines represent DCs electroporated without siRNA (dotted line, open circle), with MED GC control siRNA (dotted line, open circle), PD-L1 siRNA 2 (solid dark grey line) and PD-L2 siRNA 2 (solid light grey line). The data are representative for 2 independent experiments on 2 different donors.

PD-L knockdown DCs show increased capacity to stimulate cytokine production in allogeneic MLR assays

To investigate whether knockdown of PD-L1 and/or PD-L2 using the selected siRNAs (i.e. PD-L1 and PD-L2 target sequence no. 2 in Table 1) improved the stimulatory capacity of DCs, allogeneic T cells were co-cultured for five days with mature PD-L knockdown DCs. Thereafter, T cell proliferation and cytokine production was analyzed. A strong and dose-dependent increase in IFN-γ production was observed upon stimulation with PD-L knockdown DCs, but PD-L knockdown DCs were not capable of improving T cell proliferation of allogeneic CD4+ T cells or CD8+ T cells (Figure 2A-B). For CD4+ T cells, IFN-γ levels were enhanced up to 7-fold by PD-L1 knockdown DCs, whereas PD-L2 silenced DCs only modestly improved IFN-γ production (Figure 2B). Interestingly, stimulation with PD-L1/L2 double knockdown DCs resulted in a 10-fold higher IFN-γ production, indicating a synergistic effect when PD-L1 and PD-L2 are silenced simultaneously. Although CD8+ T cells produced less IFN-γ than CD4+ T cells, still considerable elevation of IFN-γ levels was observed upon stimulation with PD-L knockdown DCs. Especially, PD-L1 single and PD-L1/L2 double knockdown DCs improved IFN-γ levels up to 3-fold, while the increase by PD-L2 single knockdown DCs was again modest.

In order to get more insight into the levels and kinetics of various cytokines produced by the different T cell subsets upon stimulation with PD-L knockdown DCs, co-culture supernatant was harvested over time and analyzed. Early after stimulation with DCs, allogeneic T cells started producing IL-2, while production of IFN-γ, TNF-α and IL-5 initiated...
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Immature DCs were electroporated with 0.25 nmol of each single siRNA or 0.125 nmol of each siRNA in case of double knockdown. After 2 days of maturation, DCs were mixed with allogeneic T cells. (A) At day 5 of co-culture, \(^{[3]H}\)-thymidine was added to the culture and the following day T cell proliferation capacity was determined by measuring \(^{[3]H}\)-thymidine incorporation. Data are depicted as mean + SD and are representative for 3 different donors. (B) IFN-γ levels were measured in the supernatant at day 5 of co-culture using ELISA. Data are depicted as mean + SD and are representative for 2 different donors. CPM, counts per minute. (C) Proliferation capacity was determined by measuring \(\text{IL-2 (pg/ml)}\). Days of culture

KLH-specific T cell responses are strongly augmented by PD-L knockdown DCs

To examine the stimulatory capacity of PD-L-silenced DCs in secondary responses by antigen-experienced T cells, we cultured PBMCs containing primed KLH-specific T cells together with autologous KLH-pulsed PD-L knockdown DCs. After 1-5 days, KLH-specific proliferation and cytokine production of DC-stimulated T cells was studied. Interestingly, the proliferative capacity of primed KLH-specific T cells could be significantly boosted with PD-L knockdown DCs (Figure 3A). Using PBMCs from MM patient 1, all three knockdown DC types increased KLH-specific T cell proliferation, whereas in patient 2 significant induction of proliferation was only seen upon stimulation with PD-L1/L2 double knockdown DCs. In accordance with the results found in the allogeneic MLR setting, stimulation with PD-L knockdown DCs resulted in increased cytokine production by KLH-specific T cells (Figure 3B). Stimulation with PD-L1 knockdown DCs resulted in 2-5 times higher IFN-γ levels than after co-culture with control siRNA DCs. Despite the modest elevation of IFN-γ production after stimulation with PD-L2-silenced DCs, an evident synergistic augmentation (i.e. 8-14 fold) of IFN-γ levels was observed when KLH-specific T cells were stimulated with PD-L1/L2 double knockdown DCs. Already one day after stimulation with KLH-pulsed DCs, IL-2 production was markedly elevated by all PD-L knockdown DC types (Figure 3C). Again, the most evident enhancement was seen for PD-L1 single and PD-L1/L2 dual knockdown DCs. Moreover, in PD-L single and PD-L1/L2 double knockdown DC stimulated cultures, IL-2 levels remained considerably higher up to 5 days after stimulation of the KLH-specific T cells. These data demonstrate that KLH-pulsed PD-L knockdown DCs augment proliferation and strongly increase cytokine production by primed KLH-specific T cells of DC-vaccinated cancer patients.

MiHA-specific CTL expansion and function is enhanced by PD-L silenced DCs

Recently, we have observed that activated LRH-1-specific CD8+ T cells express PD-1, and that in vitro antibody blockade of PD-1 signaling results in improved stimulation of LRH-1-specific CD8+ T cells by PD-L1-expressing DCs and myeloid leukemia cells. To investigate the effect of PD-L silencing on MiHA-specific CD8+ T cell expansion and functionality initiated by DCs, we stimulated LRH-1-specific CTL clone RP1 with PD-L knockdown DCs loaded with or without LRH-1 peptide for 1-4 days. Following DC

Figure 2 PD-L knockdown DCs show enhanced stimulation of allogeneic T cells

Immature DCs were electroporated with 0.25 nmol of each single siRNA or 0.125 nmol of each siRNA in case of double knockdown. After 2 days of maturation, DCs were mixed with allogeneic T cells. (A) At day 5 of co-culture, \(^{[3]H}\)-thymidine was added to the culture and the following day T cell proliferation capacity was determined by measuring \(^{[3]H}\)-thymidine incorporation. Data are depicted as mean + SD and are representative for 2 different donors. CPM, counts per minute. (B) IFN-γ levels were measured in the supernatant at day 5 of co-culture using ELISA. Data are depicted as mean + SD and are representative for 3 different donors. (C) Cytokines produced by CD4+ and CD8+ T cells of the same donor stimulated with allogeneic DCs at a ratio of 1:10 were analyzed simultaneously day 1-5 of the co-culture. Statistical analysis was performed using One-way ANOVA followed by a Bonferroni post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.
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production by LRH-1-specific CTL following stimulation with all three LRH-1 peptide-loaded PD-L knockdown DCs (Figure 4B). Moreover, production of granzyme B by CTL RP1 was augmented by PD-L1 silenced DCs upon peptide recognition (Figure 4C). These results indicate that PD-L silenced DCs are able to increase the proliferation rate and degranulation capacity of MiHA-specific CTLs.

Figure 3  PD-L siRNA-silenced DCs show an increased capacity to stimulate KLH-specific T cell proliferation and cytokine production in vaccinated cancer patients

Autologous PD-L knockdown DCs pulsed with or without KLH were co-cultured with PBMCs from vaccinated MM patients at a stimulation ratio of 0.1:1. (A) At day 5, [3H]-thymidine was added to the culture and the following day T cell proliferation capacity was determined by measuring [3H]-thymidine incorporation. Representative data of 2 out of 5 patients are shown and bars are depicted as mean + SD. CPM, counts per minute. (B) IFN-γ levels were measured in the supernatant at day 5 of the co-culture. Representative data of 2 out of 5 patients are shown and bars are depicted as mean ± SD. (C) IL-2 levels were measured in co-culture supernatant of day 1 and 5. Representative data of 2 out of 5 patients is shown. Statistical analysis was performed using One-way ANOVA followed by a Bonferroni post-hoc test. *P < .05, **P < .01, ***P < .001.

stimulation, we measured the proliferative capacity as well as production of IFN-γ and granzyme B by LRH-1-specific CTL RP1. After four days of co-culture, LRH-1-dependent proliferation of CTL RP1 was observed (Figure 4A). Single PD-L1 and dual PD-L1/L2 knockdown significantly enhanced DC-mediated CTL proliferation, while the effect of PD-L2 knockdown was only modest. Furthermore, we observed increased IFN-γ
PD-L knockdown DCs effectively boost ex vivo expansion and function of MiHA-specific CD8+ effector and memory T cells

In order to investigate the effect of stimulation with MiHA-presenting PD-L silenced DCs on MiHA-specific CD8+ effector and memory T proliferation, we used PBMCs from three different leukemia patients collected early after SCT and DLI or during relapsed disease. First, we cultured PBMCs of patients UPN543 and UPN640, both obtained 7 months after DLI during the effector response, with MiHA-presenting PD-L silenced DCs for 1 week. At start PBMCs contained 0.12% LRH-1-specific and 0.14% HA-1-specific CD8+ T cells, respectively. In both patients we observed augmented percentages of MiHA-specific CD8+ T cells following stimulation with MiHA-loaded PD-L silenced DCs compared to control DCs (Figure 5A-B). Although the best effects were observed upon stimulation with PD-L1/L2 double knockdown DCs, all PD-L knockdown DC types evidently increased the number of LRH-1-specific CD8+ T cells of patient UPN543 after 1 week of culture ranging from 1.5 to 2.7-fold (Figure 5C). In case of patient UPN640 the effects were even more pronounced. One week culture with PD-L knockdown DCs resulted in considerably augmented expansion of HA-1-specific CD8+ T cells of up to 12-fold for PD-L1/L2 double knockdown DCs (Figure 5D).

Next, we investigated the effect of repetitive stimulations with MiHA-presenting PD-L silenced DCs on MiHA-specific CD8+ memory T cell proliferation and function. For this, we used PBMCs from CML-AP patient UPN389 and AML patient UPN543 containing, respectively, 0.06% and 0.09% LRH-1-specific CD8+ T cells, collected at 64 (UPN389) and 71 (UPN543) months post-DLI during relapsed disease. These PBMCs were stimulated for two or three consecutive weeks with LRH-1 peptide-loaded DCs, and the number of LRH-1-specific CD8+ T cells was weekly determined by flow cytometry. Following stimulation with LRH-1 peptide-loaded control DCs, a specific increase in the percentage of LRH-1-tetramer+ CD8+ T cells of 8% was observed over time in patient UPN389 (Figure 6A). Interestingly, PD-L1 single and PD-L1/L2 double knockdown DCs improved LRH-1-specific CD8+ memory T cell expansion up to 12% and 29%, respectively. Surprisingly, lower percentages of LRH-1-specific CD8+ T cells were detected upon stimulation with LRH-1 peptide-loaded PD-L2 knockdown DCs. Since absolute T cell numbers and the percentage of CD8+ T cells were higher in PD-L silenced DC-stimulated cultures compared to T cell cultures stimulated with control DCs, we also calculated the cumulative number of LRH-1-specific CD8+ T cells over time (Figure 6B). In the first week, the fold expansion upon stimulation with PD-L1 single and PD-L1/L2 double knockdown DCs was respectively 1.6- and 4.7-times higher than that of LRH-1 peptide-loaded control DCs. Ultimately, stimulation with PD-L1 single or PD-L1/L2 double knockdown DCs resulted in a 2.8- and 23.3-times higher number of LRH-1-specific CD8+ T cells by the end of week three.

Also for AML patient UPN543, we observed a specific increase in the percentage of LRH-1-tetramer+ CD8+ T cells to 0.7% upon repetitive stimulations with LRH-1 peptide loaded DCs (Figure 6C). Silencing of PD-1 ligands on these DCs resulted in improved

Figure 5 MiHA-specific CD8+ effector T cell expansion can be enhanced by PD-L silenced DCs

Two days after electroporation with PD-L1 and/or PD-L2 siRNA, mature HLA-B7+ LRH-1 or HLA-A2+ HA-1 DCs were loaded with or without 5 µM MiHA peptide. PD-L1 knockdown resulted in <17% PD-L1- and/or <5% PD-L2- DCs. Patient PBMCs, containing MiHA-specific CD8+ effector T cells, stimulated with PD-L knockdown DCs at a ratio of 1:10 were screened after 1 week for tetramer+ CD8+ T cells using flow cytometry. The numbers in the FACS plots represent the percentage of (A) LRH-1-specific (UPN543) or (B) HA-1-specific (UPN640) CD8+ T cells in the total CD3+CD8+ T cell population. Total numbers of (C) LRH-1-specific or (D) HA-1-specific CD8+ T cells obtained after 1 week of stimulation with PD-L knockdown DCs loaded with or without MiHA peptide.

percentages of LRH-1-specific CD8+ T cells of 2.5% and 10% for PD-L single and PD-L1/L2 double knockdown DCs compared to control DCs (0.7%). In absolute numbers the advantage of PD-L knockdown DCs over control DCs in augmenting LRH-1-specific CD8+ T cell expansion is even more evident (Figure 6D). Eventually, this resulted in 13.3-, 26.7- and 61-times higher numbers of tetramer+ CD8+ T cells by PD-L1, PD-L2 and PD-L1/L2 double knockdown DCs, respectively.
numbers of LRH-1-specific CD8+ T cells of patient UPN389 obtained after 3 consecutive stimulations with PD-L knockdown DCs loaded with or without LRH-1 peptide. (C) Percentage of LRH-1-specific CD8+ T cells in PBMCs of patient UPN543. PBMCs were consecutively stimulated with PD-L knockdown DCs at a ratio of 1:0.1 and weekly screened for tetramer-positive CD8+ T cells using flow cytometry. (D) Cumulative numbers of LRH-1-specific CD8+ T cells of patient UPN543 obtained after 2 consecutive stimulations with PD-L knockdown DCs loaded with or without LRH-1 peptide. (E) Degranulation of LRH-1-specific CD8+ T cells of patient UPN389, stimulated for 2 weeks with LRH-1 peptide loaded PD-L knockdown DCs, was measured by staining for CD107a during overnight stimulation with different target cells. PBMCs were cultured 1:1 with LRH-1+ donor EBV-LCLs, LRH-1+ peptide loaded donor EBV-LCLs, LRH-1+ recipient EBV-LCLs or in medium. ND, not determined.

