Therapeutic targeting of immune escaped cancers

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Chapter 1

General introduction

Introduction

Effective immunotherapeutic strategies utilize the immune system of cancer patients to target and eradicate tumors[1,2]. Cancer associated- or cancer specific- antigens, including mutated neo-antigens, are presented in the context of MHC class I (MHC-I) at the surface of cancer cells and recognized by antigen-specific CD8 T cells[3,4]. Consequently, this recognition results in the stimulation of active T cells, to produce pro-inflammatory mediators and the release of cytotoxic granules aiming to kill the cancer cells and to eradicate the tumor[5,6]. However, cancer cells may acquire multiple resistance mechanisms to avoid recognition and killing by CD8 T cells[7,8]. This thesis evaluates how cancers avoid CD8 T cell mediated control, but more importantly, explores strategies to revert or overcome acquired resistance mechanisms which have led to the escape of these cancer cells.

CD8 T cell immunity in cancers

CD8 T cells have been described as the foot soldiers of the immune system in the war against cancer cells[9]. Increased numbers of tumor infiltrating CD8 T cells correlate with better clinical outcome in many cancers, including ovarian carcinoma and melanoma[9-11]. The CD8 T cells go through several developmental stages from thymocytes into mature CD8 T cells. Negative selection, or central tolerance, induces apoptosis of T cells with high affinity to "self" antigens, preventing the release of "self" reactive T cells into the circulation to avoid self-destruction. Premature T cells which have low affinity to "self" antigens, or do not recognize "self" antigens at all, escape central tolerance and enter the circulation as naïve T cells, until they are stimulated by professional antigen presenting cells to become activated and exert their function upon recognition of cells presenting its cognate antigen. T cells with low affinity to "self" antigens can get activated when tumors overexpress the antigen. However, peripheral tolerance for those so-called "shared antigens" is acknowledged as an important problem and limits the efficacy of immunotherapy. Immunotherapies utilizing CD8 T cells specific for "non-self" antigens therefore seem a more promising approach. Cancers with high mutational burden or cancers that have been established through infection with oncogenic viruses, like human papillomavirus or merkel cell virus, results in the presentation of many tumor-specific neoantigens and predicts for better clinical outcome[12,13]. Moreover, clinical efficacy after immune checkpoint blockade therapy is shown to be privileged toward cancers harboring mutation-derived neoantigens[14,15]. This implies that neoantigens specific T cells seem more potent for immunotherapies, and identification of tumor specific neoantigens is therefore a very important objective.

Immune checkpoints

Tight regulation of immune responses are critical for immune homeostasis and survival of the host. Uncontrolled immune responses towards pathogens and cancers can lead to tissue damage and auto-immune diseases. Therefore, immune responses are carefully regulated by stimulatory and inhibitory checkpoint molecules expressed by both immune- and antigen presenting- cells. Indeed, during cancer-associated chronic inflammation many inhibitory checkpoint molecules are upregulated on effector cells to dampen the immune response, stimulating cancer progression. In addition, inhibitory checkpoint molecules, like PD-L1, are concurrently expressed at high levels by cancer infiltrating myeloid cells, such as macrophages, neutrophils, and monocytes[16]. Many inhibitory checkpoint molecules are expressed on effector T cells, including CTLA-4, LAG-3, TIM-3, NKG2A and PD-1. Concomitantly, cancer cells also upregulate the ligands of these inhibitory checkpoint molecules like HLA-E and PD-L1/PD-L2 and bind to NKG2A and PD-1, respectively, to prevent immune activation. Blocking the inhibitory immune checkpoint "axis" using monoclonal antibodies have been very successful in the clinic in a selected group of patients[17,18]. Treatment with Pembrolizumab (anti-PD1) or Nivolumab (anti-PD1) have shown impressive overall response rates up to 57%, and progression free survival of 14.0 months in melanoma patients with high expression of PD-L1[19]. Of note, the amount of CD8 T cell infiltration and mutational load of the tumor is correlated to better efficacy of immune checkpoint blockade therapy, again underscoring the importance for CD8 T cells in this immunotherapeutic modality. Interestingly, PD-L1 negative melanomas had overall response rates of 41.3% with progression free survival of 5.3 months, indicating that blocking PD-L1 on cells other than cancer cells still had beneficial effects for the patients[20]. Understanding which subsets of cells are important to influence the delicate balance towards an active anti-tumor response is pivotal information for patient inclusion and treatment strategy.

