Innate and adaptive tumour immunity
Role of invariant Natural Killer T-cells
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María Moreno Jauge

geboren te Montevideo, Uruguay
promotoren: prof.dr. R.H.M. Verheijen
prof.dr. R.J. Scheper

copromotoren: dr. S.C.T. von Mensdorff-Pouilly
dr. H.J. Bontkes
Contents

Chapter 1
Introduction
Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells

Part I: Invariant NKT cells as enhancers of anti-tumour immunity in vitro

Chapter 2
Differential indirect activation of human invariant Natural Killer T-cells by Toll-like receptor (TLR) agonists
Submitted for publication

Chapter 3
Interferon-γ producing human invariant Natural Killer T-cells promote tumor associated antigen specific cytotoxic T cell responses
Journal of Immunology 2008; In press

Chapter 4
In vitro expanded human invariant Natural Killer T-cells promote functional activity of Natural Killer cells
Clinical Immunology 2008; In press

Chapter 5a
High level of MUC1 in serum of ovarian and breast cancer patients inhibits huHMFG-1 dependent cell-mediated cytotoxicity (ADCC)
Cancer Letters 2007; 257:47-55

Chapter 5b
Toll-like receptor agonists and invariant Natural Killer T-cells enhance antibody-dependent cell-mediated cytotoxicity (ADCC)
Cancer Letters 2008; In press

Part II: Invariant NKT cells as enhancers of anti-tumour immunity in vivo

Chapter 6a
Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity
Journal of Immunological Methods 2007; 322(1-2):70-81

Chapter 6b
Chronically stimulated mouse invariant NKT cell lines have a preserved capacity to enhance protection against experimental tumour metastases
Immunology Letters 2008; 118(1): 36-43

Chapter 7
Discussion
Harnessing invariant NKT cells for the treatment of cancer

Summary
Page

7
27
43
61
77
87
99
113
123
135
Nederlandse Samenvatting
Curriculum Vitae & List of Publications
Acknowledgements
Chapter 1

Introduction
Coordinating innate and adaptive tumour immunity:
Role of human invariant NKT cells
Cancer is the second leading cause of death in the western world after circulatory diseases. In 2006, there were approximately 1.4 and 3.2 million new cancer cases and 600,000 and 1.7 million estimated cancer deaths in the USA and Europe respectively [1;2]. Breast cancer is one of the most common cancer types among women, accounting for more than 1 in 4 diagnosed. In Europe, breast cancer is the leading cause of death from cancer in women. A fall in breast cancer mortality rates in most European countries in the 1990s was reported by several studies [3;4]. The decline in mortality rate has been attributed to the combined effect of earlier detection and improved treatment, but it was observed mainly in young women, and because of the ageing of the European population the number of deaths from breast cancer is still rising (130,000 in 2004, 132,000 in 2006) [2]. A reduction in breast cancer mortality could be achieved by the implementation of organised screening programmes, in combination with the introduction of new therapies or improvement of the treatments already in use. New therapies are also needed for ovarian cancer, which is much less frequent than breast cancer but is the most lethal gynaecological malignancy and the sixth cause of death in women in the Netherlands [5].

1. The immune system and cancer

The complex relationship between the immune system and human cancer has been thoroughly investigated for decades. Observations that immune compromised patients have a higher incidence of tumours (reviewed in [6]), reveal the importance of an effective immune response to prevent the development of malignant disease.

Dendritic cells (DC) play a crucial role in inducing an effective adaptive anti-tumour response by presenting tumour-associated antigens (TAA) in the context of Major Histocompatibility Complex (MHC) molecules to cells of the adaptive immune system, namely T cells. T cells can be differentiated into two subsets defined by cell surface molecular marker expression, i.e. CD4+ and CD8+ T cells. CD4+ T cells, known as helper T cells, are key players of the adaptive immune response because upon activation, they secrete a wide variety of Th1 or Th2 cytokines, regulating the immune response; whereas, CD8+ T cells, known as cytotoxic T lymphocytes (CTL) due to their cytolytic function, can destroy viral-infected or transformed cells. Activation of CD8+ T cells occurs through the engagement of the T cell receptor (TCR) and requires a second co-stimulatory signal provided mainly by CD4+ helper T cells or professional antigen presenting cells (APC). In this manner, CD4+ T-cells provide help for the induction of effective TAA specific MHC class I-restricted CD8+ CTL. These TAA specific CTL may subsequently eradicate tumour cells [7]. Natural Killer (NK) cells are also capable of killing certain tumours, particularly those that down-regulate MHC class I expression [8], thereby evading destruction by CTL. Failure of this tight immune surveillance, due to the lack of TAA expression, the uncontrolled rapid growth of malignant cells that may out-run the immune response, or the development of different strategies to evade the immune system, might favour immune escape, leading to immune tolerance and thus the development of malignant disease.

Is it possible to break this tolerance and induce a successful anti-tumour response? Many approaches have been developed in order to break the tolerance and induce an adequate immune response against the TAA that are presented on tumour cells, in such a way that cancer cells are selectively killed while other cells are left unaffected.
2. Immunotherapy for cancer

Various types of immunotherapy are under investigation. Non-specific immune therapies consist of a general activation of the immune system to overcome the tolerant state of the immune system towards the tumour, of which Bacillus Calmette-Guerin (BCG) injection and immunostimulatory cytokine therapy are two examples [9;10]. However, the clinical efficacy of these therapies is limited due to their lack of specificity and relatively high toxicity.

Conversely, active specific immune therapies aim at inducing the in vivo development of an immune response directed specifically against the tumour. Several immunisation strategies have been developed, including tumour-cell based vaccines, vaccines with defined TAA and DC based vaccines. The latter may be the most promising since this approach exploits the unique ability of DC to induce primary T-cell responses, and thus overcome tolerance (reviewed in [11]). Nevertheless, these approaches may be less suitable for immune compromised patients, like advance cancer patients, due to the general suppressed state of the immune system that hinders the development of a strong immune response.

Passive immune therapies can partially overcome immune suppression since the components of the immune response, such as antibodies or effector cells, are generated ex vivo or in vitro and are subsequently injected. In this category, two different approaches can be distinguished: adoptive T cell transfer and monoclonal antibody (mAb) therapy.

Adoptive T cell transfer aims at enlarging tumour-reactive T-cell populations by ex vivo expansion followed by transfer of these cells back into the patient. Defined TAA recognized by tumour-reactive T cells are crucial for this approach [12].

Monoclonal antibodies are also directed to TAA. Despite the fact that there is no single antigen which is present on all tumour types and that most TAA are also present on normal cells, mAb have been produced and tested in clinical trials with distinct success. Trastuzumab (Herceptin) is one of the most successful recombinant humanized mAb clinically applied for the treatment of breast cancer. However, only 20-30% of human breast tumours express HER2/neu the TAA which is recognized by trastuzumab. Another mAb for breast cancer immunotherapy is huHMFG-1, a humanized mAb that recognizes MUC1. Contrary to HER2/neu, MUC1 is over-expressed in virtually all breast adenocarcinomas [13]. Due to its tandem-repeated extracellular sequence, MUC1 presents multiple antigenic regions per molecule, ensuring antibody binding even in low expressing tumours. Moreover, MUC1 is overexpressed on 90% of adenocarcinomas, including ovarian cancer [14;15]. Clinical studies using mAb against MUC1, have recently been initiated and preliminary results have sometimes been inconclusive [16;17] or promising [18;19]. The MUC1 specific antibody, huHMFG-1, could therefore be a more widely applicable therapeutic agent.

So far no consensus exists as to which of these approaches is most likely to result in a major clinical benefit for cancer patients. Clinical success of the different treatment modalities most probably depends on tumour type and stage. Independent of the type of therapy, any approach should overcome immune tolerance to induce a successful immune response towards the malignant cells.

In this thesis, we explored the use 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, iNKT cells represent a promise for the development of strategies to improve anti-tumour immune responses.
3 iNKT cells

NKT cells share phenotypic properties with both T cells and natural killer (NK) cells: they express both a TCR and NK receptors (reviewed in [20]). In contrast to conventional T cells, which recognize antigens presented by polymorphic MHC molecules, NKT cells recognize glycolipid antigens presented by the monomorphic CD1d molecule [20;21]. Within this CD1d-restricted NKT cell population at least two subsets have been defined. The classical or type I NKT cell is characterized by an invariant TCR using a unique \( \text{V} \alpha_{24.J} \alpha_{18} \) in humans paired with \( \text{V} \beta_{11} \); \( \text{V} \alpha_{14.J} \alpha_{18} \) in mice, paired with \( \text{V} \beta_{2}, \text{V} \beta_{7} \) or \( \text{V} \beta_{8.2} \), and called invariant NKT cell [21]. It is also characterized by its recognition of the glycolipid \( \alpha \)-galactosylceramide (\( \alpha \)-GC). Type II NKT cells have a diverse TCR repertoire recognizing other glycolipids presented by CD1d, but do not respond to \( \alpha \)-GC. Their physiological function has been less well studied and is still only poorly understood [22]. On the other hand, iNKT cells have been studied extensively, and their role in anti-tumour immunity has been repeatedly demonstrated in various murine models.

There is some controversy about iNKT cell development, including origin, timing and selection. Like conventional T cells, iNKT cells arise in the thymus in the perinatal period and do not reach significant levels until at least three weeks after birth [23]. CD1d is required for positive selection of iNKT cells, which is mediated by thymocytes rather than by thymic epithelial cells. However, the mechanism of selection and the iNKT cell ligand involved remain unclear. Once iNKT cell have developed, the final maturation stages occur in the periphery, where the iNKT cell pool is maintained in a thymus-independent manner.

Human and murine iNKT cells can either be CD4\(^{+}\) or CD4\(^{-}\)CD8\(^{-}\) (double negative, DN) and in humans a small proportion can express CD8. iNKT cells can be detected wherever conventional T cell are found, although the ratio iNKT to T cells varies widely in a tissue-specific manner. iNKT cells represent ~0.5% of the T cell population in the blood and peripheral lymph nodes, ~2.5% of T cells in the spleen and bone marrow, and up to 30% of T cells in the liver and thymus [24]. Although the tissue distribution is less well studied in humans, iNKT cells appear to be approximately 10 times less frequent in all these locations.

3.1 iNKT cell activation

iNKT cell antigen recognition is restricted to the non-classical MHC class Ib-like molecule CD1d [25]. The CD1d molecule, consisting of a monomorphic glycoprotein heavy chain non-covalently associated with \( \beta \)2-microglobulin [26], effectively binds and presents lipid or glycolipid antigens. CD1d is widely expressed on haematopoietic cells. In mice, CD1d is constitutively expressed at low levels on lymphocytes, and it is highly expressed on a population of splenic marginal zone B cells [27], while in humans CD1d is detected on some circulating B and T cells [28]. CD1d has also been detected on APC, including monocytes, macrophages and some subsets of DC [27;29].

There are different mechanisms for iNKT cell activation depending on the nature of the ligand, which are described more in detail below.

3.1.1 Detection of exogenous antigens

Exogenous iNKT cell antigens presented by CD1d can directly activate iNKT cells. During infection, microbial-derived glycolipids can be taken up by DC and presented in the
context of CD1d to iNKT cells. Their activation is enhanced by IL-12 produced by DC (Figure 1a). These natural CD1d ligands are currently being identified. One example is lipophosphoglycan from Leishmania donovani, a component of the parasitic surface glycocalyx, which stimulates interferon (IFN)-γ secretion by hepatic mouse iNKT cells [30]. Glycosylceramides from the gram-negative bacteria Ehrlichia muris and certain Sphingomonas species, as well as phosphatidylinositol mannoside from mycobacterial membranes, are also recognized by human and mouse iNKT cells [31-33]. There is also evidence that glycolipids, possibly glycosylphosphatidylinositol from Plasmodium and Trypanosoma parasites, can be detected by iNKT cells [25]. iNKT cell stimulation by other pathogens such as Salmonella typhimurium, Staphylococcus aureus, and Pseudomonas aeruginosa has also been reported [25], but the specific CD1d ligands from these organisms have not been identified yet. These findings suggest that iNKT cells are primarily involved in the detection of bacterial and parasitic pathogens.

Finally, the glycolipid α-GC, originally isolated from the marine sponge Agelas mauritianus, but possibly from microbial origin as well, and its synthetic homolog (KRN7000) are strong ligands for iNKT cells and induce iNKT cell proliferation and activation, and secretion of a broad spectrum of cytokines [34;35].

Figure 1: Possible mechanisms of iNKT cell activation. (a) Direct iNKT cell activation via uptake of exogenous glycolipid antigen by DC and presentation in the context of CD1d. (b) CD1d positive tumour cells or tumour-associated tissue may present altered self-ligands. (c) Microbes containing TLR ligands, such as LPS, can activate iNKT cells by inducing IL-12 production by DC, which amplifies the weak response of the iNKT cell TCR to endogenous antigen–CD1d complexes. (d) Alternatively, tumour-associated inflammatory responses may induce DC to produce IL-12 or itself provide inflammatory cytokines such as IL-12 to amplify the weak TCR/CD1d signal.
3.1.2 Recognition of endogenous antigens

Invariant NKT cells are also able to detect endogenous antigens, such as tumour-derived antigens. Tumour cells or tumour-associated tissue may present altered self-ligands in the context of CD1d (Figure 1b). The ganglioside GD3, which is overexpressed by human melanoma cells, can be presented in the context of CD1d to mouse iNKT cells [36].

Recently, the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) was shown to be a potent CD1d-restricted self-ligand [37]. DCs can be induced to present iGb3 upon Toll-like receptor (TLR) ligation by gram-positive bacteria like Salmonella typhimurium [31;38]. Most recently, human DC have been reported to up-regulate the glycosphingolipid biosynthetic pathway after activation by TLR ligands [39]. In certain microbial infections, iNKT cells seem to be activated by IL-12 produced by TLR-stimulated DC in the presence of a weak CD1d/TCR signal, which is derived from the engagement of TCR to induced glycolipids presented by CD1d (Figure 1c). Similarly, iNKT cells might mediate anti-tumour responses without direct recognition of tumour-derived antigens as a result of iNKT cell activation by IL-12 (or other unknown inflammatory cytokines) produced during tumour-associated inflammatory processes (Figure 1d).

Therefore, both self- and foreign glycolipids presented on CD1d can elicit iNKT cell activation. However, it is still unclear whether iNKT cells can be directly activated by TLR ligands.

3.2 iNKT cell function in innate and adaptive immunity

Human iNKT cells acquire a memory-activated phenotype already before birth [40]. Upon stimulation by antigen recognition, iNKT cells have the remarkable capacity to rapidly produce large amounts of cytokines, e.g. IFN-γ and IL-4, leading to the trans-activation of other immune cells, such as DC, NK, T and B cells (Figure 2). In addition, the cytolytic machinery is activated.

3.2.1 Innate immunity to tumour cells

Invariant NKT cells express a wide variety of cell-death-inducing effector molecules, and murine iNKT cells have been reported to exhibit direct cytotoxicity against tumour target cells [41-44]. Freshly isolated and expanded human iNKT cells express granzyme B but not FasL [45]. However, FasL expression is inducible in human CD4+ iNKT cells after stimulation with PMA/ionomycin [46]. Human iNKT cells can express TRAIL, thus inducing apoptosis in TRAIL-sensitive cells [47]. Moreover, CD4+ iNKT cells produce TNF-α and express perforin after activation [46]. Despite their full lytic machinery, human iNKT cells have little or no direct cytolytic effect against most tumour cells because these lack CD1d and/or a strong iNKT cell ligand. Indeed, direct killing of tumour cells has predominantly been described for CD1d positive leukemic cell lines [47;48]. Tumour cell lines of different origin are only susceptible to iNKT cell mediated killing after CD1d transfection and loading with α-GC [49].
Figure 2: Cellular and molecular network activated by iNKT cells. Glycolipid ligands presented in the context of CD1d activate iNKT cells to secrete Th1 and Th2 cytokines, which trans-activate different immune cells, and up-regulate CD40L and cytotoxic molecule expression. CD40L on iNKT cells interacts with CD40 on DC, leading to DC maturation, and subsequent IL-12 secretion. IL-12 from DC induces IFN-γ production by iNKT cells, which in turn contributes to a Th1 immune response by stimulation of NK and CD8+ T cells. DC activation also leads to priming of adaptive CD4+ and CD8+ T cell responses. On the other hand, iNKT cell secretion of Th2 cytokines, i.e. IL-4, induces B cell activation.

