Novel approaches towards cancer-directed immune checkpoint inhibition
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DOI:
10.33612/diss.737906343

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2023

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Introduction to the thesis

Emily M. Ploeg
INTRODUCTION TO THE THESIS

Cancer is a complex disease characterized by uncontrolled cell growth with metastatic spread that caused over 10 million deaths worldwide in 2020 alone¹. In the Netherlands, specialists indicate that in 2023 over 110,000 people will be diagnosed with cancer, and that an average of 45,000 people will eventually succumb to this disease, as shown by data of the Dutch Cancer Society. Globally, the cancer-related mortality is expected to reach 22 million by 2030². Currently, surgery remains the mainstay of treatment for most types of advanced solid tumors, which is often combined with chemo- or radiotherapy regimes. Unfortunately, many cancer patients subsequently develop refractory relapses. Therefore, novel strategies to eliminate (refractory) cancer cells are urgently needed. In this respect, exploitation of cells and/or molecules of the human immune system to fight advanced cancer seems to be a promising approach.

The idea of using the immune system to fight cancer originates from William Bradley Coley (1862-1936), who is now hailed as the ‘Father of Cancer Immunotherapy’. Coley was an orthopedic surgeon who noticed that bone sarcoma patients exposing significant postoperative infected wounds developed spontaneous regression of unresected tumors. This observation inspired him to develop the first documented cancer immunotherapy agent widely known as “Coley’s toxin”³. Coley’s toxin contains a mixture of toxins filtered from live and heat-inactivated bacteria, such as Streptococcus pyogenes and Serratia marcescens, with the hope of inducing erysipelas and subsequently stimulate the immune system to eliminate the cancer cells. Between 1893 and 1963, thousands of patients with several types of cancers, including sarcoma, lymphoma, and testicular carcinoma, achieved durable or complete remission. However, in the early 20th century, oncologists adopted surgery, chemotherapy, and radiotherapy as standard treatments because of a lack of scientific knowledge regarding the mechanism of action and reproducibility of Coley’s toxin.

For more than 100 years, scientists have attempted to engage the immune system in order to eliminate cancer cells. Even though for some types of cancer there have been (modest) therapeutic developments, the discoveries of James P. Allison and Tasuku Honjo accelerated the progress of multiple new immunotherapeutics into clinical development. In 2018, both men were awarded with the Nobel Prize in Physiology or Medicine for their work on cancer immunotherapy, which significantly boosted the field of immuno-oncology in the current era. Their discovery of immune checkpoints CTLA-4 and PD-L1/PD-1 has fundamentally changed the outcome for a certain group of cancer patients.

Immune checkpoints are ‘control molecules’ present on various cells types, including immune cells, that regulate the immune system. These molecules consist of either stimulatory immunoreceptors or inhibitory immunoreceptors with their corresponding ligand. Normally, immune checkpoints are essential to maintain self-tolerance, preventing autoimmunity, and controlling the duration and extent of immune responses in order to minimize collateral damage to healthy tissue. However, cancer cells take advantage of these molecules as they downregulate stimulatory immune checkpoints and upregulate inhibitory immune checkpoints on their cell surface, thereby achieving immune evasion. The inhibitory immune checkpoint that has received the most attention in recent years is the programmed cell death protein 1 receptor (PD-1) and its ligand PD-L1. PD-L1 normally dampens immune responses
in a timely and localized manner by inhibiting the capacity of antigen-experienced PD-
1^{pos} cytotoxic T cells to proliferate and eliminate target cells. However, cancer cells
misuse PD-L1 by incapacitating PD-1^{pos} cytotoxic T cells in the tumor
microenvironment. PD-L1-inhibiting antibodies, such as atezolizumab, avelumab, and
durvalumab, have shown unprecedented promise in selected cancer types, particularly
in melanoma, non-small-cell lung cancer, and renal cancer. Unfortunately, even in
these cancer types, only 10%–30% of patients respond to PD-L1-blocking therapy.
Moreover, in many other common cancer types responses are rare.

