Elucidating the immunological effects of 5-Azacytidine treatment in patients with myelodysplastic syndrome and identifying new conditional ligands and T-cell epitopes of relevance in melanoma

Thomas Mørch Frøsig

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Tutors: Sine Reker Hadrup, Per thor Straten and Åsa Andersson

Official opponents: Henrik J. Ditzel, Anne Letsch and Allan Randrup Thomsen

Correspondence: Center for Cancer Immune Therapy, Department of Hematology, Herlev University Hospital, Herlev Ringvej 75, 2730 Herlev, Denmark

E-mail: thomfr@vet.dtu.dk

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IV. Frøsig TM, Yap J, Seremet T, Lyngaa R, Svane IM, thor Straten P, Heemskerk MH, Grotenbreg GM and Hadrup SR. Design and validation of conditional ligands for HLA-B*08:01, HLA-B*15:01, HLA-B*35:01, and HLA-B*44:05.
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1. INTRODUCTION

CANCER

Cancer is the leading cause of death in developed countries and worldwide only surpassed by heart diseases [1]. Also, costs of cancer are huge with estimated economic loss of $895 billion worldwide in 2008 [2]. Cancer arises from cumulated genetic and epigenetic changes in healthy cells which lead to collapse of the homeostatic mechanisms, and generate escape variants with abnormal growth behavior. Investigation of the cancer-promoting molecular mechanisms has been substantial for more than a century. The genetic basis of cancer was first discovered by Theodor Boveri in 1902 [3], and we now know a lot more about fundamental cancer development and progression. Furthermore, the development of surgery enables curing of most patients with primary cancers; for patients with metastatic disease, however, there are minimally curative treatment options and medical care primarily aims for life prolongation. This poor prognosis is caused by drug and immune pressure-induced Darwinian selection of therapy-resistant tumor escape variants and the marked intratumor heterogeneity [4]. The incidence of many cancers is associated with age and as life-expectancy increases also more people will develop and eventually die from cancer. Accordingly there is a need to enhance efficacy of cancer treatment, particularly for patients with metastatic disease.

Immune therapy has recently provided a great breakthrough in metastatic cancer treatment. In recent years several therapies stimulating the immune system has shown encouraging results. These have significantly prolonged overall survival in terminal cancer patients either by targeting the inhibitory molecules Cytotoxic T-Lymphocyte-associated Antigen 4 (CTLA4), Programmed Death 1/Programmed Death 1 Ligand (PD1/PD-L1) or by adoptive cell transfer (ACT) therapy with tumor-infiltrating lymphocytes (TILs) [5–12]. This rapid development led the leading scientific magazine “Science” to name cancer immunotherapy as “Breakthrough of the year 2013” [13] and the other top-ranging scientific journal “Nature” to make a special “Outlook” about clinical targeting of inhibitory immune molecules in the end of 2013 [14]. One of these treatments has already shown advantageous to standard therapy in a phase III clinical trial and been approved by

the American Food and Drug Association (FDA) as well as European Medicines Agency, and more are likely to be approved in the near future.

Cancer is a complex disease state in which normal cells acquire several "hallmark" capabilities during carcinogenesis. Six hallmarks were initially proposed in 2000 by Hanahan & Weinberg [15] and the concept was updated in 2011 with two emerging hallmarks and two enabling characteristics [16]. The original hallmarks were: sustaining proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis. These are all gained and lead to sustained homeostasis and overcoming of endogenous mechanisms normally inducing death of abnormal cells. In the 2011 update the emerging hallmarks deregulating cellular energetics and avoiding immune destruction were added, acknowledging the importance also of these functional capabilities. In addition genome instability and tumor-promoting inflammation were added as enabling characteristics of multiple hallmarks. The incorporation of immune-mediated tumor destruction and promotion is of special interest for the research reported in this thesis and will be discussed in more details below. Furthermore, the incorporation of genomic instability is in line with data from research within disruption of epigenetic maintenance, which is interpreted to be essential for acquiring the hallmarks of cancer. Although genomic instability is one feature, disruption of epigenetic maintenance has several other consequences [17]. Carcinogenesis can therefore be seen as a multi-step process including acquirement of the proposed hallmarks, disruption of epigenetic maintenance and escape of anticancer immunity, the latter outlined by the immunoediting concept [16–19]. The immunoediting framework and epigenetic mechanisms and their importance for carcinogenesis will be explained in details below (pages 5-7).

It should be noted, though, that the somatic mutation theory (SMT) of carcinogenesis which forms basis for the hallmark theory has been criticized by several research groups, as reviewed by Sonnenschein & Soto [20]. Instead Sonnenschein & Soto propose the tissue organization field theory (TOFT). According to the SMT theory cancer is by nature a genetically based disease with net cell numbers in default (healthy) state being static, while the disease induces excessive proliferation compared to the healthy counterparts. On contrary, the TOFT theory claims cancer is a tissue-based disease with net cell numbers in default state increasing as cells proliferate, while the disease induces abnormal tissue organization compared to the healthy counterparts. One example of a flaw in the SMT theory is, according to Sonnenschein & Soto, the fact that benign tumors share five of the original hallmarks with malignant cancers, only lacking the ability for metastatic growth; in their analysis this suggests that the SMT theory is too simplistic [20, 21]. In addition they question if conclusions from *in vitro* cell cultures and *in vivo* mouse experiments can indeed be applied to human patients, arguing that cancer induction is non-comparable in these situations.

Focus for this review is metastatic melanoma and myeloproliferative diseases.

MALIGNANT MELANOMA

The incidence of malignant melanoma is rapidly increasing with annually more than 1800 cases in Denmark [22]. This cancer also occurs in younger people contrary to most other cancers that primarily affect the elderly. Patients with early stage disease (stage I or II according to the American Joint Committee on Cancer staging system) have a good prognosis and are usually cured surgically, while patients whose melanomas have spread to nearby lymph nodes (stage III) or distant to other organs (stage IV) have a very poor prognosis. Stage IV melanoma patients have a 5-year overall survival of 15-20% [23]. Until recently none of the conventional treatments in Denmark being chemotherapy, low-dose Interleukin (IL)-2 and Interferon-α either alone or in combination, radiation and surgery, had shown prolongation of overall survival in this patient group. Of notice, high-dose IL-2 treatment which was approved in the US in 1998, but not in Denmark, showed 13% overall response rate and a 4% complete response rate in a long-term follow-up study of treated metastatic melanoma patients [24].

Metastatic melanoma treatment completely changed after a phase III clinical trial showed the CTLA4 inhibitor Ipilimumab to be superior to a peptide vaccine [5], and Ipilimumab was subsequently approved by FDA in 2011 (as Yervoy®, Bristoll-Myers Squibb). In parallel, another phase III clinical trial showed a promising 48% response rate after treatment with the BRAF (B Rapidly Accelerated Fibrosarcoma) mutation inhibitor Vemurafenib (Zelboraf®, Roche) in melanoma patients harboring the V600E mutation; this group forms approximately half of the patients. These responses from the Vemurafenib treatment, however, turned out to be short-lasting and disease rapidly progressed once the protective effect was gone [25]. On contrary, ACT of TILs has shown encouraging results and also induces long-lasting responses in a subgroup of patients. The first report of ACT of TILs after a non-myeloablative lymphodepleting conditioning regimen showed regression of metastatic lesions in terminal melanoma patients, and was published in 2002 [26]. A follow-up study reported a 49% response rate with non-myeloablative conditioning alone and up to 72% with extra total body irradiation [27]. Additionally, clinical trials targeting the PD1/PD-L1 immune blockade pathway have shown promising results, with one trial (PD-1 inhibitor) showing response rates up to 52% and median progression-free survival for more than 7 months [6].

Common for all these melanoma treatments, except the BRAF mutation antibody, is the aim to stimulate anti-tumor CD8 T cells, either by expanding the CD8 pool (ACT) or by blocking the immune inhibitory pathway (CTLA4/PD1/PD-L1 inhibitors). The actual T-cell restriction elements recognized on the tumor cells in conjunction with the Major Histocompatibility Complex (MHC) molecules are, however, not known very well. With this knowledge it would be possible to specifically target or select tumor-specific T cells, potentially with enhanced clinical efficacy as a consequence. Numerous melanoma-associated antigens and their derived T-cell epitopes have been described. These are, however, distributed on 37 Human Leukocyte Antigen (HLA, human MHC) class I molecules and as 45% are restricted to HLA-A2, only very few T-cell epitopes have been identified for each of the remaining HLA molecules [28]. It is accordingly necessary to identify T-cell epitopes restricted to other HLA molecules than HLA-A2; we worked to this aim in Study I, reported in Paper I. Also, it has been questioned if T cells recognizing epitopes from the hitherto identified melanoma-associated antigens represent the actual tumor-reactive T cells [29]. We identified a new immunological target, the protein Nodal, reported in Paper II. We did this by investigating blood from melanoma patients, but as increased expression of Nodal has been described in several other cancers [30–33] it might be a universal tumor-associated antigen (TAA).

MYELOID NEOPLASMS

Myeloid neoplasms are clonal hematological disorders characterized by ineffective hematopoiesis and abnormal cell differentiation. In healthy individuals hematopoietic stem cells mature to the different cell populations along the lymphoid or myeloid cell lineages. Immature hematopoietic stem cells, however, may obtain a neoplastic phenotype at various levels of stem-cell development. Dysplasia of stem cells in the myeloid lineage, myelodysplastic syndrome (MDS), results in different clinical manifestations covered by the term "myeloproliferative" [34]. Chronic myelomonocytic leukemia (CMML), in which monocytosis occurs in peripheral blood with resulting excessive numbers of monocytes, was recently removed from the group of MDS and placed in a new group of myelodysplastic-myeloproliferative diseases by the World Health Organization [35]. Both MDS and CMML can be perceived as pre-leukemic diseases which potentially progress to acute myeloid leukemia (AML) with a poor prognosis, but importantly MDS can also be asymptomatic with no effect on mortality. The annual incidence in Denmark is 400 and is associated with age [36]. MDS can be cured by allogeneic bone marrow or stem cell transplantation following a myeloablative conditioning, but this is only possible in younger patients with good performance status, and even in these patients this treatment has a high mortality and relapse rate [37, 38]. It is thus only recommended in patients with higher-risk MDS, defined as intermediate-2 and high-risk MDS by the International Prognostic Scoring System, and AML [39]. Also, non-ablative hematopoietic transplantation has arisen as a possibility, though; this strategy can be applied also to patients with poorer performance status and has shown the ability to induce clinical responses [38].

In 2002 the cytosine analogue 5-Azacytidine for the first time showed prolongation of overall survival in patients with higher-risk MDS as compared to best supportive care in a phase III clinical trial [40]. It was subsequently approved for use in all subtypes of myeloproliferative disorders (as Vidaza®, Celgene Corporation). Due to the crossover design and lack of an active comparator these data needed confirmation in a following trial which compared 5-Azacytidine treatment with other chemotherapies, known as the AZA-001 study [41]. 5-Azacytidine induces a late clinical response in some patients [40, 42, 43], and this has led to speculations that immune-mediated mechanisms could be involved as immune modulatory interventions often have a slower onset of efficacy than direct cytotoxic drugs [44].

5-Azacytidine is a hypomethylating agent incorporated into the DNA upon replication and thus inhibits activity of the enzyme DNA methyltransferase. Furthermore, the degree of aberrant methylation increases from low-risk MDS to AML and was also increased, although not significantly (P=0.076), in CMML patients compared to a control group [45]. It has been shown that tumor suppressor genes are methylated and hence silenced in cancer patients [46]. 5-Azacytidine's clinical effect is partly explained by unsilencing of tumor suppressor genes, but treatment with 5-Azacytidine moreover upregulates the expression of cancer-testis antigens (CTAs) [47, 48]. These are particularly interesting in an immunological contexture due to their selective expression in cancers and immune-privileged areas in the adult. Also, the existence of CTA-specific CD8 T cells has previously been shown in a wide variety of cancers [49–53]. Specifically two studies in MDS patients treated with 5-Azacytidine in combination with the histone deacetylation inhibitor Valproate and the immune modulatory agent Lenalidomide, respectively, showed induction of CTA-specific T cell responses [51, 54].

In Study III we wanted to investigate a possible 5-Azacytidine-mediated induction of CTA-specific T cells in MDS, CMML and AML patients, and examine if these cells could be responsible for an enhanced direct anti-tumor activity. In addition, we measured for possible effects of 5-Azacytidine on the general effector cells: CD4 and CD8 T cells and Natural Killer (NK) cells; and inhibitory immune populations: myeloid-derived suppressor cells (MDSCs) and regulatory CD4 T cells (Tregs). We did this to investigate if combined treatment with 5-Azacytidine and immune therapy would potentially be beneficial. Results from this study are provided in Paper III.

CELLULAR IMMUNITY

The task for our immune system is to defend us against invading pathogens and ensure homeostasis once abnormalities are detected and eradicated. It is efficient and very specific in destroying most foreign organisms attacking, for example viruses, bacteria and parasitic worms. Perhaps the most important subsets for immune-mediated killing of intracellular pathogens or cancer cells are T cells and NK cells. T cells are activated through recognition of foreign peptides, or T-cell epitopes, in complex with MHC molecules presented on the cell surface of antigen-presenting cells.