3.2.2 Cytokine secretion

The main function of iNKT cells lies in the orchestration 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, rather than being effector cells themselves. Owing to the broad spectrum of cytokines, iNKT cells have the capacity to enhance host immunity to microorganisms and cancer as well as to prevent autoimmunity (reviewed in [50]). The question remains as to how iNKT cell activation results in different cytokine secretion patterns, leading to such diverse immune responses. Experimental evidence provides several possible explanations, which are not mutually exclusive. 1) Direct ex vivo analyses suggest that the different iNKT cell subsets secrete a different cytokine profile. The CD4+ iNKT cell subset produces both pro-inflammatory cytokines (TNF-α, IFN-γ) as well as anti-inflammatory cytokines (IL-4, IL-13), whereas the DN and CD8+ iNKT cell subsets produce primarily Th1 cytokines [46,51-53]. 2) The quality of the TCR signal, hence the nature of iNKT cell ligand, may influence the cytokine profile produced. For example, OCH, an α-GC analogue with shortened sphingosine, induces preferential production of IL-4 over IFN-γ [54], while a C-glycoside analogue of α-GC, α-C-GC, induces preferential production
of IFN-γ over IL-4 [55]. 3) The integration of signals from different types of receptors can influence the pattern of cytokine produced, e.g. IL-12 can selectively stimulate IFN-γ production by iNKT cells in the absence of α-GC [56;57]. 4) The context in which the iNKT cell ligand is presented has an influence on the pattern of cytokine produced. In this line, previous exposure to α-GC in the context of non-professional APC seems to favor a Th2 response [58;59]. The resulting cytokine response has an effect on nearly every haemopoietic cell type, including DC, NK cells, and B and T lymphocytes, as discussed below (Figure 2).

3.2.3 Cross-talk with DC

DC maturation is a crucial event of the induction of most adaptive immune responses. The term maturation refers to an intricate differentiation process whereby DC respond rapidly to an environmental stimulus and become capable of eliciting an adaptive immune response. The type of stimulus determines the program of DC differentiation and the subsequent host immune response. Upon maturation, surface co-stimulatory molecules are up-regulated, cytokines and chemokines are secreted, antigen presentation is enhanced, and migration to secondary lymphoid tissues is initiated.

Upon recognition of the CD1d presented molecules, e.g. microbial derived glycolipids, iNKT cells become activated and release cytokines, such as IFN-γ. In this process, they can induce DC maturation, which in turn expands the numbers and function of both innate and adaptive lymphocytes. Invariant NKT cell-induced DC maturation has been documented in vitro and in vivo after iNKT cell stimulation by α-GC presented on CD1d by DC [60;61]. Upon activation, iNKT cells up-regulate CD40L, which interacts with CD40 on DC to induce DC maturation as evidenced by increased expression of CD86, IL-12 production, and priming of T cell responses [61-64] (Figure 2).

In turn, matured DC also stimulate iNKT cells for sustained innate immune responses [62]. IL-12, which is produced by maturing DC, enhances iNKT cell activation and cytokine secretion. The interaction between iNKT cells and DC results in the induction of antigen-specific, IFN-γ-producing CD4+ and CD8+ T cells [65]. Thus, both innate responses and primary adaptive immune responses are augmented as a consequence of DC maturation by iNKT cells.

3.2.4 Activation of T cells

Activated iNKT cells in turn promote T cell activation without proliferation; activation markers such as CD69 are transiently up-regulated [66]. In particular, α-GC-activated iNKT cells induce CD4+ T cell activation, as reflected by up-regulation of CD69 expression and IFN-γ secretion [59]. Moreover, activated iNKT cells can affect the cytokine profile elicited by helper T cells, thus modulating the immune response [67]. In addition, iNKT cells can also provide effective help for CD8+ T cells [66]. α-GC-activated iNKT cells enhance CD8+ T cell activation, as evidenced by CD69 up-regulation, IFN-γ production and cytolytic function [68]. IL-12 is considered to be a crucial signal α-GC-mediated induction of functional antigen specific Th1-type and CTL responses [66;69]. Indeed, α-GC acts as an adjuvant in vivo that can increase the magnitude of the CD8+ T cell responses to protein antigens [70;71].

In contrast to the vast number of studies in mice showing enhanced antigen specific T-cell activation by α-GC and iNKT cells, the limited data on human iNKT cells demonstrate inhibition rather than enhancement of antigen specific CTL responses in vitro. Isolated human
CD4+ and CD8+ iNKT cells suppressed the expansion of antigen specific CTL by type 2 cytokine (IL-4, IL-5 and IL-10) production and lysis of APC and activated T-cells respectively [72]. Further studies to elucidate the role of human iNKT cells on tumour-specific CTL responses should clarify this dichotomy.

3.2.5 Activation of NK cells

NK cells are innate immune effectors that have critical cytolytic activity against infected or transformed cells, and they also produce immunoregulatory cytokines, including IFN-γ and tumour necrosis factor (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines [65;73]. NK cell function is regulated by a complex balance of inhibitory and activating signals that are generated by an array of different cell-surface receptors after engagement by their specific cellular ligand. For example, NK cells sense MHC class I–deficient tumour cells and mediate spontaneous cytotoxicity against them. Another way of mediating tumour cell killing is through the IgG receptor, FcγRIII (CD16), which recognizes IgG antibodies bound to tumour cells and triggers the release of cytotoxic granules leading to tumour cell killing, in a process known as antibody-dependent cell-mediated cytotoxicity (ADCC). This is one of the mechanisms by which several mAb used for cancer immunotherapy (e.g. the earlier mentioned trastuzumab and huHMFG-1) induce cancer cell death.

In mice, IFN-γ production resulting from iNKT cell activation promotes a prompt NK cell activation, including proliferation, up-regulation of CD69 expression, additional IFN-γ secretion and increase in cytotoxic activity [74]. Likewise, activated human iNKT cells have also been shown to promote human NK cell activation, as defined by an increase in CD69 expression, but not CD25 [49]. Moreover, NK cytotoxic function can be promoted by iNKT cell production of IL-2 [75]. Further studies to elucidate the mechanism of NK cell activation by human iNKT cells should help to improve iNKT cell-based anti-tumour therapies.

3.2.6 Activation of B cells

Rather than activating NK and T cells, iNKT cell secretion of Th2 cytokines, i.e. IL-4, induces B cell activation [58]. Activation of iNKT cells by α-GC promote a transient up-regulation of CD69 activation marker on B cells [65], in the absence of proliferation [76]. In addition, activation of iNKT cells enhances antibody responses to protein antigens in vivo [77]. Similarly, it has been reported that human iNKT cells help B cells to produce Ig in vitro [78].

3.3 Regulation of iNKT cell activity

As massive iNKT cell-derived cytokine release (“cytokine storm”) may profoundly influence the subsequent adaptive immune response, iNKT cell function has to be tightly regulated. Type II NKT cells may act as regulatory cells. Indeed, in vitro, type II NKT cells stimulated with sulfatide-loaded APC could suppress the proliferation and cytokine production by type I NKT cells upon stimulation with α-GC-loaded APC [78]. Furthermore, type II NKT cells repress iNKT cell-mediated anti-tumour responses. Data with culture supernatants and anti-cytokine antibodies suggest that the suppression is not simply cytokine mediated [79]. Thus, it is plausible that type I and II NKT cells might interact reciprocally to
repress each other’s function (principally type II can suppress type I NKT cells, while type I NKT cells seem to have a moderate effect on type II NKT cells), as a negative feedback loop to regulate immune responses.

In addition to type II NKT cells mentioned above, recent studies revealed the involvement of CD4⁺ CD25⁺ FoxP3⁺ regulatory T (Treg) cells in counter-acting iNKT cell function. It was reported that Treg cells can suppress the proliferation, cytokine production, and cytolytic activity of iNKT cells [20;35]. However, the precise mechanism of interaction between Treg and iNKT cells has not yet been revealed.

3.4 iNKT cell in anti-tumour immune responses

In spite of the low iNKT cell frequency within the T lymphocyte population, iNKT cells exert a critical influence on various components of the immune system as discussed above. Due to their ability to rapidly secrete high levels of a broad range of both pro- (Th1) and anti- (Th2) inflammatory cytokines within a few hours after activation [44;80], iNKT cells have the capacity to regulate the immune responses against cancer among others, by trans-activating down-stream immune cells.

3.4.1 Natural immune response

iNKT cells are thought to play an important physiological role in the immunosurveillance of cancers. iNKT cell-mediated anti-tumour immune responses can be initiated by iNKT cell recognition of endogenous antigens, most probably tumour-derived glycolipids, presented in the context of CD1d. The iNKT cell-derived IFN-γ, in turn, induces NK cell and CTL lytic activity against tumour cells (Figure 3a). Experiments using iNKT cell-deficient mice (Jα18⁻/⁻ mice) indicated a critical role of iNKT cells in the protection from spontaneous tumours chemically induced with methylcholanthrene (MCA) [80]. Furthermore, protection against MCA-induced tumour growth could be restored by adoptive transfer of iNKT cells isolated from wild-type animals. Protection depends on CD1d, IFN-γ production by iNKT cells, and NK and CD8⁺ T-cell function [44], although the role of CD8⁺ T cell is still controversial [80]. Perforin is involved, but not essential, in rejection of MCA-induced tumour [81;82]. It will be of great interest to determine whether some tumours express glycolipid ligands that stimulate iNKT cell activity.

Paradoxically, CD1d knock-out mice, which lack CD1d-restricted NKT cells, have heightened immunity to certain tumours, namely 15-12RM and CT26. In these experimental models CD4⁺ CD1d-restricted NKT cells and IL-13 have been shown to be responsible for the suppression of acquired immunity against tumour by inhibiting CTL-mediated tumour immunosurveillance [83]. In a subsequent study it became clear that the enhanced anti-tumour immunity was due to the deletion of the suppressive type II NKT cell subset, rather than type I NKT (iNKT), in these CD1d knock out mice. Jα18⁻/⁻ mice, in which specifically type I NKT cells but not type II NKT cells are deleted, lacked the enhanced immune surveillance and developed tumours [65;84-86].

3.4.2 α-GC-mediated anti-tumour immune response

The capacity of in vivo α-GC-activated iNKT cells to enhance protection against experimental tumours has been studied extensively (Figure 3b). Administration of α-GC leads
to rapid Th1 and Th2 cytokine production by iNKT cells, followed by a profound but transient depletion of spleen and liver iNKT cells [86-89]. Several groups demonstrated that systemic injection of α-GC or α-GC-loaded DC to activate iNKT cells leads to the inhibition of metastasis formation predominantly via the down-stream activation of NK cells [90]. In vivo experiments using NKT cell-, NK cell-, IFN-γ- and RAG-deficient mice demonstrated that sequential production of IFN-γ by iNKT cells and NK cells is required to mediate α-GC-induced anti-metastatic effect [44;90]. Endogenous IL-12 is important for an optimal effect [88]. Consequently, the anti-metastatic effect of α-GC can be enhanced by combined treatment with IL-12 [85;90]. In contrast, IL-4, the major Th2 cytokine produced by iNKT cells, is not required for α-GC-mediated anti-metastatic function. Neither FasL, perforin nor TRAIL are required for α-GC-mediated anti-metastatic function [66;86]. Some studies also implicate CD8+ T cells in the anti-metastatic activity of α-GC [61;91].

Figure 3: Proposed models for iNKT cell-mediated tumour rejection in vivo. (a) In natural rejection, iNKT cells can mediate anti-tumour responses in the absence of exogenous stimulation. iNKT cells may be capable of responding to tumour-derived glycolipid antigens, since protection is dependent on CD1d. The natural role of iNKT cells is IFN-γ-mediated. NK cell function and possibly CD8+ T-cell recognition of tumour peptides are involved in immunosurveillance. (b) In α-GC-mediated rejection, α-GC is presented by CD1d and can induce potent iNKT-cell-dependent tumour rejection. This process is IL-12- and IFN-γ-dependent. The significance of CD8+ T cells in iNKT cell induce tumour rejection is not yet clear. (c) In IL-12-mediated rejection, exogenously administered IL-12 can act on effector cells including iNKT and NK cells to mediate tumour rejection. IL-12 induces IFN-γ production by iNKT and NK cells. Evidence exists for both perforin-dependent and IFN-γ-dependent effector mechanisms, and the relative roles of the effector cells and associated molecules are likely to vary in a dose-dependent manner. In these three models, the effector role of macrophages (MØ) has yet not been confirmed. IFN-γ may act directly to inhibit tumour growth, tumour-associated angiogenesis or the activation of other effector cell types. It is also likely that others, yet unknown, iNKT cell-derived factors may also be important in these models.
Furthermore, murine studies combining i.v. free α-GC treatment with protein vaccination demonstrated that α-GC acts as an adjuvant for the induction of antigen specific CD4⁺ and CD8⁺ T cell mediated immunity [92]. This adjuvant effect may relate to the fact that α-GC activated iNKT cells can enhance the maturation of DC that subsequently are more efficient in (cross)-priming CD4⁺ and CD8⁺ T cells [93;94].

3.4.3 IL-12-mediated anti-tumour immune response

Some studies suggested that iNKT cells are also essential for the anti-metastatic activity of IL-12, while others found that NK cells and perforin are sufficient. There is evidence that IL-12 exerts anti-tumour effect in a dose-dependent activation of both iNKT and NK cells [93]. In these studies, a lower dose of IL-12 promotes a greater relative role of iNKT cells, whereas at a higher IL-12 dose, NK cells mediate an anti-tumour response independently of iNKT cell activation (Figure 3c). Adoptive transfer of iNKT cells, but not NK cells, to NK and NKT cell-deficient mice restored the anti-tumour response mediated by low dose of IL-12 [94]. This mechanism was IFN-γ-dependent. Thus, low dose of IL-12 induces iNKT cell activation and IFN-γ secretion, which may act directly on tumour cells whilst activating NK cells to mediate tumour cell killing in a perforin-dependent manner [95].

The role of iNKT cells in mediating the anti-tumour activity of other cytokines remains to be established.

3.4.4 Role of human iNKT cell in anti-tumour immune responses

Despite some minor phenotypic and numeric discrepancies, the function of iNKT cells appears to be greatly conserved between species. Indeed, CD1d and relevant TCR regions are highly conserved between mice and human [96]. Similar to observations in mice, stimulation of human iNKT cells results in a rapid expansion and cytokine production [97-105]. The findings from pre-clinical studies illustrate the potential of iNKT cells as regulator of the tumour immune response and suggest the benefit of exploiting iNKT cells for the treatment of cancer. To date, inconsistent data has been obtained from studies in cancer patients, from mostly relatively small and sometimes poorly defined cohorts. Some investigators found that iNKT cells in peripheral blood of cancer patients were numerically and/or functionally compromised, whereas others observed no differences compared to healthy controls.

Since most of our knowledge of iNKT cells was acquired from animal models, and because a few important discrepancies between mouse and human iNKT cell function have been observed, further investigation on the role of human iNKT cells in tumour immunity is imperative before iNKT cell-based immunotherapies can be implemented successfully.
4 Outline of this thesis

The objective of our studies was to test the relevance of human iNKT cells in the activation of NK and T cells, on their turn promoting anti-tumour immune responses. We analysed the effectiveness of iNKT cells to enhance the efficacy of tumour cell killing in human \textit{in vitro} studies. We then hypothesized that iNKT cell transfer might represent an effective immunotherapeutic approach, and developed murine iNKT cell lines that proved to be efficient in promoting anti-tumour immunity \textit{in vivo}.

Part I: Human iNKT cells as enhancers of anti-tumour immunity \textit{in vitro}

In chapter 2, iNKT cell activation by different TLR agonists is analyzed. The iNKT cell TLR profile was characterized by RT-PCR. Various TLR ligands that may exert an adjuvant function on iNKT cell activation were investigated. In addition, effects of cross-talk between DC and iNKT cells upon TLR-triggering were addressed.

In chapter 3, the effects of iNKT cells on the induction of tumour-specific CTL is described. Next to tumour antigen specific CTL response induction, in chapter 4 it was analysed whether human iNKT cells can enhance NK cell functional activity as an approach to improve anti-tumour responses. In these chapters \textit{in vitro} expanded iNKT cells were used to enhance antigen specific CTL responses and NK cell functional activity respectively. Furthermore, the mechanism of NK cell activation by iNKT cells was investigated.

In chapters 5a and 5b, we aimed at validating adoptive transfer of human iNKT cells as an approach to improve the efficacy of cancer immunotherapies in an \textit{in vitro} ADCC model. For this, huHMFG-1, a monoclonal antibody against MUC1 that is currently evaluated in clinical trials as a potential immunotherapy for breast cancer, was used. Identification of NK cells as the main effector cell mediating huHMFG-1-dependent tumour cell killing is described in chapter 5a. The effect of iNKT cells on the efficacy of NK cells to induce huHMFG-1 mediated ADCC is described in chapter 5b.

Part II: Invariant NKT cells as enhancers of anti-tumour immunity \textit{in vivo}.

In this part of the thesis, the therapeutic potential of autologous adoptive transfer of iNKT cells for the treatment of cancer is addressed \textit{in vivo}.

The generation and characterization of mouse long term cultured iNKT cell lines is described in chapter 6a. In chapter 6b, we confirm the potential of these mouse iNKT cell lines as an anti-tumour immune therapeutic approach in a melanoma experimental model.