Finally, we studied the functional capacity of the expanded LRH-1-specific CD8+ T cells of patient UPN389 after two weeks of stimulation with LRH-1 peptide-loaded PD-L knockdown or control DCs. These DC-stimulated CD8+ T cell cultures were incubated overnight with different target cells in the presence of anti-CD107a to determine the percentage of degranulating CD107a+ LRH-1-specific CD8+ T cells. We observed that stimulation with receptor EBV-LCLs and LRH-1 peptide-loaded donor EBV-LCLs resulted in >75% CD107a+ LRH-1-specific CD8+ T cells (Figure 6E), whereas stimulation with donor EBV-LCLs or medium resulted in <25% non-specific degranulation of the LRH-1-specific CD8+ T cells. A slight difference in degranulation capacity was seen between the LRH-1-specific CD8+ T cells stimulated with PD-L knockdown DCs in comparison to control DCs. Together, these results demonstrate that MiHA-specific CD8+ effector and memory T cell expansion can be efficiently boosted using PD-L knockdown DCs and that these expanded MiHA-specific CD8+ T cells have the capacity to degranulate upon recognition of MiHA+ target cells.

Discussion

Alloreactive CD8+ T cells targeting MiHAs on malignant cells of the recipient play a key role in GVT immunity after allo-SCT and DLI. However, MiHA-specific CD8+ T cell responses induced post-transplantation are in a substantial number of patients not sufficient to sustain complete remission of the malignant disease. It is evident that distinct mechanisms are involved in dampening anti-tumor T cell responses, which may allow malignant cells to escape immune destruction. Among these mechanisms, T cell inhibition or even exhaustion due to signaling of the PD-1/PD-L pathway may contribute to abrogation of immune responses by limiting the expansion and functionality of CD8+ T cells targeting MiHAs on malignant cells of the recipient.

Figure 6

PD-L knockdown DCs boost the proliferative capacity and function of MiHA-specific CD8+ memory T cells

Two days after electroporation with PD-L1 and/or PD-L2 siRNA, mature HLA-B7+ LRH-1+ DCs were loaded with or without 5 µM LRH-1 peptide. PD-L knockdown resulted in <5% PD-L1+ and/or <2% PD-L2+ DCs. (A) PBMCs of patient UPN389, consecutively stimulated with PD-L knockdown DCs at a ratio of 1:0.1, were weekly screened for tetramer-positive CD8+ T cells using flow cytometry. The numbers in the FACS plots represent the percentage of LRH-1-specific CD8+ T cells in the total CD3+CD8+ T cell population. Data are representative for 2 independent experiments. (B) Cumulative

Finally, we studied the functional capacity of the expanded LRH-1-specific CD8+ T cells of patient UPN389 after two weeks of stimulation with LRH-1 peptide-loaded PD-L knockdown or control DCs. These DC-stimulated CD8+ T cell cultures were incubated overnight with different target cells in the presence of anti-CD107a to determine the percentage of degranulating CD107a+ LRH-1-specific CD8+ T cells. We observed that stimulation with receptor EBV-LCLs and LRH-1 peptide-loaded donor EBV-LCLs resulted in >75% CD107a+ LRH-1-specific CD8+ T cells (Figure 6E), whereas stimulation with donor EBV-LCLs or medium resulted in <25% non-specific degranulation of the LRH-1-specific CD8+ T cells. A slight difference in degranulation capacity was seen between the LRH-1-specific CD8+ T cells stimulated with PD-L knockdown DCs in comparison to control DCs. Together, these results demonstrate that MiHA-specific CD8+ effector and memory T cell expansion can be efficiently boosted using PD-L knockdown DCs and that these expanded MiHA-specific CD8+ T cells have the capacity to degranulate upon recognition of MiHA+ target cells.

Discussion

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knockdown of PD-L on DC augments T cell function following stimulation of OVA-specific CD4+ T cells. Here, we investigated whether PD-L1 and PD-L2 knockdown in DCs results in improved T cell stimulation. We silenced PD-L1 and PD-L2 expression on monocyte-derived DCs using Stealth Select siRNA duplexes, which have high specificity, longevity and stability, and limited induction of non-specific cellular stress response pathways. Optimization experiments revealed that electroporation of iDCs compared to mDCs resulted in more efficient siRNA-mediated knockdown of PD-L2 and especially PD-L1 (data not shown). Therefore, we decided to continue with iDC electroporation followed by two days of maturation using the conventional cytokine cocktail containing IL-1β, IL-6, TNF-α and PGE2. Testing of three different siRNA sequences demonstrated that PD-L2 expression levels could be efficiently down-regulated by all three duplexes following one electroporation, although duplex number 2 located on the boundary of exon 2 and 3 was the most efficient PD-L1 siRNA sequence. Our results contrast to findings reported by Breton et al., who described that a two-step electroporation method was necessary to obtain sufficient PD-L1 knockdown on monocyte-derived DCs. The difference in efficacy could be related to the used PD-L1 siRNA sequence or different electroporation conditions. Our selected PD-L1 and PD-L2 siRNAs showed specific effects for the targeted proteins and did not affect DC maturation or expression of co-stimulatory molecules. Furthermore, the siRNA-mediated PD-L1 knockdown lasted for at least 5 days. After identifying the most efficient siRNAs and electroporation protocol, we determined the stimulatory capacity of PD-L knockdown DCs in primary and secondary antigen-specific T cell responses.

PD-L silenced DCs significantly increased cytokine production of CD4+ and CD8+ T cells in allogeneic MLR assays, however no improved effect of PD-L knockdown DCs on the induction of proliferative CD4+ and CD8+ T cell responses was observed. In the early phase of T cell stimulation we observed increased production of IL-2, and in the later phase enhanced production of IFN-γ, TNF-α and IL-5, but not IL-4. These data indicate that PD-L silenced DCs improve CD4+ Th1 and CD8+ T cell responses. Other studies reported increased T cell proliferation and cytokine production during primary allo-MLR responses following PD-L blockade using antibodies, especially upon initiation by weaker DCs such as iDCs and IL-10 pretreated mDCs. However, more profound stimulatory effects by PD-L1 and PD-L2 blockade were observed on memory and recently activated T cells during secondary immune responses. Therefore, we tested our PD-L knockdown DCs also in an antigen-experienced setting using PBMCs of MM patients containing KLH-specific T cells after vaccination with autologous KLH-pulsed DCs. Although effects vary to some extent, we observed in five different patients significant improvement of KLH-specific T cell proliferation after stimulation with KLH-pulsed PD-L silenced DCs. Furthermore, PD-L knockdown significantly enhanced IFN-γ and IL-2 production by the KLH-specific T cells. Interestingly, the effects of PD-L2 silenced DCs were very modest, while simultaneous knockdown of PD-L1 and PD-L2 on DCs resulted in a synergistic improvement of T cell cytokine production. A similar phenomenon was observed by Keir et al. following stimulation of OVA-specific CD4+ T cells with PD-L1/PD-L2 double-blocked DCs in the presence of OVA- peptide. This suggests that PD-L1 and PD-L2 can exert similar effects via overlapping pathways.

In order to investigate whether our PD-L knockdown DCs were capable of improving MiHA-specific CD8+ T cell responses, we used LRH-1-specific CTL RP1 and PBMCs containing MiHA-specific CD8+ effector or memory T cells, collected from three leukemia patients early after DU or years later during relapsed disease. In this MiHA-specific setting, LRH-1 peptide-loaded PD-L knockdown DCs improved the expansion rate of LRH-1-specific CTL RP1. In addition, IFN-γ and granzyme B levels produced by this CTL were significantly higher after stimulation with PD-L knockdown DCs. Most importantly, we demonstrated that LRH-1- and HA-1-specific CD8+ T cells could be more efficiently expanded by MiHA peptide-loaded PD-L knockdown DCs during both effector and memory T cell responses. After the first stimulation with DCs differences were still modest for some patient samples, but upon restimulation PD-L knockdown DCs profoundly improved the proliferative capacity of the MiHA-specific CD8+ T cells. Surprisingly, in patient UPN389 LRH-1-specific CD8+ memory T cell expansion was lower after stimulation with PD-L2 knockdown DCs than with control DCs. We are very intrigued by this observation, especially since PD-L1/L2 double knockdown DCs are evidently the best stimulator cells. It could be that under certain conditions PD-L2 acts as a co-stimulatory molecule by a PD-L1-independent mechanism, as shown by others, and that by siRNA-mediated silencing we inhibited LRH-1-specific T cell activation. Another explanation could be that PD-L2 knockdown interferes with the postulated PD-L2 reverse signaling, resulting in DCs with lower stimulatory capacity. Finally, we showed that the expanded LRH-1-specific CD8+ T cells have the capacity to degranulate upon recognition of MiHA-expressing target cells, demonstrating that these cells have competent cytotoxic functions.

In conclusion, we demonstrated that efficient, specific and long-lasting silencing of PD-L1 and PD-L2 can be achieved by single siRNA electroporation of monocyte-derived DCs. We showed that PD-L knockdown DCs profoundly boost the proliferative capacity of antigen-experienced T cells specific for KLH and MiHAs. Moreover, both in primary allo-MLR and secondary antigen-specific T cell responses, PD-L knockdown DCs strongly enhanced cytokine production. Together, these findings indicate that PD-L siRNA-
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References


Improving dendritic cell vaccine immunogenicity by silencing PD-1 ligands using siRNA-lipid nanoparticles combined with antigen mRNA electroporation

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Abstract

Dendritic cell (DC)-based vaccination boosting antigen-specific immunity is being explored for the treatment of cancer and chronic viral infections. Although DC-based immunotherapy can induce immunological responses, its clinical benefit has been limited, indicating that further improvement of DC vaccine potency is essential. In this study, we explored the generation of a clinical-grade applicable DC vaccine with improved immunogenic potential by combining PD-1 ligand siRNA and target antigen mRNA delivery. We demonstrated that PD-L1 and PD-L2 siRNA delivery using DLin-KC2-DMA-containing lipid nanoparticles (LNPs) mediated efficient and specific knockdown of PD-L expression on human monocyte-derived DCs. The established siRNA-LNP transfection method did not affect DC phenotype or migratory capacity, and resulted in acceptable DC viability. Furthermore, we showed that siRNA-LNP transfection can be successfully combined with both target antigen peptide loading, as well as mRNA electroporation. Finally, we demonstrated that these PD-L silenced DCs loaded with antigen mRNA superiorly boost ex vivo antigen-specific CD8+ T cell responses from transplanted cancer patients. Together, these findings indicate that our PD-L siRNA-LNP modified DCs are attractive cells for clinical-grade production and in vivo application to induce and boost immune responses not only in transplanted cancer patients, but likely also in other settings.

Introduction

For more than 15 years, the immunotherapeutic potential of ex vivo-generated dendritic cells (DCs) has been explored for the treatment of cancer and chronic viral infections4-6. DCs are the most potent professional antigen-presenting cells (APCs) of the immune system, and effectively initiate and reactivate T cell-based immune responses. For that reason, DCs are considered to be the best means to improve antigen-specific T cell responses in vivo. However, so far clinical effects of DC vaccination therapy have been limited7-8. Despite the documented induction of antigen-specific T cell responses in DC-vaccinated patients, the magnitude of the immune response, the functionality of the boosted T cells and the induction of long-lived memory T cells need to be enhanced. Therefore, further improvement of DC vaccine potency is essential to improve the clinical potential of DC-based immunotherapy.

Ex vivo-generated DC vaccines display high expression of peptide-HLA complexes and many accessory molecules involved in cell adhesion, cell migration and co-stimulation4. However, DC maturation with pro-inflammatory cytokines and/or toll-like receptor ligands also strongly up-regulates co-inhibitory molecules, including PD-L1 and PD-L29. The balance in expression levels and interactions between these co-stimulatory and co-inhibitory ligands on APCs with their counter-receptors on T cells, determines the activation state of the T cells. By favorably modulating this balance toward increased co-stimulation, the potency of DC vaccines could be enhanced. In this regard, we recently showed that by silencing the co-inhibitory molecules Programmed death ligand-1 (PD-L1) and PD-L2 using siRNAs, DCs with strongly improved stimulatory potential could be generated9. These PD-L knockdown DCs robustly boosted ex vivo expansion of both KLH-specific CD4+ Th1 responses, as well as minor histocompatibility antigen (MiHA)-specific CD8+ effector-memory T cells from transplanted cancer patients. It is generally accepted that these MiHAs, expressed by the patient’s malignant cells, are the key antigens in anti-tumor immune responses after allogeneic stem cell transplantation (allo-SCT)10-12. In order to further increase DC immunogenicity we exploited MiHA-encoding mRNA electroporation of mature donor-derived DCs, that allows natural and long-lasting presentation of antigenic peptides by both HLA class I and II molecules7. We and others observed that mRNA-loaded DCs efficiently stimulate specific CD8+ T cell responses13-15. Furthermore, to generate a superior DC vaccine with high stimulatory potential and capable of eliciting a robust MiHA-specific immune response, it is desirable that PD-L siRNA delivery and MiHA mRNA loading are combined. However, mRNA electroporation is optimal at the mature DC (mDC) stage13, while PD-L siRNA electroporation must be performed at the immature DC (iDC) stage to efficiently prevent PD-L1 and PD-L2 up-regulation during maturation7. Since double electroporation is undesirable due to substantial loss of DC yield, we explored a non-viral clinical-grade applicable siRNA transfection method based on lipid nanoparticles (LNPs). Using ionizable cationic lipids,
siRNAs can be efficiently encapsulated in LNPs. Due to the weak basicity of the lipid headgroups, these nanoparticles exhibit a nearly neutral charge at physiologic pH. Additionally, distearoylphosphatidylcholine (DSPC), cholesterol and polyethylene glycol (PEG)-lipid are incorporated for LNP stabilization and size control. Importantly, these LNP formulations have been proven to be well tolerated, and efficient vehicles for low-dose in vivo gene silencing in animals. Furthermore, LNPs efficiently delivered siRNAs into murine and non-human primate APCs in both healthy and diseased states.