CD8 T cells are licensed for recognition, clonal expansion and specific killing through co-stimulatory signals provided by dendritic cells or CD4 T helper cells. NK cells, on contrary, possess activating and inhibitory receptors on the cell-surface and kill cells with no or altered MHC expression. Thus NK cell killing is rather unspecific and CD8 T cells and NK cells act in a complementary fashion. These potent effector cells are kept in check in part by inhibitory cell populations, most importantly Tregs. As a subtype of the CD4 population, phentotypically being $CD25^{high}CD127^{-}FoxP3^{+}$ (forkhead box P3), Tregs dampen CD8 T cell expansion and cytotoxicity and prevent autoimmunity e.g. via decreasing the accessible amount of IL-2, the cytokine necessary for proliferation and differentiation of CD8 cells to become true killer cells. Also several other regulatory T cell populations exist, but these are outside the scope of this review.

All three subsets of T cells mentioned are present in the peripheral blood and at the tumor site in cancer patients [55]. Presence of CD4 helper and CD8 T cells are believed to be beneficial to the host by mediating anti-tumor reactivity, while Treg numbers are significantly increased in most terminal cancers and associated with a poor prognosis [56, 57]. Indeed, the ratio between CD8 T cells and Tregs present in the tumor microenvironment showed correlation to overall survival in melanoma [58]. However, in a few cancers an opposite correlation was found, possibly due to the Treg-mediated dampening of inflammation in these cancers [57]. Importantly, inflammation is known to promote carcinogenesis [59], as will be discussed further below.

Another important cell subset accumulating at the tumor site and in the blood of terminal cancer patients are the MDSCs. These inhibit the CD8 T cells by various mechanisms, including recruitment of Tregs and secretion of reactive oxygen species and inducible nitric oxide (reviewed in [60]). Both Tregs and MDSCs have furthermore been shown to be engaged by the tumor cells [57, 61]. The tumor cells utilize many different mechanisms to avoid recognition by T cells; among these are down-regulation of MHC expression and in this situation NK cells may exert their functions [62]. Additionally, other mechanisms exist to inhibit T-cell function, for instance upregulation of different inhibitory molecules on the tumor-cell surface, including PD-1, PD-L1 and CTLA4 [63].

These have all recently been targeted clinically with great success, as noted above.

ANTIGEN PRESENTATION AND T-CELL RECOGNITION

T cells recognize peptide-MHC complexes on the cell-surface of foreign cells. These peptides are processed intracellularly and originate from endogenous antigens, shown in figure 1a, or exogenous antigens, shown in figure 1b. Intracellular proteins are degraded to short peptides by the proteasome, transported by the transporters associated with antigen presentation (TAP1/2) and loaded on mature MHC class I molecules in the endoplasmic reticulum. Further, peptide-MHC complexes are transported to and presented on the cell-surface; this is known as the classical MHC class I pathway and is shown in figure 1a. Exogenous antigens can also be transported to the endoplasmic reticulum by phagosomes, endocytosed to the cytosol and then be processed and presented on the cell-surface; this is known as cross-presentation through the MHC class I pathway and is shown in figure 1b. Cross-presentation requires professional antigen-presenting cells, most often believed to be dendritic cells, and is fundamental in acquiring anti-tumor T-cell immunity. CD8 T cells directly recognize cancer cells through their surface-expression of TAA-derived epitopes, but need licensing from cross-presenting dendritic cells [55]. Cross-presentation is generally believed to be responsible for induction of tumor-specific T-cell immunity, but it has been questioned as an experimental artifact based on its low efficiency under physiological conditions compared to direct presentation [64].

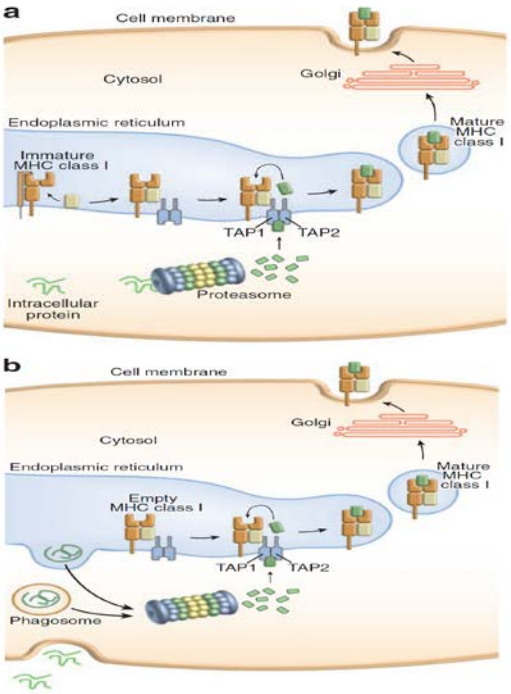

Figure 1, a) The classical MHC class I pathway of endogenous antigen processing and presentation on the cell surface. b) Cross-presentation of exogenous antigen through the MHC class I pathway. The machinery shown in a) occurs in most cells, while the pathway in b) is believed to be exclusive to professional antigen presenting cells. The figure is further explained in the text.

Adapted by permission from Macmillan Publishers Ltd: Journal of Investigative Dermatology (Andersen MH, Schrama D, Thor Straten P, Becker JC (2006) Cytotoxic T cells. J Invest Dermatol 126:32–41. doi: 10.1038/sj.jid.5700001, copyright [55].

Specifically, T-cell recognition is mediated by T-cell receptor (TCR) binding to the peptide-MHC complex. The TCR is made up of polymorphic α- and β-chains and estimated just below 10^8 different αβ TCRs exist in the human repertoire of naïve T cells [65]. This is a low number compared to the estimated more than 10^{15} different decamer peptides which can be presented by the MHC molecule, and it was estimated that every TCR are capable of recognizing more than 10^6 different peptide-MHC complexes [66]. Most of these peptides, nonetheless, are related and TCR cross-reactivity between unrelated pathogens is rare (approximately 1/30,000) [67]. Therefore, the T-cell recognition can be regarded as very specific.

Cancer antigens

Central and peripheral tolerance mechanisms in the immune system ensure that T cells recognizing self-antigens are eradicated. Cancer cells can be recognized and killed by endogenous T cells as they have an alternating pattern of antigen presentation compared to healthy cells, as explained above. van der Bruggen and colleagues identified the first TAA in 1991 [68], and since much research has been conducted to identify TAAs and their derived T-cell epitopes present on the tumor cell-surface. An extensive screening of published TAAs revealed a total of 573 T-cell epitopes distributed on 230 TAAs [28]. This knowledge about the exact T-cell restriction element is essential in monitoring anti-cancer T cell responses to increase understanding of what determines frequency and efficiency of these. Also, in a therapeutic context, knowledge of the actual tumor-specific T cells provides basis for targeted therapy with potential clinical benefits.

Previously most effort was put into identification of TAAs shared between patients, but the new possibility of exome sequencing has facilitated studies of specific immune-reactivity against mutation antigens unique for the individual patient. These studies revealed that part of the patients' T-cell repertoire recognized epitopes from these antigens [69, 70]. Also a one-patient case-study compared T-cell reactivity between shared and mutation antigens and provided evidence for a dominant T-cell recognition of mutation antigen-epitopes in melanoma [71]. These have thus been suggested to be more important targets than the shared antigens, at least in mel

anoma [29]. This approach is obviously more relevant in cancers with a high mutational load as melanoma than in cancers with lower mutational load as is the case for hematological diseases [72]. It should be realized, though, that the majority of cancer mutations are likely "passenger" mutations and although T cell responses against epitopes from these proteins will potentially affect cancer growth, escape variants may occur (as reviewed in [73]). This is similar to what has been observed for shared antigens.

Shared TAAs can be divided in groups according to their expression in healthy and tumor tissue. Recent screenings of specific T cell populations within TILs from melanoma patients against all known T-cell epitopes restricted to HLA-A1, -A2, -A3, -A11 and -B7 showed that most detected responses were against epitopes derived from differentiation antigens. This was despite the fact that the majority of T-cell epitopes included were derived from over-expressed antigens [28, 74]. Differentiation antigens are antigens related to the lineage from which the primary cancer originates. The latter group, conversely, is antigens over-expressed in cancer (often functionally relevant), but also widely present in healthy tissues. A third group is the CTAs. This group is

particularly interesting in regard to immune therapy, by being selectively expressed in cancer and immune-privileged areas and given that several lines of evidence showed the existence of CTA-specific T-cell immunity (e.g. [49, 50, 75]). Also, over-expressed TAAs essential for cancer cell continued growth have been identified, including human telomerase reverse transcriptase (hTERT), survivin and Wilm's tumor protein (WT1) [76–78]. It is believed that targeting of these antigens would not lead to tumor escape variants as down-regulation of the antigens in question would lead to inhibited growth properties. Lastly, antigens expressed by virus-induced cancers have been identified, including proteins associated with Human Papilloma Virus [79].

Humans harbor three distinct loci encoding MHC class I molecules, HLA-A, -B and -C, each with two alleles, enabling expression of up to six different HLA molecules in a given individual. Furthermore, each of these loci is highly polymorphic, thus providing huge diversity in the HLA-expression among individuals. Hundreds of different HLA subtypes exist and the HLA-A and -B have by bioinformatic clustering been grouped into 5 and 7 supertypes, respectively, with only modest overlap in peptide presentation [80]. Although some subtypes are highly frequent, for example HLA-A*02:01 in the Caucasian population in Western Europe and HLA-A*24:02 in Amerindian and Australasian Aboriginals [81], it is required to identify T-cell epitopes for at least every supertype and preferentially also for the majority of allele subtypes to enable tracking of specific T cell responses in all patients and healthy donors.

DETECTION OF ANTIGEN-SPECIFIC T CELLS IN THE PRESENT STUDIES

To increase understanding of T-cell recognition it is essential to examine patient blood for exact T-cell recognition, independent of tissue type. The development of fluorochome-coupled streptavidin-conjugated MHC multimers for tracking of antigen-specific T cells by flow cytometry changed the landscape of immunology when introduced in 1996 [82]. This technology has increased our understanding in all areas of T-cell immunology. MHC multimer staining of cells combined with a panel of antibodies to identify the CD8 T cell population represents an attractive method to measure specific CD8 T cells in a complex cell sample. By using MHC multimers for flow or mass cytometry it is possible to get a detailed description of specific T cell populations, but it requires the generation of MHC multimers.

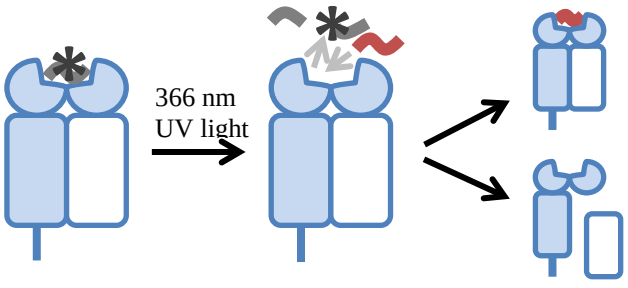

Figure 2, The UV light-mediated peptide-exchange method for generating MHC monomers with new specificities. UV light (366 nm) exposure cleaves the conditional ligand (gray with dark star), which are substituted by a rescue peptide (red) for forming an MHC complex with new specificity (top right). If no rescue peptide is present, the MHC complex degrades (bottom right).
Figure generated by the author.

To create peptide-MHC monomer complexes, recombinant heavy chain is refolded together with β2-microglobulin and the peptide of interest followed by multimerization with fluorochrome-coupled streptavidin-conjugates. Refolding is cumbersome and until the development of the UV light-mediated peptide-exchange method large screenings of specific T cell populations were not possible. This method takes advantage of conditional ligands situated in the MHC binding pocket and cleavable upon exposure to UV light (366 nm) [83]. Upon cleavage of the conditional ligand the MHC molecule degrades if not rescued by substituting the conditional ligand with a "rescue" peptide, as shown in figure 2. Large MHC monomer libraries with different peptide specificities can be produced within hours with this method [83]. Use of this method requires design of conditional ligands for every HLA molecule in question and in Study IV, reported in Paper IV, we extended the library by designing conditional ligands for use with HLA-B*08:01, HLA-B*15:01, HLA-B*35:01 and HLA-B*44:05.

THE IMMUNOEDITING FRAMEWORK

The idea that immunity and carcinogenesis are closely related was first proposed by Burnet and Thomas in the 1950's. Their theory about cancer immune surveillance suggested that cancerous cells are eradicated by the immune system before they become clinically apparent. Consequently, development of evident cancer is the result of escape from immune control, and the formal theory of cancer immune surveillance was proposed in 1970 [84]. Since then intensive research has aimed clarifying the interplay between the immune system and carcinogenesis. In 2002 Schreiber and colleagues (Dunn et al., Nat Immunol) provided a more thorough description of what they named cancer immunoediting [85]. This unifying model describes three phases from immune-mediated elimination of cancer cells (former immune surveillance), to an equilibrium in which the immune system keeps the tumor cells in check, and a final phase where certain tumor cells escape immune control and become clinically visible. Proof for cancer immunoediting was first shown experimentally in 2001 using a mouse model [86]. The hypothesis was further evaluated and confirmed by large amounts of experimental data in the following decade and these were gathered in two landmark reviews in 2011 [18, 87], the same year as "avoiding immune destruction" was included as a hallmark of cancer development [16]. The cancer immunoediting theory has recently been updated with new data [19] although these did not cause major changes to the hypothesis as proposed in 2011. Below and in figure 3 the process of cancer immunoediting is described with focus on the cell populations investigated in this thesis.