Over the past two decades, a broad range of different immune checkpoint
proteins have been identified, including CD73 and CD47, which are currently being
explored as potential targets in cancer immunotherapy. Currently, the FDA has
approved 14 different immune checkpoint antagonists for more than 12 different types
of cancer. Additionally, over 18 immunostimulatory agonists are currently under
investigation.

OUTLINE OF THE THESIS

In Chapters 2 and 3, we pre-clinically evaluated novel inhibitors of the CD73-
adenosine immune checkpoint. CD73 is a cell-surface-expressed ecto-enzyme that is
key to maintaining immune system homeostasis through the hydrolysis of autocrine
and paracrine danger signals conveyed by extracellular ATP (eATP) into anti-
inflammatory adenosine (ADO). ADO molecules engage the immunosuppressive
actions of ADO receptors on various locally present immune cells, thereby providing a
self-limiting mechanism for the timely local resolution of the immune response.

Owing to intrinsic high metabolic stress, numerous cancer cell types excrete
remarkably high levels of pro-inflammatory eATP, which is rapidly converted into anti-
inflammatory ADO by concurrently overexpressing CD73. Subsequently, diffusion of
tumor-produced ADO molecules results in the formation of a potent
immunosuppressive ‘halo’ that acts not only locally in the tumor microenvironment,
but also outside of the tumor site. Consequently, a halo of ADO molecules chronically
suppresses the anticancer immune response, which promotes the induction of immune
tolerance, immune escape, and subsequent cancer progression.

Strategies to inhibit the enzyme activity of CD73 have shown promising
preclinical capacity to overcome ADO-mediated immunosuppression in a variety of
cancer types. This includes the use of antagonistic CD73 antibodies like oleclumab,
CD73-knockdown by short interference RNAs (siRNA), and small-molecule inhibitors
(SMI) like adenosine 5'-(α,β-methylene)diphosphate (APCP) or derivatives thereof,
which act as competitive inhibitors of the enzyme activity of CD73. Currently,
several multicenter trials are ongoing to evaluate the clinical potential of CD73-
inhibiting antibodies or SMI in patients with advanced solid malignancies, including
various carcinomas. Unfortunately, recent early-on midterm reports of CD73-inhibiting
antibody oleclumab indicate that its efficacy appears modest at best. Of note, no
clinical trial results have been published on CD73-SMI.

Unfortunately, CD73-inhibiting strategies may suffer from ‘on-target/off-
tumor’ binding to CD73 molecules present on normal cells, which limits their CD73-
inhibitory activity at the tumor site(s). Consequently, inhibition of the CD73 immune
checkpoint may result in the generalized activation of T cells, potentially leading to life-
Chapter 1

threatening immune-related adverse events analogous to those associated with PD-1/PD-L1 and CTLA-4 immune checkpoint inhibitors.

Additionally, in Chapter 2, we describe how we serendipitously discovered that the treatment of carcinoma cells with antagonistic CD73 antibody oleclumab promoted several pro-oncogenic features of cancer cells, including the upregulation and phosphorylation of the Epidermal Growth Factor Receptor (EGFR), tumor cell proliferation, and resistance to cytotoxic agents and ionizing radiation. To address these issues, we designed a novel tetravalent bispecific antibody (bsAb), designated bsAb CD73xEGFR, engineered to inhibit CD73 pro-oncogenic activities in an EGFR-directed manner. BsAbs are a promising class of immunotherapeutics with the potential to improve the clinical efficacy and safety of immune checkpoint inhibitors. Moreover, tetravalent bsAbs are known to have enhanced avidity towards surfaces that simultaneously expose both target antigens of interest, as they have up to four binding sites available for the enhancement of functional interactions. In this respect, tetravalent bsAbs appear better suited to improve the efficacy and selectivity directed at the CD73 immune checkpoint on cancer cells.