In this study, we explored the development of a clinical-grade applicable human DC vaccine with improved immunogenic potential by combining PD-L siRNA and target antigen mRNA delivery. We identified the best siRNA sequences and LNP formulation that efficiently silenced PD-L1 and PD-L2 in monocyte-derived DCs with relatively low cell toxicity at the optimal ex vivo dose. Furthermore, we observed that this LNP-mediated siRNA delivery method affected neither DC phenotype, nor their migratory capacity. Importantly, we demonstrated that PD-L siRNA-LNP transfected DCs loaded with either MiHA peptide or mRNA are superior APCs, strongly augmenting the expansion of functional MiHA-specific CD8+ T cells from transplanted cancer patients ex vivo. These findings indicate that the combination of siRNA-LNP and target antigen mRNA transfection could be a major improvement of current DC vaccines to boost effector-memory T cell responses in cancer patients. Importantly, all materials used to deliver siRNAs and mRNAs have already been approved for use in the clinical setting; therefore, the combination should be easily adopted for human use.

**Materials and Methods**

**Patient and donor material**

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats (N=4; Sanquin blood bank, Nijmegen, the Netherlands) or apheresis material of allogeneic donors (N=7). Furthermore, we used PBMCs from patients who developed MiHA-specific T cell responses post-transplantation. Pt, patient; AML, acute myeloid leukemia; CML-AP, chronic myeloid leukemia-accelerated phase; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; SCT, stem cell transplantation; DLI, donor lymphocyte infusion, given at 4-10 months after allo-SCT.

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Characteristics of patients with a hematological malignancy displaying MiHA-specific CDB+ T cell responses post-transplantation. Pt, patient; AML, acute myeloid leukemia; CML-AP, chronic myeloid leukemia-accelerated phase; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; SCT, stem cell transplantation; DLI, donor lymphocyte infusion, given at 4-10 months after allo-SCT.

Carlsbad, CA, USA) according to the manufacturer’s protocols at 0.1 nM and 10 nM concentrations. mRNA levels for the appropriate transcript were quantified 24h after transfection by Q-PCR and normalized to GAPDH mRNA. Duplexes showing best knockdown at both concentrations were selected for 12-point dose-response ranging from 10 nM down to 0.01 pM to determine IC50 value for these siRNA duplexes. The best duplex for PD-L1 with the sequence 5’-AGAccuuGUAcuuuAAAdTsdT-3’ (sense), 5’-UUUUGaAGuAuuAaAGGUCuIdTsTdT-3’ (anti-sense) and the best duplex for PD-L2 with the sequence 5’-AuAAcGcGucuuGcAAAdTsdT-3’ (sense) and 5’-UUUUGaAaACaAcGCGCuGluIdTdTsTd-3’ (anti-sense) were selected for scaling up, LNP formulation and subsequent ex vivo work. As a negative control a siRNA duplex for Luciferase was used, with the sequence 5’-cucuAGucuAGuAucuuGAdTsTd-3’ (sense) and 5’-UCgaAgAGuAUCuACUGaAaGAdTsTd-3’ (anti-sense). Small case represents 2’-O-methyl modified residues.

**Preparation of LNPs and siRNA encapsulation**

The selected PD-L1 and PD-L2 siRNA duplexes were incorporated in the nanoparticles as published previously. In brief, LNPs were prepared with the ionizable cationic lipid DLin-KC2-DMA (KC2) or DLin-MC3-DMA (MC3), DSPC, cholesterol, and PEG2000-C-DMG using a spontaneous vesicle formation procedure as previously described. The LNPs had a component molar ratio of ~50/39.7/10.3 or ~50/38.5/1.5 (cationic lipid/DSPC/cholesterol/PEG2000-C-DMG). The final lipid/siRNA weight ratio was ~10:1. The particle size of LNPs was determined by dynamic light scattering (Zetasizer Nano, Malvern, UK) and the mean diameter was in the range of 60-80 nm for 1.5% PEG-LNPs and ~120 nm for 0.3% PEG-LNPs. siRNA content was determined by HPLC (anion-exchange column, Dionex PA-200) at 260 nm, and siRNA entrapment efficiency was determined by the Quant-iT RiboGreen RNA assay (Invitrogen).
Monocytes were isolated from PBMCs via direct magnetic labeling with CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) or via plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). IDCs were generated by culturing monocytes in X-VIVO-15 medium (Lonza, Verviers, Belgium) supplemented with 2% human serum (HS; FAA laboratories, Pasching, Austria), 500 U/ml IL-4 (ImmunoTools, Friesoythe, Germany) and 800 U/ml GM-CSF (ImmunoTools). After 3 days, IDCs were transferred to 6-wells plates and pre-incubated with 125-500 nM PD-L1, PD-L2 or Luciferase control siRNA-LNPs (Alnylam) in the absence of HS. After 4 hours, X-VIVO-15/14% HS medium containing 1000 U/ml IL-4 and 1600 U/ml GM-CSF was added and cells were further cultured at 0.5 × 10⁶/ml. At day 7, cells were again incubated for 4 hours with 125-500 nM PD-L1, PD-L2 or Luciferase control siRNA-LNPs, after which maturation was induced in X-VIVO-15/2% HS containing 500 U/ml IL-4, 800 U/ml GM-CSF, 5 ng/ml IL-1β, 15 ng/ml IL-6, 20 ng/ml TNF-α (all ImmunoTools) and 1 μg/ml PGE2 (Pharmacia & Upjohn, Bridgewater, NJ, USA). At day 9, mDCs were harvested, phenotyped and used in functional assays.

In vitro mRNA transcription and DC loading
IVT mRNA was generated using the mMessage mMachine T7 Ultra Kit according to the manufacturer’s instructions (Ambion-Applied Biosystems, Foster City, CA). Briefly, cDNA encoding the MiHAs P2XS or truncated HMHA1, or the tumor-associated antigen (TAA) MAGE3, was subcloned into the pGEM4Z/A64 vector. The resulting vectors were linearized using restriction enzyme NotI. IVT mRNA was transcribed from the T7 promoter site in the linearized template DNA by incubation at 37°C for 2 hours in a reaction mixture containing T7 RNA polymerase. After purification, the mRNA was quantified by spectrophotometry and checked by agarose gel electrophoresis. mRNA storage was in aliquots at -80°C. Before mRNA electroporation, DCs were washed twice with phenol red free Optimem buffer (Gibco Invitrogen). Cells were resuspended in 200 μL Optimem buffer and transferred to a 4 mm gene pulser cuvette (Biorad, Hercules, CA, USA) containing 20 μg mRNA. DCs were electroporated at 200-300 Volt, 150 μF using a Biorad Genepulser II (Biorad). Directly after electroporation, cells were resuspended in 4 ml pre-warmed phenol red free X-VIVO 15/6% HS and incubated at 37°C for 1 hour before further use. For KC2 washout experiments, DCs were resuspended in X-VIVO/2% HS and washed twice in phenol red free Optimem buffer according to our standard operating procedure (SOP) used in clinical DC vaccination trials. Subsequently, DCs were resuspended in 200 μl Optimem buffer and added to 4 ml pre-warmed phenol red free X-VIVO 15/6% HS. Finally, DCs were washed twice in PBS/5% human serum albumin.

LC/MS/MS assay for KC2 quantification
Levels of KC2 in DCs, culture media and washouts were quantified by using LC/MS/MS tandem mass spectrometry. Cell pellets were resuspended in 500 μL of PBS, and lysed by Tissuelyser LT (QIAGEN, Valencia, CA, USA). Subsequently, KC2 was extracted from 50 μL of lyed cell suspension, culture and wash supernatants with acetonitrile/isopropanol (v/v 50:50) containing 50 ng/ml of IS (Internal Standard). After centrifugation at 4000 rpm for 10 minutes, the supernatant was transferred to a clean 96-well plate for LC/MS/MS analysis using a Phenomenex Luna silica column (2.0 × 50 mm, 5 μm) with an isocratic mobile phase (90% acetonitrile/isopropanol (v/v 50:50)/10% 10 mM ammonium acetate) at 40°C and a flow rate of 0.7 ml/min for 6 min at 10 μl sample volume injection. All MS/MS experiments were performed with an API 5000 (AB Sciex, Foster City, CA, USA) with electrospray ionization source. KC2 was monitored using the MS/MS transition of 642.6 to 116.1 in positive mode. The calibration curve ranged from 0.5 to 1000 ng/ml with a lower limit of quantification of 0.5 ng/ml.

Flow cytometry
Phenotype and maturation state of DCs were analyzed by staining with the following antibodies: anti-CD14 (clone TUK4), anti-CD83 (clone HB15a), anti-CD80 (clone MAB104), anti-CD86 (clone HAS-287, all from Beckman Coulter, Fullerton, CA, USA), anti-PD-L1 (clone MIH1), anti-PD-L2 (clone MIH18, both from Becton Dickinson, Franklin Lakes, NJ, USA), anti-CCR7 (clone 150503, R&D Systems, Abingdon, United Kingdom), anti-HLA-ABC (clone W6/32, Dako), anti-HLA-DR (clone Immu357, Beckman Coulter) and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2b PE (Beckman Coulter). The mean PD-L fluorescence intensity (MFI) was corrected for the MFI of the isotype control antibody (ΔMFI), subsequently the relative PD-L knockdown efficiency was calculated as follows: (ΔMFI) PD-L LNP treated DCs / (ΔMFI Control LNP treated DCs) × 100.

LRH-1- and HA-1-specific T cells were detected by staining cell suspensions with PE-labeled tetramers containing the corresponding MHX peptide (LRH-1/HLA-B7-TPNQRQWNVC-HA-1/HLA-A2: VLHDDLLLEA). Tetramers were kindly provided by prof. dr. Frederik Falkenburg (Department of Hematology, Leiden University Medical Center, Leiden, the Netherlands). T cell cultures were incubated with 0.15-0.2 μg tetramer for 15 min at room temperature. Subsequently, cells were labeled with the appropriate concentrations of anti-CD8 (clone LTB8, Prommune, Oxford, United Kingdom) and anti-CD3 (clone UCHT1, Beckman Coulter) for 30 min at 4°C. After washing with PBS/0.5% bovine serum albumin (BSA, Sigma, St Louis, MO, USA), cells were resuspended in washing buffer containing 0.1% 7-amino-actinomycin D (7-AAO, Sigma). Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

To determine the expression levels of MAGE-3 upon electroporation, DCs were stained with specific antibodies using an indirect labeling approach. DCs were washed twice with PBS, and fixed during 10 minutes at room temperature in 4% cold paraformaldehyde solution. After washing with PBS, cells were resuspended in 0.1% saponin buffer and incubated for 30 minutes at 4°C with mouse-anti-hMAGE3 (clone 57B, kindly provided by prof. dr. Giulio Spagnoli, University Hospital Basel, Basel, Switzerland). Then, DCs were washed with 0.1% saponin buffer and, subsequently, incubated with 1100 diluted goat-
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Migration assay
Chemotaxis of DCs in response to CCL21 (ligand for CCR7 chemokine receptor) was measured in triplicate, in 24-wells plates containing transwell inserts with 5 µm pores (Corning Costar). IMDM/10% HS containing 0, 10 or 100 ng/ml CCL21 (Immunotools) was added to the lower compartment in a total volume of 600 µL, and 1 x 10^6 DCs in 100 µL were loaded into the inserts. After 2 hours of incubation at 37°C, DCs were harvested from the lower compartment and migration was quantified using the Coulter FC500 flow cytometer by acquiring events for 90 seconds.

MiHA-specific T cell expansion and degranulation assays
MiHA-specific CD8+ memory T cells present in PBMCs from transplanted patients 1-5 (Table 1) were stimulated for one to two consecutive weeks ex vivo with PD-L silenced DCs loaded with MiHA peptide or mRNA. Mature allogeneic PD-L knockdown or control DCs, cultured from apheresis material of a HLA-B7* or HLA-A2* HA-1 individual, were either loaded with 5 µM MiHA peptide for 30 minutes at 37 °C or electroporated with 20 µg MiHA mRNA. PBMCs and DCs were subsequently co-cultured at a ratio of 1:0.1 in 2 ml Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 10% HS in 24-wells plates (Corning-Costar). After 5 days, IMDM/10% HS containing 50 U/ml IL-2 (Chiron, Emeryville, CA, USA) and 5 ng/ml IL-15 (Immunotools) was added. At day 7, cells were harvested, counted and the percentage of MiHA-specific CD8+ T cells was determined using flow cytometry.