Finally, in the concluding chapter 7, the different therapeutic approaches to exploit iNKT cells to improve the anti-tumour immunity are discussed. Evidence is provided that autologous adoptive transfer of iNKT cells represents a promising approach for the treatment of cancer.
References


Part I: Invariant NKT cells as enhancers of anti-tumour immunity \textit{in vitro}

Chapter 2

Differential indirect activation of human invariant Natural Killer T-cells by Toll-like receptor (TLR) agonists

María Moreno, Berber M. Mol, Silvia von Mensdorff-Pouilly, René H.M. Verheijen, Esther C. de Jong, B. Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

\textit{Submitted for publication}
Abstract

Activation of CD1d restricted invariant Natural Killer T (iNKT) cells leads to a swift and potent cytokine response and trans-activation of dendritic cells (DC), NK and T cells. During an infection, iNKT cells can be directly activated by bacterial glycosphingolipids presented by CD1d on DC. It is not clear whether other bacterial products can activate iNKT cells via Toll-like receptors (TLR). Here we have examined TLR expression by iNKT cells and evaluated the ability of various TLR ligands to activate iNKT cells. Although human iNKT cells express all TLR, apart from TLR8, they did not respond directly to TLR ligands. However, iNKT cells became activated when total PBMC were stimulated with TLR ligands triggering TLR2/6, 7 and 8, and 9, but not TLR3, 4 and 5. TLR-stimulated monocyte-derived DC promoted iNKT cell activation and, in turn, these activated iNKT cells further enhanced DC maturation. IFN-γ production by iNKT cells and subsequent IL-12 production by DC was dependent on TCR triggering by α-galactosylceramide. Thus combinations of TLR2/6, TLR7, 8 or 9 agonists with α-galactosylceramide may act as strong adjuvants for immunotherapy by enhancing type 1 cytokine production through combined and reciprocal activation of iNKT cells and DC.

Keywords: human, invariant NKT cells, Toll-like receptor, monocyte-derived dendritic cell, cell activation

Abbreviations: α-GC: α-galactosylceramide; iNKT: invariant natural killer T-cells; mDC: myeloid dendritic cells; moDC: monocyte-derived dendritic cells; pDC: plasmacytoid dendritic cells; PGN: peptidoglycan
Introduction

Invariant NKT (iNKT) cells are T lymphocytes, characterized by an invariant TCRα chain gene rearrangement (Vα24-Jα18 paired with Vβ11 in humans) and co-expression of NK cell receptors [1]. They recognize glycolipid antigens in the context of the non-polymorphic, MHC class I-like molecule CD1d via their highly restricted TCR repertoire. iNKT cells are considered to be auto-reactive recognizing endogenous lipids [2], but also bacterial derived glycolipids [3]. Upon activation, iNKT cells rapidly secrete both type 1 (e.g. IFN-γ, TNF-α) and type 2 (e.g. IL-4, IL-13) cytokines. iNKT cells activated by the strong synthetic glycolipid α-Galactosylceramide (α-GC) presented by CD1d on dendritic cells (DC) have the capacity to enhance host immunity to tumour cells as well as to micro-organisms.

Bacterial glycosphingolipids presented by CD1d on DC can activate iNKT cells via their TCR, without the need of endogenous antigens [4-7]. Alternatively, iNKT cells can be indirectly activated by micro-organism derived toll-like receptor (TLR) ligands, such as LPS, mediated DC activation, amplifying the weak responsiveness of iNKT cells to endogenous glycolipids [7;8]. A possible third pathway of iNKT cell activation by micro-organisms would be directly through TLR triggering on the iNKT cell.

TLR recognize different pathogen-associated molecular patterns (PAMPs) and trigger innate and adaptive immune responses. The TLR-family consists of at least 11 members, although the function of TLR10 is not yet clear and TLR11 appears not to be functional in humans. Bacterial components are mainly recognized by cell surface TLR, for example, bacterial lipopeptides are recognized by the heterodimers TLR1/2 and TLR2/6, LPS by TLR4, and flagellin by TLR5. Endosomal-associated receptors are triggered by viral products, i.e. TLR3 by dsRNA, TLR7 and 8 by ssRNA, and TLR9 by hypomethylated CpG DNA (reviewed in [9]). TLR are mainly expressed by antigen presenting cells, but also by NK cells and cells of the adaptive immune response, such as T and B lymphocytes [10]. Upon TLR ligand stimulation, DC maturation is induced. This process takes place either directly through TLR triggering on DC, or indirectly though cytokines released by other TLR-activated cells. Induction of effector function of T cells depends on TLR-induced DC maturation and proinflammatory cytokine secretion, including IL-12 [11]. Activated T cells play an important role in this process providing key stimuli to DC, through e.g. CD40/CD40 L interactions [12], which are required for the enhanced release of proinflammatory cytokines upon microbial stimulation [13].

In this study, we have examined TLR expression by iNKT cells and evaluated the ability of different TLR ligands to directly or indirectly trigger human iNKT cells.

Materials and Methods

Media and reagents

IMDM (Cambrex, Verviers, Belgium) was supplemented with 10% Foetal Calf Serum (Perbio, Helsingborg, Sweden) for culture of peripheral blood mononuclear cells (PBMC) and DC derived from monocytes (moDC), or 8% human pooled serum (Sanquin, Amsterdam, The Netherlands) for the culture of iNKT cells. Both media were supplemented with 100 IU/ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100µg/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy) and 0.01 mM β-mercaptoethanol (Merck, Darmstadt, Germany).
### Table 1: TLR ligands

<table>
<thead>
<tr>
<th>TLR</th>
<th>TLR agonist</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 2/6</td>
<td>PGN</td>
<td><em>S. aureus</em></td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>TLR 3</td>
<td>Poly (I:C)</td>
<td>Synthetic viral dsRNA</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>TLR 4</td>
<td>LPS</td>
<td><em>E. coli</em></td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>TLR 5</td>
<td>Flagellin</td>
<td><em>S. typhimurium</em></td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>TLR 7 and 8</td>
<td>CL075</td>
<td>Synthetic viral ssRNA</td>
<td>0.72 μg/ml</td>
</tr>
<tr>
<td>TLR 9</td>
<td>ODN A</td>
<td>Synthetic bacterial CpG DNA</td>
<td>5 μg/ml</td>
</tr>
</tbody>
</table>

The following TLR agonists were used: peptidoglycan (PGN) from *Staphylococcus aureus* (10 μg/ml), polionosinic-polycytidylic acid [Poly (I:C), 25 μg/ml] and lipopolysaccharide (LPS, 1 μg/ml) (Sigma-Aldrich, Buchs, Germany); flagellin from *Salmonella typhimurium* (5 μg/ml) and CL075 (3M-002, 0.72 μg/ml) (InvivoGen, San Diego, CA, USA); and human specific stimulatory CpG ODN type A (ODN A 2216, 5 μg/ml) (Coley Pharmaceuticals, Düsseldorf, Germany) (Table 1). TLR concentrations used were determined based on literature for optimal NK cell activation and moDC maturation.

**Isolation of peripheral blood mononuclear cells (PBMC) and PBMC stimulation**

PBMC were isolated from buffycoats from healthy donors obtained from the Blood Bank (Sanquin, Amsterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The number of cells was determined and PBMC were cryopreserved in separate batches until use. The use of healthy donor blood has been approved by the local ethical committee.

PBMC cultures containing 4 x 10^6 cells/ml were stimulated with different TLR agonists or combination of those for 48h. In some experiments, PBMC were stimulated with TLR ligands in combination with 100 ng/ml α-GC (KRN7000, kindly provided by Dr Shigeyuki Yamano, KIRIN Brewery, Gunma, Japan). PBMC were incubated in the absence of any stimulus or with 100 U/ml IL-2 as negative and positive controls, respectively.

**moDC cultures and iNKT cell expansion**

To set up moDC cultures, 50 x 10^6 PBMC were allowed to adhere in a T75 flask for 60-90 minutes. Adherent monocytes were subsequently cultured in the presence of 1000 IU/ml GM-CSF (Schering-Plough, Kenilworth, NJ, USA) and 10 ng/ml IL-4 (R&D Systems, Abingdon, UK) for 7 days. Maturation was induced by 48hs culture in the presence of 30% v/v monocyte conditioned medium (MCM) and 50 ng/ml TNF-α (Strathmann Biotech, Hanover, Germany) as a positive control [14] or with different TLR agonists (as specified above).

Vα24^+ Vβ11^+ iNKT cells were enriched from PBMC by positive selection using the iNKT cell specific antibody 6B11 (BD Biosciences, San Jose, CA, USA) and anti-mouse Ig-coated magnetic beads mAb (Milteny Biotec, Bergisch Gladbach, Germany) by MACS sorting. iNKT cells were expanded by weekly stimulation with irradiated autologous PBMC at a 1:1 iNKT : PBMC ratio in the presence of 100ng/ml α-GC, 40 IU/ml recombinant human (rh) IL-2, 5 ng/ml rhIL-7 and 5 ng/ml rhIL-15 (all purchased from Strathmann Biotech, Hanover, Germany). After 2 expansion rounds, purity of iNKT cells was more than 90%, and the cells exhibited an activated phenotype, expressing higher levels of CD69 and in particular CD25, compared to resting iNKT cells.
In order to analyze the effects of TLR-matured moDC on iNKT cells and vice versa, moDC were pulsed with the α-GC or vehicle for 2h. After α-GC pulsing, moDC were washed and cultures containing 2x10^5 in vitro expanded iNKT cells and 2x10^5 pulsed autologous moDC were set up. After 4h, iNKT cells were analyzed for IFN-γ secretion by intracellular cytokine staining (ICCS). On day 2, supernatants were harvested for IFN-γ and IL-12 determination by ELISA and iNKT cells and moDC were characterized.

Surface marker expression on iNKT cells and moDC

FITC-, PE- PerCP-Cy5- or APC-labelled isotype controls and mouse mAbs to CD1a, CD3, CD4, CD14, CD25, CD40, CD69, CD80, CD86, iNKT TCR (6B11) (BD Pharmingen, Heidelberg, Germany), CD83, Vα24 and Vβ11 (Immunotech, Marseille, France) were used to determine the phenotype of iNKT cells and moDC. The CD1d specific clone 51.1.3 was a kind gift from Dr Mark Exley, Harvard Medical School, Boston, MA. Cells were incubated with the antibodies for at least 30 min at 4ºC. Flow cytometry was performed on a FACSCALIBUR™ apparatus and data were analyzed using CellQuest™ software (BD Biosciences, San Jose, CA, USA). Mean fluorescence index was calculated as follows: MFI = mean fluorescence intensity marker / fluorescence intensity isotype.

IFN-γ intracellular cytokine staining (ICCS)

Intracellular IFN-γ staining was performed using the BD cytofix/cytoperm plus kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. After 5 h of iNKT cell stimulation with moDC, GolgiPlug was added to each well (0.1% v/v) and after 4 hours, cells were washed and stained. iNKT cells were stained with FITC-labelled 6B11 and APC-labelled CD3 respectively, followed by intracellular staining with PE-labelled anti-IFN-γ (BD Bioscience, San Jose, CA, USA).

IFN-γ and IL-12 secretion

For determination of cytokine secretion, supernatants were harvest after 5 days of culture. Supernatants were analyzed for IFN-γ by ELISA using PeliKine Compact human IFN-γ ELISA kit (Sanquin, Amsterdam, The Netherlands) according to manufacturer’s recommendations. IL-12 p70 determination by ELISA was performed as described before [15].

Real-time quantitative RT-PCR analysis of TLR expression on iNKT cells

iNKT cells were purified from PBMC by MACS sorting as described above, followed by FACS sorting based on forward side scatter and their CD3, Vα24 and Vβ11 expression, and divided into CD4- and CD4+. In some cases, iNKT cells were expanded for 2 weeks before FACS sorting was performed. mRNA was extracted using TRIzol™ Reagent (Invitrogen Life Technologies, The Netherlands) according to the manufacturer's instructions. Isolated RNA was treated with DNase (RQ1 DNase; Promega, The Netherlands) to eliminate possible contamination with genomic DNA. mRNA was reverse transcribed using the kit “RevertAid H minus First Strand cDNA” (Fermentas, Germany) and quantified by real-time quantitative PCR (iCycler iQ MultiColor Real-Time PCR Detection System; Bio-Rad Laboratories, The Netherlands) with specific primers [16] and general SYBR green (Bio-Rad...
Laboratories, The Netherlands) fluorescence detection. mRNA expression of each sample was normalized to GAPDH [17].

**Statistics**

Normality of distribution of the data was analysed with the Kolmogorov-Smirnov test and Q-Q plots. Wilcoxon signed rank test or Student T-test for paired samples were performed to analyse iNKT cell phenotype, as well as IFN-\(\gamma\) production. A two-tailed p value of < 0.05 was considered significant. All data were analysed using the SPSS 14.0 software.

**Results**

*PBMC stimulation with different TLR agonists (PGN, CL075 and ODN A) induces iNKT cell activation and IFN-\(\gamma\) secretion*

Effect of TLR stimulation on iNKT cell activation was studied by stimulating PBMC from healthy donors with different TLR agonists (PGN ligand for TLR2/6, Poly (I:C) for TLR3, LPS for TLR4, flagellin for TLR5, CL075 for TLR8 and to a lesser extend for TLR7, and ODN A for TLR9, see Table 1). No significant changes were observed in the proportion

![Figure 1](image-url)

Figure 1: CD69 expression by iNKT cells upon PBMC stimulation with different TLR agonists. PBMC were cultured for 48h in medium (no stimulus) or in the presence of PGN, Poly (I:C), LPS, Flagellin, CL075 or ODN A. a) CD69 expression on iNKT cells (defined as CD3\(^+\) 6B11\(^+\) cells). FACS plots from one representative experiment out of 6 performed are shown. Numbers in the upper right corner represent the percentage of CD69\(^+\) iNKT cells (mean of 6 experiments). Results of all donors represented as: b) % CD69\(^+\) iNKT cells of total iNKT cells and c) Mean fluorescence intensity (MFI) of CD69 expression on iNKT cells. Horizontal lines represents mean. * Paired Student’s T-test: p<0.05
of iNKT cells (0.07 ± 0.05 % of 6B11+ cells among the CD3+ T cell population). However, iNKT cell activation was observed upon TLR stimulation in the absence of α-GC. PGN, CL075 and ODN A significantly induced an increase in CD69 expression on iNKT cells (fig. 1a), both the number of iNKT cells expressing CD69 (% CD69+ iNKT cells, fig. 1b, paired Student’s T-test, p = 0.011, 0.008 and 0.003, respectively), as well as the intensity of CD69 (MFI CD69 on iNKT cells, fig. 1c, paired Student’s T-test, p = 0.021, 0.026 and 0.003, respectively) were increased.

In addition to iNKT cell activation, the effect of TLR triggering on IFN-γ production was also addressed. After 48h, supernatants were harvested and IFN-γ production was determined by ELISA. Although there was a large variation between donors, IFN-γ secretion was consistently increased in supernatants of PBMC stimulated with PGN, CL075 and ODN A (fig. 2, Wilcoxon signed rank test, p=0.008, 0.016, 0.008 respectively), the ligands which significantly induced an increase in CD69 expression on iNKT cells as well. A trend in an increased IFN-γ production was observed after Poly (I:C), LPS and flagellin stimulation. Intracellular cytokine staining (ICCS) 24h after treatment of PBMC with the different TLR ligands revealed that PGN, CL075 and ODN A induced IFN-γ production by NK cells, as well as by CD3+ T cells (in all cases less than 3%); but hardly any IFN-γ production by iNKT cells was observed (data not shown).

**Increased activation of iNKT cells after combined TLR stimulation**

To determine whether combined TLR triggering had an additive and/or synergistic effect on iNKT cell activation, PBMC from two different donors were cultured for 48h with combinations of PGN, CL075 and ODN A (the TLR ligands that induced the strongest IFN-γ
secretion and iNKT cell activation). Combined TLR stimulation had a synergistic effect on IFN-γ production (fig. 3a). The percentage of iNKT cells remained unaltered after combined TLR agonist stimulation (data not shown), but their activation status was increased. While the proportionate increase in CD69 expressing iNKT cells was limited after combined TLR triggering (data not shown), there was an additive, though not synergistic, effect on the level of CD69 expression (fig. 3b).

We have shown iNKT cell activation by TLR agonists in the absence of exogenous iNKT cell TCR ligand. Since iNKT cells can be activated by α-GC, we tested the effect of combined α-GC and PGN, CL075 or ODN A stimulation. There was a synergistic increase in IFN-γ secretion when CL075 or ODN A, but not PGN stimulation was combined with α-GC (fig. 3c), suggesting that upon CL075 and ODN A stimulation, iNKT cells are directly or indirectly involved in IFN-γ secretion. Moreover, a slight additional effect of α-GC on CD69 expression by iNKT cells was observed (data not shown). The effect on iNKT cell activation may be underestimated due to experimental limitations to detect α-GC-activated iNKT cells. iNKT cell determination was performed using an antibody against the invariant TCR, which is internalized after α-GC stimulation, as seen by a reduction in 6B11+ iNKT cell number 48h after α-GC stimulation. Yet, the few detectable iNKT cells, which did not internalize their TCR, showed an increase in CD69 expression.

