Summary

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promotie 23 mei 2008
Invariant CD1d restricted natural killer T (iNKT) cells share phenotypic properties with both T-cells and natural killer (NK) cells; i.e. expression of a T-cell receptor (TCR) together with NK receptors (reviewed in [1]). In contrast to conventional T cells, that can recognize antigens presented by polymorphic MHC molecules, iNKT cells express a canonical TCR-Vα- chain (Vα24.Jα18 in humans, preferentially paired with Vβ11; Vα14.Jα18 in mice, paired with Vβ2, Vβ7 or Vβ8.2) and as such recognize glycolipid antigens presented by the monomorphic CD1d molecule. The glycolipid α-galactosylceramide (αGalCer) originally isolated from the marine sponge *Agelas mauritianus* is a strong synthetic ligand for iNKT cells, which induces iNKT cell proliferation and activation and secretion of both Th1 and Th2 cytokines. Glycolipids derived from e.g. *Ehrlichia muris*, *Sphingomonas capsulata* or *Borrelia burgdorferi* and a (currently challenged [2;3]) endogenous ligand, the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3), have also been demonstrated to trigger iNKT cell activation *in vitro*. This implies that microbial and endogenous glycolipids exist which may control iNKT cell activation *in vivo* [4-9].

The main function of iNKT cells lies in regulation of immune responses through the production of a wide variety of both pro-inflammatory (th1) and anti-inflammatory (th2) cytokines very swiftly upon their activation. Owing to this broad range of cytokines, iNKT cells have the capacity to enhance host immunity to microorganisms and cancers as well as to prevent autoimmunity [Reviewed in [10]]. Human and mouse iNKT cells can either be CD4+ or CD4-CD8- (double negative (DN)) and in humans a small proportion can express CD8. Direct *ex vivo* analyses suggested that CD4+ iNKT cells produce both pro-inflammatory cytokines (TNFα, IFNγ) as well as anti-inflammatory cytokines (IL-4, IL-13) whereas the DN and CD8+ iNKT cell subsets, primarily produce Th1 cytokines [11-14].

Studies in several tumor models provided the insight that systemic αGalCer injection activates iNKT cells leading to the inhibition of metastasis formation [15;16]. The therapeutic effects could be enhanced by combining αGalCer treatment with IL-12 [17]. In addition, subsequent studies have shown anti-tumor effects of adoptively transferred iNKT cells into tumor inoculated iNKT cell deficient recipients. These effects depended on the iNKT subtype in combination with systemic αGalCer treatment [18] or *in vitro* activation with IL-12 but not recombinant Th1 cytokine treatment [19]. Although the anti-tumor effects were mostly NK-cell dependent, T-cell dependent effects have also been described [20]. *In vitro* studies have shown that iNKT cells can indeed enhance NK cell activation as well as alloresponses dependent on αGalCer presented by dendritic cells (DC) [21;22]. In line with these findings, adoptive transfer of αGalCer pre-pulsed DC induced strong anti-tumor responses in various tumor models [23;24]. Interestingly, iNKT cells also play an important role in immuno-surveillance in the absence of αGalCer, as shown by studies in iNKT-deficient mice. These mice were found to be more susceptible to chemically induced sarcomas, while protection could be restored by adoptive transfer of iNKT cells derived from wild-type animals. Protection depended on CD1d, IFN-γ production by iNKT cells, and NK and CD8 T-cell function [25].

These intriguing findings from pre-clinical studies prompted several groups to study iNKT cells in human cancer patients. Inconsistent data were obtained from these studies from mostly relatively small and sometimes poorly defined cohorts, with some investigators stating that iNKT cells in peripheral blood of cancer patients were numerically and/ or functionally compromised whereas others observed no differences compared to healthy controls [26-32].