T cell cultures of patient 5 stimulated for two consecutive weeks with P2XS mRNA-loaded PD-L knockdown DCs were used in a CD107a degranulation assay to determine the cytolytic capacity upon recognition of the LRH-1 peptide. Cells were overnight restimulated with 5 µM cognate peptide in the presence of CD107a antibody (clone HA43, Becton Dickinson) and 25 U/ml IL-2. The following day, antigen-specific CD8+ T cell degranulation was determined by analyzing the percentage of CD107a+ cells within the LRH-1-tetramer+ CD8+ T cell population using flow cytometry.

Statistics
To determine statistical differences, a two-sample two-tailed t-test assuming independent samples was used. P-values <0.05 were considered significant.

Results
LNPs containing KC2 most potently deliver siRNAs into monocyte-derived DCs, mediating efficient PD-L1 and PD-L2 silencing
To generate a DC vaccine lacking PD-1 ligand expression in combination with endogenous MiHA peptide presentation, we developed a clinical-grade and versatile application for siRNA transfection of ex vivo-generated DCs. In previous work, we showed efficient, specific and long-lasting silencing of PD-L1 and PD-L2 in human monocyte-derived DCs using siRNA electroporation of iDCs. However, this strategy is incompatible with MiHA mRNA electroporation at the mDC stage. Therefore, we investigated PD-L siRNA delivery using different LNP formulations containing cationic lipids KC2 or MC3 in combination with high (1.5%) or low (0.3%) PEG concentrations. These formulations were tested for their knockdown efficiency by adding 125-500 nM of siRNA-LNPs at days 3 and 7 of DC culture. Following 48 hours of maturation, LNP treated versus untreated mature DCs were analyzed for PD-L1 and PD-L2 expression using flow cytometry. A siRNA concentration-dependent decrease in PD-L1 and PD-L2 expression was observed, with a more pronounced knockdown effect for LNPs containing cationic lipid KC2 compared to MC3-LNPs (Figure 1A-B; Supplementary Figure 1). Furthermore, KC2-LNPs containing low concentrations of PEG were slightly more efficient in silencing PD-L1 and PD-L2 than 1.5% PEG-containing KC2-LNPs. However, in contrast to the KC2-1.5% PEG LNPs that retained a particle size of 60-80 nm, the formulation with 0.3% PEG turned out to be unstable as the particle size (~120 nm) increased 1.6 ± 0.07 fold in 4 months (data not shown). The siRNA encapsulation of KC2-LNPs remained >90%, and no changes were observed between the 0.3% and 1.5% PEG particles. We chose to continue subsequent experiments with 250 nM of PD-L1 and 125 nM of PD-L2 KC2-1.5% LNPs, referred to as siPDL-LNPs from now on, based on the superior combination of efficacy and stability. Collectively, these data clearly show that LNP-mediated siRNA delivery is an effective method for silencing of PD-L1 and PD-L2 on human monocyte-derived DCs.

siPDL-LNPs treated DCs show a mature phenotype and good migratory capacity toward CCR7
To study the influence of LNP-mediated siRNA delivery on DC characteristics, we analyzed DC viability, phenotype and migratory capacity following transfection. At days 3 and 7, 250 nM PD-L1 and 125 nM PD-L2 or 375 nM control siRNA-containing KC2-1.5% PEG LNPs was added to the DC culture. Subsequently, maturation was induced and DCs were examined at day 9. Combined delivery of PD-L1 and PD-L2 siRNAs resulted in efficient knockdown of both target molecules, i.e. 91.7 ± 1.2% for PD-L1 and 86.4 ± 2.2% for PD-L2 (Figure 2A). Although siRNA transfection appears non-toxic to ex vivo-generated DCs relative to other methods, treatment with KC2-1.5% PEG LNPs resulted in a somewhat reduced DC viability (79.4 ± 3.7% in case of PD-L1/L2 silencing) compared to untreated
DCs (94.1% ± 0.69%; P < 0.01; Figure 2B). To exclude negative effects on DC maturation and phenotype, surface expression levels of various maturation and stimulatory molecules were determined. As shown in Figure 2C, all DCs displayed high expression of CD83, CD80, CD86 and HLA class I and II molecules. Neither the siRNAs themselves, nor the transfection procedure with the KC2-LNPs, affected expression levels of the molecules analyzed. Next, we also examined surface expression of lymph node homing receptor CCR7 and migration toward increasing concentrations of its ligand CCL21. The dotted line indicates the MFI of the isotype controls. (B) Viability was determined by trypan blue exclusion. Data are expressed as mean + SEM of 9 independent experiments. (C) Expression of maturation and co-stimulatory molecules by DCs was analyzed using flow cytometry and calculated as follows: ΔMFI PD-L knockdown DCs / ΔMFI Control LNP treated DCs) x100.

MiHA-specific T cell expansion is enhanced by siPDL-LNP treated DCs loaded with MiHA peptide

Next, we examined the stimulatory capacity of siPDL-LNP treated DCs on MiHA-specific CD8+ T cell expansion by loading PD-L knockdown DCs with 5 µM HA-1 peptide. Subsequently, DCs were cultured with PBMCs from two allo-SCT patients containing HA-1-specific CD8+ effector-memory T cells (Table 1). At start, PBMCs of patient 1 contained 0.03% HA-1-specific T cells, and one week stimulation with PD-L1/L2 silenced DCs resulted in a substantial increase in both the percentage and absolute number of HA-1-specific T cells.
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CD8+ T cells as compared to stimulation with control siRNA-LNP or untreated DCs (Figure 3A-B). PBMCs of patient 2, containing 0.02% HA-1-specific CD8+ T cells, were stimulated for two consecutive weeks with HA-1 peptide-loaded DCs. In the first week following stimulation with PD-L1/L2 double knockdown DCs, the percentage and absolute number of HA-1-specific CD8+ T cells increased respectively 1.9- and 1.3-fold compared to control siRNA-LNP DC stimulation (Figure 3C-D). Notably, the effects were more pronounced after the second week of stimulation, when siPDL-LNP silenced DCs further boosted HA-1-specific T cell proliferation 6.1-fold compared to 1.1-fold by control siRNA-LNP treated DCs. These data demonstrate that LNP-mediated transfection of PD-L1 and PD-L2 siRNA results in DCs with enhanced T cell stimulatory potential.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative KC2 level</th>
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<tbody>
<tr>
<td>Culture supernatant</td>
<td>100%</td>
</tr>
<tr>
<td>Wash supernatant 1</td>
<td>16.36% ± 1.38%</td>
</tr>
<tr>
<td>Wash supernatant 2</td>
<td>3.00% ± 0.34%</td>
</tr>
<tr>
<td>Wash supernatant 3</td>
<td>0.95% ± 0.09%</td>
</tr>
<tr>
<td>Wash supernatant 4</td>
<td>0.40% ± 0.03%</td>
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Removal of excess KC2-lipid from the DC product was examined using HPLC-VLC. KC2 levels in the wash supernatant were calculated relative to the original KC2 level in the culture supernatant (Mean ± SEM). N=2.

Figure 4

siRNA-LNP treated DCs can be efficiently loaded with antigen-specific mRNA using electroporation

At days 3 and 7 of culture, 250 nM PD-L1 + 125 nM PD-L2 siRNA-containing KC2-1.5% PEG-LNPs was added if indicated. Mature DCs were loaded with 5 µM HA-1 peptide and cultured at a ratio of 0.1:1 with patient PBMCs containing low numbers of HA-1-specific CD8+ T cells for 1-2 weeks. (A) After one week, cells were analyzed for tetramer-positive CD8+ T cells using flow cytometry. The numbers in the FACS plots represent the percentage of HA-1-specific CD8+ T cells within the total CD3+CD8+ T cell population. Data of one representative patient (Patient 1) are shown. (B) Total number of HA-1-specific CD8+ T cells of Patient 1 after one week of stimulation with PD-L silenced DCs loaded with or without HA-1 peptide. (C) The percentage and cumulative number of HA-1-specific CD8+ T cells of Patient 2 obtained after stimulation with PD-L silenced DCs loaded with or without HA-1 peptide.
Excess siRNA-LNP is efficiently removed from the DC product upon 2 wash steps

Eventually, we aim to develop a clinical-grade applicable DC vaccine with improved immunogenic potential. Because some toxicities attributable to the lipid formulation were observed in preclinical animal models at doses well above efficacious ones (unpublished observations), it is important to know the amount of lipid taken up by the DCs, as well as extracellular residual lipid levels after washing the LNP treated DC end-product. Therefore, we followed the SOP applied in our DC vaccination trials to harvest mDCs following maturation to quantify the amount of lipid remaining in the cells and washout supernatants. We observed that after the first wash already 83.6% ± 2.0% of the KC2 lipid was removed from the DC product (Table 2). The second wash resulted in further reduction to 3.0% ± 0.3% of the original KC2-lipid level present during DC culture. Importantly, about 99% of the KC2 was removed from the cells with subsequent washing. Furthermore, in the DCs on average only 1.1% ± 0.4% of the added KC2-lipid was detected. Together, this shows that the DC end-product, including trace amounts of extracellular residual lipids, contains less than 2% of the ex vivo added KC2-lipid, indicating that excess siRNA-LNPs are efficiently removed from the DC end product following the established washing procedure.

siRNA-LNP treated DCs can be efficiently loaded with target antigen mRNA using electroporation

Because of the observed effect on the DC viability following siRNA-LNP treatment (Figure 2B), we had to optimize the electroporation settings for target antigen mRNA loading to maximally reduce harm to the DCs. Hence, siRNA-LNP treated DCs were electroporated with antigen mRNA at different voltages and T cell stimulatory potential of the DCs was examined. Electroporation of target antigen mRNA at 300 Volt and 150 μF resulted in reduced cell viability and loss of the advantage of PD-L knockdown DC stimulation on T cell expansion (Figure 4A). However, the voltage to 200 Volt, resulted in improved function of the PD-L silenced DCs as compared to untreated DCs. Importantly, lowering the voltage to 200 Volt did not affect mRNA loading efficacy in general, as untreated DCs demonstrated similar stimulatory capacity with all voltages tested (Figure 4A). Notably, further voltage reduction to 100 Volt was not beneficial for mRNA uptake and processing (data not shown). Because MiHA mRNA loading cannot be visualized by flow cytometry due to the lack of appropriate antibodies, we also electroporated siRNA-LNP treated DCs with the TAA MAGE3 and performed FACS analysis. One hour after electroporation, both siRNA-LNP treated and untreated DCs showed high MAGE3 protein expression (Figure 4B). Together, these results demonstrate that LNP-mediated siRNA delivery can be efficiently combined with target antigen mRNA electroporation, while preserving appropriate DC numbers and good functionality.

Figure 5

The expansion of highly functional MiHA-specific CD8+ T cells is boosted by MiHA mRNA loaded PD-L silenced DCs

At days 3 and 7 of culture, 250 nM PD-L1 + 125 nM PD-L2 or 375 nM negative control siRNA-containing KC2-15% PEG-LNPs was added when indicated. Mature DCs were electroporated at 200 Volt with 20 μg MiHA encoding mRNA and cultured at a ratio of 0.1:1 with patient PBMCs containing low numbers of MiHA-specific CD8+ T cells for 1-2 weeks. (A) One week after each stimulation, cells were analyzed for tetramer-positive CD8+ T cells using flow cytometry. Data of one representative patient (Patient 5) having a LRH-1-specific CD8+ T cell response are shown. (B) Total number of LRH-1 specific CD8+ T cells of Patient 5 after 1-2 consecutive weeks of stimulation with PD-L silenced DCs loaded with P2XS miRNA (C) To combine data of all patients, the increase in absolute number of MiHA-specific CD8+ T cells after stimulation with PD-L silenced DCs was calculated relative to control siRNA-LNP DC stimulation. Data of 5 and 4 patients are shown for week 1 and 2, respectively. Lines indicate the mean. (D) Degranulation of LRH-1-tetramer negative and positive CD8+ T cells of Patient 5, stimulated for two weeks with P2XS mRNA loaded PD-L knockdown DCs, was measured by staining for CD107a during overnight restimulation with 5 μM LRH-1 peptide. Statistical analysis was performed using a two-sample two-tailed t-test assuming independent samples. *P <0.05, **P <0.01.