Figure 3: PBMC stimulated with combinations of different TLR agonist and α-GC. PBMC were cultured for 48h in medium (no stimulus) or in the presence of PGN (P), CL075 (C) or ODN A (O) or a combination of these. a) IFN-γ secretion in supernatants was determined by ELISA. b) CD69 expression level (MFI) on iNKT cells (defined as CD3+ 6B11+ cells) was analysed by flow cytometry. Graphs show results of 2 individual experiments: donor 1 (0.14% iNKT cells, left panel) and donor 2 (0.13% iNKT cells, right panel). c) PBMC cultured for 48h in medium (no stimulus) or in the presence of PGN, CL075 or ODN A in the absence (open bars) or presence (closed bars) of the iNKT cell ligand, α-GC. IFN-γ secretion in supernatants was determined by ELISA. The graphs show the results of 2 individual experiments: donor 3 (0.19% iNKT cells, left panel) and donor 4 (0.14% iNKT cells, right panel).
iNKT cells express different TLR, but do not respond directly to TLR agonist stimulation

To determine whether iNKT cells can be directly activated by TLR ligands, we have analyzed TLR mRNA expression by iNKT cells. Purified iNKT cells from healthy donors (fig. 4a, n=9, purity 93.3 - 100.0%) expressed TLR1, TLR2, TLR4, TLR6, TLR7 and TLR9 mRNA, whereas TLR3, TLR5 and TLR10 mRNA expression was low and TLR8 mRNA was absent (fig. 4b). With the exception of TLR6 and TLR7, expression levels are relatively low compared to APC [18]. Ex-vivo isolated and purified iNKT cells (n=4) showed the same TLR expression profile as in vitro expanded iNKT cells (n=5). Moreover, there was no difference in TLR expression profile between CD4+ (n=4) and CD4- (n=3) iNKT cell populations.

Despite the fact that iNKT cells express TLR mRNA, isolated iNKT cells did not respond directly to the different TLR agonists. No increase in iNKT cell activation status (CD25 and CD69 expression, n=4), cytokine production (IFN-γ and IL-4, n=6), proliferation (n=3) or enhancement of survival (n=2) was observed upon TLR agonist stimulation (data not shown). Furthermore, no effects were observed when TLR triggering was combined with TCR (anti-CD3) and/or co-stimulatory molecule (anti-CD28) triggering or in the presence of suboptimal concentrations of IL-2 (data not shown). Of note, all TLR ligands tested have previously been shown to activate NK cells (at the concentrations used in this study).
Figure 5: TLR-stimulated moDC induce iNKT cell activation. moDC were cultured in medium (no stimulus) or in the presence of different TLR agonists. As positive control, moDC maturation was induced with MCM and TNF-α. a) Phenotype of moDC after 48h. FACS plots show one representative experiment out of 4 performed. Grey histograms represent cells stained with the CD86, CD80, CD83 or CD40 specific antibodies, open histograms (bold line) represent cells stained with the appropriate isotype control. Numbers in the upper right corner represent the mean of MFI of 4 experiments performed. b) Immature, TLR-stimulated and MCM/TNF-α-matured moDC were pulsed with α-GC or vehicle, and subsequently co-cultured with in vitro expanded autologous iNKT cells. After 4h, IFN-γ secretion by iNKT cells was analysed by ICCS. iNKT cells stimulated with HeLa cell line transfected with CD1d were used as a positive control. The graph shows the results of one experiment out of 2 performed. c) After 48h, supernatants were harvested and IFN-γ secretion was determined by ELISA. Results of one representative experiment out of 4 performed are shown. Intracellular IFN-γ production and IFN-γ secretion results shown are from the same donor for comparison (donor 5). iNKT cell activation was determined by d) CD69 and e) CD25 expression on iNKT cells by flow cytometry. Results are shown as mean ± SEM (n=5).

TLR-stimulated monocyte-derived DC (moDC) induce IFN-γ secretion by iNKT cells

Human DC respond to different TLR agonists. As iNKT cells are unresponsive to direct TLR stimulation but are activated upon PBMC stimulation with different TLR ligands, TLR effects may occur indirectly through myeloid DC (mDC) or plasmacytoid DC (pDC) present in PBMC. In order to study the role of mDC, isolated and in vitro expanded iNKT cells were co-cultured with TLR-matured autologous moDC, as a model for mDC. We focused on PGN, CL075 and ODN A, which were the most effective in inducing IFN-γ secretion and activating iNKT cells. As positive control, moDC maturation was induced with MCM and TNF-α. PGN and CL075 promoted moDC maturation as evidenced by up-regulation of CD80, CD83, CD86 and CD40 expression (fig. 5a), but not IL-12 secretion (data not shown). As expected, ODN A had no effect since moDC lack TLR9, and CpG stimulation was used as a negative control next to medium control (no stimulus) in subsequent experiments. Of note, CD1d expression remained unchanged upon TLR-triggering (data not shown).
To analyse whether TLR agonists could have an indirect effect on iNKT cells through DC, TLR-stimulated moDC were pulsed with α-GC or vehicle, and co-cultured with in vitro expanded iNKT cells. After 4h, α-GC-pulsed, but not vehicle-pulsed, moDC induced IFN-γ secretion by iNKT cells as determined by ICCS (fig. 5b). iNKT cell IFN-γ production was also detected by ELISA in supernatants harvested at 48h (fig. 5c). IFN-γ secretion correlated with DC maturation status; MCM/TNF-α, PGN and CL075 induced the strongest moDC maturation and the highest IFN-γ production, whereas ODN A that did not induce moDC maturation had little or no effect on IFN-γ secretion by iNKT cells as compared to immature moDC. However, all moDC, irrespective of their initial maturation status (immature, TLR-stimulated and mature), induced phenotypic activation of iNKT cells, as determined by increase in CD69 and CD25 expression (fig. 5d and 5e). While CD69 up-regulation appeared to be α-GC-independent, CD25 expression was higher in the presence of α-GC-loaded compared to vehicle-loaded moDC, mature moDC (PGN, CL075 and MCM+TNF-α) in particular.

\[\text{Figure 6: iNKT cells promote further activation of TLR-stimulated moDC. Immature, TLR-stimulated and MCM/TNF-α-matured moDC were pulsed with α-GC, and subsequently co-cultured with iNKT cells. a) Phenotype of differentiated moDC after 48h. Results are shown as mean ± SEM (n=3). * Paired Student’s T-test: p<0.05. b) After 48hs, supernatants were harvested and IL-12 secretion was determined by ELISA. The graphs show the results of 2 individual experiments: donor 6 (left panel) and donor 7 (right panel).}\]
Maturation of moDC is enhanced by iNKT cells

Next to the effect of TLR-stimulated moDC on iNKT cells, the effect of pre-activated iNKT cells on TLR-stimulated moDC was also studied. Phenotypical characterization of TLR-activated moDC was performed after 48h of co-culture with iNKT cells. Interaction between moDC and iNKT cells led to further moDC maturation, independent of the moDC initial maturation status (fig. 6a) and of the presence of α-GC (data not shown). Up-regulation of CD40, CD80, CD83 and CD86 expression occurred on α-GC-, as well as vehicle-, loaded moDC and on immature, as well as mature moDC. Apart from the increased expression of maturation marker on moDC induced by iNKT cells, iNKT cells also triggered IL-12 production by TLR-activated moDC (fig. 6b). This IL-12 production was α-GC–dependent. Thus the reciprocal phenotypic activation of iNKT cells and (TLR) matured DC is independent of α-GC, while the reciprocal induced cytokine production (i.e. IFN-γ and IL-12 respectively) is α-GC dependent.

Discussion

Invariant NKT cells are thought to regulate immune responses against various microbial infections. Here we investigated the effect of various TLR agonists, isolated either from bacteria or synthetic compounds based on ligands found in both viruses and bacteria, on iNKT cell activation. PGN, CL075 and ODN A, the ligands for TLR2/6, TLR7 and 8, and TLR 9 respectively, strongly induce in vitro activation of human iNKT cells. Since the iNKT cell activation was observed upon stimulation of whole PBMC, the mechanism behind the iNKT cell activation we observed is not yet clear. To determine whether iNKT cells could be directly stimulated by TLR ligands, we first investigated TLR expression in human iNKT cells and demonstrated mRNA expression of all TLR with the exception of TLR8. The TLR expression profile we observed here was different from the profile previously described by Marschner et al. [19]. In contrast to our observations, they observed high TLR 5 and low TLR 4 and 6 expression levels. However, in that particular study purified Vα24+ CD3+ cells were used, which contained only 40% actual Vβ11+ iNKT cells and 60% contaminating conventional T cells. Indeed, TLR expression of this population was similar to that of conventional T cells described by Hornung et al. [10]. With the exception of TLR6 and TLR7, expression levels are relatively low (up to 100-fold lower) compared to APC, such as Langerhans cells, dendritic cells and moDC [18]. Despite expression of most TLR at the mRNA level, various in vitro assays could not demonstrate direct TLR triggering on iNKT cells. Our results are in agreement with previous studies that demonstrate a lack of iNKT cell responsiveness to TLR4, 7, 8 and 9 agonists [19-21]. Agonists of intracellular TLR, such as poly(I:C) for TLR3, CL075 for TLR7 and 8, or ODN A for TLR9, would most likely activate iNKT cells indirectly through phagocytic cells. On the other hand, cell surface expressed TLR should in principle be responsive to TLR agonists and activate iNKT cells. The lack of responsiveness to these ligands may be related to 1) the absence of accessory molecules on iNKT cells, such as CD14 that belongs to the LPS-receptor complex, 2) absent or low TLR protein expression, 3) the intracellular localization of the TLR proteins that can be modified upon activation, as previously reported for TLR2 and 4 in activated T cells [22], 4) presence of negative regulatory mechanisms that attenuate TLR signalling [23], which may be active as a feedback mechanism to maintain immunological balance, or 5) requirement of co-stimulatory molecules for TLR triggering of iNKT cell activation, as many TLR have been shown to do in T cells [22;24;25], although we and others [21] could not confirm this latter hypothesis.
The present results show that stimulation of PBMC with PGN, CL075 and ODN A induces a significant up-regulation of the activation marker CD69 on iNKT cells and an increase in IFN-γ production, suggesting an association between iNKT cell activation and IFN-γ secretion. This observation is supported by the results obtained with the other TLR agonists; LPS induced a modest increase in iNKT cell activation and IFN-γ production, while Poly (I:C) and flagellin, which did not lead to iNKT cell activation, hardly induced IFN-γ production. Activated iNKT cells are known to be involved in the early production of large amounts of IFN-γ. However, we could not detect any IFN-γ in iNKT cells by ICCS shortly after stimulation, the few IFN-γ+ cells detected were NK or conventional T cells (less than 3%, data not shown). Since iNKT cells typically comprise less than 0.2% of the total CD3+ T cell population, reliable IFN-γ staining of activated iNKT cells is difficult. The fact that the IFN-γ production by PBMC was synergistically increased by combining ODN A or CL075 stimulation with the iNKT cell antigen, α-GC, indicates that IFN-γ production was mediated by iNKT cells, if not produced by iNKT cells (fig. 3c). Marschner et al. [19] also observed a synergistic effect on IFN-γ secretion when NK-cell enriched PBMC were stimulated with ODN A, which can activate pDC but not TLR9 negative mDC, combined with α-GC. They demonstrated that ODN A is required to induce pDC-derived type I IFN secretion, which in turn promotes mDC maturation, enhancing α-GC presentation by mDC (but not by pDC as they lack CD1d), and thus promoting iNKT cell IFN-γ secretion. Since TLR7 ligands, like CL075, have been shown to activate pDC as well, the same sequence of events may take place upon combined stimulation with α-GC, resulting in increased IFN-γ secretion by iNKT cells. On the other hand, α-GC had no additive effect on PGN induced IFN-γ secretion. This may be explained by an upregulation of the glycosphingolipid synthesis pathway by PGN, resulting in enhanced recognition of CD1d-associated endogenous lipids by iNKT cells [21;26] and subsequent iNKT cell IFN-γ secretion, without the need for α-GC. PGN, CL075, Poly (I:C) and flagellin are all known mDC activating TLR agonists, however only PGN and CL075 showed a strong effect on iNKT cell activation when added to total PBMC. This may be explained by the complex combination of activating and inhibiting signals induced by TLR agonists. For example, next to DC maturation and CD4+ T cell activation, flagellin has also been shown to activate suppressor functions of Tregs [27], while LPS has been shown to induce Treg expansion and enhance their suppressive function [28]. In contrast, triggering of TLR2 induces Treg proliferation accompanied by transient loss of suppressive activity [29;30]. Moreover, TLR8 activation by CpG poly-G oligonucleotides reverses the suppressive function of Tregs [31]. Based on these observations, PGN, CL075 and CpG may not only activate DC but also temporally inactivate Tregs in total PBMC, allowing for iNKT cell activation. Of note, two independent groups have shown that iNKT cells can be indirectly activated by LPS, which induces IL-12 production by DC, amplifying the weak responsiveness of iNKT cells to endogenous glycolipids [7;8]. However, we did not observe iNKT cell activation upon LPS stimulation. This discrepancy could be due to different experimental settings. While the mentioned studies were performed using isolated DC and iNKT cell populations, we stimulated total PBMC with LPS allowing, as discussed above, for activation of e.g. Tregs that may impair iNKT cell activation [32].

Since PGN and CL075 induced the strongest iNKT cell activation (next to ODN A) and are known to activate mDC, interactions between PGN- or CL075-stimulated mDC and isolated and expanded iNKT cells were studied. Indeed, PGN- and CL075-stimulated mDC induce phenotypic activation of human iNKT cells. Functional activity as measured by IFN-γ production was observed only when DC were pulsed with α-GC. Although Raftery et al. [33] have recently shown modulation of CD1d expression, i.e. CD1d up- and down-regulation upon TLR7- and TLR2-triggering respectively, we have not been able to detect any variation...
in CD1d expression. This discrepancy may be due to different moDC preparations (2 day vs 6 day immature moDC) or the different ligands and ligand concentrations used. Our results are in agreement with other previous reports demonstrating no changes in CD1d expression in human DC matured with TNF-α and CD40L, pDC-derived cytokines upon CpG stimulation or directly with a broad panel of TLR agonists [19;21;34]. In addition to iNKT cell activation by moDC, iNKT cells could in turn induce further phenotypic maturation of TLR-matured DC independent of α-GC, which is in agreement with the observations of Hermans et al. [35] who demonstrated that DC maturation triggered by monophosphoryl lipid A, a TLR4 agonist, could be enhanced by iNKT cells. As we observed for cytokine production by iNKT cells, full DC activation as measured by IL-12 was dependent on α-GC as well, which is most likely related to the α-GC dependent iNKT cell-derived IFN-γ. Our results support the following sequence of events: TLR triggering induces phenotypic maturation of DC without IL-12 production, subsequent iNKT cell CD28-CD86/CD80 interactions induce further phenotypic maturation while additional TCR-CD1d/α-GC interactions trigger IFN-γ production by iNKT cells. This IFN-γ subsequently augments the IL-12 production by the DC induced by CD40/CD40L interactions, which in turn further enhances iNKT cell activation. This is supported by the fact that no IFN-γ secretion is observed in the absence of iNKT cell ligand, and that IFN-γ production is related to the initial maturation state, i.e. expression level of CD86 and CD80, of moDC (fig. 5). Recently, it has been demonstrated that activation of human APC by TLR ligands modulates glycosphingolipid synthesis pathway, resulting in enhanced recognition of CD1d-associated lipids by iNKT cells [21;26]. The α-GC dependency we observe here may be due to the fact that these iNKT cells were generated by repeated α-GC stimulation. However, Vincent and co-workers [36] have previously demonstrated that IL-12 production induced by self-reactive iNKT cells was dependent on α-GC. Thus iNKT cell induced IL-12 production by DC may depend on strong iNKT cell TCR agonists such as α-GC.

In conclusion, our in vitro results demonstrate a differential indirect activation of iNKT cells by TLR ligands. Combinations of TLR2/6, TLR7, 8 or 9 rather than TLR3, 4 or 5 agonists with α-GC may act as strong adjuvants for immunotherapy by enhancing iNKT cell activation and type 1, IFN-γ mediated immune responses.

Acknowledgements

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References


Chapter 3

Interferon-γ producing human invariant Natural Killer T-cells promote tumor associated antigen specific cytotoxic T cell responses

María Moreno, Johan W Molling, Silvia von Mensdorff-Pouilly, René HM Verheijen, Erik Hooijberg, Duco Kramer, Anneke W Reurs, Alfons JM van den Eertwegh, B Mary E von Blomberg, Rik J Schepfer, Hetty J Bontkes

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Abstract

CD1d restricted invariant Natural Killer T (iNKT) cells can enhance immunity to cancer or prevent autoimmunity, depending on the cytokine profile secreted. Anti-tumor effects of the iNKT cell ligand, αGalactosylceramide (αGC) and iNKT cell adoptive transfer have been demonstrated in various tumor models. Together with reduced numbers of iNKT cells in cancer patients, which have been linked to poor clinical outcome, these data suggest that cancer patients may benefit from therapy aiming at iNKT cell proliferation and activation. Here we investigated the effects of human iNKT cells on antigen specific CTL responses. iNKT cells were expanded using αGC pulsed allogeneic DC derived from the acute myeloid leukemia cell line MUTZ3, transduced with CD1d to enhance iNKT cell stimulation, and with IL-12 to stimulate type 1 cytokine production. Enhanced activation and increased IFNγ production was observed in iNKT cells, irrespective of CD4 expression, upon stimulation with IL-12 over-expressing DC. IL-12 stimulated iNKT cells strongly enhanced the MART1 specific CD8+ CTL response, which was dependent on iNKT cell derived IFNγ. Furthermore, autologous IL-12 over-expressing DC, loaded with antigen as well as αGC were superior in stimulating both iNKT cells and antigen specific CTL. This study shows that IL-12 over-expressing allogeneic DC expand IFNγ producing iNKT cells, which may be more effective against tumors in vivo. Furthermore, the efficacy of autologous antigen loaded DC vaccines may well be enhanced by IL-12 over-expression and loading with αGC.

