Chapter 10

Summary and future perspectives

Summary

Cancer immunotherapy increased survival of patients with advanced stages of several tumor types, although not all patients respond. Several immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), programmed cell death protein (PD-1)/programmed death-ligand 1 (PD-L1) and lymphocyte activation gene-3 (LAG-3) have been approved for the treatment of patients with various tumors. Furthermore, many novel immunotherapeutic agents are being developed. These agents have very different molecular structures and characteristics which may influence their pharmacokinetics. Immune targets are expressed in tumor tissues and by various components of the immune system. Therefore, the whole-body distribution of novel cancer immunotherapies is often unknown. Information on whether these therapies reach the tumor in a sufficient dose may be a biomarker of response. For this reason, there is growing interest in understanding the in vivo behavior of immunotherapeutic agents.

PD-L1 expression, microsatellite-instability (MSI)/defective mismatch repair (dMMR), and tumor mutational burden (TMB) are clinically used biomarkers. However, the immune response to checkpoint inhibition is highly dynamic and complex, and currently there is no biomarker available that predicts with high certainty the effectiveness of immune checkpoint inhibitor therapy. This may be partly explained by the fact that treatment decisions are often made using information obtained from immunohistochemistry analysis in a single tumor biopsy. Heterogeneity in target expression and drug uptake between tumor lesions within one patient are not considered, hampering the selection of patients that may benefit from immunotherapies. Here, we demonstrate how non-invasive molecular imaging of immunotherapeutic drugs radiolabeled with a suitable positron emission tomography (PET) isotope such as zirconium-89 (^{89}Zr) may help. PET imaging using ^{89}Zr-radiopharmaceuticals can provide insight into tumor target heterogeneity and whether tumors are reached in sufficient dose. Therefore, the research performed in this thesis aimed to develop ^{89}Zr-radiopharmaceuticals to advance the development of novel cancer immunotherapies and explore their use as a biomarker of response. **Chapter 1** introduces the studies described in this thesis.

Chapter 2 comprises a literature review exploring the current clinical use of radiopharmaceuticals based on antibodies. We identified 58 ongoing clinical trials that studied radiolabeled antibodies or antibody-based constructs, including full-sized monoclonal antibodies and antibody fragments such as nanobodies, minibodies, Fab-fragments, and bispecific T cell-engaging antibodies (BiTEs). A range of radioisotopes is used to study whole-body distribution and tumor-targeting. ^{89}Zr was the most frequently applied positron emitter for radiolabeling of antibodies. This may be due to its availability, radioactive half-life that matches the time most

antibodies need to reach the tumor, and feasibility for production and radiolabeling under good manufacturing practice (GMP) conditions. Most clinical trials in this field are conducted in relatively small groups of patients within one center. This precludes translation of results from clinical trials to daily practice. We found that 11 out of 26 antibodies or antibody-related drugs radiolabeled with PET isotopes were investigated in the multicenter setting. Larger studies will require the harmonization of radiolabeling and imaging procedures across centers. To facilitate this, manufacturing procedures for several ^{89}Zr-labeled antibodies were validated to obtain GMP grade ^{89}Zr-radiopharmaceuticals for administration to patients in **chapters 3, 4, 6, 8 and 9**.

Radioimmunotherapeutic (RIT) agents are antibody-based radiopharmaceuticals loaded with a therapeutic α- or β^--emitting radionuclide to selectively eradicate tumors. The RIT agent lutetium-177 (^{177}Lu)-labeled NNV003 consists of an antibody that targets the CD37 receptor expressed on B cells in non-Hodgkin's lymphoma (NHL) patients. In **chapter 3**, we developed ^{89}Zr-NNV003 and investigated whether ^{89}Zr-NNV003 PET imaging of tumor-bearing mice can predict whole-body distribution of ^{177}Lu-NNV003 radioimmunotherapy. PET imaging revealed ^{89}Zr-NNV003 accumulation in REC1 human B cell NHL tumors over time. This was not observed for indium-111 (^{111}In)-labeled IgG control molecule, indicating that the observed tumor uptake is specific for CD37. Also, ex vivo quantification showed a 2.8-fold higher tumor uptake and 4.8-fold higher tumor-to-blood ratio for ^{89}Zr-NNV003 than for ^{111}In-IgG. In RAMOS human Burkitt's lymphoma tumor-bearing mice, we found higher ^{89}Zr-NNV003 tumor uptake compared with ^{111}In-IgG tumor uptake at 10 μg, 25 μg and 100 μg protein doses. In the RAMOS tumor model, ^{89}Zr-NNV003 and ^{177}Lu-NNV003 showed similar ex vivo normal organ and tumor uptake. ^{89}Zr-NNV003 imaging can therefore help to predict the whole-body distribution of ^{177}Lu-NNV003 accurately. This data may enable clinical evaluation of ^{89}Zr-NNV003 PET imaging as a tool to select patients eligible for ^{177}Lu-NNV003 radioimmunotherapy.