MiHA mRNA loaded PD-L knockdown DCs effectively boost the proliferative capacity of highly functional MiHA-specific CD8+ effector-memory T cells

To investigate the stimulatory potential of siPD-L-LNP treated DCs electroporated with MiHA encoding mRNA, we stimulated PBMCs of 5 allo-SCT patients containing either HA-1- or LRH-1-specific CD8+ effector-memory T cells for one to two consecutive weeks with these DCs. In Figure 5A, one representative patient is shown. At start, this patient had 0.02%
PD-L silencing in DC using siRNA-LNP

Discussion

Although the immune system is capable of eliciting immune responses toward chronic virally infected cells and cancer, the quantity and functionality of these responses is most often insufficient or hampered, contributing to disease progression\(^{34,35}\). To eventually cure these patients, boosting of antigen-specific effector and memory T cell responses in vivo is essential. As DCs are the most potent professional APCs, they are considered to be an ideal cell-based therapy to enhance immune responses\(^{13,15}\). Especially, in the setting of allo-SCT, DC-based vaccination exploiting hematopoietic-restricted MiHAs as tumor target antigens is a promising strategy to selectively boost donor T cell-mediated graft-versus-tumor (GVT) immunity, without evoking severe graft-versus-host-disease (GVHD)\(^{24,28}\). During the last decade, clinical DC vaccination strategies have been widely explored. In various hematological and metastatic solid cancers, vaccination with ex vivo-generated DCs loaded with tumor antigens has been reported to induce anti-tumor T cell responses and tumor regression\(^{14,27}\). However, overall response rates and durable responses are still limited, implicating that further improvement of DC vaccine potency is crucial to increase clinical benefit.

The efficacy of DC vaccines is dependent on a variety of variables, including DC type, maturation status, antigen loading strategy, dosing and frequency of the vaccine injections, and the route of administration\(^{24,25}\). The most commonly used cells for generating high numbers of mature DCs ex vivo, are monocytes\(^{3}\). We and others have shown that upon electroporation of monocyte-derived DCs with target antigen mRNA, antigen-specific CD8\(^+\) T cell responses can be efficiently boosted\(^{35,37}\). The advantage of using mRNA instead of peptide loading is that the antigen epitopes are naturally processed, and a variety of different epitopes are long-term presented by both HLA class I and class II molecules\(^{35,35,37}\). In that way, the breadth of the elicited immune response could be enlarged. Besides the recognition of the HLA/antigen complex by the T cell receptor, T cells require co-stimulatory signaling to become activated and exert their effector function. However, antigen-specific T cells can become functionally impaired over time both in chronic viral infections and in cancer\(^{24,25}\). It has been shown that these antigen-specific T cells up-regulate various co-inhibitory molecules like Programmed death-1 (PD-1) due to chronic antigen exposure. Continued downstream signaling of these molecules eventually results in T cell exhaustion. We previously demonstrated that the PD-1/PD-L pathway is involved in the functional inhibition of MiHA-specific T cell responses post-transplantation, and that blockade of the PD-1 receptor, using a humanized blocking antibody, in combination with DC stimulation boosted the ex vivo expansion of these MiHA-specific T cells, especially in relapsed allo-SCT patients\(^{35}\). Importantly, in two recently published reports on anti-PD-1 (BMS-936,558) and anti-PD-L1 (BMS-936,559) blocking antibody therapy in solid cancers, durable tumor regression was observed with objective clinical responses in 18-28% and 6-17% of the patients, respectively\(^{36,37}\). However, in vivo treatment of allo-SCT patients with these blocking antibodies might bring high risks of inducing GVHD. Therefore, we developed a more specific approach using siRNA electroporation to silence the co-inhibitory molecules PD-L1 and PD-L2 in DCs. In this way, we obtained a DC vaccine with an improved stimulatory phenotype, that strongly enhanced antigen-specific T cell proliferation and cytokine production (IFN-γ, IL-2, TNF-α and IL-5) ex vivo\(^{3}\). To eventually generate a superior DC vaccine with high stimulatory potential, and capable of eliciting a robust MiHA-specific immune response, it is desirable to combine PD-L siRNA delivery and MiHA mRNA loading.

Lipid nanoparticles are attractive agents to deliver specific target siRNAs into ex vivo-generated human DC vaccines in a non-viral manner. Importantly, these LNPs can be generated in compliance with good manufacturing practice (GMP) guidelines, and all materials required have been approved for use in the clinical setting. Recently, Basha et al\(^{35}\) demonstrated that LNPs are competent in vivo siRNA delivery reagents for silencing target genes in murine DCs. An advantage of using LNPs, compared to for instance...
poly(lactic-co-glycolic acid) particles, is that due to the ionizable cationic lipids and nearly neutral charge, these particles can be efficiently loaded with siRNA. Furthermore, these cationic lipids competently mediate siRNA delivery across the cellular membrane. Moreover, these LNPs have been proven safe and efficient vehicles for low dose in vivo gene silencing in animals. Here, we first tested 4 formulations composed of different ionizable cationic lipids (KC2 vs. MC3), containing either high or low PEG concentrations, for their transfection efficiency of human monocyte-derived DCs. Treatment with the KC2 siPDL-LNPs resulted in specific and efficient silencing of PD-L1 and PD-L2. Furthermore, no effect on DC maturation phenotype was observed, although DC viability and in some donors also migratory capacity were somewhat reduced upon treatment with KC2-1.5% PEG LNPs. As the effect on DC viability already indicates, the cell integrity may be affected to a minor extent by LNP-mediated siRNA delivery. Especially, for target antigen mRNA loading of siPDL-LNP treated DCs, milder electroporation settings had to be applied due to reduced DC viability and stimulatory potential at the standard voltage of 300, in contrast to untreated DCs. Notably, lowering the electroporation voltage did not impair mRNA processing and peptide presentation, while the highly stimulatory function of the PD-L knockdown DCs was retained.

In accordance with our previous findings, exogenous loading of siPDL-LNP silenced DCs with MiHA peptide strongly augmented the expansion of MiHA-specific CD8+ effector-memory T cell responses in allo-SCT patients. Moreover, we showed that siPDL-LNP treated DCs can also be effectively electroporated with MiHA-encoding mRNA. These improved DC products potently boosted the proliferation of antigen-specific CD8+ T cells.

After one week of stimulation with PD-L silenced DCs increased numbers of MiHA-specific CD8+ T cells were observed, as also been reported by Breton et al. Interestingly, these PD-L silenced DCs are also superior in boosting MiHA-specific T cell expansion from the naive repertoire of MiHA donors (unpublished observations). In addition, we observed that the growth advantage was even further augmented upon repetitive stimulations with PD-L knockdown DCs. The amplification of the response upon restimulation with PD-L silenced DCs could be attributed to various mechanisms. During T cell activation PD-1 expression levels are up-regulated, which would normally mediate dampening of the T cell response. However, since our DCs no longer express the PD-1 ligands, the expanded MiHA-specific T cells remain in a highly activated state. Furthermore, Karwacz et al. reported that interference with PD-1/PD-L1 signaling inhibits TCR down-regulation following T cell activation, also rendering T cells in a hyperactivated state. It is likely that because of these mechanisms, the expanded T cells responded better to the restimulation with the highly immunogenic PD-L knockdown DCs. Importantly, we demonstrated that these expanded MiHA-specific CD8+ T cells efficiently degranulate upon antigen restimulation. Soon these findings will be implemented in a clinical phase I/II proof-of-concept trial, in which patients treated with HLA-matched partial T cell-depleted allo-SCT will receive DLI in combination with vaccination of PD-L1/L2 silenced donor DCs loaded with hematopoietic-restricted MiHAs. Due to their restricted expression on the recipient’s normal and malignant hematopoietic cells, T cell responses against these MiHA vaccine targets will likely induce GV/T immunity without causing severe GvHD. Importantly, our data indicate that the overall dose of the KC2 lipid in the DC end-product is less than 2%, as only a limited part of the LNPs are taken up by the DCs during ex vivo culturing, and most residual lipids are efficiently removed by washing the cells. This corresponds to a calculated maximum dose of 0.125 µg KC2 lipid per kilogram body weight in our patients, which is far lower than the No Observed Adverse Effect Level (NOAEL) of 1.0 mg/kg in preclinical animal studies (unpublished observations).

In conclusion, we developed a new clinical-grade applicable DC vaccine with improved immunogenic potential by combining PD-1 ligand siRNA and target antigen mRNA delivery. We demonstrated efficient and specific silencing of PD-L1 and PD-L2 on DCs using siRNA-LNPs, without affecting the phenotype or migratory capacity of the DCs. Furthermore, we showed that LNP-mediated siRNA delivery can be successfully combined with target antigen mRNA electroporation. Finally, we demonstrated that these PD-L silenced DCs loaded with MiHA mRNA have superior stimulatory potential and effectively boost ex vivo antigen-specific CD8+ T cell responses in transplanted cancer patients. Together, these findings indicate that our siPDL-LNP modified DCs are attractive cells for clinical-grade production and in vivo application to induce and boost immune responses in the allo-SCT setting, and likely also in other cancers or viral infections.

Acknowledgements
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References


Supplementary Figure 1
PD-L1 and PD-L2 are efficiently silenced upon LNP-mediated siRNA delivery
At days 3 and 7 of culture, siRNA-LNPs were added. Subsequently, DCs were cultured in maturation medium containing IL-1β, IL-6, TNF-α, and PGE2, and after 2 days PD-L expression levels were analyzed by flow cytometry. Surface expression of PD-L1 and PD-L2 is depicted for one representative donor out of 3 experiments following addition of 500 nM PD-L1 or PD-L2-siRNA-LNP (solid line), 500 nM control (dotted line) as compared to isotype control staining (filled grey). Four different siRNA-containing LNP formulations composed of either cationic lipid KC2 or MC3 in combination with 1.5% or 0.3% PEG were tested. The numbers in the plots represent the mean fluorescence intensity of PD-L1 and PD-L2.
Summary, general discussion and future perspective
Summary and general discussion

Allogeneic hematopoietic stem cell transplantation (SCT) is a powerful treatment option for patients suffering from a hematological malignancy, like leukemia, lymphoma and myeloma\(^1\). The therapeutic efficacy can be attributed to the graft-versus-tumor (GVT) effect, constituted by alloreactive cytotoxic T lymphocytes (CTL) which recognize minor histocompatibility antigens (MiHAs) expressed by the malignant cells\(^2\). These MiHAs are polymorphic human leukocyte antigen (HLA)-presented peptides which can elicit powerful alloreactive T cell responses. It has been demonstrated that emergence of MiHA-specific T cells precedes clinical remission in patients treated with donor lymphocyte infusions (DLI)\(^3\). While some MiHAs are exclusively expressed by malignant hematopoietic cells, others are also expressed by healthy tissues. These latter MiHAs can mediate graft-versus-host-disease (GVHD), which is a common cause of post-transplant morbidity and mortality due to alloreactive T cell responses recognizing healthy tissues, particularly the skin, liver and gastrointestinal tract. Therefore, it is desirable to direct GVT immunity toward MiHAs selectively expressed by the residual hematopoietic cells of the patient, including the tumor cells, to minimize the risk of GVHD induction.

The introduction of allogeneic SCT and DLI has greatly improved clinical response rates and overall survival in patients with aggressive hematological cancers. However, many patients relapse eventually. Despite immune surveillance by long-lived MiHA-specific memory T cells, these alloreactive T cells often fail to respond efficiently to the recurring tumor cells\(^4\). The failure to launch productive anti-tumor immune responses is the sum of various alterations in antigen presentation, antigen recognition, T cell activation and T cell survival, resulting in tumor cells with an immunosuppressive phenotype and surrounding tissue\(^5\). Hence, it is crucial to gain more knowledge on these immune escape mechanisms exploited by the tumor cells in order to develop new and improved adjuvant immunotherapies that can be applied post-transplantation to boost GVT immunity and prevent or counteract tumor recurrences. In this thesis, the importance of genetic MiHA disparities and the resulting donor T cell responses on post-transplant outcome has been studied. Furthermore, we investigated the involvement of co-inhibitory molecules in the functional impairment of MiHA-specific T cell responses, which could contribute to immune evasion of hematological cancers. Finally, we developed new and improved immunotherapeutic approaches that interfere with these negative co-signaling pathways to enhance MiHA-specific T cell expansion and functionality in patients treated with allogeneic SCT.

Being the dominant target antigens in HLA-matched allogeneic SCT, MiHAs play a pivotal role both in GVT responses and in GVHD. Precise understanding of the MiHA-specific T cell responses involved in these processes may not only lead to a better prediction of clinical outcome after allogeneic SCT, but also provides a rationale for specific selection of the most potent MiHAs in post-transplant immunotherapeutic strategies. In Chapter 2, we evaluated whether disparities in MiHAs are associated with improved clinical outcome.
post-transplantation. In this retrospective analysis 327 patients have been included, who received partial T cell-depleted allogeneic SCT because of a hematological malignancy. In a comprehensive statistical analysis we observed that DNA mismatches in autosomal-encoded MiHAs are associated with increased relapse-free survival in patients treated with a sibling graft. Especially, multiple myeloma patients showed a lower incidence of relapse and increased relapse-free survival when transplanted with a MiHA-mismatched sibling stem cell graft. Moreover, mismatches for the ubiquitous Y chromosome-derived MiHAs resulted in a higher incidence of acute GVHD (grade 3-4), while autosomal MiHA mismatches, ubiquitous or restricted to hematopoietic cells, were not associated with severe GVHD. In addition, we assessed the in vivo presence of MiHA-specific T cell responses post-transplant using a dual-color tetramer analysis. We observed considerable differences among the studied MiHAs in their capability to induce in vivo T cell responses (i.e. 0-60%). Especially, T cells specific for the hematopoietic-restricted MiHAs HA-1, HA-2, PANE1, LRH-1 and ACC-1 and the ubiquitous HY-encoded antigens were most frequently observed. It could be that our data underestimate the real frequency of MiHA-specific T cell responses in patients as detection of tetramer-positive T cells is dependent on several variables, including timing of blood sampling after allogeneic SCT (effector versus memory phase), possible masking by other (unknown) MiHAs, and localization of the T cells (peripheral blood versus tumor site). Nevertheless, patients in whom we could detect MiHA-specific T cells, including those T cells recognizing ubiquitous MiHA like HY, showed significantly better relapse-free survival in the first years after allogeneic SCT. Importantly, this was not associated with an enhanced incidence or severity of GVHD, indicating that not only ubiquitous antigen expression, but also inflammation in and homing to GVHD-prone tissues is required for the induction of GVHD. Together, these findings provide further evidence that MiHAs play a dominant role in the beneficial GVT immunity and imply that patients could benefit from transplantation with a MiHA-mismatched stem cell graft. Furthermore, these data provide a rationale to induce and boost GVT immunity towards autosomal MiHAs, with a hematopoietic-restricted expression pattern, to improve outcome after HLA-matched allogeneic SCT.