Healthy cells are continuously exposed to carcinogens, radiation and viral infections which potentially lead to genetic and epigenetic alteration. The outcome of this "mutation pressure" can be development of abnormal cell growth. Due to internal mechanisms of the cell machinery, e.g. senescence and induction of apoptosis, however, this rarely results in abnormal cell growth as touched upon above [15]. To become truly neoplastic, the cell needs fulfilling the requirements for most of the hallmarks, although not necessarily the ability to invasiveness. If so, it can still be eliminated by extrinsic tumor suppression prior to progression to clinically evident disease, shown in figure 3, bottom left. CD4, CD8 T cells and NK cells are all expected to be major effector cells in this phase by protecting the host through antigen-specific and -unspecific killing of immunogenic transformed cells. Direct evidence regarding the T cells in this phase was recently shown [88, 89]. Less immunogenic tumor subpopulations may, however,

persist in an equilibrium state with the host's immune system. In this phase tumor cells do not outgrow, but are potentially shaped, or edited, in phenotype to be capable of avoiding the immune pressure. Surprisingly only the adaptive immune system has been found to be important in this phase [90], shown in figure 3, bottom middle.

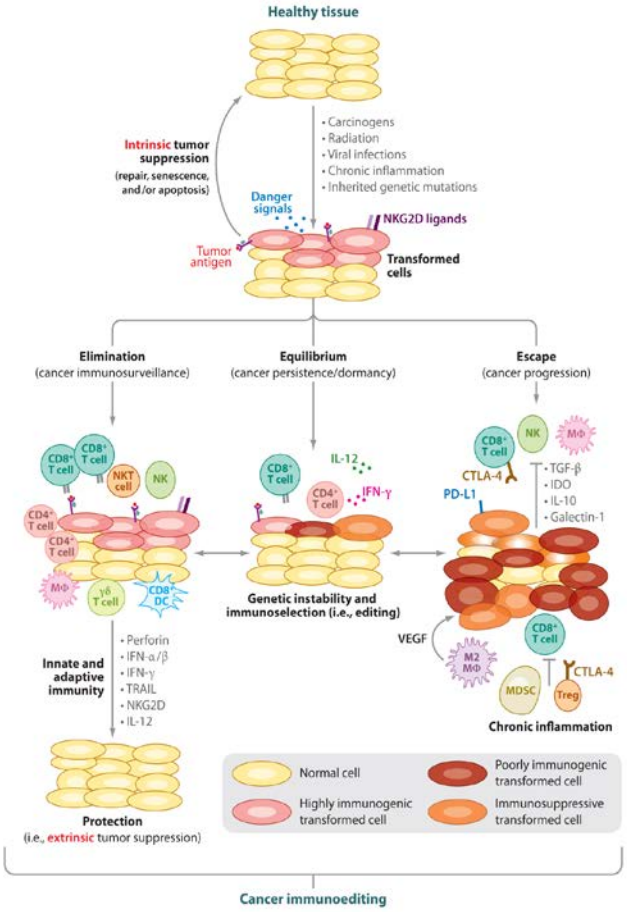

Figure 3, The cancer immunoediting process. When the intrinsic tumor suppressive mechanisms are overcome through various mechanisms, the abnormal cells might be eliminated by the host immunity and return to a healthy state; this is known as extrinsic tumor suppression. If not completely eradicated an equilibrium state between carcinogenesis and tumor suppression occurs. Here, the cancer persists, but do not ou grow; this is known as tumor dormancy. Tumors in this state are edited by the immune system and poorly immunogenic variants might arise, capable of escaping the host immunity. These will grow unhindered and the now clinically manifested cancer progresses. Due to chronic inflammation various host immune cells will promote further progression in this phase.

Adapted from Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ (2011) Natural innate and adaptive immunity to cancer. Annu Rev Immunol 29:235–71. doi: 10.1146/annurev-immunol 031210-101324 [87]. Reproduced with permission of Annual Reviews in the format Republish in a journal/magazine via Copyright Clearance Center.

Tumor cells are kept in check by immune effector cells, and the so-called dormant tumor cells may persist in this phase for years or even for the full life-length of the host [18]. Both immunosuppressive and poorly immunogenic transformed cells can be part of the tumor mass in the equilibrium phase and these may escape immunity to the final phase of cancer progression. Here, the cancer cells recruit inhibitory cells, as Tregs and MDSCs, and create a heavily immune-suppressed microenvironment, shown in figure 3, bottom right. In this fashion the immune system edits the tumor cell phenotype and induces tumor escape variants by Darwinian selection, through eradication of the most immunogenic tumor cells and leaving the cells with a poorly or non-immunogenic phenotype. These tumor escape variants may mutate even further to become fully "invisible" to recognition by the immune system, and may eventually metastasize.

This process should be kept in mind when designing new cancer treatments. Controlling cancer cell growth by stabilization of the equilibrium phase might be a favorable outcome, instead of accelerated induction of drug resistance and immune escape by treating with a very aggressive regimen. Escape variants might still outgrowth from the equilibrium phase, known clinically as progression from minimal residual disease, but presumably after an increased period of non-clinical manifestation of disease. Immune therapy has the potential to maintain cancer cells in the equilibrium phase and has also been shown to induce long-term responses with possible cure of patients [91].

The process of immunoediting is one mechanism by which immunity and carcinogenesis have an intimate relationship; the other is inflammation. Several types of inflammation can promote carcinogenesis, including therapy-induced, environmentally caused, chronic inflammation caused by infections or autoimmunity and tumor-associated inflammation (reviewed in [59]). Cancer stroma has been described as a "wounds that never heal" [92], and the chronic inflammation-like cancer microenvironment promotes carcinogenesis. The cancer microenvironment has been described important in all phases of carcinogenesis and promotion also prior to clinical manifestation of disease, and thus contributes in all three phases of the cancer immunoediting model [59, 87]. Cancer cells are not autonomous; they need stroma cells to form a tumor. Some of the stroma cells are cells of the immune system, but other cells may play equally important roles. Thus, tumor cells exploit normal cells to support their growth [93].

Tumor infiltration by adaptive immune effector cells, on contrary, has shown to be positively correlated to survival in several solid tumors, e.g. colon cancer [94], melanoma [95] and multiple myeloma [96]. Although phase III data are still lacking, the use of expanded TILs (being CD4 and CD8 cells) in melanoma treatment has shown impressive response rates (e.g. [26, 97]).

EPIGENETIC MECHANISMS

Carcinogenesis and its interaction with the immunoediting process and inflammation have been described above, and the final contribution arises from disruption of the epigenetic mechanisms. The term "epigenetic" refers to changes in the gene expression not defined in the DNA sequence; most investigated have been DNA methylation, post-translational modifications of the histone complex (e.g. acetylation) and micro-RNA expression [98]. As epigenetic changes are heritable and can survive numerous rounds of cell division (at least in regard to methylation, extensively reviewed in [99]), they can promote clonal outgrowth of cancer cells similar to genetic mutations directly in the DNA sequence. Targets for methylation in mammalian DNA are cytosine bases in CpG-rich regions. In these regions, cytosine and guanine are found on the same strand of DNA or RNA and are connected by a phosphodiester bond. In cancer, *de novo* methylation, initiated by still unknown processes, occurs at CpG-rich regions at the

transcription promoters. Accordingly methylation silences gene expression of i.e. tumor suppressor genes.

Epigenetic dysfunction is believed to be relevant in carcinogenesis of all cancers and could be essential for acquiring all of the hallmarks of cancer [16, 17, 100]. Abnormal stem cell epigenetic modification, however, has shown especially important in myeloproliferative diseases [101]. Also, methylation status was shown to be correlated to MDS progression [45], and the abnormal cell differentiation, a characteristic feature of most cases of MDS, is believed to be caused by defects in the epigenetic machinery. Finally, the clinical efficacy of the hypomethylating agents 5-Azacytidine [41] and 5-Aza-2'-deoxycytidine (Decitabine, Dacogen®, MGI Pharma) [102] suggests the importance of gene promoter hypermethylation in the pathogenesis of MDS. Further, a recent pilot study reporting treatment of AML patients with a new cytosine analogue, Sapacitabine (Cyclacell Pharmaceuticals Inc.), revealed promising data for hypomethylating treatment in this patient group [103].

Apart from inactivating gene transcription, hypermethylation in CpG-rich regions also promotes carcinogenesis by inducing point mutations and by causing genetic instability, shown in figure 4. Methylated cytosine may deaminate to thymine and act as an endogenous mutagen, causing disruption of genes involved in DNA repair or cell growth and survival [100]. Importantly, although CpG-rich regions are hypermethylated in cancer, there is a state of *global hypo*-methylation. This leads to genetic instability and breakage of chromosomal regions with consequently even more genetic instability [100, 104].

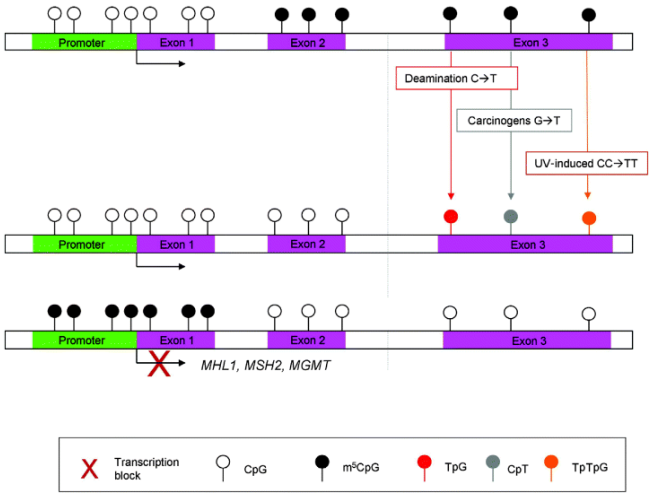

Figure 4, Epigenetic processes leading to hypermethylation of promoter regions in parallel with global hypomethylation. Methylated cytosines within the exons may lead to genetic instability, facilitating mutations by spontaneous deamination to thymine, by changing the absorption wavelength of cytosine leading to CC → TT mutations and by binding to carcinogens from tobacco smoke leading to ^{5}mCG → CT mutations. If promoter methylation of DNA repair genes (e.g. MHL1, MSH2 and MGMT) occurs, mutations induced by other mechanisms remain uncorrected. C: cytosine, T: thymine, G: guanine, CpG: cytosine-phosphodiester-guanine. ^{5}m: methylated, TpG: thymine-phophodiester-guanine, CpT: cytosine-phophodiester-thymine and TpTpG: thymine-phophodiester-thymine-phosphodiester-guanine.

Adapted by permission from Grønbaek K, Hother C, Jones PA (2007) Epigenetic changes in cancer. APMIS 115:1039–59. doi: 10.1111/j.1600-0463.2007.apm_636.xml.x [100]

IMMUNE THERAPY OF METASTATIC MELANOMA

Active immune therapy of cancer has been struggled with for many years, especially in metastatic melanoma, and for many years high-dose IL-2 administration was the only FDA approved immune therapy treatment for this patient group. IL-2 does enhance overall survival in a small fraction of patients (10-20%), but long-term remission is only seen in 3-5% of the patients and the high dose used has significant and severe side effects [24, 105].

To develop other treatment modalities for melanoma patients numerous clinical trials of vaccinations with peptides or dendritic cells has been conducted. The latter cells were encoded with for example TAA mRNA or naked DNA or loaded with different substances, including peptides from TAAs or tumor lysate. Although these vaccinations frequently induced specific T cell responses, clinical efficacy was generally poor, with most responders obtaining stable disease and only a few partial or complete responses [105–107]. Also, only a few trials included in a comprehensive review of dendritic cell vaccinations showed correlation between immunological responses and overall survival [105]. Important exceptions to this pattern is a recent trial with survivin epitopes and an older trial with tyrosinase and gp100 peptides [108, 109]. Importantly, in the survivin trial immune monitoring was performed as most common through analysis of peripheral blood samples. In the latter trial, however, the presence of antigen-specific T cells in skin biopsies from delayed-type hypersensitivity sites correlated with clinical outcome.

More promising results have been obtained with ACT of rapidly expanded TILs administered upon non-myeloablative lymphodepletion of the patients. It should be emphasized that the lymphodepleting regimen provided prior to ACT of TILs, being chemotherapy and possible additional total body irradiation, dramatically enhanced the clinical response rate. In 1994, the first data of ACT with TILs in human melanoma patients along with high-dose IL-2 treatment, but no prior lymphodepletion, was published [110]. Although significant clinical responses superior to IL-2 treatment alone were observed in this trial, the efficacy of treatment remarkably increased upon additional lymphodepleting treatment. An average of 49% overall response rate, which could be increased to 72% by intensive total body irradiation, and prolongation of overall survival was obtained in the trials incorporating lymphodepletion conducted by Rosenberg's group at the Surgery Branch, National Cancer Institute [26, 27, 111]. These encouraging results were reproduced by our group, an Israeli center and a group at MD Anderson Cancer Centre, using the ACT method with slight modifications [12, 112–114]. Also interesting is the high proportion of long-term responders following this treatment strategy, which lead to the suggestion that ACT of TILs could be a curative melanoma therapy [11, 91].

