Abstract

The immune system can defeat invading pathogens and abnormal cells in the organism. Therefore, the highly selective recognition of antigens must be strictly controlled in a multi-step process preventing the development of autoimmune reaction against healthy cells. Regardless of the source of activation, the immune response must be silenced at some point to prevent prolonged immune response. Immune checkpoint proteins, such as PD-1, which are present on the surface of T and B cells, NK cells, and macrophages act as a negative regulator of immune response. The interaction of PD-1 with its ligand PD-L1 inhibits T cell activation, proliferation, and cytotoxic functions leading to suppression of immune response. The PD-L1 receptor is found on the surface of not only tumour cells but also immune cells residing in the tumour site. Activation of the PD-1/PD-L1 axis leading to the development of an immunosuppressive environment that promotes further tumour growth is also widely observed in many types of tumours. Thus, immunotherapy focused on targeting immune checkpoints, including the PD-1/PD-L1 axis, is one of the most important achievements in oncology in recent years. Immunotherapies mobilize the patient's immune system for elimination of tumour cells, with the greatest contribution of monoclonal antibodies, and anti-PD-1 and anti-PD-L1 antibodies have revolutionised the treatment of certain types of cancer. However, there are some limitations in the use of therapeutic antibodies related to pharmacokinetics and insufficient tumour tissue penetration, which may sometimes weaken the response to the treatment. Thus, new generations of PD-1/PD-L1 axis inhibitors need to be developed.

This work presents three alternative strategies based on active and passive cancer immunotherapy mechanisms directed against the PD-1/PD-L1 axis.

The first strategy consists in the use of *Salmonella enterica* subsp. *enterica* serovar Typhimurium bacteria as a vector for secreted fusion proteins that block the PD-1/PD-L1 interaction. This strategy is based on the *S*. Typhimurium ability to reach the tumour tissue in response to chemotactic signals, e.g., the cell content released from necrotic tumour cells. The presence of bacteria producing a therapeutic agent increases its concentration locally in the tumour tissue and triggers the immune response in the tumour site. Thus far, the Cellular Biochemistry Department research group has attempted to obtain the S. Typhimurium VNP20009 therapeutic strain with the ability to secrete soluble PD-1 using T3SS. However, the

chosen experimental setup was unsuccessful. At that time, I proposed the use of the flagellar protein FliC secretion signal for secretion of PD-1 extracellular domain using F-T3SS in my previous work. Herein, I demonstrate an alternative idea of using the bacterial motion flagellar system for efficient secretion of the PD-1/PD-L1 inhibitory protein.

Due to the unsatisfactory results using FliC secretion signal, an alternative solution that utilizes the flagellar system of the *S*. Typhimurium VNP20009 therapeutic strain and the wild-type LT2 strain was developed to secrete HAC-V protein (modified PD-1 domain), which has potential to interact with PD-L1. To accomplish this, deletion of selected genes coding the flagellar system protein was performed. To estimate the effect of mutations on the VNP20009 and LT2 strains, the bacterial viability and growth curve as well as the infectiveness of the monocytic-macrophage RAW264.7 cell line were tested. Furthermore, plasmids encoding fusion protein sequences intended for secretion were introduced into mutant strains. Each fusion protein consists of a domain of FlgL, FlgD, or full-length FlgM protein providing a secretion signal for the flagellar system and HAC-V protein binding PD-L1. The analysis of the fusion protein secretion showed that, among all the LT2 strains, ΔfliCΔflgK bacteria secreted the FlgD-HAC-V protein. The results presented in this thesis indicate that the use of the flagellar system to secrete fusion proteins inhibiting the PD-1/PD-L1 axis is feasible; however, it is limited to the wild-type LT2 strain. This result may be useful for further optimization of the presented system.

The second strategy presented in this thesis is based on activating the immune response against cancer cells using ssDNA aptamer molecules. This study involved the characterization of novel aptamers that recognize PD-1/PD-L1 complex proteins. The level of binding of the aptamers to cells presenting PD-1 or PD-L1 on the surface were analysed in *in vitro* studies. The biological activity of the aptamers as a potential inhibitor of the PD-1/PD-L1 axis and the cytotoxic effect of the aptamers on the tested cells were investigated. The study results indicated that the aptamer against PD-L1 (L2c2s) binds to the surface of aAPC/CHO-K1 cells overexpressing human PD-L1 (hPD-L1) as well as to human cell lines. The P2c2s aptamer and its truncated form p34 (recognizing PD-1) bind specifically to the surface of the tested Jurkat T cells overexpressing PD-1. The aptamer truncation involving deletion of nucleotides that do not contribute to specific binding to PD-1 resulted in a slight increase in the aptamer binding to the PD-1 cell receptor. However, all the tested aptamers exerted no biological effect

inhibiting the formation of the PD-1/PD-L1 complex in the cellular system. Therefore, since the aptamers did not exhibit cytotoxicity towards the tested cells, an alternative application for diagnostic and imaging purposes has been proposed.

The last strategy of PD-1/PD-L1 inhibition presented in this work is focused on the use of macrocyclic peptides. Macrocyclic peptide p104 binding PD-L1 was biochemically and structurally characterized. Macrocyclic peptides seem to be a promising alternative to monoclonal antibodies. The p104 peptide presented in this work belongs to the poorly characterized class III of macrocyclic peptides developed by Bristol Myers Squibb (BMS). In this group, macrocyclic peptides are exclusively composed of proteinogenic amino acids, whereas peptides of two previous classes (I and II) contain modified amino acids. This work investigates the hypothesis that the dualistic nature of the PD-L1 binding previously observed for p101 is a common feature for all macrocyclic peptides from class III of BMS. First, the PD-L1/p104 complex formation was confirmed by NMR analysis, whereas the ability of peptide p104 to inhibit the PD-1/PD-L1 complex was confirmed by an *in vitro* cell-based assay. Peptide p104 showed no cytotoxicity to the tested cells. Neither the solution nor the crystallographic studies confirmed the PD-L1/p104 bifurcation interaction, suggesting that the phenomenon of bifurcation interaction of peptides from class III with PD-L1 is not a characteristic feature of the whole group. The structural analysis revealed that p104 mostly interacted with PD-L1 via hydrophobic contacts, but some electrostatic interactions were also observed. Additionally, the π-sulphur interaction observed between $_{PD\text{-}L1}$Met115 and $_{104}$Phe3 was found to support the macrocycle-receptor binding. Previously, this interaction was only observed during p71 peptide (class II) binding. The elucidation of the mechanism of PD-L1/p104 complex formation has expanded the characterisation of class III of BMS macrocyclic peptides. This study may be helpful in the future design and optimization of other peptides targeting the PD-1/PD-1 axis with increasingly improved therapeutic properties.