Despite the powerful aspects of immune responses and presence of anti-tumor memory, most often tumor cells are able to evade immune recognition and destruction. Mechanisms exploited by tumor cells that impair T cell-mediated immunity include disruption of antigen presentation, down-regulation of HLA molecules, secretion of immunosuppressive cytokines, as well as recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells. In the last decade, another powerful immunosuppressive mechanism gained much attention: the repressive action of co-inhibitory molecules, such as CTLA-4, PD-1 and BTLA. The role of these co-inhibitory molecules in the immune escape of hematological malignancies is reviewed in Chapter 3. The balance in positive and negative co-signals ultimately determines the activation state of the tumor-reactive T cells. In Chapter 4, we investigated whether the negative regulatory Programmed death (PD-)1 pathway is involved in the functional impairment of MiHA-specific T cell immunity after allogeneic SCT. With flow cytometrical analysis we demonstrated that immature leukemic progenitor cells show high PD-L1 expression in combination with low PD-L2, CD80 and CD86 expression following IFN-γ and TNF-α exposure. Leukemia cells of relapsed patients also exhibited high PD-L1 levels under inflammatory conditions. In addition, we showed that MiHA-specific effector and memory T cells have elevated levels of PD-1, indicating that negative signaling via PD-1 can occur in these patients. Stimulation of MiHA-specific T cells with MiHA-peptide loaded dendritic cells (DCs) or acute myeloid leukemia (AML) cells in the presence of clinical grade anti-PD-1/PD-L1 blocking antibodies resulted in augmented proliferation and IFN-γ production by these T cells. Importantly, we showed that PD-1 blockade had a significantly superior effect on dysfunctional MiHA-specific T cells from relapsed patients. Collectively, these findings show that the PD-1/PD-L signaling pathway suppresses MiHA-specific CD8+ T cells responses. Therefore, PD-1 blockade may be an attractive approach to boost GVT immunity in patients with persistent or relapsed disease.

Besides PD-1, more co-inhibitory receptors may be involved in the immune escape of hematological cancers. Another interesting candidate, recently described to be implicated in the escape of melanoma cells from T cell immunity, is B- and T-lymphocyte attenuator (BTLA)18. Hence, we studied in Chapter 5 whether BTLA also contributes to suppression of MiHA-specific T cell immunity in hematological malignancies. We showed that MiHA-specific CD8+ effector-memory T cells highly express BTLA. The ligand of BTLA, herpes virus entry mediator (HVEM), is part of an intricate signaling network as it has also other binding partners that distinguishively mediate T cell responses, amongst which CD160 and LIGHT (discussed in Chapter 3). Therefore, we analyzed hematopoietic tumor cells for the presence of BTLA, HVEM, CD160 and LIGHT. Importantly, both primary leukemia and myeloma cells displayed high HVEM expression. BTLA expression, however, was higher on myeloma cells than on leukemia cells, while LIGHT and CD160 were present at low levels on both tumor types. The observation of BTLA-positive tumor cells directed us to investigate whether T cells could express HVEM, which was indeed the case. These findings indicate that a bidirectional HVEM-BTLA signaling route between tumor cell and T cells can occur. To investigate the effect of BTLA signaling on MiHA-specific T cell function, we stimulated these T cells with MiHA-peptide loaded DCs in the presence of an antagonistic anti-BTLA antibody. This resulted in a significant augmentation of MiHA-specific CD8+ T cell expansion. Furthermore, we showed that these MiHA-specific T cells are highly competent in proliferation, degranulation and cytokine production upon antigen reencounter. Interestingly, some patients responded better to BTLA blockade, while others showed more pronounced effects following PD-1 blockade. Unfortunately, we could not predict the response to either blocking antibody as we were unable to correlate the effects on T cell proliferation with receptor expression levels on the MiHA-specific T cells. Impairment via these receptors might depend more on the amount
of intracellular signaling it has experienced, than the expression of the receptor as such, as suggested for PD-1\(^1\). In addition, we investigated whether an additive effect could be observed by combined blockade of BTLA and PD-1. However, we did not observe an unambiguous additive effect on MiHA-specific T cell proliferation, suggesting that these receptors share common intracellular signaling pathways. Further research into the distinct roles of BTLA, PD-1 and other co-inhibitory molecules is needed to understand the functional state of the impaired MiHA-specific T cells. In addition, it would be interesting to investigate the effect of BTLA signal transduction into the tumor cells. In conclusion, we show that next to PD-1, BTLA is also involved in MiHA-specific T cell inhibition, though the relative contribution of these two receptors to functional impairment of GVT-specific responses differs between patients.

The findings described in Chapter 4 and 5 demonstrate that both PD-1 and BTLA interactions hamper GVT immunity, providing a rationale for incorporating co-signaling blockade against these receptors in post-transplant immunotherapy. Recently, two key reports were published on anti-PD-1 (BMS-936,588) and anti-PD-L1 (BMS-936,559) blocking antibody therapy in solid cancers\(^2\). Here, durable tumor regression was observed with objective clinical responses in 18-28% and 6-17% of the patients, respectively. Moreover, a relative limited number of the patients (9-14%) showed grade 3-4 drug-related adverse events. Also treatment with the anti-CTLA-4 blocking antibodies ipilimumab (MDX-010) and tremelimumab (CP-675,206) mediated objective clinical responses and prolonged survival in solid cancers, though 10-25% of the patients developed severe treatment-related adverse events. Therefore, in vivo treatment of allogeneic SCT patients with PD-1 blocking antibodies might deteriorate GVHD, because of the highly activated immunological state post-transplant due to chemotherapy-induced tissue damage and inflammation, especially in GVHD-target tissues such as skin and gut. Releasing immune checkpoints at this time could be dangerous, as T cells might home to the inflamed alloantigen-expressing GVHD sites and destroy healthy cells. Therefore, we developed another immunotherapeutic strategy in Chapter 6. Here, we investigated whether we could improve MiHA-specific T cell activation by stimulation with PD-L1 and PD-L2 silenced monocyte-derived DCs. Electroporation of PD-L siRNA sequences into immature DCs, resulted in efficient, specific and long-lasting silencing of PD-L1 and PD-L2. These PD-L silenced DCs strongly augmented proliferation and cytokine production of keyhole limpet hemocyanin (KLH)-experienced CD4\(^+\) T cells. Furthermore, PD-L silenced DCs showed superior potential to boost the expansion of MiHA-specific CD8\(^+\) effector and memory T cells from leukemia patients early after DLI and later during relapsed disease. Together, these data demonstrate that PD-L siRNA electroporated DCs are highly effective in enhancing T cell proliferation and cytokine production, and hence attractive cells for the induction and boosting of GVT immunity in cancer patients. To generate a clinical-grade applicable DC vaccine with further improved immunogenic potential, we studied in Chapter 7 the combination of PD-1 ligand siRNA and target antigen mRNA delivery. The advantage of using mRNA instead of peptide loading, is that the antigen epitopes are naturally processed, and a variety of different epitopes are long-term presented by both HLA class I and class II molecules\(^3\). In that way, the breadth of the elicited immune response could be strongly enlarged. While PD-L siRNA electroporation must be performed at the immature DC stage, to efficiently prevent the up-regulation of PD-L1 and PD-L2 during maturation, mRNA electroporation is most optimal at the mature DC stage. Hence, we explored a non-viral siRNA transfection method based on lipid nanoparticles (LNPs), which can be safely applied in the clinical setting. We demonstrated that PD-L siRNA delivery using DLin-KC2-DMA-containing LNPs mediated most efficient and specific knockdown of PD-L expression on monocyte-derived DCs and this siRNA-LNP transfection could be successfully combined with both target antigen peptide loading, as well as mRNA electroporation. Finally, we demonstrated that these PD-L silenced DCs loaded with antigen mRNA potently boosted antigen-specific CD8\(^+\) memory T cell responses from transplanted cancer patients, especially after consecutive stimulations. The amplification of the response to restimulation with PD-L silenced DCs could be attributed to various mechanisms. During T cell activation PD-L1 expression levels are up-regulated, which would normally mediate dampening of the T cell response. However, since our DCs no longer express the PD-1 ligands the expanded MiHA-specific T cells remain in a highly activated state. Furthermore, Karwacz et al. reported that interference with PD-1/PD-L1 signaling inhibits TCR down-regulation following T cell activation, also rendering the T cells in a hyper-activated state\(^4\). It is likely that because of these mechanisms, the expanded T cells responded better to the restimulation with the highly immunogenic PD-L knockdown DCs. Collectively, these findings indicate that our PD-L silenced DCs loaded with MiHA mRNA are attractive cells for clinical-grade production and adjuvant immunotherapy to competently induce and boost GVT immunity after allogeneic SCT.

Future perspective

The adaptive immune system is a powerful defense mechanism against cancer due to the formation of tumor-reactive immune memory, thereby providing long-term surveillance and, ideally, protection against tumor recurrences. Nevertheless, tumor cells exploit a plethora of escape mechanisms to avoid recognition and attack by the immune system. Hence, it is important to develop more potent immunotherapeutic strategies in conjunction with approaches that interfere with these immunosuppressive pathways (Figure 1). Interference with negative co-signaling pathways is one of the possible strategies to improve anti-tumor immunity. In solid cancers, monotherapy with blocking antibodies targeting CTLA-4, PD-1 or PD-L1 mediates tumor regression and improves overall survival\(^5\). However, not all patients gained clinical benefit and individual responses are hard to
gain more knowledge on the downstream pathways of different co-inhibitory receptors to evaluate their importance in suppression and exhaustion of the immune system by hematological malignancies. Furthermore, more research on the application and timing of blocking antibodies in patients with hematological malignancies is warranted.

In addition to alleviation of negative signaling pathways exploited by the tumor cell, it is also essential to activate MiHA-specific T cell responses in vivo. As DCs are the most potent professional APCs, they are considered to be the ideal means to enhance anti-tumor immunity. However, despite the induction of tumor-reactive T cell responses and tumor regression following DC vaccination, overall clinical response rates and durable responses are limited. Therefore, further improvement of DC vaccine potency is crucial to increase the clinical benefit. In order to enhance the immunogenic potential of monocyte-derived DCs, we silenced the co-inhibitory molecules PD-L1 and PD-L2 and pulsed the DCs with MiHA-encoding mRNA. Based on their superior capacity to boost MiHA-specific T cell responses ex vivo, especially in a second or third round of stimulation, we will start soon with a clinical phase I proof-of-concept trial in MiHA-mismatched patients. Based on the immunogenic MiHA disparity rates, hematopoietic-restricted expression pattern and effectiveness in inducing T cell responses post-transplant, we will select 5 MiHA vaccine targets (i.e. HMHA1, MYO1G, P2XS, ARHGDIB and BCL2A1) allowing vaccination of approximately 30% of the patients treated with HLA-matched allogeneic SCT. As their expression is restricted to the recipient’s normal and malignant hematopoietic cells, T cells recognizing these MiHA vaccine targets will likely only induce GVT responses without causing severe GVHD. These mRNA-pulsed PD-L1 silenced DCs can be administered at late time-points after allogeneic SCT to reactivate MiHA-specific memory T cell responses, but can also be exploited together with pre-emptive DLI to boost naïve MiHA-specific CD8+ T cells and thereby induce MiHA-based immunity shortly after transplantation.

Another application with our MiHA-loaded PD-L1 silenced DCs is the rapid ex vivo expansion of naïve MiHA-specific T cells present in DLIs for adoptive transfer purposes. As shown in Figure 2A-B, ex vivo stimulation of MiHA donor lymphocytes with MiHA peptide-loaded DCs results in efficient induction of the corresponding MiHA-specific T cell responses. Importantly, PD-L1 silenced DCs potently boost the expansion of naïve HA-1-specific CD8+ T cells up to 41%, compared to 1.6% in case of two weeks of stimulation with control DCs (Figure 2C-D). Nevertheless, it is crucial to prevent these cells from becoming quickly exhausted due to terminal differentiation. Recently, Gattinoni et al. described the identification of a stem cell memory T cell population (TSCM) that exhibits stem cell features, like a naïve phenotype and high potency for self-renewal, in combination with memory characteristics, including a high proliferative potential and polyfunctionality. These cells showed superior anti-tumor immunity in humanized mice. Importantly, by stimulation of T cells in the presence of small molecule inhibitors directed against glycogen synthase kinase-3β (TWS119) in the Wnt-pathway or Akt inhibitor VIII, these T cells retained a naive-like T cell phenotype (TSCM) and personal communication).