T cell epitopes associated with impaired peptide processing

The presentation of antigens in MHC-I molecules depend on the processing of endogenous proteins into short peptides. Proteasomes chop proteins into small peptides which are translocated into the endoplasmic reticulum (ER) lumen through the transporter associated with antigen processing (TAP). TAP is a component of the peptide-loading complex (PLC). The PLC complex consists of beta-2 microglobulin (β2m), calreticulin, ERAAP, TAP, Erp57, tapasin, and MHC-I, and is of utmost importance for the stabilization and loading of empty MHC-I molecules with a peptide. After stable binding with a high affinity peptide, the peptide/MHC-I molecule is transported to the cell surface through the Golgi apparatus[21]. Limiting the supply of peptides transported from the cytosol into the ER lumen

by downregulating the peptide pump TAP strongly reduces the presentation of antigens in MHC-I molecules, and is often observed in cancer cells that escape immune recognition[22-25]. Interestingly, when cancer cells down modulate TAP, a novel subset of non-mutated neoantigens is allowed to be presented in MHC-I molecules. These antigens are called TEIPP (T cell epitopes associated with impaired peptide processing) and are exclusively presented by cancer cells with TAP-deficiency (reviewed in[26]). However, TAP-deficient cancer cells presenting TEIPP antigens usually have poor MHC-I presentation, limiting the endogenous immune responses. Therefore, to exploit TEIPP antigens for immunotherapy, TEIPP specific T cells need to be therapeutically stimulated to fully exploit their potential[27]. Elegant studies have shown the strength of TEIPP targeted immunotherapy in pre-clinical mouse studies[27,28]. A powerful strategy to target TEIPP in a clinical setting is by synthetic long peptide (SLP) vaccination[29] by which TEIPP peptides are administered, processed, and presented by host dendritic cells to induce TEIPP T cell immunity. However, since TEIPP antigens are TAP-independently processed by nature, feeding TAP-proficient host dendritic cells with TEIPP SLPs elongated with flanking sequences might not lead to efficient cross-presentation and consequent T cell activation. Therefore, an optimized dendritic cell vaccination strategy is necessary to fully exploit these tumor specific TEIPP antigens presented on MHC-I^{low} TAP-deficient cancers.

Interferon-gamma signaling pathway

Interferon gamma (IFNγ) is a pro-inflammatory cytokine and is critical for anti-tumor immunity[30-32]. It is the only member of the type II class of interferons[32], and is mainly secreted by NK cells, as well as type 1 CD4 T cells and CD8 T cells when they become activated. The IFNγ pathway is activated through binding of the cytokine with the heterodimic IFNγ receptor (IFNγR1/IFNγR2) and has many consequences for effector cells. Upon activation, phosphorylated JAKs induce the phosphorylation and nuclear translocation of STAT1, followed with binding to Interferon-gamma activated sequences (GAS). Consequently, transcription of many immunoregulatory genes are increased, including interferon regulatory factor-1 (Irf-1), Tap-1/2, and MHC-I[33]. Loss-of-function mutations in the IFNγ signaling pathway have all been associated with impaired therapeutic anti-tumor responses and reduced overall survival after checkpoint therapy. *In vivo* CRISPR-Cas9 studies identified PTPN2 as a negative modulator for the IFNγ signaling pathway by inducing dephosphorylation of STAT1 and JAK1. Additionally, many other genes have been discovered that negatively impact the sensitivity of cancer cells to respond to IFNγ in cancer cells and thereby inhibit the response to immunotherapy[34,35]. These studies underscore the essential role of this signaling pathway for immunotherapy[36-38]. Strategies to increase the responsiveness of cancer cells to IFNγ could lead to increased immunotherapeutic success.

Cancer metabolism and hypoxia

Cells depend on nutrients to sustain viability and to maintain their cellular functions. Two main pathways are involved in the generation of energy for the cell, namely glycolysis and oxidative phosphorylation (OXPHOS). Glucose is transported into the cell through the glucose transporter GLUT-1 where it is catabolized into pyruvate, a molecule important for feeding the TCA cycle. When ample oxygen is available, the metabolites produced in the TCA cycle are utilized in the electron chain transport complexes to generate energy. However, as tumors progress, oxygen becomes a limiting factor and cancer cells start to secrete angiogenic stimuli, including VEGF, to stimulate to growth of new blood vessel. In turn, the development of such highly disorganized vascular network in the tumor microenvironment limits the influx oxygen and ultimately results in severe hypoxia completely restricting OXPHOS metabolism in solid tumors.

