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Therapeutic targeting of immune escaped cancers

Marijt, K.A.

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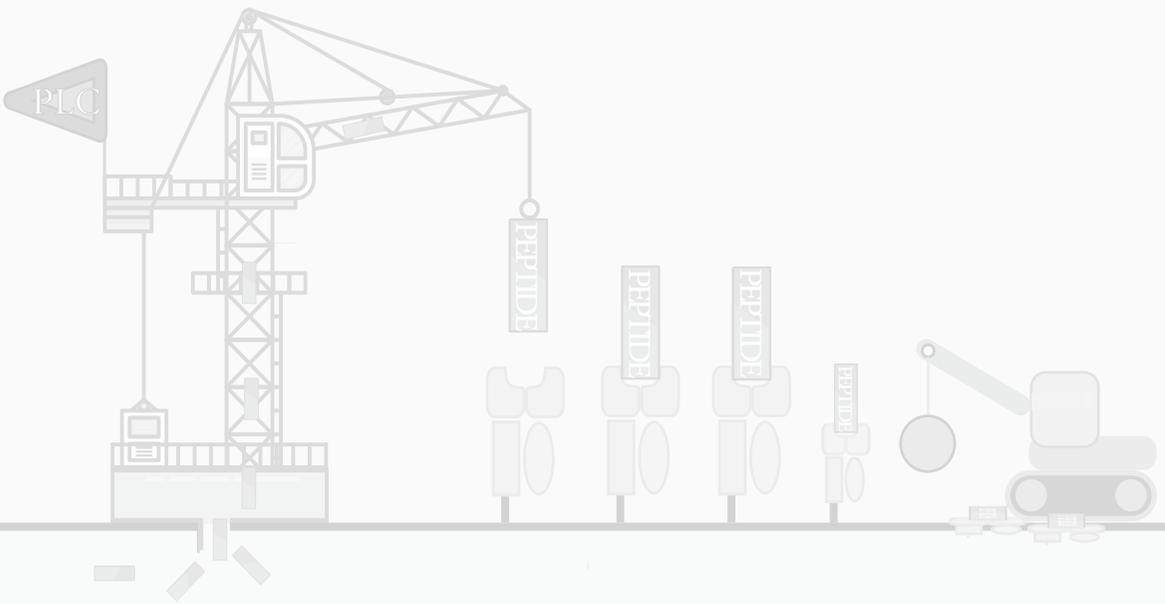
Author: Marijt, K.A.

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Chapter 8

General discussion



General discussion

MHC class I (MHC-I) antigen presentation plays a pivotal role in anti-tumor immunity. High surface expression of MHC-I molecules is generally correlated with high CD8 T cell infiltrate and improved overall survival in many cancers, including melanoma, lung carcinoma, and ovarium carcinoma¹⁻⁴. In contrast, partial (>50% of cells) or complete loss of MHC-I surface expression is associated with lower overall survival and primary resistance to multiple types of immunotherapy in many cancer patients⁵⁻⁸, underscoring the importance of MHC-I presentation for spontaneous and therapy-induced anti-tumor immunity. Expression of additional molecules in the tumor microenvironment (TME), such as PD-L1 and HLA-E inhibitory checkpoints, further shape immune responses. The presence of immune cells and the expression of immune-related genes together determine the ‘immune landscape’ or ‘immune contexture’ of cancers, while the local production of interferons (IFN) strongly impacts this environment⁹. Notably, immune contexture also includes infiltrating myeloid cells, such as tumor associated macrophages which also highly express PD-L1 immune suppressive molecules (**chapter 2**). Although MHC-I and PD-L1 are both regulated by the IFN pathway, an in-depth study on immune escape of NSCLC showed that the expression of co-inhibitory markers and the loss of MHC-I expression are two independent mechanisms of immune evasion¹⁰. This classifies tumors into different “types” depending on their surface expression of MHC-I and PD-L1^{10,11}. The differential expression of MHC-I and PD-L1 suggests that immune escape of cancer cells occurs through a multitude of distinct “hard-wired” and “soft-wired” modifications¹²⁻¹⁴ and knowing which of the mechanisms underlie immune escape determines which immunotherapeutic strategy has the most potential for clinical success. Here, we discuss several acquired immune resistance mechanisms and possible therapeutic strategies to overcome the resistance for immunotherapies.