Keywords: human; T cells, Cytotoxic, Cytokines, Cell activation, Tumor Immunity

Abbreviations: αGC: α-galactosylceramide; DC: dendritic cells; iNKT: invariant natural killer T-cells; iNKTIL-12: IL-12 stimulated iNKT cells; IRES: internal ribosome entry site; MART-1: melanoma antigen recognized by T cells; M3: acute myeloid leukemia cell line; CD1d transduced M3: M3CD1d; IL-12 and CD1d double transduced M3: M312CD1d; NGFR: nerve growth factor receptor; TAA: tumor associated antigen; Tm: tetramer.
**Introduction**

Invariant Natural Killer T (iNKT) cells are T lymphocytes, characterized by an invariant TCRα chain gene rearrangement (Vα24-Jα18 paired with Vβ11 in humans) and co-expression of NK cell receptors (1). iNKT cells recognize glycolipid antigens in the context of the non-polymorphic, MHC class I-like molecule CD1d via their highly restricted TCR repertoire. iNKT cells are considered to be primarily auto-reactive recognizing endogenous lipids (2), but can also recognize bacterially derived glycolipids, suggestive for a role in antimicrobial defense (3). The synthetic glycolipid αGalactosylceramide (αGC) induces activation and proliferation of iNKT cells in vitro as well as in vivo. The main physiological function of iNKT cells remains to be elucidated, but based primarily on murine studies, modulation of innate and adaptive immune responses through activation or elimination of dendritic cells (DC) is thought to be the main function of iNKT cells (4-9). Upon activation, iNKT cells rapidly secrete both type 1 (e.g. IFNγ, TNFα) and type 2 (e.g. IL-4, IL-13) cytokines. Owing to this broad spectrum of cytokines, iNKT cells have the capacity to enhance host immunity to microorganisms and cancer, as well as to prevent autoimmunity. This has been experimentally demonstrated in various animal models and is also strongly suggested by low numbers of circulating iNKT cells in patients suffering from autoimmune diseases or cancer (10-14). We have recently demonstrated that a severe circulating iNKT cell deficiency predicts poor clinical outcome in head and neck squamous cell carcinoma patients (15). In addition, increased iNKT cell infiltration at tumor sites is associated with prolonged survival in colon cancer and neuroblastoma patients (16,17). These data suggest a critical contribution of iNKT cells to anti-tumor immune responses in humans. Subtypes of iNKT cells, based on CD4 expression, have been shown to express different cytokine profiles when analyzed directly ex vivo. Human CD4+ iNKT cells produce both type 1 and type 2 cytokines, whereas CD8+ and CD4+CD8+ double negative iNKT cells primarily produce type 1 cytokines (18,19). Although iNKT cells possess the full lytic machinery, direct killing of tumor cells has predominantly been described for CD1d positive leukemic cell lines (20,21). Tumor cell lines of different origin were only susceptible to iNKT cell mediated killing after CD1d transfection and pulsing with αGC (22). The anti-tumor effect of αGC in various tumor models has been shown to primarily depend on IL-12 (23-25). iNKT cells induce IL-12 production by DC through CD40 ligation and IFNγ production (26). IL-12 is a strong NK cell activator and is also considered to be a crucial third signal for induction of functional antigen specific type 1 Th cell and CTL responses (27). In contrast to the vast number of studies in mice showing enhanced antigen specific T-cell activation by αGC and iNKT cells, the limited data on human iNKT cells demonstrate inhibition rather than enhancement of antigen specific CTL responses in vitro. Isolated human CD4+ and CD8+ iNKT cells suppressed the expansion of antigen specific CTL by type 2 cytokine production and lysis of APC and activated T-cells respectively (28,29). Here we expanded and stimulated human iNKT cells using αGC pulsed allogeneic DC derived from the acute myeloid leukemia cell line MUTZ3, transduced with CD1d to enhance iNKT cell stimulation, and with IL-12 to stimulate IFNγ production. These MUTZ3 variants provided us with a standardized unlimited source of precursors to generate DC expressing CD1d and secreting IL-12. Effects of IL-12 on CD4+ and CD4+ iNKT cell activation and cytokine production were analyzed, as well as the effect of IL-12 stimulated iNKT cells on tumor associated antigen (TAA) specific CTL responses induced by autologous MoDC. In a more physiological and clinically relevant set-up, it was examined whether iNKT cells in total PBL can also enhance CTL expansion. To this end PBL were cultured with αGC/MART1 double loaded, IL-12 over-expressing, autologous MoDC to simultaneously stimulate MART1 specific CTL and iNKT cells.
Materials and Methods

Media, reagents and cell lines

Recombinant human TNFα (50 ng/ml), IL-7 (5 ng/ml), IL-15 (5 ng/ml) and IL-2 (10 or 50 IU/ml) were purchased from Strathmann Biotech, Hanover, Germany. Recombinant human GM-CSF (Schering-Plough, Kenilworth, NJ) was used at 100 ng/ml; IL-4 (R&D systems, Abingdon, UK) was used at 10 ng/ml; IFNγ (Biosource, Camarillo, CA) was used at 400 U/ml; blocking antibodies against IL-4, IFNγ and IL-10 (R&D systems) were used at 4 μg/ml. IMDM (Cambrex, Verviers, Belgium) was supplemented with 10% FCS (Perbio, Helsingborg, Sweden), for culture of the melanoma cell lines Mel-JKO and Mel-AKR, the EBV-LCL JY and CD1d transfected Hela cells (a kind gift of Dr M. Kronenberg, LIAI, San Diego, CA), 8% human pooled serum (Sanquin, Amsterdam, The Netherlands) for culture of iNKT cells or Yssels supplement (30) and 1% human AB serum (ICN Biomedicals, Zoetermeer, The Netherlands) for CTL cultures. The CD34⁺ human acute myeloid leukemia cell line MUTZ3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was cultured in MEM-α medium containing ribonucleosides and deoxyribonucleosides (Life Technologies, Paisley, UK) supplemented with 20% FCS (Perbio) and 10% 5637 (ATCC, Manassas, VA, USA) conditioned medium. All media were supplemented with 100 IU/ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 μg/ml streptomycin sulphate (Radiapharma-Fisiopharma, Naples, Italy), 2.0 mM L-glutamine (Invitrogen, Breda, Netherlands) and 0.01 mM 2-mercapo-ethanol (Merck, Darmstadt, Germany). FITC-, PE- PerCP Cy5- or APC-labeled isotype controls and mouse mAbs to CD1a, CD1d (clone CD1d42), CD40, CD80, CD86, iNKT TCR (6B11), CD8, CD4, CD3, CD69, CD161, CTLA4, CD25, IFNγ, IL-4, IL-10 (BD Pharmingen, Heidelberg, Germany), CD83, Vα24, Vβ11 (Immunotech, Marseille, France), Granzyme B (Sanquin) and CD56 (IQ products, Groningen, The Netherlands) were used to determine the phenotype of iNKT cells and DCs. The CD1d specific clone 51.1.3 was a kind gift from Dr Mark Exley, Harvard Medical School, Boston, MA. Mean fluorescence index was calculated as follows: MFIndex = mean fluorescence intensity marker / fluorescence intensity isotype.

Retroviral transduction

The human CD1d (Open Biosystems, Huntsville, AL) and the IL-12 elastin cassette containing the p35 and p40 subunits of IL-12 joined together by a flexible linker (Invivogen, San Diego, CA) open reading frames were cloned into the Moloney murine leukemia virus-based retroviral vector LZRS (31). IL-12 was inserted into the multiple cloning site (mcs) of the bicistronic LZRS vector containing an internal ribosome entry site (IRES) followed by the truncated version of the nerve growth factor receptor (ΔNGFR) (LZRS-IL-12-IRES-ΔNGFR) (32). CD1d was cloned behind the IRES sequence (LZRS-mcs-IRES-CD1d). The constructs were transfected into the packaging cell line Phoenix-A using lipofectamine (Invitrogen), retroviral supernatant was produced, followed by retroviral transduction of MUTZ3 as described previously (33). Briefly, 5x10⁵ MUTZ-3 cells, were resuspended in retroviral supernatant supplemented with 10% 5367 conditioned medium and transferred to a fibronectin (RetroNectin; Takara, Otsu, Japan)-coated well of a non-tissue-culture-treated 24-well plate (BD Biosciences). Plates were centrifuged, followed by 5h incubation at 37°C. The next day, retroviral transduction was repeated. NGFR-specific (Chromoprobe, Apts, CA) and CD1d specific (clone CD1d42, BD Biosciences, Heidelberg, Germany) antibodies were used to analyze transduction efficiency and isolate transduced cells by flow cytometry. IL-12
transduced cells were transduced with LZRS-mcs-IRES-CD1d after sorting of NGFR positive cells to obtain double transduced MUTZ3 cells.

Expansion of iNKT cells and MART126-35A27L peptide specific CD8+ T-cells

DC derived from monocytes (MoDC) and wild-type MUTZ3 (M3) as well as CD1d (M3CD1d) and IL-12 and CD1d double transduced (M312CD1d) cells were generated as described previously (34). Maturation was induced by 48 hours culture in the presence of 30% v/v MCM and TNFα as described (35). iNKT cells were enriched from 500x10^6 PBMC, isolated from buffy coats from healthy blood donors obtained after informed consent, by positive selection using the iNKT cell specific antibody 6B11 (BD Pharmingen) and anti-mouse Ig coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS sorting. iNKT cells were expanded by weekly stimulation with irradiated αGC (KRN7000, kindly provided by Dr. Shigeyuki Yamano, KIRIN Brewery, Gunma, Japan) pulsed (100μg/ml) mature M3CD1d-DC in the presence of IL-2 (50U/ml), IL-15 and IL-7. If necessary, iNKT cells were further purified by MACS sorting (>90%). To enhance type 1 cytokine production, iNKT cells were stimulated for 5 days with αGC pulsed mature M312CD1d-DC. To study proliferation of iNKT cell lines in response to various DC types, iNKT cell lines (>90% pure) were labeled with CFSE (1 μM; Molecular Probes, Eugene, OR) for 10 minutes at 37°C, washed twice in ice-cold PBS and cultured in medium (supplemented with IL-2, IL-7 and IL-15) only or in the presence of αGC pulsed MoDC or M3, M3CD1d or M312CD1d derived DC. Intensity of CFSE was measured or cell counts were performed after 1 week culture. The relative CFSE intensity on 6B11 positive iNKT cells was calculated as follows: mean CFSE fluorescence intensity of iNKT cells cultured with DC / mean CFSE fluorescence intensity of iNKT cells cultured in medium.

CD8β-positive CTL precursors were isolated from buffy coats of HLA-A2.1-positive healthy donors using a CD8β specific antibody (Beckman Coulter, Marseille, France) by MACS sorting. Mature MoDC were pulsed with the HLA-A2.1 restricted epitope MART1_{26-35}A27L or transfected with mRNA encoding a minigene of this epitope ubi(MART)4 mRNA or with ubi(MART)4-2A-IL12 mRNA, encoding both the MART minigene and IL-12. In vitro transcription and transfection methods were described in detail previously (35). After αGC and peptide pulsing or mRNA transfection, DCs were washed and multiple bulk cultures containing 0.5-1x10^6 CD8β T-cells or PBL and 0.5-1x10^5 αGC and peptide pulsed or mRNA transfected DCs were set up. 1000-2000 (corrected for purity) autologous iNKT and IL-12 stimulated iNKT cells (iNKT/IL-12) were added as indicated. 0.25-0.5x10^6 irradiated autologous CD8β T-cell/iNKT cell/monocyte depleted PBL were added as helper cells to the CD8β cultures. The next day IL-7 was added. After 10 days, T-cells were analyzed for specificity using PE- and/ or APC-labeled HLA-A*0201 tetramers (Tm) presenting the MART1_{26-35}A27L epitope (35). On day 10 the bulk cultures were restimulated with DCs and irradiated autologous CD8β T-cell/iNKT cell/monocyte depleted PBL, fresh iNKT cells were added to the appropriate wells, the next day IL-2 (10U/ml) was added. Tm staining and functional analysis was performed after 7 days. In order to analyze the involvement of soluble factors, some experiments were done in trans-wells (0.4μm pore size, Corning Life Sciences, Schiphil-Rijk, The Netherlands) or in the presence of cytokine blocking antibodies as indicated.
Expansion of iNKT cells and MART1_{26-35}A27L peptide specific CD8+ T-cells from melanoma patients

Thirty to forty ml of blood were drawn from four patients (two HLA-A2.1 positive and two HLA-A2.1 negative) with metastasized melanoma WHO stage 0 or 1. Patients did not receive any immune suppressive agents or chemotherapy in the four weeks preceding blood sampling. iNKT cells were enriched from isolated PBMC and expanded and stimulated as described above. iNKT cells form HLA-A2 positive patients were added to co-cultures of their autologous CD8β T-cells and allogeneic MART peptide, αGC pulsed, HLA-A2 matched MoDC. The iNKT cells derived from the HLA-A2 negative patients were added to co-cultures of allogeneic HLA-A2 positive, healthy donor derived CD8β T-cells and MART peptide, αGC pulsed MoDC. The medical ethics committee of the VU Medical Center, Amsterdam, The Netherlands, approved the study and all patients gave informed consent.

Functional assays

Seven days after the second DC stimulation the T-cell bulk cultures were harvested and pooled per condition, and incubated with target cells (JY, MART1_{26-35}A27L pulsed JY, Mel-JKO or Mel-AKR). iNKT cells were harvested 5 days after stimulation with M3CD1d-DC or M312CD1d-DC and incubated with vehicle or αGC pulsed target cells (Jurkat, Daudi or Hela-CD1d). Intracellular IFNγ/IL-4 staining was performed using the BD cytofix/cytoperm plus kit (BD Biosciences) according to the manufacturer’s instructions. One hour after the start of the stimulation, GolgiPlug was added to each well (0,1% v/v). After 5 hours cells were washed and stained. CTL cultures were stained with APC-labeled tetramers and PE-labeled CD8 followed by intracellular staining with FITC-labeled anti-IFNγ. iNKT cells were stained with PE-labeled 6B11, PerCPCy5-labeled CD3 and APC-labeled CD4 followed by intracellular staining with FITC-labeled anti-IFNγ or anti-IL-4. Cytolytic activity of the iNKT cells to Daudi and Jurkat cells was determined using a standard chromium release assay as described (36).

Statistics

When n≥5, parametric tests were used after confirmation of Gaussian distribution (normal distribution was confirmed in all cases); non-parametric tests were used when n<5. Differences in iNKT cell characteristics after culture with or without IL-12 were analyzed with either the parametric two-sided paired Student’s T-test, or the non-parametric Wilcoxon rank sum test. Differences in MART1 specific CTL frequencies were analyzed with either a two sided Student’s T-test or a two sided Mann Whitney U (MWU) test.

Results

Dendritic cells derived from CD1d transduced MUTZ3 support iNKT cell expansion

αGC loaded autologous DC efficiently induce iNKT cell proliferation in vitro (37). Since iNKT cells are restricted by the non-polymorphic CD1d molecule, allogeneic DC may be used to induce iNKT cell proliferation and activation, precluding the generation of autologous MoDC for each donor. Previously we have shown that the human acute myeloid leukemia cell line MUTZ3 (M3) can be induced to differentiate into immature and mature DC upon cytokine stimulation. These DC display the full range of functional MHC-mediated
antigen processing and presentation pathways and produce relatively low levels of IL-12 upon CD40 triggering (38). To further promote CD1d antigen presentation and type 1 cytokine production by iNKT cells, CD1d and IL-12 were introduced into M3 cells by retroviral transduction. CD1d expression was analyzed on wild-type M3, CD1d transduced M3 (M3CD1d), IL-12/CD1d double transduced M3 (M312CD1d) and monocytes before and after generation of mature DC using two antibodies (figure 1a and b). Expression levels were low to undetectable on wild-type M3 precursors, and their derived mature DC. Monocytes, M3CD1d and M312CD1d precursors strongly expressed CD1d (particularly demonstrated with the 51.1.3 clone, the CD1d42 clone worked less well in our hands, figure 1b), which was reduced upon DC differentiation. Thus both natural and ectopic CD1d expression was reduced upon DC differentiation. The 51.1.3 clone (more so than the CD1d42 antibody) has some cross-reactivity with CD1b, and it can therefore not be excluded that the remaining staining detected on mature DC is in fact CD1b. However, the αGC dependent iNKT cell proliferation observed with αGC pulsed mature DC (figure 1c and d; table 1), strongly suggests that the staining observed with 51.1 on mature DC is, at least in part, CD1d.