Generating energy from glucose through glycolysis can occur without the use of oxygen, although much less efficiently. Interestingly, cancer cells often exploit glucose metabolism, even in the presence of oxygen. This is called the "Warburg effect". Although not exactly known why cancer cells utilize this inefficient energy pathway, data suggests that biomolecules generated when catabolizing glucose, supports the high proliferative capacity of cancer cells and thereby tumor progression. High glucose consumption rates and an inefficient vascular network, ultimately results in a tumor microenvironment which is deprived from both oxygen and glucose.

The harsh metabolic environment limits effector functions of many infiltrating immune cells that depend on glucose as their main source of energy. For example, studies have shown that "unused" GAPDH, as a result of glucose deprivation and inactive glycolysis metabolism, binds to the 3' UTR of the IFNy gene and thereby inhibits transcription of the inflammatory cytokine. In contrast, regulatory T cells survive much better in glucose deprived conditions as they can generate energy from fatty acid metabolism. These changes translate into a highly suppressive metabolic tumor microenvironment and is an important strategy how cancer cells can escape immune recognition.

Scope of this thesis

Great progress has been made in improving clinical responses with immunotherapy. However, it has also led to the increased awareness of the ability of cancer cells to acquire immune-escape mechanisms. It appears that the next challenge in immunotherapy could be the uncovering and understanding of secondary resistance mechanisms. This might result in novel immunotherapeutic strategies to overcome the discouraging results of immunotherapy on patients with immune escaped cancers.

The ligands for several inhibitory checkpoint molecules expressed by T cells can be constitutively or adaptively expressed by both tumor cells and myeloid cells. In **chapter 2**, we dissected the role of PD-L1 expression on either host stromal cells or cancer cells and their effects on clinical outcome during anti-PD-1 therapy in mouse tumor models. To summarize, we first showed that lack of PD-L1 expression on cancer cells greatly delayed cancer outgrowth compared to PD-L1 WT cancer cells. A cumulative anti-tumor effect was observed in mice harboring PD-L1 negative tumor cells and were treated with PD-L1 blocking therapy, suggesting that PD-L1 on host stromal cells contribute to the inhibition of tumor reactive T cells.

Studies have shown that metabolic alternations in the tumor microenvironment creates a harsh environment for immune cells to exert their anti-tumor functions. However, not much is known about how metabolic stress affects the immunogenicity of cancer cells. In **chapter 3** we explored the effects of nutrient deprivation on antigen presentation by cancer cells[39,40]. We found that oxygen- and glucose-deprivation strongly led to reduced MHC-I presentation and abrogated CD8 T cell recognition of cancer cells. Mechanistically, oxygen- and glucose-deprivation decreased the ability of cancer cells to respond to IFNy via increased PI3-kinase pathway signaling with the result that the cancer cells failed to activate the antigen processing pathway. Pharmacological inhibition of the PI3-kinase pathway resulted in restored MHC-I expression and improved CD8 T cell recognition.

In **chapter 4**, we review the current knowledge on the unique aspects of TEIPP cancer antigens, in order to set the stage for our efforts to exploit TEIPP antigen-based immunotherapy in patients. **Chapter 5** describes a hybrid forward-reversed immunological screen to identify human TEIPP antigens. In short, the human proteome was scrutinized for peptide sequences from proteins with high predicted HLA-I binding affinity and an ER-localization. This hit list was matched with a database of peptides which were exclusively eluted from human cancers but not healthy cells. For one lead candidate (p14), we isolated CD8 T cell clones specific for this antigen, which recognized various TAP-deficient cancers, including lymphomas, renal cell carcinomas, colon carcinomas, and melanomas but did not respond

to normal cells.

In **chapter 6**, we describe an approach to apply the SLP vaccination platform for the induction of TEIPP-specific T cells. We were able to induce TAP-dependent processing of the epitope without losing the specificity of the activated TEIPP T cells. This resulted in expansion of TEIPP-specific T cells and efficient immune responses towards TAP-deficient melanomas. An overview how TEIPP targeted immunotherapies may come in the clinic can be read in **chapter 7**.

Collectively, this thesis covers multiple topics about acquired immune resistance mechanisms by cancer cells and how we can counteract them. A general discussion on how therapeutic strategies targeting multiple aspects of these immune resistance mechanisms may lead to improved immunotherapies, can be read in **Chapter 8.**

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