Importance of IFN-signaling for T cell immunity

The IFN γ signaling pathway is essential for regulating innate and adaptive immune responses¹⁵. The absence of IFN γ production or cellular responsiveness sensitizes the host for microbial infection, supporting the importance of the cytokine IFN γ . Of note, IFN γ also plays important roles in inhibiting tumor angiogenesis¹⁶, and directly has cytotoxic and cytostatic effects delaying tumor progression¹⁷. IFN γ is mainly secreted by activated type-I T cells and NK cells upon recognition of their target cells. IFN γ binds to the IFN γ -receptor, activating the IFN γ signaling pathway by phosphorylation of the JAKs and STATs, nuclear translocation, and binding of STAT1 homodimers onto interferon-gamma activation sequences. This triggers the transcription of many immunoregulatory genes, including the expression of antigen presentation molecules like MHC-I, but also immune-inhibiting receptors such as

PD-L1. Therefore, the exact downstream effects of IFN γ is context dependent and essential for regulating the efficacy of an anti-tumor immune response.

Cancers with high PD-L1/MHC-I expression are likely IFN γ -sensitive tumors. These tumors usually have a preexisting intratumoral T cell population, but are limited in exerting their effector function as a result of high inhibitory checkpoint expression. Patients with such tumors are most likely responders to PD-1/PD-L1 blockade mono-therapy, since alleviation the PD-1 inhibitory signals will reinvigorate preexisting anti-tumor T cell immunity.

However, cancers with defects in the IFN γ signaling pathway or downstream effector genes will not increase surface expression of MHC-I upon IFN γ stimulation, and will theoretically display weaker T cell reactivity to their tumors. As a consequence, reduced clinical response rates to checkpoint blockade therapies are indeed observed in this category of patients⁶. Moreover, two studies implicated the crucial role of proficient IFN γ signaling in melanoma patients who acquired resistance to PD-1 or CTLA-4 blockade therapies^{18,19}, and in patients who never responded to therapy²⁰. Loss-of-function mutations in the IFN γ -receptor, and Jak1/ Jak2 caused the lack of response in these patients. Alternatively to these hard-wired defects, *in vivo* CRISPR-Cas9 studies have identified APLN2, PTPN2, and LNK as negative modulators of the IFN γ signaling, also limiting the efficacy of immune responses²¹⁻²³. For patients with IFN γ -insensitive tumors, restoring the IFN γ signaling would be essential in order to fully benefit from immunotherapy, including checkpoint blockade therapies. Recently, using MEK inhibitors to increase IFN γ -sensitivity of cancer cells received considerable attention for immune combination therapies²⁴⁻²⁶. Inhibition of MEK signaling results in downregulation of LNK expression, increases the IFN γ signaling, and restores MHC-I presentation^{23,25,26}. Similarly, inhibition of EGFR, upstream of MEK, also results in increased sensitivity to IFN γ and higher levels of MHC-I after IFN γ stimulation^{27,28}. However, by enhancing the IFN γ signaling, it concurrently increases the expression of the inhibitory immune checkpoint receptor PD-L1, still limiting an effective immune response²⁴. Therefore, IFN γ amplifying therapeutics, like EGFR and MEK inhibitors, need to be combined with immune checkpoint blockade therapy to become optimally effective as treatment for IFN γ -insensitive cancers, as suggested by several pre-clinical^{24,26,29} and clinical studies³⁰.

PI3K inhibitors for combination immunotherapy

Cancer cells deprived from oxygen and glucose similarly lose their ability to perceive an active IFN γ response, resulting in low MHC-I and PD-L1 expression. These metabolically stressed cancers can therefore also be classified as IFN γ -insensitive, MHC-I^{low} tumors (**chapter 3**) and are also likely to benefit from therapies that aim to increase MHC-I presentation in combination with checkpoint blockade therapy. We experimentally showed that inhibi-

tion of PI3K signaling restored IFN γ signaling, increased MHC-I presentation, and improved T cell recognition *in vitro* of cancer cells in nutrient-deficient as well as nutrient-proficient conditions. Of note, although PI3K and MEK are downstream of EGFR signaling, they are part of two different pathways and execute distinct effector functions³¹. Therapeutic inhibition of PI3K signaling in combination with immunotherapy could therefore be an interesting new strategy to improve anti-tumor responses where IFN γ sensitivity is abrogated.