Mature MoDC, M3-DC, M3CD1d-DC and M312CD1d-DC expressed similar high levels of CD40, CD80, CD83 and CD86 [not shown and (38,39)]. IL-12 transduced M3 precursors (M312CD1d) released 495 (SD 192) pg IL-12 / 2x10^5 cells / 24h and M312CD1d-DC produced 1600 (SD 586) pg IL-12 / 2x10^5 cells / 24h.

**Figure 1:** Dendritic cells derived from CD1d transduced MUTZ3 support iNKT cell expansion. CD1d expression by MUTZ3 (M3), CD1d transduced M3 (M3CD1d), IL-12 and CD1d transduced M3 (M312CD1d) and monocytes (Mo) precursors (prec) and their derived mature DC (DC) using the a) CD1d42 and b) 51.1.3 antibodies. Grey histograms represent cells stained with the CD1d specific antibodies, open histograms (bold line) represent cells stained with the appropriate isotype control. Numbers in the upper right corner represent the mean fluorescence index (mean of 3 experiments). c) Example of CFSE dilution of an iNKT cell line after 7 days culture with the indicated αGC pulsed mature DC. The vertical dotted line represents mean CFSE fluorescence of the medium control. d) relative CFSE intensity (mean CFSE fluorescence intensity of iNKT cells cultured with DC / mean CFSE fluorescence intensity of iNKT cells cultured in medium) of iNKT cell lines after culture with the indicated αGC pulsed DC (mean ±SD of 4 independent experiments).
Next it was investigated whether ectopic CD1d and IL-12 expression had any effect on iNKT cell proliferation. Four iNKT cell lines were labeled with CFSE and cultured in medium supplemented with cytokines in the absence or presence of the different αGC pulsed DC. CFSE dilution as a measure for proliferation was determined after 1 week of culture (figure 1c). Proliferation rate was very low in response to M3DC, but high in response to M3CD1d-DC, M312CD1d-DC and allogeneic MoDC, demonstrating that the increased ectopic CD1d expression in M3DC lead to enhanced iNKT cell proliferation (figure 1c and d). These results were confirmed with 4 other iNKT cell lines; 1.5x10⁶ cells were stimulated with αGC pulsed DC and counted after one week (Table 1). Proliferation induced by M3-DC was increased after CD1d transduction and CD1d transduced M3-DC were as effective as MoDC to induce iNKT cell proliferation. Based on these data, M3CD1d-DC and M312CD1d-DC were used to expand iNKT cells.

### Table 1: Dendritic cells derived from CD1d transduced MUTZ3 support iNKT cell expansion.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>iNKT cell line</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<td>M3DC</td>
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<td>4.4</td>
<td>2.5</td>
<td>2.8</td>
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<tr>
<td>M3CD1dDC</td>
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<td>8.8</td>
<td>4.4</td>
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<tr>
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<td>9.6</td>
<td>3.2</td>
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</tr>
<tr>
<td>MoDC</td>
<td>NT</td>
<td>8.4</td>
<td>9.2</td>
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</table>

* iNKT cell counts (x10⁶) after 1 week stimulation with the indicated DC pulsed with αGC. All cultures were started with 1.5x10⁶ iNKT cells. All iNKT cells were >90% pure before and after culture.

**Stimulation with M312CD1d-DC induces iNKT cell activation and increased IFNγ production**

Several investigators have demonstrated that CD4⁻ and CD4⁺ iNKT cells represent different subsets with different cytokine secretion patterns when isolated directly ex vivo: CD4⁻ iNKT cells produce predominantly type 1 cytokines and CD4⁺ iNKT cells produce both type 1 and type 2 cytokines (18,19). Here, we investigated whether this also holds true after in vitro expansion using IL-12 producing DC. iNKT cells isolated from peripheral blood were expanded for 2-3 weeks using αGC pulsed M3CD1d-DC followed by a 5 day culture with αGC pulsed M3CD1d-DC or M312CD1d-DC. The proportion of CD4⁺ iNKT cells was similar in cultures stimulated with or without IL-12 [67% (range 10-82%) and 68% (range 10-86%) respectively]. Stimulation with M312CD1d-DC induced increased activation as measured by CD161, CD56, CD25 and intracellular CTLA4 and Granzyme B expression compared to M3CD1d-DC (0.001<p<0.015 for all parameters, figure 2a). The increase was observed in both CD4⁻ and CD4⁺ iNKT cells (not shown). Rather than inducing a type 1 skewed cytokine secretion profile, i.e. increase in IFNγ and a decrease in IL-4 production, IL-12 stimulation resulted in increased numbers of IFNγ (p=0.0003) producing iNKT cells, without significantly changing the number of IL-4 producing iNKT cells (figure 2b). The effect of IL-12 was the same on both CD4⁻ and CD4⁺ iNKT cells (figure 2c and d). The modulation of cytokine production by IL-12 resulted in a slight increase in the ratio of IFNγ and IL-4 producing iNKT cells, which was statistically significant for the whole population and for CD4⁻ iNKT cells, and borderline significant for the CD4⁺ iNKT cell population (p<0.08) (figure 2e). In addition, a very small but distinct population of IL-10 producing iNKT cells appeared after stimulation with M312CD1d-DC (figure 2f). Stimulation with
M312CD1d-DC induced expression of the lymphoid homing receptor CD62L, known to be expressed on early effector CD8$^+$ and central memory conventional T-cells, on a subpopulation of both CD4$^+$ and CD4$^-$ iNKT cells (figure 2g). The increased expression of Granzyme B by M312CD1d-DC stimulated iNKT cells (figure 2a) did not lead to enhanced killing of CD1d$^+$ target cells: Jurkat (figure 2h), MOLT-4 and CD1d transfected Hela (not shown). Killing of CD1d$^+$ target cells (Daudi, HL60, Hela) was always low (<5%) and could not be enhanced by IL-12 (not shown).

**Figure 2**: Stimulation with M312CD1d-DC induces iNKT cell activation and increased IFN$\gamma$ production. iNKT cells were expanded for 2-3 weeks using M3CD1d-DC followed by 5 days culture with either M3CD1d-DC (open bars) or M312CD1d-DC (closed bars). Phenotype of the resulting iNKT cell lines was determined by FACS analysis after gating on CD3$^+$6B11$^+$ iNKT cells. All iNKT cell lines were >90% pure. a) Mean fluorescence index (mean ± SD of 6-8 individual experiments) of activation markers (CD161, CD56, CTLA4, Granzyme B and CD25). Percentage of IFN$\gamma$ and IL-4 producing cells (mean ± SD of 8 individual experiments) among b) all iNKT cells, c) CD4 negative and d) CD4 positive iNKT cells. e) ratio of IFN$\gamma$ and IL-4 producing iNKT cells (mean ± SD of 8 individual experiments). f) stimulation with IL-12 induces a small increase in IL-10 producing iNKT cells (mean ± SD of 4 individual experiments). g) increase in number of iNKT cells expressing CD62L after stimulation with IL-12 (mean ± SD of 6 individual experiments). P values are the result of a paired two-sided Student’s T-test. h) No increase in αGC dependent killing of CD1d positive targets cells (Jurkat) by iNKT cells stimulated with IL-12 over-expressing DC.
**Figure 3**: *IL-12 stimulated iNKT cells significantly enhance expansion of MART-1 specific CTL.* a) iNKT cells and IL-12 stimulated iNKT cells (iNKTIL-12) were added (0.2%) to multiple bulk co-cultures (n=8) of isolated CD8β T-cells and autologous MoDC pulsed with MART1 peptide alone or with peptide and αGC. Percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the mean, p values are the result of a two sided student’s T-test. b) Induced CTL were functional and produced IFNγ in response to HLA-A2.1 positive, MART1 peptide loaded, JY cells and the MART1 and HLA-A2.1 positive melanoma cell line (Mel-AKR), MART1 negative JY and MART1 positive but HLA-A2.1 negative Mel-JKO cells are not recognized. Percentage of IFNγ positive Tm positive T-cells is shown. Results shown are from one representative donor out of 4 tested. c, d) Melanoma patient derived, IL-12 stimulated iNKT cells (iNKTIL-12) were added (0.2%) to multiple bulk co-cultures of autologous isolated CD8β T-cells and allogeneic HLA-A2 matched MoDC pulsed with MART1 peptide and αGC. The percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the median, p values are the result of a two sided Mann-Whitney U-test. e, f) Melanoma patient derived, IL-12 stimulated iNKT cells (iNKTIL-12) were added (0.2%) to multiple bulk co-cultures of healthy donor derived CD8β T-cells and MoDC pulsed with MART1 peptide αGC. The percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the median (e) or mean (f), p values are the result of a two sided Mann-Whitney U-test (e) or two-sided Student’s T-test (f).

**IL-12 stimulated iNKT cells significantly enhance expansion of MART-1 specific CTL**

To analyze the effects of iNKT cells on antigen specific CTL responses, isolated CD8β+ T-cells, depleted of iNKT cells, were co-cultured with autologous MoDC. Autologous iNKT cell lines were generated using αGC pulsed M3CD1d-DC (iNKT) and M312CD1d-DC (iNKTIL-12) as described above, and were added to CD8β+ and MoDC co-cultures. MoDC were pulsed with αGC to induce cytokine release by the added iNKT cells and/or MART1 peptide to stimulate TAA specific CTL. Specific CTL frequencies were measured using tetramer (Tm) staining after two *in vitro* stimulations as described previously (35). Addition of iNKT or iNKTIL-12 lead to a significant increase in MART1 specific CTL (p<0.02 and p<0.0005 respectively; figure 3a). The iNKT cell effect was αGC dependent; addition of iNKT cells had no effect when MoDC were pulsed with peptide alone without αGC. The MART1 specific CTL were functionally active as demonstrated by specific IFNγ production.
by Tm positive CD8+ T-cells in response to either peptide pulsed targets or endogenously MART1 expressing melanoma cells. However, addition of iNKTIL-12 did not increase the effector function of the induced MART specific CTL (figure 3b). Peptide titration assays revealed no difference in functional avidity (not shown). To study the clinical relevance of this finding, iNKTIL-12 were generated from four melanoma patients as described above. Since the amount of blood that could be obtained was not sufficient to generate sufficient numbers of autologous MoDC, CD8β T-cells from two HLA-A2+ patients were stimulated with allogeneic HLA-A2 matched MoDC in the absence or presence of iNKTIL-12 (figure 3c and d). The two other melanoma patients were HLA-A2 negative and therefore their iNKTIL-12 cells were added to co-cultures of CD8β and MoDC of HLA-A2+ healthy donors (figure 3e and f).

As was observed with the healthy donor derived iNKT cells, addition of melanoma patient derived iNKTIL-12 cells lead to a significant increase in MART specific CTL in three out of the four donors tested (p<0.03, figure 3c, e and f). In one of the HLA-A2 positive donors a high proliferation of presumable alloresponsive CD8 T-cells was observed, which resulted in overall relatively low percentages of MART specific CTL. However, an increased, although not statistically significant, percentage of MART specific CTL was observed when iNKTIL-12 cells were added (p=0.11, figure 3d).

**Figure 4:** iNKT cell mediated help is IFNy dependent. a) Multiple bulk co-cultures (n=6 / condition) of isolated CD8β T-cells and autologous MoDC pulsed with MART1 peptide and αGC were set up in a trans-well system (-iNKT). IL-12 stimulated iNKT cells were added either to the lower well (+iNKTlow), to the upper well alone (+iNKTup) or in the presence of αGC pulsed MoDC (+iNKTup +DCup). Percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the mean. Results shown are from one representative donor out of three tested, p values are the result of a two sided student’s T-test. b) Multiple bulk co-cultures (n=4/condition) of isolated CD8β T-cells and autologous MoDC pulsed with MART1 peptide and αGC were set up in the presence or absence of IL-12 stimulated iNKT cells (iNKTIL-12) in the presence of blocking antibodies against IFNy, IL-4, IL-10 or isotype controls. Percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the median. Results are from one donor out of two tested, p values are from a two-sided Mann-Whitney U-test. c) Multiple bulk co-cultures (n=6/condition) of isolated CD8β T-cells and autologous MoDC pulsed with MART1 peptide and αGC were set up (-), recombinant IFNy or iNKTIL-12 cells were added. Percentage of Tm positive CD8+ T-cells is shown, horizontal lines represent the mean, results are from one donor out of three tested, p values are the result of a two sided Student’s T-test.
**iNKT cell mediated help is IFNγ dependent**

To analyze whether the enhanced expansion of MART1 specific CTL in the presence of iNKT<sup>IL-12</sup> cells was dependent on cell-cell contact or soluble factors, co-cultures were set up in trans-wells. Lower wells contained CD8<sup>+</sup> T-cells and peptide and αGC pulsed MoDC (-iNKT; figure 4a). iNKT<sup>IL-12</sup> were added to the lower wells (+iNKT<sub>low</sub>), the upper wells alone (+iNKT<sub>up</sub>) or together with αGC pulsed MoDC (+iNKT<sub>up</sub>+DC<sub>up</sub>). Similar CTL frequencies were obtained when iNKT cells were added to the lower wells and to the upper wells as long as αGC presenting DC were also present to trigger the iNKT cells, suggesting that soluble factors were responsible for the observed enhanced effects (figure 4a). Since iNKT<sup>IL-12</sup> cells provoked the strongest effect and contained high numbers of IFNγ, IL-10 and IL-4 producing cells, MART1 CTL stimulation cultures were performed in the presence of iNKT<sup>IL-12</sup> cells and IFNγ, IL-10 or IL-4 blocking antibodies. Blocking of IFNγ, IL-4 and IL-10 had no effect on specific CTL frequencies in the absence of iNKT cells (figure 4b). However, the enhanced effect of iNKT<sup>IL-12</sup> cells was completely blocked by anti-IFNγ, while anti-IL-4 antibodies did not have any effect. Interestingly, in the presence of anti-IL-10 antibodies the CTL frequencies increased even further (figure 4b). Finally, to confirm that IFNγ can indeed enhance the MART1 specific CTL response, soluble recombinant IFNγ was added to co-cultures and had similar enhancing effects as iNKT<sup>IL-12</sup> (figure 4c).

αGC and MART1 double loaded IL-12 over-expressing MoDC induce activation of iNKT cells and increased MART1 specific CTL expansion

In order to examine whether IL-12 stimulated iNKT cells in PBMC physiologically enhance CTL expansion, cultures were started from PBL and αGC/MART1 double loaded MoDC. Multiple bulk cultures of total monocyte depleted PBL were stimulated with autologous mature MoDC, transfected with MART1 minigene ubi(MART)4 mRNA or ubi(MART)4-2A-IL-12 mRNA (35), either loaded with vehicle or αGC. Bulk cultures stimulated with αGC loaded MoDC contained significantly higher numbers of iNKT cells, which were further increased when DCs were transfected with IL-12 mRNA (figure 5a). As expected and previously described by us (35), stimulation with IL-12 over-expressing MoDC leads to an increased MART1 specific CTL response (figure 5b). Pulsing with αGC without IL-12 over-expression had no significant effect, however IL-12 over-expressing DC pulsed with αGC induced significantly more MART specific CTL as compared to either IL-12 over-expressing DC or αGC pulsed DC (figure 5b).

**Discussion**

Here we demonstrate that healthy donor derived, expanded iNKT cells, and IL-12 stimulated iNKT cells in particular, enhance antigen specific CTL responses in vitro. This effect was dependent on αGC induced IFNγ production. We have previously demonstrated, that despite impaired numbers of iNKT cells in cancer patients, IFNγ production by the remaining iNKT cells was not affected (14). Furthermore, iNKT cells from cancer patients could be expanded and skewed to produce IFNγ in vitro, to similar levels as healthy donor derived iNKT cells (40). In line with these findings we demonstrate here that melanoma patient derived, in vitro expanded iNKT cells, enhanced melanoma antigen specific CTL as well, demonstrating their potential for the enhancement of CTL responses. Apart from IFNγ,
IL-12 can also enhance IL-4 and IL-10 production by conventional T-cells (41,42). Indeed after stimulation with IL-12 over-expressing DC, not only an increased number of IFNγ producing iNKT was detected, but a small increase in IL-10 producing iNKT cells was observed as well. The positive effect of iNKT cell derived IFNγ on CTL proliferation was not affected by concomitant IL-4 secretion; blocking of IL-10 however, further enhanced CTL expansion. Although only few iNKT cells produced IL-10 (see figure 2f), the amount of IL-10 may have been sufficient for the inhibiting effect. Alternatively, MoDC derived IL-10 induced by iNKT cell-MoDC interactions may inhibit CTL expansion. In contrast to our findings here, Osada and colleagues previously demonstrated an inhibition of MART1 specific CTL responses by expanded CD4+ iNKT cells. This inhibition was type 2 cytokine (a.o. IL-4 and IL-10) mediated. In the present study the majority of the added iNKT cells was CD4+ (80-90%). However, there are several technical aspects which may explain the discrepancies. In healthy donors the frequency of circulating iNKT cells ranges from undetectable to 0.3% of circulating T-cells. In the present study iNKT cells were added to the co-cultures approximating physiological proportions, i.e. 0.2% (1000 iNKT were added to 0.5x10E5 CD8αβ+ T-cells). The much higher dose of iNKT cells (i.e. 10%) used by Osada and co-workers may have had different effects on CTL responses. IFNγ effects may come apparent at lower doses, while inhibition by IL-4 may become effective at higher concentrations. Both Osada et al. and Ho et al. observed a decline in antigen specific CTL when free αGC and antigenic peptide were added to total PBMC and autologous DC co-cultures (28,29). It was concluded that this was due to type 2 cytokine release by CD4+ iNKT cells and lysis of APC and/or activated T-cells by CD4+CD8αα+ iNKT cells respectively. Addition of free MART peptide and αGC enables peptide presentation by (MART specific) CTL as well as αGC presentation by activated T-cells expressing CD1d (28) facilitating fratricide and iNKT cell mediated killing of MART specific CTL respectively, which may lead to reduced MART specific CTL rather than increased frequencies. Here, lysis of
activated MART1 specific CTL is less likely to occur because MART1 was either introduced by mRNA transfection or the peptide was loaded, as was αGC, on DC before addition to total PBL or isolated CD8β+ T-cells. *In vivo*, αGC loading specifically on DC also appears to be important, since in mice treatment with free αGC induced iNKT cell anergy in contrast to adoptive transfer with αGC pulsed DC, which induces prolonged iNKT cell responsiveness (43). Similarly, in humans free αGC induces immune activation only after the first injection without iNKT expansion (44), while adoptive transfer with mature αGC pulsed DC leads to a sustained expansion of iNKT cells (45). Addition of free αGC to total PBMC may lead to presentation by non-professional APC leading to iNKT cell unresponsiveness. Furthermore, to provide iNKT cell mediated help for antigen specific CTL induction *in vivo*, the same DC had to present both the peptide and the glycolipid (7,8). This is in contrast to our transwell results, where iNKT cells activated by αGC pulsed DC in the upper well could enhance CTL responses induced by antigen loaded MoDC in the lower well. This is probably due to the experimental conditions: a confined environment of a small well where cytokine concentrations remain high and can easily reach all the cells versus the *in vivo* situation where cytokine levels rapidly diffuse, and the differentially loaded DC have a low chance of ending up in close proximity to each other in the secondary lymphoid organs.