Several groups are working on optimizing this method even further. The initiation of a new protocol for rapid expansion to obtain so-called "young TILs" with no selection for cytokine secretion (as was done previously) has resulted in a faster preparation time with similar clinical outcome, enabling treatment of more patients [97, 115]. Also enriching CD8 T cells from bulk cultures prior to expansion has been evaluated *in vivo*, although with clinically disappointing results and in a very small study [116]. This suggested a net positive role for the CD4 populations within tumor-infiltrating lymphocytes. The finding was surprising as *in vitro* data showed enhanced reactivity of CD8 cells after separation from remaining T cells [117], emphasizing the difficulties in transforming *in vitro* results to the clinic and the susceptible balance between inhibitory and stimulatory CD4 T-cell subsets in these cultures. It has been shown, though, that the number of

$CD27^+CD8^+$ T cells infused was associated with the likelihood of obtaining an objective response in a long-term follow-up study covering 93 patients treated in several ACT protocols. CD27 is down-regulated upon cell differentiation, and in addition the study showed the benefits of infusing T cells with longer telomeres, another marker for less differentiated cells [11].
Additionally, IFN-γ treatment of autologous melanoma cells *in vitro* enhanced the anti-tumor T-cell reactivity by upregulating MHC class I and II expression [118]. Since none of the previous clinical evaluations was performed as randomized trials, though, the efficacy is questioned in regard to the possible selection of included patients. Therefore ACT of TILs in terminal melanoma patients is currently being tested in a European phase III clinical trial as compared to Ipilimumab, hosted by Inge Marie Svane, Center for Cancer Immune Therapy, Herlev, Denmark, and in collaboration with John B Haanen, Netherlands Cancer Institute and Robert Hawkins, Cancer Research UK Manchester Institute (Inge Marie Svane, personal communication).
Genetic engineering of TILs prior to ACT to direct the T-cell specificity of the infused cells has also been tried with remarkable results in melanoma treatment. Although promising clinical efficacy has been noted in some trials [119–121], also severe side effects have been induced with this potent treatment. These have largely arisen from the challenge of choosing the right antigen with exclusive expression on tumor cells and in non-essential tissue [122]. Side effects occurred due to recognition of either cognate antigen contained in healthy cells [119, 123] or cells expressing an unrelated, although in regard to the amino acid sequence, alike T-cell epitope [124, 125].
Finally, inhibitors of T-cell blockade molecules (CTLA4 and PD1/PD-L1) have proven very effective by prolonging overall survival in a high proportion of patients [5–8]. These were first tested as mono-therapies and currently combination therapy studies are conducted, of which the first of CTLA4 and PD1 inhibition combination has shown a possible synergistic effect [9]. The CTLA4 inhibitor Ipilimumab also induces long-term overall survival advantageous to what is seen in untreated patients, as reported 4 and 5 years after treatment initiation in two papers gathering results from in total 6 clinical studies [126, 127]. Also, the first long-term results from PD1 inhibition treatment is encouraging with a 2-year overall survival of 43% [128]. In addition, other inhibitors of T-cell blockade molecules are currently investigated as potential anticancer agents [106].

IMMUNE THERAPY OF MYELOID NEOPLASMS

Extensive clinical research has been conducted in melanoma, studied as a model disease for cancer immune therapy, and fewer data are available on treatment of myeloid neoplasms by immune modulation. Encouraging results, though, have been obtained from treatment with the immunomodulatory agents Thalidomide and its analogue Lenalidomide, used in both lower-risk and higher-risk MDS and AML. It has been shown that autoimmunity contributes to early MDS pathogenesis and possibly also transition from lower-risk to higher-risk disease. This results in increased inflammation, recruiting various cell types from the innate arm of the immune system in addition to NK cells, cytotoxic T and B cells, and at the same time decreasing the numbers of Tregs [129, 130]. The immune modulatory agents are thought to inhibit disease-induced inflammation [131]. Also, a contribution from MDSCs directly to MDS pathogenesis has recently been shown [132] and these cells are also known to be key players in inhibiting cytotoxic T cells and NK cells in other cancers [56, 133]. Immune modulatory agents do, however, also inhibit cytotoxic T cells. These are thus not optimal agents to induce effective tumor-specific T cells as required to combat cancer cells in patients with higher-risk MDS or AML.
Treatment with hypomethylating drugs, 5-Azacytidine and Decitabine, have shown the best clinical results in higher-risk MDS [41, 102]. 5-Azacytidine, though, showed superiority compared to Decitabine for MDS treatment in a meta-analysis comparing overall survival benefits [134]. These agents were not developed as immune stimulating agents, but as standard chemotherapeutic drugs with the aim of directly killing the dividing cells. Several lines of evidence now additionally indicate important immunological implications when they are used in a low-dose regimen, probably due to a hypomethylating-mediated upregulation of the expression of certain genes. Among these genes are CTAs in addition to tumor suppressors and genes associated with (normal) cell growth, differentiation and apoptotic processes [47, 48, 130].
Initial studies of 5-Azacytidine in combination with Lenalidomide and the histone deacetylase inhibitor Valproate, respectively, showed induction of CTA-specific T cell responses in human patients [51, 54]. This finding was confirmed and further extended in our study, as we also showed 5-Azacytidine-induced enhanced anti-tumor reactivity (Paper III). Also, Mikysková and colleagues has recently showed 5-Azacytidine-induced inhibition of MDSCs in mice [135]. This finding might add to the explanation of the clinical effect obtained with this agent as MDSCs were shown to contribute to induction of MDS in another study, noted above [132]. Also, expression of CTLA4, PD1, PD-L1 and PD-L2, immune checkpoint molecules investigated clinically in several cancers, was shown to be up-regulated in MDS, CMML and AML patients. This up-regulation was further enhanced by hypomethylating agents, and treatment-resistant patients showed even higher expression of these four immune blockade molecules [136]. Inhibition of these T-cell blockade molecules could be worth considering as a second line therapy after development of resistance to hypomethylating drugs in higher-risk MDS, CMML and AML, as has been reported with very encouraging results in solid cancers [5–9].

2. SUMMARY OF RESULTS

During my PhD studies I have been involved in several projects, which have resulted in the four papers referred below and a number of co-authorships.

PAPER I

Numerous melanoma-associated T-cell epitopes have been described, but 45% of these are restricted to HLA-A2 and the rest distributed on 36 different HLA molecules. The only other HLA molecule with a fair amount of described T-cell epitopes is HLA-A24, and this leaves the remaining HLA molecules with only a few described T-cell epitopes each. It is thus needed to identify melanoma-associated T-cell epitopes restricted to other HLA molecules than HLA-A2 and HLA-A24 to enable monitoring of T cell responses also in patients not expressing these HLA molecules. We used a T-cell epitope mapping platform for this purpose. We chose gp100, MAGE-A3, Mart1, NY-ESO-1, tyrosinase and TRP-2 (glycoprotein 100, melanoma antigen gene-family-A3, melanoma antigen recognized by T cells 1, New York esophageal squamous cell carcinoma-1, tyrosinase and tyrosinase-related protein-2) as these have previously been frequently recognized in HLA-A2 restricted melanoma patients.

In the amino acid sequence of these antigens we predicted by *in silico* algorithms 9- and 10-mer HLA ligands for binding to HLA-A1, -A3, -A11 and -B7. The peptides were synthesized and their affinity investigated experimentally with MHC ELISA (enzyme-linked immunosorbent assay). This resulted in a library of 127 HLA ligands which were used for UV light-mediated peptide-exchange of conditional ligands to produce MHC monomers for each of these specificities. MHC monomers were multimerized with Phycoerythrin (PE)-coupled streptavidin-conjugates for use in a T-cell enrichment procedure, with the aim of enhancing the frequency of the specific T cell responses to a detectable level. We also multimerized exchanged MHC monomers with up to 9 different fluorochrome-coupled streptavidin-conjugates in a combinatorial encoded fashion (corresponding to up to 36 different T-cell specificities) for detection of specific T cell responses. We tested peripheral blood mononuclear cells (PBMCs) from 39 melanoma patients and found 27 T cell responses, distributed on 17 different peptide-specificities. Some of these specific T cell populations were sorted by fluorescence-activated cell sorting (FACS) and expanded. For five of these peptide sequences we tested processing and presentation in HLA-expressing and K562 cells electroporated with relevant mRNA or melanoma cells and found three of these peptides to be presented on the cell-surface. Only two of these are novel T-cell epitopes as the third was previously published.

PAPER II (MANUSCRIPT IN PREPARATION)

Numerous melanoma-associated antigens have been described, but recent screenings of the T-cell recognition of melanoma-associated T-cell epitopes within TILs only established the T-cell specificity for a minor fraction of the tumor-reactive CD8 T cells. Targets for tumor rejection have not yet been defined, and it is therefore necessary to identify new antigens for immune therapeutic targeting and monitoring in cancer. The protein Nodal is correlated to aggressiveness and metastatic potential in melanoma as well as in several other cancers. Apart from this cancer-specific expression it is important during embryogenesis, but in the adult it has only been found expressed short-term during menstruation and in pancreatic islet regeneration in diabetes patients. So, this makes Nodal an ideal target for immunotherapeutic investigations, and we hypothesized that Nodal-specific T cells could be present within PBMCs from melanoma patients. We used a T-cell epitope mapping platform, as described above for Study I, to identify 32 HLA ligands restricted to HLA-A1, -A3, -A11 and -B7. Through T-cell enrichment we detected 10 Nodal-specific T cell responses distributed on 8 different peptide-specificities. Some of the specific T cell populations were sorted by FACS and expanded, and for three of these we could determine processing and presentation. One was found presented on the cell-surface of HLA- and Nodal-expressing K562 cells, and this T-cell epitope was recognized in our study both by HLA-A3 and HLA-A11 restricted CD8 T cells. We also applied the T-cell enrichment strategy on PBMCs from 11 healthy donors, but did not find any Nodal-specific T cell responses. This suggested that T-cell immunity against Nodal is restricted to cancer patients as we frequently detected Nodal-specific T cell responses within PBMCs from melanoma patients. Thus, Nodal represents a potentially important new immunological antigen in melanoma.

PAPER III

Treatment with the hypomethylating drug 5-Azacytidine has proven prolongation of overall survival in higher-risk MDS patients, but the mechanisms responsible for obtaining clinical efficacy have not been fully identified yet. Gene promoter hypomethylation enhances transcription and expression, and 5-Azacytidine up-regulates CTAs in addition to other genes, e.g. tumor suppressor genes. CTA-specific T cells have been detected in cancer patients in many previous studies, and we hypothesized that also CTAs-specific T cells would be induced by 5-Azacytidine, possibly inducing or enhancing T-cell mediated killing of tumor cells.

To test this hypothesis we collected blood samples prior to therapy and at various time points after treatment initiation from a heterogeneous cohort of 17 patients (10 with higher-risk MDS, 4 with AML, 1 with MDS/AML and 2 with CMML) treated with a cyclical 5-Azacytidine regimen. To determine if 5-Azacytidine induces CTA-specific T cells we performed a flow cytometry-based screening of the T-cell recognition of 43 CTA T-cell epitopes within PBMCs from these patients, by using combinatorial encoded MHC multimers. We found that CTA-specific T cells were present prior to therapy in the blood from 6 of 8 patients tested. These T-cell responses were further enhanced significantly at the first measured time point (3^{rd} to 5^{th} cycle), but stabilized or declined afterwards. We also isolated CD8 T cells and CD34 cells obtained prior to therapy and at a late cycle, using CD34 as a surrogate marker for myeloid blast cells. These four cell populations were co-cultured in four combinations to elucidate separately the effect of 5-Azacytidine on CD8 T cells and myeloid blast cells. We found that the therapy induced better CD8 T-cell mediated recognition of myeloid blasts; no effect was observed directly on the CD8 T cells.

To investigate 5-Azacytidine therapy for potential combination with immunotherapeutic modulation, we further investigated if the drug exerts any general effects on the immune system. We measured the absolute numbers of the general populations of the effector cells CD4 and CD8 T cells and NK cells and the inhibitory cell populations MDSCs and Tregs prior to and after therapy initiation. We found only a small effect of 5-Azacytidine on the NK cells, specifically NK cells with the inhibitory phenotype $CD56^{+}CD16^{+}CD158b^{+}$, while the remaining cell population numbers were stable. Taken together these results suggest that 5-Azacytidine primarily affects the myeloid blasts, thus enhancing their “visibility” to specific T cells.

PAPER IV

The human HLA molecule is highly polymorphic and this is challenging for MHC multimer-based detection of specific T cells and other measurements restricted to the tissue type. The UV light-induced peptide-exchange method for production of MHC monomers was first developed for HLA-A2 and has been expanded to a number of other HLA molecules. Three of the most frequent HLA-B molecules in the Caucasian population, however, were not included in these studies and we consequently aimed for identification of conditional ligands restricted to HLA-B*08:01, HLA-B*35:01 and HLA-B*44:05. We designed conditional ligands and evaluated their refolding efficiency, UV light cleavability and streptavidin-coupled multimerization by size-exclusion high-pressure liquid chromatography. We additionally demonstrated their applicability in an MHC ELISA assay and for detection of specific T cell responses by flow cytometry. Furthermore, we showed that a conditional ligand first developed for HLA-B*15:02 can be used with an HLA-B*15:01 construct and applied in the above mentioned applications. Also, we compared staining of HLA-B*15:01 PBMCs with HLA-B*15:01 and HLA-B*15:02 MHC

multimers. We found that HLA-B*15:01 virus-derived T cell responses within a normal range (up to 0.38% of the CD8 cells) were undetectable with HLA-B*15:02 MHC multimers. In a largely enriched peptide pre-stimulation culture, however, we were able to detect a minor part of the specific T cells also with HLA-B*15:02 MHC multimers.