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### Summary, general discussion and future perspective

**Figure 1** Post-transplant immunotherapeutic strategies

MiHA+ patients receive CD34+ hematopoietic stem cells from a HLA-matched MiHA+ donor. To induce GVT immunity post-transplant, patients can be treated with donor lymphocyte infusions either alone or in combination with PD-L1 silenced DC vaccines. MiHA-specific T cells could also be expanded with these DCs prior to T cell infusion (adoptive transfer). At later time-points after stem cell transplantation, PD-L1 silenced DC vaccines can be given as single treatment or in combination with antibodies that block co-inhibitory signals to boost MiHA-specific memory responses. MiHA, minor histocompatibility antigen.
 Addition of such small molecule inhibitors to \textit{ex vivo} rapid expansion cultures, could strongly improve the efficacy and functionality of tumor-reactive T cells in adoptive transfer strategies. Nevertheless, even though activated tumor-reactive T cells are administered via adoptive transfer or boosted in vivo by vaccination with PD-L silenced DCs, these expanded T cells may still become functionally impaired when they encounter residual tumor cells expressing co-inhibitory molecules. Because the administration of antagonistic antibodies blocking these negative signaling pathways could deteriorate GVHD, other adjuvant immunotherapies have to be developed to specifically interfere with co-inhibitory signaling in the tumor microenvironment. Therefore, we will investigate in a follow-up study a new immunotherapeutic approach consisting of anti-CLEC12A antibody-coated nanoparticles, loaded with siRNAs specific for these co-inhibitory molecules, to selectively target myeloid tumor cells (Figure 3). CLEC12A is an appealing targeting molecule because of its discriminative expression pattern (i.e. exclusive for hematopoietic cells of the myeloid origin, and high expression on myeloid leukemic blasts and stem cells) and internalization capacity upon antibody-crosslinking\textsuperscript{18,23}. Besides silencing co-inhibitory molecules expressed by the leukemia cells, these anti-CLEC12A coated nanocarriers can also target myeloid DC subsets in vivo. Upon internalization of nanoparticles loaded with siRNA, tumor antigen or MiHA peptide, and toll-like receptor (TLR) ligands, activated DCs with superior potential to stimulate antigen-specific T cell responses could be generated. Using this targeted approach, we aim to alleviate the inhibitory phenotype of the tumor cells thereby interfering with the functional suppression of MiHA-specific T cells, and at the same time boost the expansion of these T cells with the highly immunogenic DCs. As CLEC12A expression is restricted to cells of the hematopoietic system, the risk of GVHD induction upon nanoparticle-mediated antigen delivery is expected to be minimal. Furthermore in the allogeneic SCT setting, hematopoietic-restricted MiHAs can be selected to avoid the induction of GVHD-associated T cell responses. We believe that the development and implementation of this new immunotherapeutic strategy will greatly improve anti-tumor immunity, without inducing adverse systemic effects, and improve long-term protection by MiHA-specific memory T cells to prevent the patients from relapsing.

**Conclusion**

To conclude, in this thesis we provide further evidence that MiHA-specific T cell responses play an important role in the beneficial GVT immunity. Furthermore, we demonstrated that signaling via the co-inhibitory receptors PD-1 and BTLA contributes to the functional impairment of MiHA-specific effector and memory CD8\textsuperscript{+} T cell responses. Stimulation with MiHA peptide-loaded DCs in the presence of PD-1 or BTLA blocking antibodies reinvigorates alloreactive T cell proliferation, degranulation and cytokine production. With this research we gained more knowledge on how GVT immunity could be hampered after allogeneic SCT, which provides a rationale for the implementation of (personalized) therapies targeting negative co-signaling pathways after allogeneic SCT. Though, more research into the distinct roles of PD-1, BTLA and other co-inhibitory molecules is required to fully understand the functional state of the impaired MiHA-specific T cells, and to...
predict individual responses to such treatment. Finally, we demonstrated that by silencing PD-1 ligands, especially in combination with MiHA mRNA pulsing, we can generate highly immunogenic DCs with strong stimulatory potential. Vaccination with these superior DCs is a highly attractive treatment option to induce and boost MiHA-specific effector and memory T cell responses post-transplant. By successful translation of the research described in this thesis into proof-of-concept clinical studies we will gain important information whether GVT immunity can be boosted in a specific manner using MiHA-based DC vaccination in combination with blockade of co-inhibitory signaling. By selecting hematopoietic-restricted MiHAs as targets for therapy the risk of GVHD induction will be low, contributing to a better quality of life. In addition, the implementation of these novel therapeutic strategies holds a great promise to reduce the tumor recurrence rates, and combined with the low risk of GVHD this will eventually improve survival after allogeneic SCT.

![Figure 3](image-url)

**Figure 3** Targeting of co-inhibitory molecules on leukemic (stem) cells and DCs

Mature MiHA-presenting DCs highly express co-stimulatory molecules, but also co-inhibitory molecules. Upon stimulation of a naive CD8+ donor T cell, this T cell acquires effector functions and clonally expands. Activated T cells up-regulate expression of co-inhibitory receptors, and upon encounter of MiHA+ leukemic cells presenting the corresponding inhibitory ligands, alloreactive T cell function is impaired. To improve anti-tumor immunity, patients can be treated with donor lymphocyte infusions in combination with blockade of co-inhibitory signaling. By selecting hematopoietic-restricted MiHAs as targets for therapy the risk of GVHD induction will be low, contributing to a better quality of life. In addition, the implementation of these novel therapeutic strategies holds a great promise to reduce the tumor recurrence rates, and combined with the low risk of GVHD this will eventually improve survival after allogeneic SCT.
References


Nederlandse samenvatting
Nederlandse samenvatting

Allogene hematopoïetische stamcel transplantatie (SCT) is een krachtige behandelingsoptie voor patiënten met een hematologische maligniteit, zoals leukemie, lymfoom en myeloom. De therapeutische werking kan worden toegekend aan het graft-versus-tumor (GVT) effect, een reactie waarbij de T-cellen van de donor (alloreactieve T-cellen) minor histo-compatibiltiteit antigenen (MiHAs) op de tumorcellen van de patiënt herkennen als ‘vreemd’ en deze vervolgens aanvallen. MiHAs zijn humaan leukocyten antigeen (HLA)-gebonden polymorfe pepitden, afkomstig van eiwitten die verschillend zijn tussen een patiënt en zijn/haar donor, welke sterke alloreactieve T-cel responsen kunnen opwekken. Dit is in patiënten aangetoond dat na behandeling met een donor lymfocyten infusie (DLI), dergelijke MiHA-specifieke T-cel responsen optreden en gevolgd kunnen worden door een afname van het aantal tumorcellen (klinische remissie). Terwijl sommige MiHAs alleen voorkomen op de tumorcellen van de patiënt, komen anderen ook op gezonde weefsels en organen voor. Dit laatste type MiHAs kan hierdoor graft-versus-host-disease (GVHD), oftewel omgekeerde afstotingsziekte, veroorzaken. Dit is een ernstige en soms ook fatale complicatie bij allogene SCT, omdat het nieuwe donor afweersysteem de gezonde weefsels afstoot, met name in de huid, de lever en het maag-darmkanaal. Daarom is het belangrijk om heel gericht T-cel responsen op te wekken tegen MiHAs die enkel tot expressie komen op de tumorcellen (GVT immuniteit) om zo het risico op het optreden van GVHD te beperken.

De implementatie van allogene SCT en DLI heeft de klinische uitkomst en algehele overleving in patiënten met een agressieve hematologische kanker sterk verbeterd. Echter, bij veel patiënten komen de tumorcellen uiteindelijk terug (tumor recidief). Ondanks dat MiHA-specifieke geheugen T-cellen langdurig in het lichaam circuleren, zijn deze alloreactieve T-cellen vaak niet in staat om efficiënt te reageren op terugkerende tumorcellen. Het gebrek aan het opwekken van dergelijke productieve anti-tumor afweerreacties is de som van diverse veranderingen in antigeen presentatie, antigeenherkenning, T-cel activatie en T-cel overleving, waardoor de tumorcellen een afwieronderdrukkend uiterlijk en omgeving verkrijgen. Daarom is het cruciaal om meer kennis te vergaren over dit soort ontsnaptingsmechanismen die door de tumorcellen gebruikt worden, zodat nieuwe en verbeterde adjuvanthe post-transplantatie immuuntherapieën ontwikkeld kunnen worden om de GVHD te versterken en de terugkomst van tumorcellen te voorkomen. In dit proefschrift is het belang van genetische ongelijkheid in MiHAs tussen patiënt en donor, en het optreden van de bijbehorende alloreactieve T-cel responsen op klinische uitkomsten na allogene SCT bestudeerd. Daarnaast hebben we de betrokkenheid van co-inhibitorische moleculen in de remming van MiHA-specifieke T-cel functionaliteit onderzocht, welke kunnen bijdragen aan de ontduiking van de afweersysteem door hematologische tumorcellen. Ten slotte hebben we nieuwe en betere immuuntherapieën ontwikkeld die ingrijpen op deze negatieve co-signalingroutes om zo MiHA-specifieke T-cel aantallen en functionaliteit te verbeteren in patiënten na allogene SCT.
Aangezien MiHAs de dominante antigenen zijn in HLA-identieke allogene SCT, spelen ze een centrale rol in zowel GVT als GVHD immuunreacties. Beter begrip van de MiHA-specifieke T-cell respons die betrokken zijn bij deze processen zou niet alleen bijdragen aan een betere voorspelling van de klinische uitkomst na allogene SCT, maar ook een rationale leveren voor het specifiek selecteren van de meest potente MiHAs in post-transplantiaties immunotherapeutische strategieën. In Hoofdstuk 2 hebben we geëvalueerd of ongelijkheid in MiHAs tussen patiënten en hun donoren gecorreleerd is met een betere klinische uitkomst na transplantatie. In deze retrospectieve analyse incluerden we 327 patiënten met een hematologische maligniteit, die behandeld zijn met een deels T-cell gedeplete allogene SCT. Uit de analyses bleek dat ongelijkheid in autosomale MiHAs op DNA-niveau gecorreleerd is met een betere recidief-vrije overleving in patiënten die getransplanteerd zijn met stamcellen van een broer of zus. Met name in multiple myeloom patiënten, was de recidief-incidentie lager en was ook de recidief-vrije overleving hoger in het geval van ongelijkheid in autosomale MiHAs. Ongelijkheid voor de breed tot expressie komende Y-chromosomale MiHAs resulteerde in een hogere incidentie van acute GVHD (graad 3-4), terwijl verschillen in autosomale MiHAs niet geassocieerd waren met ernstige GVHD, ongeacht of het expressiepatroon van deze laatste groep breed was of beperkt was tot hematopoietische cellen. Daarnaast hebben we de in vivo aanwezigheid van MiHA-specifieke T-cell respons na transplantatie bepaald met behulp van een twee-kleuren tetrameer analyse. Hierbij waren aanzienlijke verschillen te zien in het vermogen van de verschillende MiHAs om ook daadwerkelijk in vivo T-cell reacties op te wekken (i.e. 0-60%). In het bijzonder T-cellen specifiek voor de hematopoietische MiHAs HA-1, HA-2, PAN1, LRH-1 en ACC-1, en de breed tot expressie komende HY-antigenen werden het vaakst gedetecteerd. Belangrijk is dat patiënten met MiHA-specifieke T-cell respons een significant betere recidief-vrije overleving hadden in de eerste jaren na allogene SCT en dat dit niet leidde tot een toenemende of incidentie of ernst van GVHD. Samen leveren deze bevindingen nader bewijs dat MiHAs een dominante rol spelen in de gehele GVHD immuunreacties en dat patiënten voordeel kunnen hebben van transplantatie met stamcellen van een MiHA-ongelijke donor. Bovendien geven deze data een rationale om GVH immuunresponsen gericht tegen hematopoietische MiHAs op te wekken en te versterken om zo de klinische uitkomst na HLA-identieke allogene SCT te verbeteren.

Ondanks de kracht van afweerreacties en de aanwezigheid van anti-tumor geheugen, zijn tumorcellen toch vaak in staat om te ontkomen aan herkenning en vernietiging door het immuunsysteem. Mechanismen die door tumorcellen gebruikt worden om T-cell gemedieerde immuuniteit te verhinderen zijn versterkte antigeen presentatie, verlaagde expressie van HLA moleculen, uitscheiding van afweeronderdrukkende cytokines, alsook het aantrekken van regulatoriöse T-cell (Tregs) en suppressieve cellen van myeloid afkomst. In het afgelopen decennium heeft ook een ander krachtig afweeronderdrukkend mechanisme veel aandacht gekregen: de remmende werking van co-inhibitoire moleculen (besproken in Hoofdstuk 3). De balans in positieve en negatieve co-signalen bepaalt uiteindelijk of tumor-reactieve T-cell geactiveerd worden. In Hoofdstuk 4 hebben we onderzocht of de negatief-regulerende Programmed death (PD)-route betrokken is bij de remming van MiHA-specifieke T-cell functie na allogene SCT. Met behulp van flowcytometrische analyses hebben we geobserveerd dat immature leukemie progenitor cellen hoge expressie hebben van PD-L1, in combinatie met lage PD-L2, CD80 en CD86 expressie na blootstelling aan IFN-γ en TNF-α. Leukemiecellen van gecideerde patiënten vertoonden ook hoge PD-L1 niveaus onder inflammatoire omstandigheden. Daarnaast hadden MiHA-specifieke effector en geheugen T-cell verhoogde PD-1 expressie, wat aangeeft dat negatieve signalering via PD-1 plaats kan vinden in deze patiënten. Stimulatie van MiHA-specifieke T-cell met MiHA-peptide beladen dendritische cellen (DCs) of acute myeloid leukemia (AML) cellen in de aanwezigheid van anti-PD-1/ PD-L1 blokkerende antilichamen resulteerde in toegenomen proliferatie en IFN-γ productie door deze T-cell. Een belangrijke observatie was dat PD-1 blokkade een significante beter effect had op disfunctionele MiHA-specifieke T-cell van gecideerde patiënten. Samen laten deze bevindingen zien dat de PD-1/PD-L1 signaleringsroute MiHA- specifieke T-cell reacties onderdrukt. Daarom zou blokkade van PD-1 een aantrekkelijke manier kunnen zijn om GVT immuuniteit te stimuleren in patiënten met gecideerde ziekte.