Treatment of various carcinoma cell types with bsAb CD73xEGFR induced rapid and prolonged co-internalization of cancer cell surface-expressed CD73 and EGFR. Moreover, treatment of tumor-bearing immunocompetent mice with bsAb CD73xEGFR outperformed oleclumab in terms of restoring the anticancer activity of ADO-suppressed T cells and enhancing the intratumoral presence of CD8^pos T cells and M1 macrophages. In addition, in vitro treatment of cancer cells with bsAb CD73xEGFR sensitized these cells to the cytotoxic activity of various chemotherapeutic agents. Moreover, when immunodeficient mice inoculated with EGFR-overexpressing tumor cells were treated with bsAb CD73xEGFR, the size of xenografted tumors was significantly reduced. In conclusion, bsAb CD73xEGFR may be of significant clinical potential for various forms of difficult-to-treat solid cancer types.

In Chapter 3, we extended our bsAb-based approach to selectively inhibit CD73 on ovarian cancer (OC) cells by constructing bsAb CD73xEpCAM. EpCAM is a well-established tumor-associated target antigen that is selectively overexpressed on the cell surface of various human carcinomas, including OC. In particular, EpCAM is selectively overexpressed in 55% to 75% of OC patients, in which overexpression correlates with decreased overall survival. In vitro treatment of OC cells with bsAb CD73xEpCAM resulted in EpCAM-directed inhibition of cancer cell surface-exposed CD73, which incapacitated OC cells to convert extracellular AMP to anti-inflammatory ADO. Importantly, bsAb CD73xEpCAM showed potent capacity to overcome the ADO-mediated suppression of T cell proliferation and reinvigorated their capacity to eliminate OC cells. Additionally, we demonstrated that bsAb CD73xEpCAM can inhibit the proliferative capacity of OC cells and sensitize these cells to cytotoxicity induced by cisplatin, doxorubicin, 5FU, and ionizing radiation. Taken together, bsAb CD73xEpCAM has multiple and possibly mutually reinforcing anticancer activities that may be applicable as an alternate and more tumor-selective immunotherapeutic
approach to overcome CD73-mediated immunosuppression in patients with refractory OC.

In Chapter 4 and 5, we describe the development and preclinical evaluation of a novel bsAb-based approach to inhibit tumor-derived exosome (TEX)-exposed immune checkpoints. Exosomes are endosome-derived small extracellular vesicles (30–150 nm) that are actively secreted by essentially all cell types, including cancer cells. Typically, TEX carry pro-oncogenic and immunosuppressive cargos that promote various pro-oncogenic features, such as angiogenesis, invasion, metastasis, and immune evasion25. Over the last years, TEX have attracted widespread attention for their potent immunosuppressive role in many cancer types26–29. Importantly, exposure of immune checkpoint molecules on TEX may represent a largely overlooked strategy of cancer cells to evade immune prosecution.

Recently, it was shown that a significant part of the immunoinhibitory activity of TEX is attributable to the exposure of CD73. Moreover, CD73-exposing TEX may fuse with the plasma membrane of (cancer) cells, providing these cells with CD73-based immunoinhibitory and pro-oncogenic activities30. Consequently, CD73-exposing TEX may prime an initially immunocompetent microenvironment and render it amenable to subsequent metastatic cell colonization and/or tumor progression. Therefore, inhibition of TEX-exposed CD73 may be a useful approach to overcome ADO-mediated immune suppression in various malignancies. Thus far, therapeutic approaches to selectively inhibit TEX-exposed CD73 have not been developed.

