Summary

Cancer is the second leading cause of death in the western world after circulatory diseases. To date, many approaches have been developed in order to induce an adequate immune response against tumour cells. In this thesis, entitled "Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells", we explored the value of a relatively recently discovered T-cell subset, the CD1d-restricted invariant Natural Killer T (iNKT) cell, in cancer immune therapy. Due to their ability to coordinate both innate and adaptive immune responses, they are promising for the development of strategies to improve anti-tumour immune responses.

In fact, pre-clinical studies illustrate the potential of iNKT cells as regulators of the tumour immune response [reviewed in **chapter 1**] and suggest the benefit of exploiting iNKT cells for the treatment of cancer. iNKT cells of cancer patients have numerical and also functional defects, i.e. impaired proliferation and cytokine secretion. Nonetheless, they still possess the capacity to proliferate and to secrete IFN-γ when properly stimulated *in vitro*. This suggests that iNKT cells of cancer patients may still be capable of enhancing anti-tumour responses after therapies aiming at their expansion and activation. However, data obtained from clinical studies attempting to exploit iNKT cells are contradictory. For implementation of iNKT cell-based immunotherapies further studies on the role of human iNKT cells in anti-tumour immunity are needed.

In the first part of this thesis, we analyzed the role of human iNKT cells as enhancer of anti-tumour immunity *in vitro*, by trans-activation of both dendritic cells (DC) and effector cells, such as NK cells and cytotoxic T lymphocytes (CTL).

In **chapter 2**, we investigated adjuvant effects of various Toll-Like Receptor (TLR) agonists on iNKT cell function, as an approach to enhance iNKT cell-based immunotherapies. First, the iNKT cell TLR profile was characterized. Although human iNKT cells express all TLR, apart from TLR8, they did not respond directly to TLR ligands. Nevertheless, iNKT cells became activated when total peripheral blood mononuclear cells (PBMC) were stimulated with TLR ligands triggering TLR2/6, 7 and 8, and 9, but not TLR3, 4 and 5. Our results suggest that the combination of TLR2/6, TLR7, 8 or 9 agonists with α-galactosylceramide (α-GC), the prototype iNKT cell stimulatory ligand, may act as a strong adjuvant for immunotherapy because any of these ligands will promote cross-talk between DC and iNKT cells. This cross-talk is evidenced by the induction of iNKT cell-derived type 1 cytokine production, IFN-γ in particular, and DC maturation.

In **chapter 3**, we investigated the effects of human iNKT cells on antigen specific CTL responses *in vitro*. iNKT cells were expanded using α-GC-pulsed allogeneic DC derived from the acute myeloid leukemia cell line MUTZ3, transduced with CD1d to enhance iNKT cell proliferation, and with IL-12 to stimulate type 1 cytokine production. Enhanced activation and increased IFN-γ production were observed in iNKT cells upon stimulation with IL-12 over-expressing DC. Via IFN-γ secretion, IL-12-stimulated iNKT cells strongly enhanced the tumour specific $CD8^+$ CTL response. In a more physiological set-up, autologous IL-12 over-expressing DC, loaded with tumour antigen as well as α-GC were superior in stimulating both iNKT cells and antigen specific CTL. Thus, human iNKT cells activated by IL-12 over-expressing, α-GC-pulsed DC, provide help for antigen specific CTL responses.

Next to tumour antigen specific CTL response induction, in **chapter 4** we investigated whether human iNKT cells could enhance NK cell functional activity *in vitro*, as an approach to improve anti-tumour responses. We found that addition of α-GC to PBMC induced iNKT cell activation but did not enhance NK cell effector functions. On the other hand, addition of

in vitro expanded, pre-activated iNKT cells to PBMC enhanced NK cell-mediated cytotoxicity in a α-GC-dependent manner. In line with the observations with iNKT cell-induced CTL responses, IFN-γ was sufficient, though in this case not required, for iNKT cell-mediated NK cell activation. These results indicate that adoptive transfer of *ex vivo* expanded and activated autologous iNKT cells, in combination with treatment with α-GC, may enhance NK cell effector functions.