Naast PD-1 kunnen ook andere co-inhibitorie receptoren betrokken zijn bij de ontsnapping van hematologische tumoren aan het immuunsysteem. Een andere interessante kandidaat, die beschreven is betrokken te zijn bij het ontsnappen aan T-cell immuunitet in melanoom, is B- en T-lymphocyte attenuator (BTLA). Daarom hebben we onderzocht of BTLA ook betrokken is bij de remming van MiHA-specifieke afweerreacties in hematologische maligniteiten. We hebben laten zien dat MiHA-specifieke effector-geheugen T-cell hoge BTLA expressie vertonen. De ligand van BTLA, Herpes virus entry mediator (HVEM), is onderdeel van een gecompliceerd signaleringsnetwerk aangezien het ook andere bindingspartners heeft die T-cell respons verschillend beïnvloeden, waaronder CD160 en LIGHT (besproken in Hoofdstuk 3). Vandaar dat we hematologische tumorcellen geanalyseerd hebben op de expressie van BTLA, HVEM, CD160 en LIGHT. interessant is dat zowel HVEM als zijn bindingspartner BTLA hoog tot expressie kwamen op primaire leukemie- en myeloomcellen, terwijl LIGHT en CD160 niveaus laag waren. Omdat de tumorcellen positief waren voor BTLA hebben we ook onderzocht of T-cell HVEM tot expressie konden brengen. Dit bleek inderdaad het geval te zijn. Deze bevindingen deden er op dat een bidirectional HVEM-BTLA signalering tussen tumorcell en T-cell kan plaatsvinden. Om te onderzoeken wat het effect van BTLA signalering op MiHA-specifieke T-cell functie is, hebben we deze T-cellen gestimuleerd met MiHA-peptide beladen DCs in de aanwezigheid van een blokkerend anti-BTLA antilichaam. Dit resulteerde in een significante vermeerdering van de aantal MiHA-specifieke T-cell. Daarnaast zagen we dat deze MiHA-specifieke T-cell uitermate bekwaam reageerden op restimulatie met antigeen door te prolifereren, degranuleren en cytokines
te produceren. Interessant is dat sommige patiënten beter reageerden op BTLA blokkade, terwijl andere sterkere effecten lieten zien na PD-1 blokkade. We hebben echter geen additief effect geobserveerd wanneer beide receptoren tegelijk geblokkeerd werden. Samengevat hebben we laten zien dat naast PD-1 ook BTLA betrokken is bij de inhibitielijn van MiHA-specifieke T-cell reacties, echter de relatieve bijdrage van deze twee receptoren aan de functionele remming van GVT afweerresponsen verschilt tussen patiënten.

De bevindingen beschreven in Hoofdstuk 4 en 5 laten zien dat zowel PD-1 als BTLA interacties GVT immuniteit hinderen, en bieden een rationale voor het toepassen van co-signalingblockade gedurende post-transplantatie immuuntherapie. Ondanks de goede resultaten behaald met blokkerende anti-PD-1, anti-PD-L1 en anti-CTLA-4 antilichamen in solide tumoren, zou behandeling met deze antilichamen in patiënten die een allogene SCT ondergaan hebben te gevaarlijk kunnen zijn. Na transplantatie bevinden de patiënten zich namelijk in een verhoogd geactiveerde immunologische status als gevolg van chemotherapie-geïnduceerde weefselschade en inflammatoire, met name in GVHD-gevoelige organen zoals huid en darmen. Het opheffen van negatieve signalering op dit moment zou gevaarlijk kunnen zijn, aangezien de T-cell en dan naar de ontstoken allo-antigen-positieve GVHD weefsels zouden kunnen migreren en daar gezonde cellen zouden kunnen vernietigen. Daarom hebben we een andere immunotherapeutische strategie ontwikkeld, die beschreven is in Hoofdstuk 6. Hier hebben we onderzocht of we MiHA-specifieke T-cell activatie konden verhogen door stimulatie met PD-L1 en PD-L2 negatieve DCs. Electroporatie van PD-L siRNA sequenties in immature DCs leidde tot efficiënte, specifieke en langdurige verlaging van PD-L1 en PD-L2 niveaus. Deze PD-L1 en PD-L2 negatieve DCs medieerden vervolgens verhoogde proliferatie en cytokine-productie door keyhole limpet hemocyanin (KLH)-specifieke T-cellen. Daarnaast vertoonden PD-L negatieve DCs een superieur vermogen in het stimuleren van MiHA-specifieke T-cell expansie in leukemiepatienten kort na DLL, en later tijdens gereciedeerd cellen. Samen laten deze data zien dat PD-L siRNA geëlectroporere DCs zeer effectief zijn in het versterken van T-cell proliferatie en cytokine-productie, en aantrekkelijke cellen zijn om de werkzaamheid van DC vaccins in kankerpatiënten te verbeteren. Om een klinisch toepasbaar DC vaccin met een verder verbeterd immunogeen potentieel te genereren, hebben we in Hoofdstuk 7 de combinatie van PD-1 ligand siRNA en antigeen mRNA toediening onderzocht. Het voordeel van het gebruik van mRNA in plaats van peptide belading is dat de antigeen epitopen op een natuurlijke manier verwerkt worden. Bovendien wordt een verscheidenheid aan epitopen langdurig gepresenteerd door zowel HLA klasse I als II moleculen. Op deze manier kan de breedte van de afweerreactie sterk vergroot worden. Terwijl PD-L siRNA electroporatie uitgevoerd moet worden op immature DCs, voordat PD-L1 en PD-L2 expressie toeneemt als gevolg van maturatie, is mRNA electroporatie het meest optimaal in het mature DC stadium. Daarom hebben we een niet-virale siRNA transfectiemethode gebaseerd op lipide nanopartikels (LNPs) onderzocht, die veilig toegepast kan worden in de klinische situatie. We hebben laten zien dat PD-L siRNA toediening met behulp van DLin-KC2-DMA-bevattende LNPs resulteerde in de meest efficiënte en specifieke verlaging van PD-L expressie niveaus op DCs en dat deze siRNA-LNP transfectie succesvol gecombineerd kan worden met zowel antigeen peptide belading alsook mRNA electroporatie. Tot slot hebben we aangetoond dat deze PD-L negatieve DCs, beladen met antigeen mRNA, krachtige antigeen-specifieke geheugen T-cell responsen stimuleren, met name na opeenvolgende stimulatierondes. Samengevat laten deze bevindingen zien dat PD-L negatieve DCs, beladen met MiHA mRNA, aantrekkelijke cellen zijn voor klinische productie als adjuvante immuuntherapie om op competente wijze GVT immuniteit te induceren en te versterken na allogene SCT.

**Conclusie**

De studies beschreven in dit proefschrift bieden aanvullend bewijs dat MiHA-specifieke T-cell reacties een belangrijke rol spelen in GVT immuniteit. Verder hebben we aangetoond dat signalering via de co-inhibitoire receptoren PD-1 en BTLA bijdraagt aan de functionele remming van MiHA-specifieke effectoren en geheugen T-cell responsen. Stimulatie met MiHA-peptide beladen DCs in de aanwezigheid van een PD-1 of BTLA blokkend antilichaam herstelt alloselectieve T-cell proliferatie, degranulatie en cytokine-productie. Met dit onderzoek hebben we meer kennis vergaard over hoe GVT immuniteit belemmerd kan worden na allogene SCT, wat een rationale biedt voor de implementatie van (persoonlijke) therapieën na allogene SCT die gericht zijn tegen deze negatieve co-signalingroutes. Echter, meer onderzoek naar de verschillen in de bijdrage van PD-1, BTLA en andere co-inhibitoire moleculen is vereist om de functionele status van geremde MiHA-specifieke T-cellen beter te begrijpen en individuele responsen op dergelijke behandelingen te kunnen voorspellen. Tenslotte hebben we aangetoond dat we door het verlagen van PD-L niveaus, met name in combinatie met MiHA mRNA belading, zeer immunogene DCs met een krachtig stimulerend potentieel kunnen genereren. Vaccinatie met deze superieure DCs is een aantrekkelijke behandelingsoptie om MiHA-specifieke T-cell activatie te verbeteren. Na transplantatie kunnen we onderzoeken of GVT immuniteit specifiek versterkt kan worden door middel van MiHA-DC vaccinatie in combinatie met blokkade van co-inhibitoire signalering. Implementatie van deze nieuwe therapeutische strategieën is veelbelovend voor patiënten na allogene SCT, om het aantal tumor recidieven te verlagen en de overleving te verbeteren.
Dankwoord

Na 4 jaar van experimenten en schrijven ben ik nu bij het einde van mijn proefschrift aangekomen en ga ik promoveren! In de voorgaande hoofdstukken heb je kunnen lezen waar ik me de afgelopen jaren mee bezig heb gehouden en een indruk kunnen krijgen van waarom ik soms ‘s avonds of in het weekend toch nog even moest werken. Hoewel mijn project voortvarend liep, zou ik zonder jullie hulp, advies en steun nergens zijn geweest. Ik ben heel trots op dit mooie eindresultaat en wil bij deze iedereen bedanken!

Een aantal mensen wil ik in de komende alinea’s in het bijzonder noemen.

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liep onze samenwerking gesmeerd en vond jij het niet erg om met mijn muizen-stress om te moeten gaan. Ook vind ik het super fijn dat ik altijd bij jou terecht kan voor advies over experimenten, de juiste woorden/zin als ik iets aan het schrijven ben, hoe ik een situatie het beste aan kan pakken, enzovoort. Ik ben daarom blij dat ook jij mijn paranorm wilt zijn.

Niet alleen Hanny heeft als analiste op mijn project gewerkt, ook een heleboel data is door Nienke gegenereerd tijdens de eerste twee jaar van mijn promotieonderzoek. Nienke, although I know your Dutch is very good I’ll write to you in English this time :). We started almost at the same time with our first job and both really had to find our way in the beginning. After a while we had our ‘own’ projects and you spent hours not only at the flowcytometer, but also isolating many, many, many DNA samples and performing series of KASPar PCRs without any complaints. Funny to mention is that you really surprised everyone regularly with a ‘different’ look (read: diversity of different contact lenses amongst which cat-eyes)! Thanks for all your help and good luck with your own carrier in science!

Wiegertul, Kelly, Sandra en Anniek, mijn mede AIO-maatjes. Samen zaten we in hetzelfde schuurtje, hoewel sommigen van ons nattere voeten kregen dan anderen tijdens de promotievaart. Toch hadden we altijd wel een reden om samen op congres te gaan, gek te doen, lekker achterover te leunen, biertjes te drinken, te eten, etc. Ik hoop dat we dit de komende jaren nog vaak zullen herhalen. Wiegertul, je hebt me wegvie gemaakt in het PD-1 project en ondanks dat we allebei heel verschillend zijn en regelmatig kunnen kibbelen, hebben we samen een paar mooie papers geschreven en blijven we ook in de toekomst samenwerken. Kelly, wij hebben samen een ander mooi, maar soms ook frustrerend, paper geschreven wat nu gelukkig gepubliceerd is in een mooi tijdschrift. Ik heb echt ontzettend veel geleerd van jouw creativiteit en voortvarendheid en ik ben nog steeds onder de indruk van de snelheid waarmee jij ideeën en artikelen op papier wilt krijgen. Hoewel ik het jammer vind dat je niet meer in Nijmegen woont en werkt, ben ik blij dat je nu de baan hebt die je ontzettend graag had. Sandra, we hebben dan misschien geen data gedeeld maar wel onze bureaus. Ook dat is wat waard, dat zonder gemopper de grens nog wel eens verschoof onder stapels data :). Toen we beiden meer zo dat ik het bij mij hoefde te doen, lekker achterover te leunen, biertjes te drinken, te eten, etc. Ik hoop dat we dit weer vaak zullen herhalen. Wiegertul, je hebt me wegvie gemaakt in het PD-1 project en ondanks dat we allebei heel verschillend zijn en regelmatig kunnen kibbelen, hebben we samen een paar mooie papers geschreven en blijven we ook in de toekomst zal het een nieuwe kruising van ideeën en stijlen zijn. Ik hoop dat we dit de komende jaren nog vaak zullen herhalen.

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Curriculum Vitae

List of Publications


*These authors contributed equally.
List of Abbreviations

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