3. DETECTION METHODS

FLOW CYTOMETRY-BASED METHODS

Data obtained in this thesis have largely been based on polychromatic flow cytometry, being a powerful method to elucidate characteristics of bulk populations of cells intra- and extracellularly. It is a high-throughput method capable of recording thousands of events per second and thus also suited for the detection of rare cell populations. Polychromatic flow cytometry experiments can generate a detailed description of the cells in question within a short time frame.

For the studies reported here, we used flow cytometry to distinguish antigen-specific T cells for the purpose of identifying new T-cell epitopes (Study I and II), for monitoring of 5-Azacytidine-induced T cell responses (Study III) and for testing of new conditional ligands (Study IV). Also, we used flow cytometry-based methods to measure cytotoxicity or up-regulation of a CD8 T-cell activity marker upon challenge with target cells. Direct cytotoxicity of CD8 T cells and NK cells was measured against HLA-expressing K562 cells electroporated with mRNA ([137], Study I and II) or K562 cells with no HLA expression (Study III), respectively. Upregulation of the activation marker CD107a on CD8 T cells was measured upon challenge with tumor cells affected by 5-Azacytidine treatment or not [138, 139]. Also we measured the general numbers of CD4 and CD8 T cells, NK cells, Tregs and MDSCs (Study III).

We used up to 14 different parameters in parallel (12 fluorochromes and forward and side scatter), but it is possible to run experiments with even more colors. Already in 2004 a 17-color system was presented [140] and later gradient-based fluorescence coding, or "bar-coding", developed. Through this method cells are marked with different concentrations of a reactive fluorescent dye, with following simultaneously detection of up to 96 different encoded populations [141, 142]. Also, the development of mass cytometry, integrating single-cell fluidics with mass spectrometry detection of rare-earth metal isotopes used for antibody tags, has enabled parallel analysis of 50 different parameters. This technique can potentially be expanded to incorporate most atoms in the periodic table [143, 144]. Recently, also spectral flow cytometry was commercialized. This method differs from conventional flow cytometry by recording the full fluorescence emission spectra. Fluorochromes with highly overlapping spectra, not possible to use in parallel with conventional flow cytometry due to compensation issues, might be possible to use simultaneously for this specialized sort of flow cytometry. The full potential of this technique has not yet been explored [145].

The static nature of the above mentioned methods, however, has been challenged by sophisticated single-cell technologies measuring dynamic properties on the single-cell level. By microfluidic methods and nanoliter-well plates it is possible to add single cells to each well in a multiplex plate. These can be characterized and describe numerous spatiotemporal dynamics, including motility, protein secretion and phenotype. Also, comparisons between single cells has revealed remarkable differences, e.g. that initiation of cytokine secretion varies among T cells in the same differential state [145].

MHC MULTIMERS AND UV LIGHT-MEDIATED PEPTIDE-EXCHANGE

The UV light-mediated peptide-exchange technology, explained page 5 and in figure 2, has been expanded to cover many HLA molecules from its first development for HLA-A2 [146–150]. This technology additionally forms the basis for combinatorial encoding of MHC multimers for high-throughput screening and monitoring by flow and mass cytometry [151–154]. Although we routinely use the 2-dimensional combinatorial encoding technique first described by Hadrup & Bakker et al. [151], it can be further multiplexed for 3-dimensional color-coding. This was also proved for in the original paper, and data analysis for this more complex situation was recently developed by Lin and colleagues in collaboration with us [155]. Finally, UV exchange of conditional ligands can be coupled to a sandwich-ELISA, enabling measurement of peptide-MHC affinity [156]. With the profound advantages of using the UV exchange technology for generation of MHC monomers it is clear that it should preferably be exploited to at least the most frequently expressed HLA molecules.

The UV exchange method was applied to generate data reported in all of the four studies included in this thesis. For studies I, II and IV it was used both for affinity evaluation of MHC monomers by the MHC ELISA and for detection of specific T cells by combinatorial encoded MHC multimers, while for Study III only the application for detection of specific T cells was utilized. For the studies I and II, we aimed to identify new T-cell epitopes and took advantage of a T-cell epitope mapping platform. This platform describes *in silico* prediction of potential HLA ligands, affinity testing by MHC ELISA and subsequent detection of specific T cell populations by combinatorial encoded MHC multimers [157]. We furthermore added PE-coupled MHC multimer-assisted enrichment of specific T cell populations, after UV exchange-mediated generation of libraries of potential T-cell epitopes in complex with the appropriate HLA molecules. The frequency of TAA-specific T cells is often low and expected to be below threshold level for the detection method. The T-cell enrichment methods was first used by Day and colleagues [158] and has further been used in T-cell epitope mapping studies similar to the studies reported in this thesis [159, 160]. Finally we used MHC multimers for sorting of specific T cells to generate highly specific T cell cultures.

T-CELL EPITOPE MAPPING PLATFORM

The T-cell epitope mapping platform and MHC multimer-assisted T-cell enrichment used in our studies enable high-throughput discovery of T-cell epitopes using panels with hundreds of peptides [157, 160]. This combination is in this regard superior to for example the enzyme-linked immunosorbent spot (ELISPOT) assay, as only a handful of peptides can be investigated simultaneously with this method. As current peptide-MHC affinity-prediction algorithms achieve far from 100% concordance between the predicted and actual affinity values (unpublished observations), the incorporation of a peptide-MHC affinity validation step is beneficial for excluding the peptides with poor binding. We used NetMHC and NetMHCpan prediction servers in combination and only selected peptides below threshold in both servers. We did this when the studies were started in 2009 and interestingly a recent study (published in 2012) comparing NetMHC, NetMHCpan and PickPocket (a prediction method for receptor-ligand interactions), identified the NetMHC-NetMHCpan combination to be superior to any other combination or use of single algorithms for predicting ligands restricted to MHC molecules included in the training set [161]. These prediction servers have now been joined and named NetMHCcons.

By this approach we might exclude peptides which could be processed and presented on the cell-surface, as previously described T-cell epitopes span a large range of different peptide-MHC affinities. The clinical relevance of such low-affinity T-cell epitopes can be questioned, however, as it seems logical that peptides with higher binding affinities would be better presented by the MHC molecule. This speculation was partly confirmed in a recent mouse study which showed that tumor relapse or regression is directly dependent on the peptide-MHC affinity [162]. Thus, even though T cells specific for TAAs are present within the tumor and fully functional, they will only be capable of killing the tumor cells if the peptide-MHC affinity is sufficient. If peptide-MHC affinity was not sufficient the tumor cells either continued growing with the same phenotype (for low-affinity interactions), or generation of escape variants occurred (with intermediate-affinity interactions). The peptide-MHC:TCR affinity is certainly also important and has been extensively investigated, but the range of this interaction is orders of magnitude smaller than the range of the peptide-MHC interaction due to the complex T-cell selection process, as outlined in a comment to the mouse study [163].

IMPORTANT THINGS TO CONSIDER FOR MHC MULTIMER STAINING AND COMPARISON WITH ELISPOT

The frequently used ELISPOT assay facilitates measurement of specific T cells with a functional characterization. Most often is interferon-γ secretion measured, but ELISPOT kits for analysis of many other cytokines, including tumor necrosis factor-α and granzyme B are readily available. Usually ELISPOT is used for analyzing only one cytokine per experiment, but the Fluorospot assay (from Mabtech Inc.) using fluorescent antibodies, enables simultaneous detection of multiple cytokines. Cytokine secretion measurement is also possible using the intracellular cytokine staining assay for flow cytometry. Although also parallel detection of MHC multimer-specific T cells and cytokine secretion in response to stimulation with peptide or target cells is feasible, however, it is hampered by stimulation-induced TCR down-regulation. Attempts to use MHC multimer-based detection of specific T cells in this situation often results in decreased frequency and mean fluorescence intensity measured compared to a direct staining with no stimulation; it is consequently suboptimal. It was recently shown that differences in HLA-subtype between HLA reagents and the HLA molecules expressed by the cells analyzed may lead to significant underestimation of the number of specific T cells. This represents an important issue since for many immunological analyses the HLA-subtypes are not determined. A comparison of staining with HLA-A*02:01 and HLA-A*02:06 MHC multimers for detection of specific T cell populations within TILs from an HLA-A*02:06 positive melanoma patient, showed remarkable differences in both the number and frequency of detected T cell responses [164]. This was observed even though the HLA-A*02:01 and HLA-A*02:06 heavy chains only differ in two amino acid positions. Also, it was found that MHC monomers were to a similar extent capable of binding the peptides in question, suggesting the staining differences were exclusively due to the binding between TCRs and peptide-MHC complexes. We observed even larger differences in the staining pattern upon comparison of staining of HLA-B*15:01 restricted T cells with HLA-B*15:01 and HLA-B*15:02 MHC multimers (Paper IV). On contrary, no differences in cytokine secretion upon peptide stimulation would presumably have been observed using the ELISPOT assay. 145 melanoma-associated peptides were included in the HLA-A*02:06 screen, however, and it had been almost impossible to perform parallel ELISPOT assays including all of these, both in regard to time and material consumption.

Lack of correlation between the responses detected with MHC multimers and ELISPOT has been demonstrated (e.g. [165, 166]), is often debated and can be attributable to a number of different things. If specific T cells are detected using ELISPOT and not by MHC multimers, it could be due to the above mentioned issue on using the right HLA subtype of the MHC multimers. Also, detection threshold might be lower for ELISPOT in regard to the frequency of specific cells and some TCR:peptide-MHC interactions may be too weak for MHC multimer staining, although the interaction is sufficient to induce cytokine secretion in the ELISPOT plate. Additionally it can be difficult to judge if an ELISPOT response is solely derived from CD8 T cells as other cells capable of producing the cytokine in question are usually present, for example CD4 T-cell recognition or NK cell-mediated interferon-γ production. Of notice, the use of dual-color codes for the detection of MHC multimer-specific T cells was estimated ten-fold more sensitive than conventional single channel staining [167].

Finally, an elegant study showed MHC multimer-based detection to be dependent on the TCR:peptide-MHC dissociation off-rate, by comparing MHC multimer-based detection of clones with same TCR avidity [166]. Consequently, the dissociation kinetics should preferably be taken into account when staining with MHC multimers. As these are rarely known, under standard staining procedures the possibility arises of missing T cell populations capable of binding to the MHC multimers. The dissociation off-rate has shown important in reaching clinical efficacy in mouse models, and a light microscopy-based assay was recently developed for determining this parameter unique for each TCR clonotype [168]. Here, the authors showed marked differences in T-cell reactivity between two T-cell lines recognizing the same peptide-MHC complex, but with different dissociation off-rates. This correlation between fast dissociation and lack of sufficient T-cell reactivity was further confirmed in human cell cultures [169]. Thus, the dissociation off-rate should ideally be taken into account when evaluating T cell responses from monitoring studies, as the largest T cell populations might also possess a fast dissociation off-rate and thus be non-functional against target cells.

The affinity between the peptide and the MHC molecule could also be expected important for obtaining a proper staining, but it seems not crucial given the frequent detection of HLA-A2/Mart1$_{26\text{-}35}$-specific T cells with MHC multimers [28, 74]. The wild-type version of this T-cell epitope has a very low binding affinity, as measured by MHC ELISA (unpublished observations), and it can be imagined that low-affinity peptides continuously bind and dissociates from the MHC complex. Importantly, this frequent detection was observed with MHC monomers generated through UV light-mediated peptide-exchange for which great excess of peptide stabilizes the complex. If this low binding affinity is responsible for the apparent lack of clinical Mart1 wildtype-mediated control of carcinogenesis remains to be established. Such an association would be in line with the above mentioned finding of correlation between peptide-MHC affinity and the regression or relapse of implanted tumors [162]. Interestingly, a prediction server called NetMHCstab for predicting peptide-MHC stability has recently been developed with the aim of predicting immunogenicity and not only affinity, and this algorithm incorporates also (predicted) peptide-MHC dissociation off-rate values [170].

If, on the other hand, MHC multimer-specific T cells are detected along with no ELISPOT response, reasons could be that the experimental circumstances are not optimal for cytokine production,

that specific T cells are anergic and not capable of producing cytokines or that they produce other cytokines than is measured for in the assay. Also, cells need to be more vigorous to be functional, than simply binding to MHC multimers for detection by flow cytometry.

MEASURING PROCESSING AND PRESENTATION OF TUMOR-ASSOCIATED ANTIGENS

To determine processing and presentation of TAAs for T-cell or NK-cell mediated cytolytic recognition we used two cytotoxicity assays, the VITAL-FR (Study I-III) and the chromium release (Study I) assays [171]. For use in a VITAL-FR experiment positive and control (negative) target cell lines are labeled with two different dyes and mixed. Effector cells are added in different effector:target ratios and remaining target cells are counted via the use of flow cytometry after 24, 48 or 72 hours of co-culturing. From the ratio between the two target cell populations the specific T-cell mediated killing can be calculated. This method has previously been used in a T-cell epitope mapping study, providing proof of the T cell specificity in regard to HLA and protein expression [159]. For use in a chromium release assay positive and negative target cell lines are labeled with ^{51}Cr, mixed with effector cells in different effector:target ratios and the ^{51}Cr release is measured with a gamma counter. Following, the specific lysis of the target cells can be calculated. This method has previously been extensively used in T-cell epitope mapping studies for the same reasons as the VITAL-FR assay and in addition for the ability to kill cancer cell lines (e.g. [172–174]).