Visualizing the in vivo behavior of immune checkpoint-targeting antibodies may help to better predict response to immune checkpoint therapy. In **chapter 4**, we radiolabeled PD-1-targeting antibody pembrolizumab with ^{89}Zr. This allowed us to study its whole-body distribution with PET imaging in humanized mice bearing human A375M melanoma tumors. PET imaging and ex vivo analysis showed high ^{89}Zr-pembrolizumab uptake in murine tissues containing human immune cells, including spleen, lymph nodes and bone marrow. This uptake was reduced 6.0-fold in the spleen by supplementation with unlabeled pembrolizumab, indicating saturation of PD-1 receptors in these tissues and specific uptake. We found modest ^{89}Zr-pembrolizumab tumor uptake: Lower than uptake in lymphoid tissues, but higher than uptake in other organs. ^{89}Zr-pembrolizumab in the blood pool was increased by unlabeled pembrolizumab, while tumor uptake was not affected. Similarly, autoradiography revealed blockade of signal by unlabeled

pembrolizumab in the spleen, but not in the tumor. Accordingly, immunohistochemistry showed PD-1 positive cells present in the spleen, while tumor tissue did not show PD-1 expression. In conclusion, ^{89}Zr-pembrolizumab PET imaging captured PD-1-mediated uptake in both tumor and normal tissues.

In **chapter 5**, we evaluated ^{89}Zr-pembrolizumab imaging as a non-invasive approach to assess tumor response to PD-1 blockade in patients with melanoma and non-small cell lung cancer (NSCLC). The optimal protein dose for PET imaging was 5 mg ^{89}Zr-pembrolizumab, and the optimal timepoint for scanning was day 7. We found that tumor ^{89}Zr-pembrolizumab uptake correlated with tumor response to anti-PD-1 antibody treatment, progression-free, and overall survival. Tumor maximum standardized uptake value (SUV_{max}) was similar in melanoma and NSCLC patients. PET imaging revealed ^{89}Zr-pembrolizumab uptake at 5 mg was highest in the spleen, and there was ^{89}Zr-pembrolizumab uptake in Waldeyer's ring, normal lymph nodes, and at sites of inflammation. This study shows that ^{89}Zr-pembrolizumab PET imaging in patients with metastatic melanoma and NSCLC is safe and feasible, but validation in larger studies is required to prove its impact on patient selection for anti-PD-1 therapy.

Combining immune checkpoint inhibitors improves the survival of patients with advanced stages of several tumor types, but can increase immune-related adverse events. The CX-072 Probody targets PD-L1 and is activated in vivo by proteases specifically present in the tumor microenvironment, thereby potentially reducing PD-L1-mediated toxicities in normal tissues. In **chapter 6**, we developed ^{89}Zr-CX-072 and investigated its conditional activation and whole-body distribution by PET imaging. ^{89}Zr-CX-072 showed accumulation human MDA-MB-231 triple-negative breast cancer (TNBC) tumors of immune-compromised mice and 2.1-fold higher tumor-to-blood ratios than ^{89}Zr-labeled non-binding control Probody. Tumor tissue autoradiography revealed high ^{89}Zr-CX-072 uptake in high PD-L1-expressing regions. Activated CX-072 species were detected in these tumors, with 5.3-fold lower levels found in the spleen. ^{89}Zr-CX-072 uptake by lymphoid tissues of immune-competent mice bearing syngeneic MC38 colon carcinomas was limited compared to ^{89}Zr-labeled normal anti-PD-L1 antibody (not protease-activatable). This preclinical data supports the idea that CX-072 accumulates specifically in PD-L1-expressing tumors.

Next, we investigated whether the tumor-specific activation we observed in mouse models is translatable to patients. In **chapter 7**, we describe a first-in-human study with PET imaging to evaluate ^{89}Zr-CX072's whole-body distribution and tumor uptake. A 10 mg protein dose resulted in sufficient ^{89}Zr-CX072 blood pool levels and was therefore considered most optimal. PET imaging showed tumor uptake was present in all patients, and the highest tumor uptake was found on day 7. After treatment with CX-072, one patient experienced stable disease,

and two patients had a partial response. PD-L1 tumor expression levels measured by IHC were high (90%) in one patient and low (≤1%) in the other patients. Highest normal tissue uptake was found in the spleen, although less than the unconditionally activated anti-PD-L1 antibody ^{89}Zr-atezolizumab. A few patients demonstrated uptake in normal lymph nodes (axillary: 57.1%, inguinal: 62.5%) and Waldeyer's ring (50%). Furthermore, ^{89}Zr-CX072 was intact (inactivated) in serum and plasma. These findings demonstrate evident ^{89}Zr-CX-072 tumor uptake, even in lesions with ≤1% PD-L1 expression, and modest uptake in normal lymphoid organs, with no unexpected uptake in other healthy tissues.