Chapter 2 of this thesis addresses this discrepancy in a study in a relatively large cohort of 120 healthy controls and 69 carcinoma patients. Results from this study demonstrated that a) iNKT cell levels in humans selectively decrease with age, most prominently in males and b) that after correction for age and gender variation, carcinoma patients had a selective numeric iNKT cell deficiency, particularly when evaluated within the circulating T cell pool [33]. Interestingly, iNKT cell numbers were not influenced by tumor type, disease stage (also reported by Motohashi et al [28] and Konishi et al [30]) or tumor de-bulking by surgery or radiotherapy, suggesting that the numeric defect was not the consequence of tumor growth.
Furthermore, there were no differences in the percentages of CD4+ and CD4– iNKT cells compared to healthy controls, and the residual iNKT cells had retained the capacity to produce IFN-γ in response to αGalCer in a direct ex-vivo ELISPOT assay. Finally, iNKT cell levels were found to be very broadly distributed in these cohorts with some cancer patients having levels comparable to age matched healthy controls.

The reduction in circulating iNKT cells of cancer patients is indicative of their alleged importance for efficient anti-tumor immune responses to occur. However, it has not been demonstrated previously, whether a reduction of circulating iNKT cells in cancer patients precedes the development of cancer and can be regarded as a risk factor. In Chapter 3 we therefore studied prospectively the relation between peripheral blood iNKT cell and frequencies and the natural course of human papillomavirus th16 (HPV16) infection, in 82 patients who participated in a non-intervention cohort study of women with abnormal cervix cytology (Molling JW et al, Int. J. Cancer in press). HPV16 infection is strongly related to the development of cervical dysplasia, and persistent HPV16 infection is associated with the development of severe cervical intraepithelial neoplasia (CIN3) which may eventually lead to cervical carcinoma. The number of iNKT cells was not related to persistent HPV16 infection or progression of cervical dysplasia towards carcinoma in situ, indicating that iNKT cells appear to play no part in this early event of HP16-persistance-induced progression to neoplasia. Of course, this does not exclude that iNKT cells could control immune responses directed at later stage malignancies.

The findings in patients with established carcinomas (Chapter 2), in conjunction with observations that iNKT cell infiltration in neuroblastoma [31] and colon carcinoma [34] was related to favorable clinical outcome, prompted us to postulate that a severe deficiency in circulating iNKT cells would be related to a poor prognosis in individual patients as compared to those with a relatively intact iNKT cell population. Indeed, as presented in Chapter 4 of this thesis, we established in a prospective study in 47 head and neck squamous cell carcinoma (HNSCC) patients that after tumor debulking by radiotherapy a severe quantitative defect in circulating iNKT cells predicted a significantly shortened disease specific survival; associated with poor locoregional control of tumor recurrence [35]. Collectively these two chapters support the alleged critical contribution of iNKT cells to anti-tumor immune responses. Furthermore, screening for iNKT cell numbers may be useful for determining which patients can benefit from immunotherapeutic adjuvant therapies aimed at reconstitution of the circulating iNKT cell pool.

Clinical phase I trials aimed at achieving the above goal have already been conducted. We have demonstrated that i.v. injection of a broad dose range of αGalCer was well tolerated in patients with solid tumors and lead to increased serum cytokine levels (IL-12, IFN-γ, TNF-α and GM-CSF) in those patients with relatively high numbers of iNKT cells [36]. However, the applied regimen resulted in loss of detectable iNKT cells from the circulation within 24 hours after αGalCer treatment. Subsequent injections of the glycolipid, in retrospect at the moment that iNKT cell numbers were not yet normalized, did not evoke the same cytokine release in these patients. Other groups have followed the strategy of delivering αGalCer to patients by loading it to immature or mature autologous monocyte derived DC prior to i.v. injection [37-39]. Again no adverse events were recorded, but with this protocol striking but transient immunological effects were observed including increases in serum IL-12 and IFN-γ, reduced levels of IL-4, activation of myeloid DC, T and NK cells, an increase in NK cell number and cytotoxicity and a potent expansion of circulating iNKT cells.