The chromium release assay can be used equally well for suspended and adherent target cells, whereas use of VITAL-FR is limited to target cells in suspension due to the need for exact counting. Advantageous for the VITAL-FR method is that each well contains both positive and control target cells and effector cells. This enables simultaneous measurement of killing of both target cell populations. Also, unspecific killing is included in the measurement since the ratio between target cells forms the basis for calculating effector-cell mediated lysis. This means that proper selection of target cell lines is fundamental, but also that the lysis measured is indeed specific. Additionally, flow cytometry is readout for the VITAL-FR assay, experiments are set up in 96-well plates and each tube only contains few cells, so it is potentially a high-throughput method if an auto-sampler for flow cytometry loading is available. Finally, the VITAL-FR method was claimed up to 30 times more sensitive than the chromium release method due to negligible spectral overlap between the two dyes and the long co-culturing time [137].

The chromium release assay possesses the important capability of measuring specific killing of adherent cancer cell lines. This can be regarded closer to the *in vivo* situation than for example the transduced and transfected cell lines we used for the VITAL-FR assay. Most melanoma cell lines, nevertheless, have been in culture for years and are not expected to fully reflect the phenotype of the cells in the original lesion. Additionally, the co-culturing of high-frequent effector and target cells in growth medium in a plastic tube does not reflect the *in vivo* situation either. It does, however, not necessarily imply that results obtained from such *in vitro* experiments are not relevant for the *in vivo* situation, as was argued by Soto and Sonneschein (page 2, [20]), but extrapolation from the *in vitro* to the *in vivo* situation is not simple.

4. DISCUSSION

DISCOVERY OF NEW MELANOMA-ASSOCIATED T-CELL EPITOPES

The net result of our T-cell epitope mapping studies is identification of three new T-cell epitopes, but they also provided information about the broadness of T-cell reactivity against the seven proteins included. In total 8 of these 25 peptide sequences recognized were tested for processing and presentation and preferably the remaining ones should also be evaluated, as a fraction of these are presumably also processed and presented. We sorted a few more specific T cell populations by FACS, but did not succeed in generating specific T cell cultures for these after sorting, and were subsequently not able to conduct functional assays. The remaining responses were detected in too low frequency to be sorted, even after the T-cell enrichment. This sorting step represents a bottleneck of the mapping platform which can only be solved by development of more sensitive functional assays or cell culturing methods with selective expansion of specific cells. In regard to the latter an interesting strategy of coupling Dextramers® with different molecules for stimulation or inhibition of specific T-cell subsets might be relevant (called Klickmers®; both Klickmers® and Dextramers ® are produced by Immudex, Copenhagen, Denmark). Dextramers® are several MHC monomers bound to a backbone and claimed to possess enhanced avidity compared to normal streptavidin-coupled MHC multimers. The usability of Klickmers® with attached IL-15 and the CD28-ligand B7.1 for T-cell enrichment similar to the techniques described herein is currently being explored in our group. It will be interesting to see if Klickmers® are capable of selectively stimulating specific T cells with resulting T cell cultures of increased specificity.

The use of ELISPOT in these screenings instead of MHC multimer-based detection by flow cytometry would possibly have revealed more or other T cell responses. For MHC multimer-based detection a certain TCR:peptide-MHC complex binding affinity is required to sustain the procedure. The addition of peptide in excess to a well in an ELISPOT assay in contrary can be expected to require less binding for the cytokine secretion to occur. Also, the above mentioned requirements for exact HLA subtype match between the cells and the MHC multimers could have influenced the possibility of detecting responses along with other matters mentioned in this section (pages 10-11). On the other hand, a recent comparison of detection of T cell responses against TAAs with ELISPOT and MHC multimers showed that the latter method revealed more responses, although still some responses were found exclusively with the ELISPOT method [165].

PROVIDING NODAL AS A NOVEL IMMUNOLOGICAL TARGET

The Nodal screening is interesting in two ways. First, expression of this protein is implicated in the metastatic process both in breast, prostate, endometrial and glioma carcinomas in addition to melanoma [30–33, 175, 176]. Our identification of Nodal as a melanoma-associated immunological target can thus potentially be broadened to these cancers. Second, the number of Nodal-specific T cell responses detected in our melanoma screening is striking; more different peptide sequences from Nodal were recognized than from any of the common and frequently recognized melanoma-associated antigens included. Also, in regard to the total number of T cell responses for each protein, Nodal-derived peptides were most frequently recognized. Of note, we included a similar number of HLA-ligands (32, 41, 32 and 29 from Nodal, gp100, tyrosinase and TRP-2, respectively).

Although we provide evidence that Nodal is an immunological target in patients with diverse tissue types, the incorporation of also HLA-A2 ligands and patients in the screening would have been beneficial. HLA-A2 is the most prominent HLA molecule in Western Europe among Caucasians and was primarily excluded from the screening of common melanoma-associated antigens due to the multiple T-cell epitopes restricted to this HLA molecule already described. The high frequency of Nodal-specific T cell responses detected in our patient cohort would most likely also apply to HLA-A2 positive patients. Furthermore, it would be interesting to investigate for the presence of specific T cells in PBMCs from patients harboring other cancers in which the metastatic potential has been linked with Nodal expression. Given that Nodal expression in adults is linked to the metastatic potential of these cancers and that we frequently detect Nodal-specific T cell responses in terminal melanoma patients, an investigation of Nodal-specific T-cell reactivity in patients with less progressed cancers is needed too. This would enable a more thorough description of the Nodal-specific T cells. If present in non-terminal patients, Nodal-specific T cells might be responsible for inhibiting metastatic spread by selectively killing clonal cancer cells capable of invasiveness. If, on the other hand, Nodal-specific T cells are only present in patients with more advanced disease, their presence might be valuable as a biomarker for aggressiveness. Nodal expression has been suggested as a biomarker for melanoma and breast cancer [33, 176], but the presence of Nodal-specific T cells might be detected earlier in progression and facilitate earlier treatment of advanced disease.

SHARED ANTIGENS VERSUS MUTATION ANTIGENS

All seven TAAs used in our T-cell epitope mapping studies are antigens shared between patients. Previous studies dissecting T-cell specificities in TILs from melanoma patients with a panel of predominantly shared antigens, referred above, found only a fraction of the anti-tumor reactivity to be attributable to T cells recognizing these epitopes [28, 74]. Also, T-cell reactivity against patient-specific unique mutation antigens was recently described and has been shown in a one- patient case-story to be superior to the T-cell reactivity against shared antigens [69–71]. This led to the suggestion that T cells specific for mutation antigens unique for individual patients could be the major anti-tumor effector cells, at least in melanoma [29]. T-cell immunity against these unique mutation antigens has recently been explored due to the newly developed method of exome sequencing, and their importance remains to be established. It should be noted, though, that these screenings of T-cell recognition in TILs from melanoma patients of shared antigens did not observe frequent T-cell recognition of T-cell epitopes from shared mutation antigens [28, 74]. It cannot be ruled out that the situation is different regarding individual mutation antigens. However, the idea that mutation antigens should be recognized by TCRs of higher affinity, and thus induce more frequent and reactive T cells due to less induced T-cell tolerance, would also be expected to apply to shared mutation antigens.

Ideally a study comparing the T-cell frequency and reactivity against as many shared and mutation antigens as possible in a group of cancer patients should be conducted. This would possibly provide evidence for the superior importance of one group of antigens. To perform such a study full coverage of the HLA molecules present in the patient population should be aimed for, and in this regard our newly developed conditional ligands for four frequent HLA-B molecules would be of use. Mutation antigens are believed to be important due to their foreignness to the host, with following expected existence of high-affine T cells which have circumvented T-cell tolerance mechanisms in the host. Virus-induced cancers are an example of a similar situation with expression of really foreign antigens, and especially Human Papilloma Virus-induced cancers have been studied in depth. These studies revealed that the occurrence of broad T cell responses recognizing the virus-derived proteins E6/E7 is not sufficient to eradicate large tumor burdens [177, 178]. Although the occurrence of Tregs most probable contributes to dampening of the anti-tumor responses, these data also suggest that targeting foreign antigens as virus-derived or mutations antigens does not with certainty lead to cancer rejection.

Significant anti-tumor reactivity has been obtained with T cells recognizing shared antigens, though. The studies of Becker et al., Inderberg-Suso et al. and Walter et al. [109, 179, 180] are interesting in showing correlation between the specific T cell responses and overall survival after vaccination of cancer patients with epitopes from survivin, hTERT and from multiple antigens identified directly on the cancer cell-surface, respectively. Both survivin and hTERT are antigens believed to be essential for sustained tumor growth, meaning that tumor variants escaping specific T-cell recognition will obtain a less-aggressive phenotype. The multiple peptides administered in the last trial were all identified directly and exclusively on the surface of tumor cells as compared to healthy counterparts. This method for epitope identification might be superior to the indirect immunology approach used in our and most other T-cell epitope mapping studies as it provides evidence that the peptide sequences are actually present on the tumor cell-surface.

The possibility of clinically targeting the most malignant cancer cells through the use of Nodal-specific T cells is tempting and should be investigated further. If so, it should be considered, though, that Nodal also has been found shortly up-regulated during the menstruation cycle, and this might give the possibility of off-target effects in younger women [31, 181]. Also, Nodal expression in the pancreas during development and at islet regeneration should be taken into account if therapeutic targeting of Nodal is investigated *in vivo* [182].

IMPLICATIONS FOR IMMUNE THERAPY OF MELANOMA

Nodal

The identity of the true rejection antigens for targeting by immune therapy has not yet been established [122], and this necessitates identification of new antigens. It should be noted, though, that there is no certainty that actual single rejection antigens exist. It is possible that targeting multiple non-rejection antigens to avoid the occurrence of tumor escape variants is a better strategy for cancer eradication.

We provided evidence that Nodal is in fact an immunological antigen frequently detected by T cells within the blood of melanoma patients. Nodal is important for the development and aggressiveness of cancer in both melanoma, glioma, breast, prostate, endometrial, pancreas and liver cancers [30–33, 175, 183, 184], and the selective expansion of Nodal-specific T cell populations might facilitate killing of the most aggressive cancer cell variants. Another possibility is the use of TCR transduction therapy with naturally occurring or affinity enhanced TCRs recognizing T-cell epitopes from Nodal prior to ACT. This sort of therapy has induced tumor regression although severe on- and off-target side effects were noted in previous studies [123–125]. Expression of Nodal in the adult seems cancer-selective, with only short-term

expression in few healthy tissues [31, 182]. This should preferably be investigated further in more tissues, but suggests that Nodal-specific TCRs would be safe to use for ACT of TCR transduced T cells. Specifically targeting Nodal-expressing cancer cells may enable us to control the remaining cancer cell subtypes by the non-Nodal-specific expanded TILs used for ACT, thus creating equilibrium between tumor growth and immunity.
A marked tumor heterogeneity has been shown in several cancers [4, 185], and thus it seems wise to target antigens like Nodal which is probably present in most cells with metastatic potential. This selective expansion could be obtained by separating Nodal-specific T cells from bulk TILs, possibly together with T cells specific for other antigens associated with tumor growth. T-cell recognition of survivin and hTERT has already suggested to be associated with clinical efficacy [109, 179]. These T cells could be separately expanded in parallel with expansion of bulk TILs for ACT. The composition of TILs are not fully established, but it is certain that a significant part of these do not react against cancer cells and instead might be passive "pass-through cells", recognizing antigens on stromal cells or virus-derived epitopes [28, 74]. Therefore a selective expansion of tumor-reactive T cells prior to infusion would probably be beneficial. In addition to the possible clinical relevance of Nodal-specific T cells, also other anti-tumor specific T cells, e.g. recognizing individual mutation-derived T-cell epitopes, are most probably relevant for obtaining a clinical benefit [28, 29, 69, 70, 74]. I would therefore suggest that expanded tumor growth-specific T cells are mixed with the remaining T cell population prior to infusion. This would result in an infusion product enriched with tumor growth-specific T cells with possible superior reactivity against the metastatic cancer cell clones compared to normal expanded TIL infusion products.
Nodal is an important factor in the transforming growth factor (TGF)-β signaling pathway and serves to provide production of this cytokine [186], which is known to suppress the function of CD4 and CD8 T cells and other effector immune cells, and to increase Treg numbers by CD4 differentiation [187]. Taken together, this suggests that Nodal-specific T cells could function to block the immune suppression in addition to their possible targeting of metastatic cancer cells. Although it is not yet established, such mechanism would have same outcome as what is believed for self-reactive CD8 T cells recognizing cells presenting FoxP3, Indoleamine 2,3 dioxygenase and PD-L1, among others [172, 174, 188, 189].