The cytokine neutralization experiments revealed that IFNγ is at least one soluble factor responsible for the enhanced proliferation of antigen specific CTL, this effect was αGC dependent. It is not yet clear how the iNKT cell derived IFNγ enhances CTL responses. It may act together with CD40 signaling provided by helper cells present in the co-cultures to enhance IL-12 production by MoDC, which is an essential third signal for the development of CTL responses. However, as we have previously demonstrated for help provided by irradiated PBL (35) or isolated NK cells (46), CTL expansion is even further enhanced by iNKT cells (figure 5b) when DC over-express IL-12. These results suggest that enhancing IL-12 secretion by DC is not the only mechanism at work here. Alternatively, IFNγ enhances CTL responses by positively affecting antigen presentation, at least in those experiments using MART1 mRNA transfection for antigen loading of DC, through up-regulation of HLA class I expression and optimizing access of antigenic peptides to HLA class I (47) or by direct signaling through the IFNγR on CD8+ T-cells (48).

MART1 presenting MoDC pulsed with αGC without IL-12 over-expression did not increase CTL expansion within PBL (figure 5b), in contrast to when expanded iNKT cells were added (figure 3a). This may be due to the type 1 skewing growth factor IL-15, which was added (next to IL-2 and IL-7) during the *in vitro* expansion of iNKT cells. This may generate iNKT cells more prone to produce IFNγ than the iNKT cells in the PBL experiments to which IL-2 and IL-7 but no IL-15 was added. This IL-15 mediated pre-activation may be necessary in the absence of IL-12 for sufficient iNKT cell derived IFNγ, while in the presence of IL-12, αGC does have an effect on CTL expansion.

The opposing effects of iNKT cells, i.e. on enhancing anti-tumor immune responses on the one hand and inhibiting auto-immunity on the other hand have been explained by the existence of iNKT cell subsets, based on CD4 and CD8αα expression, secreting distinct cytokine profiles *ex vivo* (18,19). Here we show that upon culture of human blood derived iNKT cells the distinction based on CD4 expression becomes less clear. Both CD4− and CD4+ iNKT cells produced IFNγ as well as IL-4, and IFNγ production was enhanced by IL-12 in CD4+ as well as CD4− iNKT cells, demonstrating the plasticity of iNKT cell subsets. The ratio of CD4+/CD4− iNKT cells also changed during *in vitro* culture. Human CD4− and CD4+ iNKT cells have differential homeostatic requirements, CD4− iNKT cells proliferate with a higher rate in response to IL-15, while CD4+ iNKT cells respond better to IL-7 *in vitro* (49).
Nevertheless, the CD4^-CD4^+ ratio is reduced upon culture in vitro irrespective of the cytokine used, due to a higher proliferation rate of CD4^+ iNKT cells (50), which may be due to the fact that CD4^- iNKT cells have been through more cell divisions in vivo as illustrated by a decrease in TRECs in CD4^- iNKT cells (49). IL-12 stimulation induced an overall increase in activation markers, but despite the increase in Granzyme B expression, cytolytic activity was not enhanced. This is probably due to the fact that the expression of NKG2D, which is thought to play an important role in cytolytic activity by conventional NK and T-cells (51), is not modulated by IL-12 (not shown).

In conclusion, IFNγ production by iNKT cells can be increased, irrespective of CD4 expression, by stimulating with IL-12 producing DC. These iNKT cells enhance antigen specific CTL expansion in an IFNγ dependent fashion. Phase I studies aiming at increasing iNKT cell numbers and activation by i.v. injection of αGC, αGC pulsed DC or in vitro expanded iNKT cells were shown to be safe (44,45,52). Both, vaccination with αGC pulsed mature DC and iNKT cell adoptive transfer, resulted in an increase in circulating iNKT cell numbers and IFNγ producing PBMC but no clinical responses were observed. Using the approach described here, the efficacy of both treatment modalities may be enhanced. In vitro expansion of iNKT cells using IL-12 over-expressing (allogeneic) DC will lead to an iNKT cell population for adoptive T-cell transfer which is superior in providing help for antigen specific CTL. However, additional treatment with αGC, presented by DC, may be needed to re-activate the iNKT cells in vivo. An even more effective form of iNKT cell mediated immunotherapy would be vaccination with antigen and αGC loaded, IL-12 over-expressing, DC. This approach is expected to lead to an increased anti-tumor immune response mediated by TAA specific CTL as well as IFNγ producing iNKT cells.

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References


Chapter 4

In vitro expanded human invariant Natural Killer T-cells promote functional activity of Natural Killer cells

María Moreno, Johan W. Molling, Silvia von Mensdorff-Pouilly, René H.M. Verheijen, B. Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

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Abstract

Invariant Natural Killer T (iNKT) cells play a pivotal role in cancer immunity through trans-activation of effector cells via swift cytokine secretion. In mice, iNKT cell activation by α-Galactosylceramide (α-GC) induces potent NK cell-mediated anti-tumour effects. Here we investigated whether human iNKT cells could enhance NK cell functional activity in vitro. iNKT cell activation by α-GC treatment of peripheral blood mononuclear cells (PBMC) was not sufficient to enhance NK cell effector functions. However, addition of in vitro expanded iNKT cells to PBMC enhanced NK cell-mediated cytotoxicity in an α-GC-dependent manner. NK cell activation by iNKT cells was primarily mediated by soluble factors, and could be enhanced by the NK cell activating cytokine IL-21. These results suggest that adoptive transfer of ex vivo expanded iNKT cells will enhance NK cell function and is expected to enhance the efficacy of cancer immunotherapy, particularly in combination with IL-21 and α-GC.

Keywords: human, invariant NKT cells, α-Galactosylceramide, NK cells, cytotoxicity, interferon-γ

Abbreviations: α-GC: α-galactosylceramide; CTL: cytotoxic T lymphocytes; DC: dendritic cells; IFN-γ: interferon-γ; iNKT: invariant natural killer T-cells; moDC: monocyte derived dendritic cells; NK: natural killer cells; PBMC: peripheral blood mononuclear cells; TNF-α: tumour necrosis factor-α
Introduction

Invariant Natural Killer T (iNKT) cells are T lymphocytes characterized by an invariant TCR α chain gene rearrangement (Vα24-Jα18 paired with Vβ11 in humans) and co-expression of NK cell receptors (1). This invariant TCR recognizes glycolipid ligands, such as the putative endogenous ligand isoglobotrihexosylceramide (iGb3), bacterial glycosphingolipids or the artificial ligand α-Galactosylceramide (α-GC), in the context of the monomorphic CD1d antigen-presenting molecule (2-7). Upon stimulation, iNKT cells have the capacity to rapidly produce large amounts of cytokines e.g. IFN-γ and IL-4 (1), leading to the trans-activation of other immune cells, such as dendritic cells (DC), natural killer (NK), T and B cells (8-11). In mice, iNKT cells play an important role in tumour immunosurveillance (8;12-17). In vivo iNKT cell activation by α-GC or α-GC-loaded DC can induce potent anti-tumour immune responses in various tumour models by NK cell trans-activation (18-22).

In humans, we and others have demonstrated that cancer patients have reduced circulating iNKT cell numbers (23-27). In addition, we recently observed in a prospective study that a relatively large circulating iNKT cell pool predicts favourable clinical outcome in individuals with squamous cell carcinoma of the head and neck (28). These findings suggest that cancer patients might benefit from immunotherapy aimed at the expansion of their peripheral blood iNKT cell pool.

We have recently demonstrated that human iNKT cells can enhance tumour antigen specific cytotoxic T lymphocyte (CTL) responses (29). Next to CTL, human NK cells have a high cytolytic potential against tumour cells. NK cells discriminate between normal and tumour cells due to the loss of NK cell inhibiting MHC class I molecules (30). Since MHC class I loss renders tumour cells invisible to MHC class I restricted CTL (31), it is relevant to examine whether human iNKT cells not only enhance tumour antigen specific CTL responses but also induce NK cell functional activity. Here, we investigated the effect of human iNKT cell activation on phenotypic and functional NK cell activation.

Materials and methods

Cell lines and culture media

IMDM (Cambrex, Verviers, Belgium) was supplemented with 10% Foetal Calf Serum (Perbio, Helsingborg, Sweden) for culture of peripheral blood mononuclear cells (PBMC), monocyte-derived DC (moDC) and the NK-sensitive K562 (ATCC, Manassas, VA, USA) cell line, or 8% human pooled serum (Sanquin, Amsterdam, The Netherlands) for the culture of iNKT cells. Both media were supplemented with 100 IU/ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100μg/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy) and 0.01 mM β-mercaptoethanol (Merck, Darmstadt, Germany).

Isolation of peripheral blood mononuclear cells (PBMC), NK cell purification, iNKT cell expansion

PBMC were isolated from buffycoats from healthy donors obtained from Sanquin Blood Bank (Amsterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The number of cells was determined and PBMC were cryopreserved in separate batches until use. To generate moDC, 50x10^6 PBMC were allowed to adhere in a T75 flask for 60-90 min. Non-adherent cells were washed away by
rinsing the flask with PBS. Adherent monocytes were then cultured in the presence of 1000 IU/ml GM-CSF (Schering-Plough, Kenilworth, NJ, USA) and 10 ng/ml IL-4 (R&D Systems, Abingdon, UK) for 7 days. Maturation was induced by 48hs culture in the presence of 30% v/v monocyte conditioned medium (MCM) and 50 ng/ml TNF-α (Strathmann Biotech, Hanover, Germany) as described (32).

Vα24+ Vβ11+ iNKT cells were enriched from PBMC by positive selection using the iNKT cell specific antibody 6B11 (BD Biosciences, San Jose, CA, USA) and anti-mouse Ig-coated magnetic beads mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS sorting. The non-selected PBMC (iNKT cell-depleted PBMC, containing < 0.00% iNKT cells among CD3+ lymphocytes) were cryopreserved until use. The selected iNKT cells were expanded by weekly stimulation with irradiated autologous PBMC at a 1:1 iNKT : PBMC ratio in the presence of 100ng/ml α-GC (KRN7000, kindly provided by Dr Shigeyuki Yamano, KIRIN Brewery, Gunma, Japan), 40 IU/ml recombinant human (rh) IL-2, 5 ng/ml rhIL-7 and 5 ng/ml rhIL-15 (all purchased from Strathmann Biotech, Hanover, Germany).

NK cell enrichment was performed by negative selection using Easysep human NK cell enrichment kit (StemCell Technologies, Grenoble, France) according to manufacture’s recommendations. Achieved purity was higher than 99.5%.

**Evaluation of NK cell activation by iNKT cells**

Donor PBMC (5x10^6 cells/ml) were cultured in the presence or absence of 100 ng/ml α-GC. *In vitro* expanded iNKT cells were added to iNKT cell-depleted PBMC at different percentages, as specified, and cultured for 5 days. In some experiments, IL-21 (25 ng/ml) was added to the cultures on day 2.

In order to analyse the involvement of soluble factors, 2x10^6 NK cells were cocultured with 2x10^5 iNKT cells, 2x10^5 α-GC-pulsed moDC or both in a trans-well system (0.4μm pore size, Corning Life Science, Schiphol-Rijk, The Netherlands) or in the presence of 400 IU/ml of IFN-γ (Biosource, Camarillo, CA, USA), or 4 μg/ml IFN-γ neutralizing antibody (R&D Systems, Abingdon, UK), for 5 days.

**NK cell effector function analysis**

After 5 days of iNKT cell-mediated stimulation of either PBMC or NK cells, NK cell effector functions were analysed. Cytolytic activity of the NK cells to K562 cells was assessed using a standard chromium release assay. Briefly, 1x10^5 K562 target cells were labelled with 100 μCi of Na_2[^51]CrO_4 (Amersham, Bucks, U.K.) for 2h at 37°C and washed extensively. Effector PBMC were harvested, washed and added to 5x10^3 target cells at the indicated E:T ratios in triplicate wells of a round-bottom 96-well plate (Nunc). After 18h incubation at 37°C, 50 μl of the supernatant was harvested, and its radioactive content was measured. The percentage specific lysis was defined as follows: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x 100%.

Alternatively, NK cell cytotoxic capacity was determined in a CD107a (LAMP1) translocation assay (33). NK cells (1x10^5 cells) were cultured in the presence of PE-labelled mouse anti-human CD107a (BD Biosciences, San Jose, CA, USA) and 4μM monensine in the absence (background translocation) or presence of K562 target cells (0.5x10^5 cells). After 5 hours, cells were washed and counterstained with CD3 and CD56 for at least 30 min at 4°C. Flow cytometry was performed on a FACSCALIBUR™ apparatus and data were analysed using CellQuest™ software (BD Biosciences, San Jose, CA, USA). The percentage of
CD107a positive NK cells and the mean fluorescence index (MFI) of CD107a expression induced by K562 were calculated as follows:

\[
\% \text{CD107a}^+ = \% \text{CD107a}^+ \text{upon K562 stimulation} - \% \text{CD107a}^+ \text{background}
\]

\[
\text{MFI} = \frac{\text{mean fluorescence intensity upon K562 stimulation}}{\text{background mean fluorescence intensity}}.
\]

**Cytokine detection**

For determination of cytokine secretion, supernatants were harvested after 5 days of culture. Supernatants were analysed for IL-2, IFN-\(\gamma\) and TNF-\(\alpha\) by ELISA using the appropriate PeliKine Compact human ELISA kit (Sanquin, Amsterdam, The Netherlands) according to the manufacturer’s recommendations.

**Surface marker expression on iNKT and NK cells**

FITC-, PE-, PerCP-Cy5- or APC-labelled isotype controls and mouse anti-human 6B11, CD3, CD4, CD16, CD25, CD69 (BD Bioscience, San Jose, CA, USA), V\(\alpha\)24, V\(\beta\)11 (Immunotech, Marseille, France) and CD56 (IQ products, Groningen, The Netherlands) were used to determine the phenotype of NK and iNKT cells by flow cytometry. Cells were incubated with the antibodies for at least 30 min at 4°C. Flow cytometry was performed on a FACSCALIBUR™ apparatus and data were analysed using CellQuest™ software (BD Biosciences, San Jose, CA, USA).

**IFN-\(\gamma\) and TNF-\(\alpha\) intracellular cytokine staining (ICCS)**

Intracellular IFN-\(\gamma\) and TNF-\(\alpha\) staining was performed using the BD cytofix/cytoperm plus kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer’s instructions. After 5 days of iNKT cell-stimulation of NK cells, GolgiPlug was added to each well (0.1% v/v) for 5h. iNKT and NK cells were washed and stained with FITC-labelled 6B11 and PerCP-Cy5-labelled CD3, and APC-labelled CD56 respectively, followed by intracellular staining with PE-labelled anti-IFN-\(\gamma\) or PE-labelled anti-TNF-\(\alpha\) (both from BD Bioscience, San Jose, CA, USA).