PI3K signaling is important for cancer cells, but is also essential for homeostasis of immune cells. The PI3K family can be divided into three classes, namely class I, II and III³². In our studies we used LY294002 and wortmannin (**chapter 3**), which are pan-PI3K inhibitors, inhibiting all classes of the PI3K family. It is important to determine which PI3K family is responsible for the observed MHC-I effects, since inhibition of different PI3K classes has differential effects on immune cells and therefore also may affect the therapeutic strategy. Several studies suggested that class I PI3K isoforms directly affect innate and adaptive immune cells. Selective inhibition of the gamma isoform of PI3K (PI3K γ), highly expressed in infiltrating tumor associated myeloid cells, restored sensitivity to immune checkpoint blockade as a result of reduced immune suppression by M2-like macrophages in tumors^{33,34}. Moreover, inhibition of the PI3K isoform delta (PI3K δ) has been linked to break regulatory T cell mediated immune tolerance³⁵. Interestingly, *ex vivo* inhibition of PI3K and its downstream effector AKT were shown to increase the formation of memory-like CD8 T cells and prolonged anti-tumor responses^{36,37}. However, class I PI3K signaling also plays critical roles during development and differentiation of CD4 and CD8 T cells³⁸. Inhibiting PI3K isoform gamma or delta during thymopoiesis stops T cell development at the CD4/CD8 double negative stage³⁹, while PI3K γ is critical for exerting T cell function in matured T cells⁴⁰. Interestingly, inhibition of AKT or mTOR, which are the main effector molecules of class I PI3K, did not restore MHC-I surface expression on tumor cells under oxygen and glucose deprived conditions (**chapter 3**, data not shown) and perhaps other PI3K classes or other non-canonical PI3K-signaling pathways⁴¹ play important roles at the intersection of the PI3K and STAT1 axis. Class II PI3K was shown to be essential in TCR mediated activation of T cells by modulating the activation of potassium ion channels^{42,43}. In cancers, increased expression of class II PI3K (PI3K-C2 β) was suggested to be implicated in MEK1/2 activation, which implies that class II PI3K might have a role in MEK1 mediated suppression of MHC-I presentation^{23,44,45}. Class III PI3K, or Vps34, plays a critical role in autophagy and endocytosis and is important for in the intracellular trafficking of the IL7-receptor in tumor cells⁴⁶. Mice lacking Vps34 in T cells displayed a decreased number of T cells, suggesting that Vsp34 is an important regulator of naive T cell homeostasis, modulating IL-7R α trafficking, signaling, and recycling.

Collectively, understanding the distinctive roles of the several isoforms of PI3K is critical for development of effective combination therapies, since modulation of the activity of these PI3K isoforms has differential effects on either tumor cells and host T cells. A delicate balance of single or combinatory specific PI3K inhibitors might be required to ensure improved IFN γ signaling and MHC-I presentation without compromising the effector function of immune cells. Therefore, the use of PI3K-signaling blocking compounds in combination therapies should be carefully evaluated with respect to their effects on T cells.

Immunotherapy using TEIPP antigens

Tumors with minimal MHC-I expression, irrespective of PD-L1 expression, might be a witness of previous ongoing immune response that resulted in acquired immune escape by downmodulation of MHC-I antigen presentation, making the tumor “invisible”. Soft-wired loss of TAP function is often observed in cancers that lost their antigen presenting capabilities, especially in lung carcinomas, and melanomas^{47,48}. Single agent checkpoint blockade therapy will most likely not result in an effective anti-tumor response, given the fact that the tumor (neo)antigens, that were initially recognized by tumor-reactive TILs, are not presented anymore by the immune escaped MHC-I^{low} cancer cells. Other T-cell based immunotherapeutic strategies, including dendritic cell vaccination, adoptive T cell therapy, and TCR-tg T cell transfers which target conventional tumor antigens are probably also not effective, because in all cases the presentation of their targeted (neo)antigens depends on TAP function²². Therefore, identification of alternative tumor antigens specifically presented on MHC-I^{low}, antigen processing-deficient cancer cells is an important objective. We designed a novel strategy to successfully identify those tumor antigens have resulted in the identification of so-called TEIPPs (**chapter 4**). TEIPPs are a subset of non-mutated antigens solely presented on MHC-I^{low} cancers as a result of downregulated TAP1/2 function⁴⁹. TEIPP-presenting cancer cells typically have no TEIPP specific T cell infiltrate given the lack of sufficient MHC-I presentation which limits T cell activation through direct tumor recognition⁵⁰. However, vaccination strategies were capable of igniting strong TEIPP-directed T cell immunity, and a multitude of studies in TEIPP animal models^{50,51} motivated the search for human TEIPP antigens (**chapter 5**). After identification of 16 novel HLA-A*0201 binding TEIPP epitopes, we now look forward to therapeutic exploitation of these novel tumor antigens.