Therefore, in Chapter 4, we studied whether we could exploit bsAb CD73xEpCAM (described in Chapter 3) to achieve selective inhibition of exosome-exposed CD73. We selected bsAb CD73xEpCAM for this purpose as EpCAM is not only highly expressed on the cell surface of various carcinomas31 but also on carcinoma-derived TEX32,33. Indeed, bsAb CD73xEpCAM demonstrated potent capacity to selectively inhibit the enzyme activity of CD73 exposed on TEX that were derived from EpCAM-expressing cancer cells. BsAb CD73xEpCAM showed remarkable capacity to restore the anticancer activity of T cells that were incapacitated by TEX that exposed CD73. Intriguingly, antagonistic CD73 antibody oleclumab had no or very limited capacity to inhibit TEX-exposed CD73. To the best of our knowledge, this is the first report of a bsAb-based approach that selectively and potently inhibits immune suppression mediated by both cancer cell- and TEX-exposed CD73.

Recently, it was reported that TEX can expose high levels of immune checkpoint protein PD-L1, which potently inhibits the activity of anticancer T cells34–36. Moreover, TEX-exposed PD-L1 was shown to confer resistance to currently used PD-L1-inhibiting antibodies. We (and others) revealed that the clinically used PD-L1-inhibiting antibodies avelumab, durvalumab, and atezolizumab fail to effectively inhibit TEX-exposed PD-L1. This may explain (at least in part) why the majority of cancer patients remain unresponsive to current immune checkpoint-inhibiting antibodies37.

In Chapter 5, we report on bsAb PD-L1xEpCAM, that was designed to inhibit TEX-exposed PD-L1 in an EpCAM-directed manner. In contrast to conventional PD-L1-blocking antibodies avelumab, durvalumab, and atezolizumab, bsAb PD-L1xEpCAM potently inhibited both cancer cell surface-exposed and TEX-exposed PD-L1, and does so in an EpCAM-directed manner. BsAb PD-L1xEpCAM showed potent capacity to
overcome immune suppression mediated by PD-L1\textsuperscript{pos}/EpCAM\textsuperscript{pos} TEX derived from various cancer cell lines and patient-derived colorectal cancer cells. Taken together, bsAb PD-L1xEpCAM may be useful to devise a novel treatment modality for carcinoma patients who remain unresponsive to the currently used conventional PD-L1-inhibiting antibodies.

In Chapter 6, we extended our bsAb-based approach to selectively inhibit the CD47 immune checkpoint on carcinoma cells by constructing bsAb CD47xEGFR. CD47 is a multifunctional pentaspan transmembrane glycoprotein that is expressed on virtually all normal cell types. One of its most prominent functions involves interaction with signal regulatory protein alpha (SIRPa), a cell surface glycoprotein expressed by various types of phagocytes\textsuperscript{38}. CD47-SIRPa interaction initiates a signal transduction cascade that result in the inhibition of the phagocytic activity of, e.g. macrophages and dendritic cells (DCs)\textsuperscript{39}. In this respect, CD47-SIRPa interaction has been hailed as a “Don’t eat me” immune checkpoint that serves to prevent the untimely phagocytic removal of normal healthy cells. Unfortunately, a broad variety of hematologic and solid malignancies appear to misuse the CD47-SIRPa immune checkpoint by overexpressing CD47, thereby evading phagocytic elimination and subsequent immunogenic processing of neoantigens\textsuperscript{40}. Currently, several CD47-blocking antibodies are being evaluated in clinical trials, alone or in combination with therapeutic anticancer antibodies (NCT02953509, NCT02953782, and NCT03558139).

However, the clinical efficacy of current CD47-blocking antibodies is anticipated to be hampered by the wide-spread expression of CD47 on normal cells which may limit sufficient antibody accretion at the tumor site(s)\textsuperscript{41–43}. Moreover, the lack of cancer selectivity of current monospecific CD47-blocking antibodies may result in a generalized blockade of CD47 present on normal cells which in turn may promote cross-presentation of self-antigens, thereby increasing the risk of breaking self-tolerance and inducing unpredictable immune-related adverse events. In this respect, a bsAb-based approach may be a suitable strategy to inhibit the CD47-SIRPa immune checkpoint in a more tumor-restricted manner. EGFR appears to be particularly suitable tumor target for inhibiting CD47 in a more tumor-directed manner (as shown in Chapter 2).