In addition to predicting response, PET imaging can be used to study the effect of different binding affinities on distribution to tumors and immune tissues of a bispecific antibody. In **chapter 8**, we evaluated the in vivo distribution of ^{89}Zr-labeled bispecific antibody ERY974, which simultaneously targets CD3 on T cells and glypican 3 (GPC3) on tumor cells. In immunodeficient mice bearing GPC3-expressing tumors, ^{89}Zr-ERY974 tumor uptake was GPC3-dependent and specific over ^{89}Zr-labeled control antibodies, bispecific antibody targeting CD3 and the non-mammalian protein keyhole limpet hemocyanin (KLH) or KLH-targeting antibody. In humanized mice, ^{89}Zr-ERY974 tumor uptake was ~3.5-fold higher than in immunodeficient mice, suggesting that human immune cells facilitate ^{89}Zr-ERY974 tumor uptake. We found a preferential distribution of ^{89}Zr-ERY974 to tumor areas containing CD3-expressing T cells by using autoradiography. Additionally, high ^{89}Zr-ERY974 uptake was observed in spleen and lymph nodes. We concluded that ^{89}Zr-ERY974 can be used to study ERY974's whole-body distribution in patients to support its clinical development.

Furthermore, PET imaging can provide information on tumor immune status. For example, the amount of tumor infiltrating CD8$^+$ T cells due to immune checkpoint inhibition may be a predictive factor of response. In **chapter 9**, we performed a first-in-human PET imaging study with ^{89}ZED88082A to visualize CD8$^+$ T cells in patients with solid tumors. With an optimal ^{89}ZED88082A protein dose of 10 mg, spleen uptake was observed within 1 h. On day 2, there was ^{89}ZED88082A uptake in all normal lymphoid tissues and tumor lesions across the body, which was variable between and within patients. We found that ^{89}ZED88082A tumor uptake was predictive for progressive disease versus no progressive disease and overall survival. Also, SUV_{max} was higher in dMMR tumors and in lesions with an immunohistochemical stromal/inflamed phenotype. Furthermore, tumor autoradiography correlated with CD8 expression. After 2 cycles of atezolizumab treatment, PET imaging showed no change in the geometric mean of ^{89}ZED88082A tumor uptake, but revealed temporal heterogeneity in individual lesions, independent of tumor response. This study showed an excellent correlation between ^{89}ZED88082A tumor uptake and CD8 expression upfront PD-L1 immune checkpoint inhibitor therapy, and suggests large heterogeneity in CD8 presence during treatment.

Future perspectives

Preclinical development of ^{89}Zr-radiopharmaceuticals

Studying the in vivo behavior in a mouse model may be informative when developing a radiopharmaceutical that can predict drug distribution in patients. A preclinical study allows for the use of appropriate control molecules, frequent blood sampling, serial PET scans, precise measurements of ex vivo uptake per organ and a controlled environment. In this thesis (**chapters 5 and 7**) we showed for two ^{89}Zr-radiopharmaceuticals, ^{89}Zr-pembrolizumab and ^{89}Zr-CX-072 respectively, how clinical study design might benefit from observations in mouse models.

It is challenging to select a mouse model in which, besides tumor targeting, immune cells can be studied. The translatability of results from mouse studies will increase if immunotherapeutic antibodies are evaluated that are cross-reactive with mouse and human targets. However, most immunotherapeutic antibodies are specific to human targets. The use of surrogate molecules reactive with murine target can help to overcome this issue. Alternatively, immune-compromised mice can be reconstituted with human immune cells by engraftment with human peripheral blood mononuclear cells (PBMCs) or human CD34^{+} hematopoietic stem cells (HSCs). In this thesis (**chapters 4 and 8**), we demonstrated the utility of humanized mice to study the whole-body distribution of immune-targeting antibodies. While this model likely reflects the presence of multiple hematopoietic cell lineages, homing of these cells to immune tissues is hampered. The development of customized CRISPR/Cas9-based mouse models that allow for knock-in or knock-out of specific immune target(s) may support the creation of a more suitable mouse model and advance the clinical translation of results obtained in such a model.