In **chapters 5a and 5b**, we aimed at validating the previously suggested approaches, i.e. TLR agonist adjuvant function and adoptive transfer of human iNKT cells, to improve the efficacy of monoclonal antibody-based cancer immunotherapies. In **chapter 5a**, we described the model we employed for these experiments: huHMFG-1, a monoclonal antibody against MUC1 that is currently evaluated in clinical trials as a potential immunotherapy for breast cancer. huHMFG-1 exerts *in vitro* tumour cell killing through antibody-dependent cell-mediated cytotoxicity (ADCC). We identified NK cells as the main effector cell mediating huHMFG-1-dependent tumour cell killing. In **chapter 5b** we studied the effect of TLR agonists and iNKT cells on the efficacy of NK cells to induce huHMFG-1-mediated ADCC. Analogous to NK cell activation, we found that addition of *in vitro* expanded iNKT cells in combination with α-GC, but not addition of free α-GC, to PBMC enhanced ADCC. Furthermore, huHMFG-1-mediated tumour killing was enhanced through PBMC stimulation with TLR ligands triggering TLR2/6, 7 and 8, and 9, the same TLR agonists that induced the strongest iNKT cell activation. These results suggest that autologous adoptive transfer of *ex vivo* expanded iNKT cells or administration of TLR agonists that induce iNKT cell and NK cell activation as adjuvants, may improve the efficacy of NK cell-mediated antibody-based tumour immunotherapies.

The results demonstrated the value of iNKT cells to enhance the efficacy of anti-tumour effector cells in humans *in vitro*. Adoptive iNKT cell transfer is one approach to achieve this effect *in vivo*. However, the effect of long-term *in vitro* stimulation with DC pulsed with the strong agonist α-GC on *in vivo* iNKT cell functionality has not previously been investigated. In the second part of this thesis, the therapeutic potential of autologous adoptive transfer of chronically stimulated iNKT cells for the treatment of cancer was addressed *in vivo*.

In **chapter 6a**, we described the generation and characterization of long term cultured mouse iNKT cell lines. Using α-GC-loaded, IFN-γ/LPS-mature D1 DC cells we generated highly pure long-term oligoclonal mouse iNKT cell lines from iNKT cells isolated from spleen. These iNKT cell lines retained their capacity to recognize α-GC/CD1d complexes and release substantial amounts of Th1 and Th2 cytokines (IFN-γ, GM-CSF, IL-4, IL-5, IL-6 and IL-10) upon stimulation with α-GC.

In **chapter 6b**, we checked whether the capacity of these iNKT cells to enhance anti-tumour responses *in vivo* remained unaffected. Using a melanoma experimental model, we proved that *in vitro* cultured mouse iNKT cell lines were still capable of enhancing NK cell-mediated protection against B16.F10 experimental lung metastases, upon their adoptive transfer into wild-type mice shortly after tumour injection, confirming their potential as anti-tumour immune therapeutic approach.

In the last part of this thesis (**chapter 7**), the different therapeutic approaches to exploit human iNKT cells to improve anti-tumour immunity are discussed. Results from the studies described in this thesis and from various other investigators indicate that autologous adoptive transfer of *in vitro* expanded iNKT cells represents a potentially valuable approach for the treatment of cancer. *In vitro* expansion of iNKT cells using IL-12 over-expressing

(allogeneic) DC will lead to an iNKT cell population for adoptive transfer which is superior in secreting type 1 cytokines, providing help for antigen specific CTL, and inducing NK cell activation. Of note, additional treatment with α-GC, preferably presented by DC, may be needed to re-activate the iNKT cells *in vivo*. In conclusion, vaccination with antigen and α-GC- loaded, IL-12 over-expressing DC combined with adoptive transfer of *ex vivo* expanded iNKT cells may result in a clinical benefit for cancer patients, since this approach is expected to lead to an increased anti-tumour immune response mediated by NK cells and tumour specific CTL, as well as IFN-γ producing iNKT cells.