The other melanoma-associated antigens included in the studies
The relevance of HLA-A2 restricted T-cell epitopes from MAGE-A3, gp100, tyrosinase and NY-ESO-1 for melanoma regression was shown in previous *in vivo* and *in vitro* TCR transduction studies in human patients, mice and cell lines, respectively [75, 119, 190, 191]. Human TRP-2 has to my knowledge not been investigated in such trials, but did in its murine form protect mice from B16 melanoma development after an DNA plasmid vaccination [192]. Our identification of HLA-A3 restricted T-cell epitopes from the differentiation antigens gp100 and tyrosinase are believed to possess the same importance. Affinity enhanced TCRs recognizing these epitopes would probably induce the same levels of clinical efficacy in HLA-A3 melanoma patients. Also at least a fraction of the remaining peptide sequences we found recognized in Study I are expected to contribute to anti-tumor reactivity. Selective BRAF inhibition upregulates the expression of melanoma differentiation antigens and induces specific T cell responses *in vitro* [193]. Differentiation antigens is the antigen class most frequently recognized within TILs from melanoma patients [28, 74], and these T cells may contribute to anti-tumor reactivity, also in regard to the impressive, although short-lasting clinical efficacy of BRAF inhibitors [25]. This suggests a strategy of selective BRAF inhibition to enhance the frequency of T cells specific for differentiation antigens prior to ACT. Potentially 5-Azacytidine treatment could additionally be included either prior to or after ACT to enhance the expression of CTAs. Treatment with these agents is expected to increase recognition of T-cell epitopes derived from the differentiation antigens and CTAs and might in turn enhance the cytotoxic reactivity against these.
The peptide sequences that were found relevant for T-cell recognition in Study I and II could also be included in a T-cell epitope panel for pre-isolation of melanoma-specific T cells prior to expansion of TILs for use in ACT. Such a pre-isolation step should obviously include all known T-cell epitopes, but a drawback is that our knowledge in this area is still limited [28, 74]; therefore all TILs from the initial lesion should be infused. It might, however, be beneficial to expand selectively the T cell populations known to recognize tumor cells and thus decrease the proportion of e.g. virus-specific T cells in the final infusion product. Upon unselected expansion it has been shown that the frequency of virus-specific T cells increases while the frequency of tumor-specific T cells decreases [28]. This strategy for optimizing the ACT approach could be combined with the above mentioned strategy for selectively enhancing the T cell populations recognizing antigens associated with tumor growth. To identify and isolate T cells specific for these melanoma-associated T cell epitopes, preferably combinatorial encoded MHC multimers for flow or mass cytometry could be utilized for high-throughput analyses [151–153]. An advantage of the current ACT strategy is that it is not HLA-restricted, and if such a selective expansion step using MHC multimer screening should be incorporated it would be important to cover the majority of HLA molecules present in the patient cohort. This would introduce the need for conditional ligands to a broad selection of HLA molecules, and our designed conditional ligands would greatly contribute to reaching this goal.
In addition to their possible relevance for therapy, the identified peptide sequences of relevance in melanoma should also be included in immune monitoring studies, to possibly increase our understanding of anti-tumor T-cell immunity in general and the identity of tumor rejection antigens in particular. Especially the immunological mechanism related to the encouraging clinical results of blocking the inhibitory molecules CTLA4 and PD-1 on T cells should be investigated further with a panel of T-cell epitopes, e.g. by flow or mass cytometry-mediated detection of specific T cells with MHC multimers. This would make possible subsequent ACT with selectively expanded TILs or tumor-specific T cells selected from peripheral blood once patients progress clinically on the blockade inhibitors. Blockade of these inhibitory molecules on T cells prior to ACT, e.g. by genetic engineering could also increase the frequency and/or reactivity of tumor-specific T cells within TILs. In the latter case, I expect the checkpoint inhibitor blockade therapy should be short-term, thus introducing ACT prior to the induction of tumor variants capable of escaping the T cells activated by the blockade inhibition.
Recently a method for TCR extraction from rare cell populations down to precursor frequencies of 0.00005% of the CD3 population was published [194]. This method takes advantage of MHC multimer-based pre-enrichment and single-cell sorting with following polymerase chain reaction (PCR) clonotype mapping, and enables characterization of the full-length TCR sequences from single T cells. The applicability of this method is thus also further

broadened from the possibility of producing MHC multimers restricted to more HLA molecules through the UV exchange method.

The above suggested therapeutic strategies could enhance clinical efficacy in the treatment of late stage melanoma patients. Immune therapy may, however, also be of use in patients with earlier disease. Intensive lymphodepletion enhances efficacy of ACT [27], but also induces considerable side effects prior to treatment necessitating that the patients are hospitalized for weeks. Instead, vaccination with dendritic cells pulsed with antigen or peptide or solely peptide vaccinations may be a good alternative to ACT, at least at earlier stages of disease. These vaccinations have frequently induced specific T cell responses in melanoma patients [105, 195], and although generally not very effective in terminal patients, they might induce sufficient anti-tumor reactivity in patients with less tumor burden and likely more immunogenic tumors. Also data from one study suggested that peptide-induced T-cell immunity prolonged the relapse-free period in the context of minimal residual disease [196]. If Nodal-specific T cells contribute to hindering of the metastatic process, a vaccination approach including peptides from Nodal might be beneficial in controlling tumor growth in these patients. This vaccination should also include HLA matched peptides from other antigens to induce a broad immune response. Immune vaccinations could additionally be of relevance in the prevention of relapse after for example chemotherapy by controlling minimal residual disease, consistent with the equilibrium phase in the immunoediting framework [18].

IMPLICATIONS FOR IMMUNE THERAPY OF MYELOPROLIFERATIVE DISEASES

The characterization of tumor-specific immunity in the blood stream of patients with myeloproliferative diseases is difficult compared to characterizing the TIL compartment in solid cancers. The PBMC compartment contains to a very large extent T cells with no relation to tumor, while TILs are obtained from the tumor tissue and, although a fraction of these are not directly tumor-specific as discussed above, to a larger extent can be regarded as related to the tumor. Also, melanoma is highly mutated while hematological cancers have a much lower mutation load [72]. The direct correlation between mutational load and immunogenicity is hard to establish, but there are some indications for a causal relationship (as discussed in [73]).

We showed that 5-Azacytidine treatment mediated tipping of the immunological balance through enhanced T-cell mediated anti-tumor reactivity and induction of CTA-specific T cell populations among patients with MDS, CMML and AML. We showed that 5-Azacytidine selectively affects the myeloid blasts and enhanced the frequency of CD8 T cells expressing the marker for release of cytotoxic granula CD107a after co-culture. We were, however, not able to show that the CTA-specific T cells were indeed responsible for this enhanced T-cell reactivity, and this should be further investigated. Also, the patients were not systematically clinically evaluated rendering correlation between immune reactivity and clinical performance impossible. The 5-Azacytidine-mediated induction of CTA-specific T cells is in line with previous results from combination treatments with the histone deacetylase inhibitor Valproate and the immune modulatory agent Lenalidomide [51, 54].

The involvement of the immune system in myeloproliferative diseases is well established, although not fully understood. Our pending clinical trial of 5-Azacytidine and CTA-peptide vaccination in higher-risk patients with myeloproliferative diseases might reveal the contribution of these CTA-specific T cells to the clinical outcome. We could not formally correlate the enhanced anti-tumor CD8 T-cell reactivity with the induction of CTA-specific T-cell immunity due to limited amounts of material. Therefore, from the data included in Paper III, we cannot rule out that the enhanced reactivity observed in the co-culture experiments could be directed against other antigens, e.g. patient-specific mutation antigens. Also, a previous study suggested that Decitabine additionally acts directly on the tumor cells also in low concentrations, along with its role as a demethylating agent [197]. 5-Azacytidine might possess the same action, and this direct cytotoxic action could lyse tumor cells recognized by the T cells, thus removing target cells for the induced CTA-specific T cells.

The coming clinical trial will be a non-randomized phase I trial, though, and we will not be able to conclude the superiority of the combination treatment compared to 5-Azacytidine treatment alone. Peptide vaccination with WT1 peptide in AML patients has previously suggested clinical efficacy and shown correlation between decreased mWT1 and progression-free survival [198]. Also in a one AML patient case-study WT1 peptide vaccination induced complete remission, probably due to the generation of a predominant T-cell clone detected within both blood and bone marrow of this patient [199]. These studies both demonstrate that immune responses can be induced through peptide vaccinations in this patient group.

The pathogenesis of myeloproliferative diseases demonstrates the complex role for the immune system in the interplay with carcinogenesis, described above. Early stage disease is characterized by decreased numbers of Tregs and an active adaptive immune system due to excessive apoptotic activity. The excessive autoimmune activity contributes to disease progression by elimination of haematopoietic progenitor cells. Late stage disease, on contrary, is characterized by increased numbers of Tregs and a general state of immune inhibition, like in many other cancers. Also frequency and reactivity of NK cells and CD8 T cells in these patients, among several other immune populations, are known to be inverted from early to late stage disease (reviewed in [130]). Our findings for 5-Azacytidine treatment are thus exclusively expected to be relevant for patients with late stage disease. Nevertheless, 5-Azacytidine may also be of benefit in lower-risk disease as a phase II clinical study showed induction of a significant proportion of complete and long-term responders in this patient group [200]. The possible immunological role for 5-Azacytidine in early stage patients, however, remains to be established [201].

It has been shown *in vitro* that expression of FoxP3 is regulated by promoter methylation [202, 203], and thus also *in vivo* demethylating treatment might induce Tregs. Further, *in vivo* mouse studies showed Decitabine-mediated induction of FoxP3 on naïve T cells and CD4 T helper cells, respectively, thus converting these functionally and phenotypically to Tregs [204, 205]. 5-Azacytidine treatment might induce the same effect in human patients. Both increasing and decreasing numbers of Tregs have been reported upon treatment with 5-Azacytidine prior to allogeneic stem cell transplantation in AML patients and in one high-risk MDS patient, respectively [206, 207].

We found no significant changes in the absolute numbers of Tregs in late stage patients upon 5-Azacytidine treatment and did not investigate their function. Importantly, it has previously been found that 5-Azacytidine impairs the function of Tregs in higher-risk MDS patients [208]. If this effect also occur in lower-risk stage patients remains to be established. Additionally, we found a 5-Azacytidine-mediated decrease in numbers of NK cells specifically

with the inhibitory phenotype $CD56^+CD16^+CD158b^+$, which could potentially be detrimental in early stage patients by enhancing NK cell-mediated killing of hematopoietic progenitor cells. It should be noted, though, that this effect was only modest in absolute numbers (although significant, $p<0.05$) and not present in regard to the proportion of $CD158b^+$ cells in the $CD56^+CD16^+$ population.
5-Azacytidine has been shown to upregulate the T-cell blockade molecules PD-1, PD-L1 and CTLA4 [136], a potential drawback effect for treatment of higher-risk disease in addition to the immune-stimulatory effects. A currently recruiting trial of epigenetic treatment with 5-Azacytidine and the histone deacetylase inhibitor Etinostat preceding treatment with Nivolumab (PD-1 inhibitor) in non-small cell lung cancer addresses this issue (ClinicalTrials.gov identifier: NCT01928576). Preliminary data suggests enhanced clinical efficacy compared to Nivolumab treatment alone (as presented at the annual meeting of Society of Immunotherapy of cancer 2013, National Harbor, MD, USA, Jon Bjørn, personal communication). This effect may be due to 5-Azacytidine-induced CTA-up-regulation with following increased number of myeloid blast-specific T cells, as we showed. The 5-Azacytidine analogue Decitabine also showed up-regulation of HLA-A1 and -A2 molecules, though, and if this holds true for 5-Azacytidine and other HLA molecules this effect could be responsible for enhancing T-cell reactivity against other TAAs too [209]. In addition, 5-Azacytidine-induced up-regulation of PD-1 can be blocked by Nivolumab with following clinical beneficial effects. Immunological outcome of this trial remains to be established. Furthermore, it could be interesting to study the effect of combined 5-Azacytidine and PD-L1 or CTLA4 blockade, or even a combination therapy of 5-Azacytidine and blockade of both of these molecules. Importantly, PD1 and CTLA4 blockade combination therapy has shown impressive results and it might be possible to further enhance the clinical outcome [9].
5-Azacytidine also inhibits accumulation and suppressive capacity of MDSCs due to induction of apoptosis and differentiation along the myeloid lineage in a mouse study by Mikysková and colleagues [135]. We measured the absolute counts of monocytic MDSCs and did not observe any effects of treatment in our patients, though, but this can be due to our analysis on cryopreserved material. We excluded measurement of the polymorphonuclear MDSC subset (phenotypically $CD3^-CD19^-CD56^-HLA\text{-}DR^-CD33^+CD11b^+CD14^{low}CD15^{high}$) as it has been questioned if the frequency of these cells remains stable upon cryopreservation [210, 211]. Mikysková and colleagues measured the effect of 5-Azacytidine on the total population of $CD11b^+Gr\text{-}1^+$ cells, including both the monocytic and the polymorphonuclear subsets of MDSCs in the mouse [135]. A potential selective effect on one of these subsets can therefore not been interpreted from their data. They apparently exclusively used fresh material for these analyses.
Promoter hypermethylation with following silencing of gene transcription occurs in most cancers [17], and thus treatment with hypomethylating agents could potentially be beneficial also under other conditions. The use of 5-Azacytidine in cancer treatment is extensively tested in clinical trials (see http://www.clinicaltrials.gov/ct2/results?term=azacytidine&Search=Search). Interestingly, expression of Lefty, the inhibitor of the Nodal protein, are known to be silenced in melanoma cells through promoter methylation [212]. It was previously shown that exposure of aggressive melanoma cells to an embryonic stem cell environment, with Lefty expressed, served to inhibit the metastatic potential of these melanoma cells [213]. As a consequence, it can be speculated that also hypomethylating treatment, for example with 5-Azactydine, would result in increased expression of Lefty and following inhibition of Nodal and the metastatic potential in melanoma cells.
CTA-specific T cell responses and upregulation of CTAs on cancer cells have been detected in patients with several different cancer types [52, 53, 214]. We showed that 5-Azacytidine treatment increases the population of CTA-specific T cells, although only transiently, and we speculated if this effect could be boosted by a peptide vaccination in parallel. We will soon have the chance to test this strategy in a clinical trial including patients with myeloproliferative diseases, and it could be interesting to also investigate this combination treatment in other cancers.
The 5-Azacytidine-mediated induction of CTA-specific T cells could additionally be relevant for ACT, both for patients with myeloproliferative diseases and with other cancers. The strategy could be based on the isolation of (specific) T cells from the blood for direct expansion with following infusion after a non-myeloablative lymphodepleting regimen. This regimen could be similar to the approach for ACT in melanoma patients [91], but aiming for depletion of also the abnormal cells in addition to the inhibitory immune cell subsets. If 5-Azacytidine is applied prior to this step, it would increase the population of CTA-specific T cells with potential enhanced clinical outcome.
The origin of myeloproliferative diseases is the bone marrow, though, and abnormal cells should preferably be targeted directly in this compartment. The bone marrow is a secondary immunological active compartment in addition to its well-known status as a primary immune organ with development and maturation of B cells. It thus also harbors significant amounts of T cells and dendritic cells able of taking up TAA, which could potentially be used in an immunotherapeutic context [215]. Interestingly the CD4:CD8 T-cell ratio in the bone marrow is 1:2, thus opposite of what is found in the blood [215], and homing of tumor-specific central memory CD8 T cells to the bone marrow has been shown previously [216, 217]. Furthermore, bone marrow-derived memory CD8 T cells showed long-term potent anti-tumor reactivity in a mouse study, which suggested control of dormant tumor cells situated in the bone marrow by these memory immune cells [218]. It should be noted, though, that one-third of the bone marrow CD4 cells in healthy individuals are Tregs whose suppressive function is even superior to blood-derived Tregs. Additionally, the bone marrow contains suppressive mesenchymal stem cells and a massive fraction of MDSCs [215]. CD8 T cells derived from the bone marrow, however, showed anti-tumor and anti-viral reactivity superior to blood-derived and similar to spleen-derived CD8 cells, respectively [216, 219, 220]. These bone marrow-derived CD4 and CD8 T cells could be used in an ACT approach for treating hematological cancers and other cancers with established bone marrow metastases, preferably in combination with drug-mediated inhibition of Tregs and MDSCs. Further addition of a pre-treatment step with 5-Azacytidine, to upregulate CTA-expression and induce specific CD8 cells, would possibly increase the anti-tumor reactivity for this treatment strategy. It might be a challenge, though, to infuse sufficient numbers of T cells to the bone marrow.
Finally, 5-Azacytidine therapy prior to treatment with CTA-TCR affinity enhanced transduced T cells could be a tempting possibility, thus upregulating CTA-expression on the tumor cells with following possible enhanced anti-tumor T-cell reactivity once the transduced T cells are administered. Data from recent clinical trials, however, has questioned the use of T-cell epitopes from CTAs for such therapy, at least regarding MAGE-A3, due to on-target effects (although directed against other of the highly ho-