Radiopharmaceuticals to study drug behavior

Cancer immunotherapies may be more effective when multiple immune targets are combined. Therefore, antibody-based constructs in development are getting more complex. For example, next to full-sized monospecific antibodies, multispecific antibodies such as bispecific antibodies are gaining interest. These include bifunctional (bivalent, trivalent or tetravalent) and trifunctional antibodies, and trispecific antibodies. In this thesis (**chapter 8**), we demonstrated how PET imaging could provide insight into the in vivo behavior of ^{89}Zr-ERY974, a bispecific antibody simultaneously engaging T cells and tumor cells with different binding affinities. Furthermore, we used PET imaging to reveal how the PD-L1 targeting prodrug CX-072's whole-body distribution is affected by its in vivo activation (**chapter 6 and 7**).

For these engineered biomolecules, in vivo behavior and pharmacokinetics are not readily predicted based on molecular structure. Our studies showed that whole-body distribution

of immune-targeting antibodies do not just depend on target expression in the tumor, but is primarily determined by their presence in healthy organs of the immune system. In addition, target affinity, Fc receptor affinity, target internalization after antibody-binding, and molecular weight are all considered determinants of whole-body distribution for a specific immunotherapeutic drug. In this setting, PET imaging may be a valuable tool to support immunotherapeutic drug development.

Radiopharmaceuticals to monitor response to immunotherapy

The development of novel immunotherapeutic drugs is costly, and many patients have to participate in clinical studies. Furthermore, not all patients respond to immunotherapy, while they are all at risk for developing adverse events. Therefore, strategies to optimally select those patients most likely to benefit from immunotherapy are urgently needed. PET imaging of radiolabeled immunotherapeutic drugs can help visualize drug distribution throughout the body and inform on whether it reaches the tumor. Earlier, ^{89}Zr-atezolizumab imaging was able to predict tumor response, progression-free survival and overall survival in a small clinical study. We found that ^{89}Zr-pembrolizumab imaging also predicted response to PD-1 blockade and survival in a small number of patients, as described in **chapter 5**. Larger studies are required to obtain the definitive impact of this approach.

The presence of T cells and potentially other immune cells in the tumor may be a biomarker of response. We have limited tools available for monitoring the immune status of metastatic cancers, and current methods include blood- or biopsy-based measurements of T cells. Still, these techniques do not reflect the dynamic and spatial information that is required to monitor immune responses to therapeutic intervention. In this thesis (**chapter 9**), we non-invasively visualized CD8$^+$ T cells in cancer patients with one-armed antibody ^{89}ZED88082A using PET imaging. ^{89}ZED88082A tumor uptake before anti-PD-L1 antibody treatment correlated with disease outcome (progressive vs non-progressive) and overall survival. This indicates that PET imaging is a suitable strategy for visualizing tumor immune status and may be used for patient selection.

Immunotherapeutic drugs targeting T cells are gaining interest. They include T cell-expressed inhibitory receptors: T cell immunoglobulin and mucin domain-3 (TIM-3) and T cell immunoglobulin and ITIM domain (TIGIT), as well as inhibitory ligands in the B7 family: B7-H3, B7-H4 and B7-H5. Furthermore, immune cell-based therapies such as chimeric antigen receptor (CAR) T cells are being evaluated in clinical trials. Knowledge of both target and drug distribution in tumors and normal tissues will increase our understanding of the antitumor immune response. Compiling this data in a warehouse of clinical trials results may help to select the most optimal strategy for patient stratification and prediction of treatment response

to immunotherapy.

Implementation of ^{89}Zr-radiopharmaceuticals in clinical practice

To enable the administration of ^{89}Zr-radiopharmaceuticals to patients, a GMP-compliant manufacturing process needs to be developed. This process must be robust and reproducible, and quality control of the radiolabeled product must comply with a predefined list of specifications. In this thesis (**chapters 5, 7 and 9**) we showed that insight into the whole-body distribution of a new specialized designed medicine can be obtained already in a small number of patients. However, validation in larger studies is often required to prove the predictive value of PET imaging for patient selection. To achieve this, radiolabeling and imaging procedures must be standardized across centers. Sharing GMP-compliant manufacturing procedures for ^{89}Zr-radiopharmaceuticals will support clinical implementation. Also, setting up the transport of ^{89}Zr-radiopharmaceuticals to other centers is essential.

There is a growing need for patient selection, and ^{89}Zr-radiopharmaceuticals can potentially serve as a companion diagnostic to guide the administration of cancer immunotherapies to patients and help with clinical decision making. Knowledge from multiple disciplines, including medical specialists, pharmacists, nuclear physicians, and radiochemists, needs to be combined to organize the development of ^{89}Zr-radiopharmaceuticals from bench to bedside. Technological advances may allow broad application of ^{89}Zr-radiopharmaceuticals. For example, a whole-body PET scanner provides images with higher resolution and requires a lower radioactive dose, enabling potential use in the detection of small lesions, long-term response monitoring and follow-up, and administration to children.