In this respect, we designed bsAb CD47xEGFR, which has binding capacity for both CD47 and EGFR and is equipped with a fully functional human IgG1 FC effector domain. BsAb CD47xEGFR selectively induced phagocytic removal of CD47\textsuperscript{pos}/EGFR\textsuperscript{pos} cancer cells and endowed neutrophils with capacity to kill these cancer cells by trogoptosis; an alternate form of antibody-dependent cellular cytotoxicity (ADCC) that disrupts the target cell membrane. Moreover, bsAb CD47xEGFR selectively enhanced phagocytosis and immunogenic processing of CD47\textsuperscript{pos}/EGFR\textsuperscript{pos} cancers cells ectopically expressing viral protein CMV\textsubscript{pp65}. Taken together, bsAb CD47xEGFR has multiple mutually reinforcing anticancer activities that are not available in any of the currently available conventional CD47-blocking antibodies and as such may be useful to reduce on-target/off-tumor effects, enhance cancer cell elimination by ADCC, and promote adaptive anticancer immune responses.

In Chapter 7, we report on a hitherto unrecognized direct immunoinhibitory feature of cancer cell-expressed CD47. We uncovered that in response to IFN\textgamma released during
cognate T cell immune attack, cancer cells dynamically enhance CD47 cell surface expression, which coincides with acquiring adaptive immune resistance toward proapoptotic effector T cell mechanisms. Since we demonstrated that CRISPR/Cas9-mediated CD47-knockout rendered cancer cells more sensitive to cognate T cell immune attack, we used bsAb CD47xEGFR (equipped with IgG2s Fc domain) to induce rapid and prolonged cancer cell surface displacement of CD47 by internalization. Indeed, treatment of CD47pos cancer cells with bsAb CD47xEGFR potently enhanced susceptibility to cognate CD8pos T cells. This approach may open up alternate avenues for CD47-blocking approaches in cancer immunotherapy with potentially enhanced efficacy and reduced off-target side effects.

So far, we used bsAbs to selectively inhibit the CD73, PD-L1, or CD47 immune checkpoint on cancer cells (and/or corresponding TEX). In Chapter 8, we demonstrate that selective inhibition of immune checkpoints can also be achieved through the use of small molecule inhibitors (SMIs). In particular, we developed a CD73-SMI, designated compound X, that can be remotely activated near or at the tumor site, which may be useful for enhancing its clinical efficacy while mitigating ‘on-target/off-tumor’ side effects. Switching on the bioactivity of a CD73-SMI by an external light stimulus may allow selective activation of its therapeutic action in a spatiotemporally controlled manner. Of note, this Chapter is partly under embargo until the patent is filed, any information related to CD73-SMI compound X that could affect the patenting process has been deliberately removed.

Compound X consists of a small molecule CD73-inhibitor which we chemically modified with a NVOC-based photoremovable protecting group (PPG). PPGs are sometimes also revered to as photoreleasable, photocleavable, or photoactivatable protecting groups, which provide spatial and temporal control over the activity of a therapeutic compound. Modification with a PPG is frequently revered to as ‘caging’ to designate that the compound is functionally inactivated by this PPG. The NVOC-based PPG we applied was designed to rapidly dissociate from compound X upon radiation with light of 365 nm. Light-mediated removal of this PPG should result in the rapid restoration of the CD73-inhibitory activity. Indeed, compound X regained potent CD73 inhibitory activity upon irradiation with light of 365 nm. Importantly, light-activated compound X showed remarkable capacity to restore the anticancer activity of ADO-suppressed cytotoxic T cells. This novel approach may pave the way for the construction of analogous near-infrared activatable small-molecule immune checkpoint inhibitors so that spatiotemporal activation can be achieved during cancer immunotherapy at more clinically relevant tissue penetration depths.

In Chapter 9, we provide a summary of the thesis and in this context elaborate on future perspectives.

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