Vaccinating strategies with TAP-independent TEIPP peptides could initiate a CD8 T cell response of MHC-I^{low} cancers. Indeed therapeutic intervention via dendritic cell vaccination or therapeutic peptide vaccination resulted in priming of TEIPP-specific T cells and induced efficient homing to TAP-deficient MHC-I^{low} tumors in mouse models⁵¹. It can be envisioned that this results in increased local IFN γ levels in the tumor microenvironment. As most

TAP-deficient cancers typically have “soft-wired” epigenetic alterations^{47,48}, the epigenetic inhibition of TAP could be unleashed by this IFN γ and re-activate the antigen processing machinery. This would theoretically result in reduced presentation of TEIPP antigens and re-presentation of conventional tumor antigens, which may restore the efficacy of TILs responding to the conventional antigens. Alternatively, one could also add conventional tumor antigens to such TEIPP vaccines as this would also stimulate the generation of TAP-dependent peptide specific CD8 T cells and could boost the efficacy of the vaccine. This concept has been attractively shown by the group of Dr. Mami-Chouaib, where they combined long peptides of conventional tumor antigens together with a TEIPP peptide. With this combination vaccine they were able to strongly inhibit outgrowth of an immune escaped TAP-deficient lung carcinoma in a mouse model (**chapter 7**)⁵². Combinatorial vaccination therapy with checkpoint blockade therapy, or TCR gene transfers should be explored to fully take advantage of the re-activation of anti-tumor responses toward immune escaped cancers. In **chapter 6** we showed an approach for the development of a TEIPP vaccine as synthetic long peptide (SLP) and also demonstrated that gene transfer of a TEIPP TCR confers their unique specificity.

The exact threshold for decreased TAP function in cancer cells that is required to optimally present TEIPPs remains to be determined. This makes it difficult to predict how many cancer patients are susceptible to TEIPP targeted therapy. Analyses of *in vitro* cultured human cancer cell lines for TEIPP presentation, revealed that two out of six cancers were spontaneously recognized by TEIPP specific T cells. In four other cancer lines, further inactivation of TAP was necessary for proper TEIPP recognition (**chapter 5**). One cancer line, strongly recognized by TEIPP T cells, had relatively low levels of TAP, underscoring the importance of TAP downmodulation for efficient presentation of TEIPP. In a cohort of 135 lung cancer samples, approximately 53% and 32% displayed low to intermediate expression levels of TAP2, respectively⁵². For melanoma, TAP downregulation is observed in 50% of the primary lesions and a remarkable 83% in metastatic lesions⁵³, suggesting that a significant proportion of cancer patients might be eligible for TEIPP therapy. However, TEIPP antigen expression, proficiency of other components of the MHC-I antigen presentation pathway, and presence of IFN γ in the TME influencing TAP expression are additional factors expected to strongly influence efficient TEIPP presentation. Notably, when both antigen processing and MHC-I expression are reduced, for example in the case of nutrient deprived IFN γ -insensitive B16F10 cancer cells, no increased recognition was observed by TEIPP specific T cells, although the cancer cells were not expressing TAP proteins (data not shown). Interestingly, the nutrient deprived cancer cells were equally well recognized by TEIPP T cells as their proficient counterparts, suggesting that TEIPP peptides were not hampered in their presentation, in contrast to conventional cancer antigens (data not shown). In addition, IFN γ stimulation of cancer cells with hard-wired loss-of-function TAP mutations were bet-

ter recognized by TEIPP specific T cells, probably due to increased MHC-I gene transcription. Therefore, it seems that the ratio of TAP-mediated influx of peptides and availability of empty MHC-I molecules in the ER are important parameters for presentation of TEIPP antigens. Further studies should provide answers on the percentage of cancer patients that are sensitive for TEIPP therapy.