An alternative strategy would be the adoptive transfer of high purity and well defined autologous iNKT cells. This would facilitate manipulation of the functional aspects of these cells in vitro (e.g. Th1/Th2 cytokine profile, the capacity to home towards tumor sites or tumoricidal potential) and to control these aspects before transferring the iNKT cells into the patient. Furthermore, this approach would enable to ascribe any immunological or clinical effects observed to the injected iNKT cells. In Chapter 5 of this thesis the development of a robust method to expand peripheral blood iNKT cells of healthy controls as well as advanced cancer patients in vitro is presented. Furthermore, the polarization of the obtained iNKT cells...
towards a Th1 cytokine profile by stimulation with mature αGalCer loaded monocyte derived DC (moDC) in the presence of IL-15 [40] is demonstrated. These Th1 polarized iNKT cultures secreted large amounts of the pro-inflammatory cytokines IFN-γ, TNF-α and GM-CSF. Importantly, although some cancer patient derived iNKT cell cultures showed a delay in the proliferative response this could be overcome by repeated stimulation via this protocol. The direct injection of lymphocytes highly enriched for iNKT cells as a result of ex vivo expansion has already been demonstrated to be well tolerated [41]. However, although the approach of treating patients with high purity Th1 polarized iNKT cell cultures is very appealing, intensive long term culture with repeated in vitro stimulation would be required. This might eventually lead to a loss of in vivo function, e.g. as a result of selection for those cells that only respond to the very strong in vitro signals delivered by αGalCer.

To address this issue in mouse tumor models, we developed a method to generate from mouse spleen highly pure, long-term and chronically stimulated oligoclonal murine iNKT cell lines representative of in vivo iNKT cells (Chapter 6) [42]. DC derived from the D1 dendritic cell line were loaded with αGalCer and employed to expand iNKT cells in vitro in the presence of exogenously added IL-7, which had a pronounced enhancing effect on iNKT cell expansion. Using this method up to 10^8 iNKT cells could be obtained from one spleen within 12 to 14 weeks, and cell lines could be continued for up to 24 months. Importantly, the iNKT cell lines had retained the capacity to swiftly secrete substantial amounts of both Th1 and Th2 cytokines upon in vitro activation. Next we set out to determine whether, despite of the repeated stimulation with αGalCer, these polyclonal iNKT cell lines still possessed a strong capacity to strengthen anti-tumor immune responses in vivo. Indeed, as demonstrated in Chapter 7 of this thesis, we observed that 4 out of 4 intravenously administered iNKT cell lines investigated were capable of evoking a cytokine storm, resulting in the partial inhibition of B16.F10 melanoma experimental lung metastases. In vitro and in vivo cytolytic activity of this iNKT cell line towards B16.F10 tumor cells was very low, suggesting that activation of down-stream effector cells constituted the major anti-tumor effector mechanism in this model. Depletion experiments revealed that the predominant effector cells needed for lung metastasis inhibition in our system were NK cells. Furthermore, injection of iNKT cells resulted in an increase of NK cells, but not CD8 T cells, in the lungs of mice. These findings emphasized that, after in vitro culture, mouse iNKT cells retain their function and can instruct NK cells to interfere with outgrowth of B16.F10 metastases. (JW Molling et al, submitted manuscript).

In summary, based on our epidemiologic findings in carcinoma patients, this thesis provides evidence for a distinct contribution of iNKT cells to natural anti-tumor immune responses. Furthermore, screening for iNKT cell levels may be useful for determining which patients could benefit from re-constitution of the circulating iNKT cell pool, since its size is positively related to favorable clinical outcome. Additional evidence regarding the physiological role of iNKT cells in anti-tumor responses, possible mechanisms behind this and the efforts already made to exploit their potential in cancer patients are discussed in Chapter 8. Our observations that: a) the functionality of apparently defective iNKT cells of some individual cancer patients can be restored in vitro using αGalCer loaded DC and b) long-term polyclonal mouse iNKT cell lines, repeatedly stimulated with αGalCer, retain their ability to enhance anti-tumor immune responses in vivo underscore the potential of autologous adoptive transfer of ex vivo expanded Th1 polarized iNKT cells, in conjunction with established modalities such as surgery and radiotherapy, as an immunotherapeutic adjuvant therapy against carcinomas.
Reference List