mogenous MAGE variants) in healthy cells [123]. Also off-target reactivity against cardiac cells expressing an epitope similar to the epitope in question was found after treatment with T cells recognizing a T-cell epitope from MAGE-A3 [124, 125]. This finding suggests that the issue would not necessarily solve by choosing another antigen, even though NY-ESO-1 for instance is more stringently expressed only in cancer and testis cells in the healthy individual than MAGE-A3 is [221].

IS CHARACTERIZATION OF PERIPHERAL BLOOD IMMUNE CELLS RELEVANT FOR IMMUNE CELLS IN THE TUMOR MICROENVIRONMENT?

All immune cells investigated in the studies included in this thesis were obtained from peripheral blood, like the majority of other studies aiming for a better understanding of the interplay between the immune system and cancer. Peripheral blood is easy to obtain and isolation of the mononuclear cells is done through a simple gradient separation. Considering melanoma and other solid cancers, though, the important thing is to understand how the interplay is at the tumor site. Likewise it seems obvious that the most exact description of anti-tumor immunity would be obtained from tumor-infiltrating immune cells. Comparisons of measurements obtained within PBMCs and TILs have only rarely been done, but it seems, at least in one renal cell and two colon cancer studies, as conclusions drawn correlate between analyses of TILs and PBMCs [222–224]. Not surprising, the measured therapy- or disease-induced changes in the immune cell subsets are significant to a higher degree when data obtained from TILs are used compared to data from PBMCs. Also, a study in prostate cancer showed generally no differences when analyzing PBMCs while changes in the intra-tumoral immune-infiltrate were revealed using immunohistochemistry [225]. These data emphasize that changes of immune subsets intratumorally not necessarily are detected in peripheral blood. On the other hand, no reports on changes observed in the peripheral blood and not within the tumor could be found, and thus it seems correct to state that our findings within peripheral blood can be extrapolated to the intratumoral environment. Even though we believe the peripheral blood to some extent mirror the situation in the tumor microenvironment, we performed a screen within TILs from melanoma patients for T-cell recognition of the 25 peptide sequences we found recognized in peripheral blood. For the 17 peptide sequences included in Paper I we found three responses from a screening of 30 patients. Initially we detected T cell responses after a selective T-cell enrichment strategy, and the finding that such T cell populations are also present in the TILs in detectable frequencies suggest the clinical relevance of these peptides.

In regard to our study in myeloproliferative disease patients the situation is different from the above statements, as these diseases occur in the bone marrow with following secretion of abnormal cells to the blood stream. We were thus able to measure directly the T-cell reactivity against autologous myeloid blasts within the population of mononuclear cells obtained from blood samples. Also our investigations of CTA-specific T cells and the general populations of effector and inhibitor cell populations in peripheral blood can be regarded as almost “at the tumor site”, strengthening the conclusions from these measurements. Studies of bone marrow biopsies would have been optimal, but it was not possible to obtain these in this study. This was partly because 5-Azacytidine is standard treatment provided to the patients regardless of participation in our study and the excessive pain attributed to obtaining bone marrow biopsies.

5. CONCLUSION AND FURTHER PERSPECTIVES

Immune therapy of cancer has finally reached maturation. It has now been proven that the human immune system is capable of eradicating large tumor masses under the right circumstances and also that the immune system inhibits abnormal cell growth prior to clinical manifested cancer. Still, however, not all patients benefit from these therapies and we need to further optimize the clinical efficacy. To do this, further understanding of the interplay between immunity and epigenetic mechanisms on one side and carcinogenesis on the other is needed. The results obtained during my PhD and presented in this review will contribute to reach this goal. We confirm an indirect immunological action of the hypomethylating agent 5-Azacytidine and this suggests it is worthwhile to investigate immunological effects of also other epigenetic agents. In addition, we provide the scientific community with a collection of peptide sequences relevant for T-cell recognition in melanoma patients, part of these derived from a new immunological antigen of great interest. Furthermore, our design of 4 conditional ligands expands the possibility of measuring specific T cells within all fields of immunology with a subsequent improved understanding of the immunological mechanisms.

The immunological action of 5-Azacytidine will soon be investigated further in a clinical phase I trial, and results from this trial might imply if CTA-specific T cells indeed contribute to the clinical efficacy of this drug. The identification of Nodal as an immunological melanoma-associated antigen implies a number of further perspectives (as mentioned in the Discussion part), and additional investigation of the importance of Nodal in other cancers as well as in early stage disease is highly warranted. Also, our description of anti-melanoma T-cell immunity in patients expressing HLA-A1, -A3, -A11 or -B7 will enable broader monitoring of this patient group in future clinical studies. The obtained knowledge for these patients might also be of use for future therapy.

6. LIST OF ABBREVIATIONS

AML = Acute Myeloid Leukemia	Mart1 = Melanoma antigen recognized by T cells 1
ACT = Adoptive Cell Transfer	MDS = Myelodysplastic syndrome
BRAF = B-Rapidly Accelerated Fibrosarcoma	MDSC = Myeloid-derived suppressor cell
CTA = Cancer-testis antigen	NK = Natural Killer
Treg = CD4 regulatory T cell	NY-ESO-1 = New York esophageal squamous cell carcinoma-1
CMML = Chronic myelomonocytic leukemia	PBMC = Peripheral blood mononuclear cell
CpG = Cytosine and guanine at same RNA/DNA strand linked by a phosphodiester bond	PE = Phycoerythrin
CTLA4 = Cytotoxic T-Lymphocyte Antigen 4	PD1 = Programmed Death 1
ELSIA = Enzyme-linked immunosorbent assay	PD-L1 = Programmed Death 1 Ligand
ELISPOT = Enzyme-linked immunosorbent spot	PD-L2 = Programmed Death 2 Ligand
FACS =Fluorescence-activated cell sorting	SMT = Somatic mutation theory
FDA = Food and Drug Association	TCR = T-cell receptor
FoxP3 = Forkhead box P3	TOFT = Tissue organization field theory

gp100 = Glycoprotein 100	TGF = Transforming growth factor
HLA = Human leukocyte antigen	TAP1 and 2 = Transporters associated with antigen presentation
hTERT = Human telomerase reverse transcriptase	TAA = Tumor-associated antigen
IL = Interleukin	TIL = Tumor-infiltrating lymphocyte
MHC = Major Histocompatibility Complex	WT1 = Wilm's tumor protein 1
MAGE-A3 = Melanoma antigen gene-family-A3	

7. SUMMARY

This review is focused on research within three different areas of tumor immunology: discovery of new T-cell epitopes and a new immunological antigen (reported in Paper I and II), elucidation of the immunological effects of treatment with a hypomethylating drug (reported in Paper III) and discovery of new conditional ligands (Reported in Paper IV). Many melanoma-associated T-cell epitopes have been described, but 45% of these are restricted to human leukocyte antigen (HLA)-A2, leaving the remaining 36 different HLA molecules with only a few described T-cell epitopes each. Therefore we wanted to expand the number of T-cell epitopes restricted to HLA-A1, -A3, -A11 and -B7, all HLA molecules frequently expressed in Caucasians in Western Europe and Northern America. In Paper I we focused on the proteins gp100, Mart1, MAGE-A3, NY-ESO-1, tyrosinase and TRP-2, all melanoma-associated antigens frequently recognized by T cells from HLA-A2 patients. On contrary, in Paper II we wanted to investigate the protein Nodal as a novel immunological target.

We took advantage of a T-cell epitope mapping platform (Hadrup et al., Methods Mol Biol, 2009) in which HLA ligands are predicted by computer-based algorithms, further tested in the laboratory by an ELISA-based method and used for flow cytometry-based detection of specific T cell responses by use of combinatorial encoded Major Histocompatibility (MHC) class I multimers. This procedure resulted in 127 (Paper I) and 32 (Paper II) confirmed HLA ligands, respectively, which we used for screening of the T-cell recognition within peripheral blood mononuclear cell samples from melanoma patients. As spontaneous tumor-specific T cell responses tend to be of very low frequency and probably below the detection threshold of the method, we incorporated a T-cell enrichment step prior to the detection of these responses. Our screening of 39 melanoma patients resulted in 26 (17 different) T cell responses against the common melanoma-associated antigens and 10 (8 different) T cell responses against Nodal. We were further able to show processing and presentation on the cell-surface in K562 and melanoma cells expressing relevant protein and HLA molecules of four of these peptide sequences from tyrosinase, gp100 (2 peptides) and Nodal, respectively. However, one of the gp100 peptides has previously been described as a T-cell epitope. In addition to identifying new melanoma-associated T-cell epitopes we could thus describe Nodal as a new immunological antigen found of relevance in melanoma patients.

In Paper III we wanted to investigate if the hypomethylating drug 5-Azactytidine (Vidaza, Celgene Inc.) modulates the immune system in patients with myeloproliferative diseases. It has previously been shown that 5-Azacytidine-mediated demethylation of gene promoter regions results in enhanced transcription and expression of tumor suppressor genes and cancer-testis antigens. Cancer-testis antigens have frequently been recognized by T cells in many cancers, and we hypothesized that 5-Azacytidine treatment in the clinic would increase their frequency with resulting enhanced anti-tumor reactivity. We investigated separately the effect on T cells and tumor cells, and found that tumor cells affected by the treatment were better recognized, resulting in higher numbers of activated T cells, than tumor cells not exposed to 5-Azacytidine. No effects were observed on the T cell population. A screen of the T-cell recognition of 43 cancer-testis antigens in blood from our patients revealed increased T-cell recognition upon start of therapy which, though, stabilized or declined at later time points. We further investigated the general immune effector and inhibitory cell populations and found only minor effects of drug exposure, suggesting that 5-Azacytidine primarily affects the tumor cells. From these results we are currently initiating a phase I clinical trial of cancer-testis antigen-peptide vaccination in combination with 5-Azacytidine therapy for patients with myeloproliferative diseases.

In Paper IV we wanted to expand the library of conditional ligands for use with the UV light-mediated peptide-exchange method. This method enables high-throughput generation of MHC class I molecules with different peptide-specificities. These MHC monomers can be multimerized and used for detection of specific T cell populations by flow or mass cytometry. The HLA molecules are highly genetically variable and this necessitates unique design of conditional ligands for each HLA molecule. Thus, to screen for the T-cell recognition in a given setting within all patients or healthy donors present in a cohort, a broad library of conditional ligands is needed. We designed and evaluated conditional ligands for HLA-B*08:01, HLA-B*35:01 and HLA-B*44:02/03/05, all HLA-B molecules present in high frequency among Caucasians. In addition, we provided proof for the use of a conditional ligand first designed for HLA-B*15:02 in complex with HLA-B*15:01. We compared the staining patterns of HLA-B*15:01 and HLA-B*15:02 MHC multimers and found remarkable dissimilarities, although the two heavy chains in these MHC molecules only differ in a few amino acid positions.

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