Alternatively, exploration how to induce TEIPP antigen presentation on cancers cells might be an interesting approach for combination immunotherapies. Interesting progress has been made in generating tools to deliver therapeutics specifically to cancer cells. This way, we could specifically target cancer cells with “tools” to temporarily abrogate TAP function in order induce susceptibility to TEIPP-specific T cells. Oncolytic viruses, which particularly replicate in cancer cells, can be armed with genetic products, like TAP-downmodulating proteins or siRNAs⁵⁴. Engineering oncolytic viruses to express siRNA's towards TAP1/2 might induce TAP-deficiency specifically in cancers cells, making those infected cancer cells vulnerable for TEIPP therapy. Alternatively, aptamers conjugated with siRNAs are also used in the clinic to specifically target cancer cells to confer cancer specificity⁵⁵. Currently, together with the group of Dr. Gilboa (Miller school of Medicine, Miami, USA) we are exploring the possibility of temporarily downregulating TAP function via siRNA in tumor cells by tumor directed targeting by nucleolin-binding aptamer conjugates. Our data show that treatment of TAP siRNA-aptamers specifically targets cancers cells, leaving healthy cells untouched. In preclinical mouse tumor models, treatment with TAP siRNA-aptamers results in strong inhibition of outgrowth of tumors and increased survival in mice⁵⁶. The temporal decrease of TAP and thus MHC-I molecules ignited a broad immune response, including TEIPP-specific T cells. Follow up studies in which aptamer-targeted vaccines are included even show more dramatic anti-tumor responses⁵⁶. These studies highlight the benefits of inducing alternative tumor antigens for effective immunotherapies of immune-escaped cancers.

Hard-wired loss of MHC class I presentation

Loss-of-function mutations in the $\beta 2m$ or HLA-A,B,C locus completely abrogates the presentation of MHC-I molecules. Especially in mismatch repair deficient (MMR-d) colon carcinomas as well as lung carcinomas, total loss of MHC-I expression is often observed^{14,57}. Several studies illustrated that cancers acquired immune resistance after combination therapies with adoptive T cell transfer and checkpoint blockade, due to loss of HLA-I and/or $\beta 2m$ ^{7,58,59}. IFN γ stimulation or therapeutics amplifying the IFN γ signaling will not restore MHC-I antigen presentation in these type of cancers, making all previously discussed therapeutic approaches ineffective. However, NK-cells are innate immune cells specialized in targeting HLA-I negative cancers cells⁶⁰. Unlike T cells, NK-cells do not have TCRs to recognize antigens, but are activated when inhibitory receptors are overruled by activating

receptors. The inhibitory receptors of NK cells bind to MHC-I molecules, which explains why NK cells mainly target MHC-I negative cells. One dominant inhibitory checkpoint molecule is NKG2A which is expressed on a majority of NK cells and limits the effector function of NK cells upon binding with non-classical MHC-I molecule HLA-E, overexpressed on most tumor cells^{61,62}. Monalizumab, an anti-NKG2A blocking antibody, enhances NK cell activity against multiple tumor cells and is currently tested in several phase II clinical trials in combination with anti-EGFR (cetuximab) or anti-PD-L1 (Durvalumab) for solid cancer patients⁶². Of note, monalizumab not only antagonized the inhibitory signal on NK cells, but also on immunotherapy activated CD8 T cells, improving immune responses through distinct mechanisms⁶³.

Another exciting development is the manufacturing of TCR gene transfers in NK cells⁶⁴. NK cells that obtained expression of a TCR, exerted the function of T cells phenotypically, metabolically and functionally, while maintaining their NK cell effector function. This way, when these tailored anti-tumor effector cells are used in (TEIPP) neoantigen mediated therapies, it might prevent cancer cells to immune escape by MHC-I loss, due to direct targeting of the NK cell effector function of these TCR-NK-cells.

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