**Statistics**

Normality of distribution of the data was analysed with Kolmogorov-Smirnov test and Q-Q plots. The non-parametric Wilcoxon Signed Ranks test was used to analyse increase in cytokine (IFN-\(\gamma\) and TNF-\(\alpha\)) production. Otherwise, a parametric paired Student’s T-test was used to analyse the differences between groups. Non-parametric correlation Spearman’s test was performed to investigate the relation between cytokine secretion and up-regulation of activation markers. \(P < 0.05\) was considered significant. All data were analysed using SPSS 14.0 software.
Results

iNKT cell activation with α-GC is not sufficient to augment NK cell activity

In vitro stimulation of PBMC, containing various numbers of iNKT cells (range 0.01-0.19% iNKT cells among CD3+ lymphocytes), with α-GC for up to 5 days induced iNKT cell activation, as demonstrated by an increased proportion of iNKT cells expressing CD69 and CD25 (p<0.001 and p=0.017, respectively) (figures 1a and b). A limited, but statistically significant NK cell activation was observed as well (p=0.024 and 0.027, respectively) (figures 1c and d). However, this limited phenotypic NK cell activation may not be biologically significant since no enhanced NK cell-mediated cytotoxicity against K562 was observed (figure 1e). Moreover, IFN-γ, as well as TNF-α, secretion was only modestly increased following α-GC treatment of total PBMC [Mean (range) 53 (0-194) vs 185 (0-768) pg/ml IFN-γ, and 11 (0-30) vs 25 (5-62) pg/ml TNF-α, in supernatants of unstimulated vs α-GC-stimulated PBMC, p>0.05]. Of note, no increase in IL-2 secretion could be detected. Thus, while addition of α-GC to total PBMC was sufficient to induce phenotypic activation of iNKT cells accompanied with a modest increase in cytokine production, this activation was insufficient to establish enhanced NK cell lytic activity.

Figure 1: Invariant NKT cell activation by PBMC stimulation with α-GC. PBMC were stimulated with 100ng/ml α-GC. Activation status of a, b) iNKT cells (defined as CD3+ 6B11+ cells) and c, d) NK cells (defined as CD3- CD56+ cells) after 5-days culture. a, c) Example dot-plots of CD69 and CD25 expression on iNKT and NK cells after 5 days without and with α-GC. The numbers in the right upper quadrant represent the % of positive cells. b, d) The graphs show the mean ± SEM of CD69 and CD25 expression of 5 independent experiments performed with individual donors, in the absence (open bars) or presence (solid bars) of α-GC. Paired Student’s T-test: *p<0.05. e) NK cell effector function was assessed by K562 cytotoxic assays. Results from one representative experiment out of 7 performed are shown.
Addition of expanded iNKT cells to iNKT-depleted PBMCs enhances NK cell cytotoxicity in an α-GC-dependent manner

We have previously developed a method to generate high purity pro-inflammatory iNKT cell lines from healthy donors in vitro (34). Using an adapted version of this method, we expanded iNKT cells from healthy donor PBMC to investigate their capacity to activate NK cells. After 2 expansion rounds, purity of iNKT cells was more than 90%, and the cells exhibited an activated phenotype, demonstrated by CD69 expression (median 7%, range 1-34%, n=5) and an increased CD25 expression (median 46%, range 32-66%, n=5), compared to resting iNKT cells (figure 1b, open bars). Expression of both activation markers could be further increased by antigen stimulation (data not shown).

To test the capacity of these pre-activated iNKT cells to activate NK cells, iNKT-depleted PBMC were co-cultured with increasing percentages (within the physiological range, i.e. 0.02%, 0.10% and 0.50%) of in vitro expanded iNKT cells (>90% pure) for 5 days. Addition of iNKT cells resulted in an iNKT cell dose-dependent increase in IFN-γ (figure 2a) and TNF-α (data not shown) secretion. Higher cytokine levels were detected in cultures containing α-GC, although there was a large variation between donors. Ex vivo stimulation of total PBMC with α-GC leads to much lower levels of IFN-γ (see previous paragraph; 185 (0-768) pg/ml), which is indicative for enhanced cytokine secretion by expanded iNKT cells compared to ex vivo stimulated iNKT cells. Addition of pre-activated iNKT cells did not modify percentage of NK cells but enhanced CD25 and CD69 expression on NK cells to a similar extend as adding α-GC to total PBMC (data not shown). Of note, after 5 days the proportion of iNKT cells in culture remained also unchanged. However, in addition to this phenotypic activation, NK cell functional activity was enhanced as well. Despite some variation in K562 lysis (5.4 - 19.9%) due to differences in NK cell frequencies between donors, the enhanced effect of the iNKT cells was observed in all donors (1.2 - 3.3 fold-increase in cytotoxicity at E:T=12.5:1). In the absence of α-GC, the addition of expanded iNKT cells to PBMC had a limited effect on NK cell cytotoxicity (figure 2b). On the other hand, NK cell ability to kill K562 was substantially augmented when PBMC were co-cultured with in vitro expanded iNKT cells in the presence of α-GC, in an iNKT cell dose-dependent manner (figure 2c). This effect is not mediated directly by iNKT cells, as they have been shown to be unable to kill K562 (25;35).

![Figure 2](image-url)

**Figure 2**: α-GC-dependent NK cell activation by expanded iNKT cells. iNKT-depleted PBMC were co-cultured with 0.02, 0.10 and 0.50% in vitro expanded iNKT cells, in the absence or presence of iNKT cell ligand. After 5 days, a) IFN-γ secretion in supernatants was determined by ELISA. Wilcoxon Signed Ranks test: *p<0.05. b, c) NK cell effector function was assessed by K562 cytotoxic assays. Results from one representative experiment out of 5 performed are shown. iNKT-depleted PBMC (open squares) were co-cultured with 0.02, 0.10 and 0.50% in vitro expanded iNKT cells (solid symbols) b) in the absence or c) presence of α-GC.
IL-21 is a strong NK cell activating cytokine. Indeed, *in vivo* activation of murine iNKT cells by α-GC injection, followed by IL-21 administration, enhanced NK cell activity (13). Therefore, we analysed whether sequential activation of human iNKT (with α-GC) and NK cells (with IL-21) could further enhance NK cell-mediated cytotoxicity. Invariant NKT-depleted PBMC were co-cultured with 0.20% *in vitro* expanded iNKT cells (>90% pure), in the presence or absence of α-GC for 5 days. At day 2, IL-21 was added to the co-cultures. The proportions of iNKT and NK cells remained unaltered. The percentage in NK cell-mediated K562 killing varied between 2.2 and 6.7%, which was modestly increased when PBMC were stimulated with *in vitro* expanded iNKT cells followed by IL-21 in the absence of α-GC in all donors tested (1.3 – 3.9 fold increase at E:T=12.5:1) (figure 3a). In the presence of α-GC, combination of both treatments induced a strong increase in NK cell cytolytic activity (5.5 - 10.3 fold increase) (figure 3b). These data show that NK cell cytotoxic function can be enhanced by sequential activation of human iNKT (with α-GC) and NK cells (with IL-21).

![Figure 3](image-url)

*Figure 3: Effect of α-GC-stimulated iNKT cells and IL-21 combination on NK cell activation. K562 cytolysis performed with a) unstimulated and b) α-GC-stimulated iNKT-depleted PBMC without (open squares) or with (solid triangles) 0.20% *in vitro* expanded iNKT cells, in the absence (solid lines) or presence (dashed lines) of IL-21. Results shown are from one representative experiment out of 3 performed.*

**IFN-γ is sufficient but not necessary for iNKT mediated NK cell activation.**

To analyse whether the enhanced activation of NK cells by iNKT cells is dependent on cell-cell contact, soluble factors or both, co-cultures were set up in trans-wells. Lower wells contained purified NK cells alone (-iNKT). Autologous *in vitro* expanded iNKT cells were added to the lower wells (+iNKT<sub>low</sub>) or to the upper wells (+iNKT<sub>up</sub>) alone or together with α-GC-pulsed moDC (+iNKT<sub>low</sub>+DC<sub>low</sub> and +iNKT<sub>up</sub>+DC<sub>up</sub>, respectively). Appropriate moDC controls were included (+DC<sub>low</sub> and +DC<sub>up</sub>). Analysis of the culture supernatants showed a significant increase in IFN-γ (Wilcoxon Signed Ranks test, p=0.018) (figure 4a), and to a lesser extent TNF-α (Wilcoxon Signed Ranks test, p=0.018) (data not shown), secretion in wells containing both iNKT cells and α-GC-pulsed moDC, compared to NK cells alone. Intracellular cytokine analysis revealed that both cytokines were produced by iNKT cells and not by NK cells (data not shown). Interestingly, IL-2 levels in supernatants dropped under these conditions (data not shown), suggesting IL-2 consumption by NK cells, iNKT cells or both. In parallel with IFN-γ secretion, NK cell activation status (figures 4b and 4c), particularly CD69 expression, was augmented when NK cells were cultured with iNKT cells in the presence of α-GC-pulsed moDC. Likewise, NK cell lytic capacity against K562...
induced by iNKT cells, determined by CD107a translocation, was also enhanced under these conditions. This was illustrated by an increase both in the percentage of NK cells that translocated CD107a (1.3–3.9 fold increase) (figure 4d) and in the amount of degranulation on a single cell level (1.4–2.5 fold increase in CD107a surface expression level) (figure 4e). NK cell activation could likely be attributed to the iNKT cells because addition of α-GC-pulsed moDC alone, either in the lower or in the upper wells, had no effect on NK cell activation.

Figure 4: NK cell activation by expanded human iNKT cells is mediated by soluble factors, the production of which is dependent on iNKT cell - α-GC-loaded moDC contact. Trans-well experiments were performed by culturing purified NK in the lower wells (-iNKT). Autologous in vitro expanded iNKT cells were added to the lower wells (+iNKTlow) or to the upper wells (+iNKTup) alone or together with α-GC-pulsed moDCs (+iNKTlow+DClow and +iNKTup+DCup, respectively). Appropriate moDC controls were included (+DClow and +DCup). After 5 days, a) IFN-γ secretion in supernatants was assessed by ELISA (mean ± SEM of 7 individual experiments). Wilcoxon Signed Ranks test: *p<0.05. NK cell activation was determined as up-regulation of b) CD69 and c) CD25 expression (mean ± SEM of 5 individual experiments). Paired Student’s T-test: *p<0.05. NK cell lytic capacity against K562 was determined as d) percentage of CD107a positive cells and e) CD107a MFI within NK cell population (mean ± SEM of 3 individual experiments).

These data suggest a role for soluble factors in iNKT cell-mediated NK cell activation. Indeed, correlation analysis showed a significant correlation between increase in IFN-γ secretion and up-regulation of CD69 expression (p<0.001) (figure 5a), as well as CD107a translocation by NK cells (p=0.001) (figure 5b). Correlation was also observed for TNF-α (p=0.003 and <0.001, respectively) (data not shown). To confirm that IFN-γ can indeed enhance the NK cell response, soluble recombinant IFN-γ was added to NK cell cultures. Indeed, addition of IFN-γ had similar enhancing effect as iNKT cells: up-regulation of CD69 and increase in lytic function in both donors tested (figures 6a and 6b). However, the enhanced effect of iNKT cells was not blocked by an IFN-γ neutralizing antibody in either of
the two donors tested (figures 6c and 6d), while IFN-γ secretion was completely inhibited (data not shown). These results suggest that beside IFN-γ, other soluble factors, such as TNF-α or IL-2, can play a role in NK cell activation.

Interestingly, higher cytokine levels, which were associated with the stronger NK cell activation, were induced when NK cells were cultured in contact with both iNKT cells and α-GC-loaded moDC compared to NK cells cultured with iNKT cells and α-GC-loaded moDC in the trans-well, indicating a role for cell-cell contact between NK and iNKT cells next to soluble factors. This is also suggested by the observation of slight, although not significant, increase in cytokine secretion, and NK cell activation, after co-culture of NK cells in contact with iNKT cells alone (+iNKTlow) in some of the donors (figure 4).

![Figure 5: Human iNKT cell-mediated NK cell activation correlates with IFN-γ cytokine secretion. Spearman’s correlation between increase in IFN-γ secretion and a) CD69 up-regulation on NK cells and b) enhanced NK cell lytic capacity against K562.](image)

**Discussion**

We investigated whether human iNKT cells activated by α-GC could promote NK cell activation, in the context of improving NK cell-mediated tumour immunotherapies. In murine models, resident iNKT cells can augment anti-tumour responses upon systemic treatment with α-GC or α-GC-loaded DC, as a result of NK cell trans-activation by iNKT cells (13;19;22). Circulating iNKT cells of patients with cancer are significantly reduced compared to healthy controls (23-27). Nonetheless, the iNKT cells in these patients still possess the capacity to proliferate and to secrete IFN-γ when properly stimulated in vitro (28). This suggests that iNKT cells of cancer patients, though reduced in number, may still be capable of enhancing anti-tumour responses in a physiological setting or after therapies aiming at their increase and activation. One approach to achieve the latter could be by systemic α-GC treatment. Here we demonstrate that stimulation of total PBMC with α-GC strongly promotes phenotypic activation of iNKT cells as evidenced by a highly increased expression of CD69 and CD25. However, α-GC induced only a modest increase in IFN-γ production. Although NK cells became phenotypically activated, functional activity of NK cells was not enhanced by this approach. In vivo, systemic treatment with free α-GC in mice induces iNKT cell anergy, rendering iNKT cells unresponsive to subsequent α-GC treatment (11). Similarly, administration of free α-GC in humans induces a transient and limited increase of cytokines (IFN-γ, TNF-α, IL-12 and GM-CSF) without enhancing NK cell cytolytic activity.
Furthermore, the transient cytokine increase was only observed in patients with normal pre-treatment iNKT cell numbers (36). These results combined with the data we present here where stimulation with free α-GC, even of PBMC derived from healthy donors with relatively high numbers of iNKT cells, is not sufficient to enhance iNKT cell numbers and NK cell-mediated cytolytic activity, exemplify that alternative treatments to expand and activate iNKT cells in vivo are warranted. This can be achieved either by vaccination with α-GC-pulsed mature moDC or by adoptive transfer of in vitro expanded iNKT cells. In this study we demonstrate an enhanced, α-GC-dependent, NK cell activation status and cytotoxic effector function upon addition of expanded and pre-activated iNKT cells. Furthermore, we demonstrate that sequential activation of in vitro expanded human iNKT cells with α-GC, followed by IL-21 treatment to mature the iNKT cell-activated NK cells into highly cytotoxic effector cells, strongly enhanced NK cell cytotoxicity. Previously, Lin et al. (37) showed upregulated CD69 expression on NK cells early after addition of in vitro expanded iNKT cells to PBMC, but CD25 expression was not affected. Here we show that in vitro expanded human iNKT cells not only promote phenotypic activation of NK cells, but induce functional NK cell activation as well. Both effects were dependent on α-GC. Transwell experiments showed that iNKT cell-mediated NK cell activation is mainly mediated by soluble factors, and correlates with cytokine (IFN-γ and TNF-α) production. Recombinant IFN-γ induced a similar level of NK cell activation as addition of iNKT cells. However, human iNKT cell-mediated NK cell activation was not solely dependent on IFN-γ, as neutralization of IFN-γ did not abrogate the effects of iNKT cells (figures 6c and b). Interestingly, when NK cells were co-cultured with iNKT cells in the presence of α-GC-loaded moDC, IL-2 completely disappeared from supernatants (data not shown), most likely due to consumption during NK cell activation. Thus other factors such as TNF-α or IL-2 may be sufficient to induce NK cell activation. A role for IL-2 was indeed demonstrated by Metelitsa et al., who showed that the iNKT cell-mediated enhancement of NK cell activity was primarily mediated by IL-2, an effect which was enhanced by IFN-γ (38). However in contrast to our findings these authors demonstrated a small but significant effect of IFN-γ neutralization while the addition of recombinant IFN-γ had only an effect when added in combination with IL-2. These differences may be explained by different dosages of recombinant IFN-γ added and the experimental conditions: i.e. the addition of iNKT cell culture supernatants versus the addition of iNKT cells in trans-wells.

**Figure 6:** IFN-γ is sufficient but not necessary for iNKT mediated NK cell activation. 2x10⁶ NK cells were co-cultured with 2x10⁵ α-GC-loaded moDC. 400IU/ml soluble recombinant IFN-γ or 2x10⁵ in vitro expanded iNKT cells were added. a) Percentage of CD69⁺ cells within NK cell population is shown. b) NK cell lytic capacity against K562 was determined as CD107a MFI. NK cell cells were cultured alone or with in vitro expanded iNKT cells in the presence or absence of α-GC-loaded moDC in a trans-well system, in the presence of 4μg/ml blocking antibodies against IFN-γ (solid bars) or isotype control (open bars). c) Percentage of CD69⁺ cells within NK cell population is shown. d) NK cell lytic capacity against K562 was determined as CD107a MFI. The graphs show the results of one experiment out of two performed.
In addition to soluble factors, direct contact-mediated interactions between NK and iNKT cells appear to play a role as well in iNKT cell-mediated NK cell activation. It is not yet clear which molecules are involved in iNKT cell-NK cell interactions, and whether TCR ligation is required. While endogenous glycolipid antigens, such as iGb3 (6), could act as iNKT cell ligand, CD1d expression on NK cells has not been demonstrated. Other co-stimulatory interactions may play a role. Of interest in this respect is the APC like properties of activated NK cells, expressing co-stimulatory molecules such as CD80, CD86 and OX40 ligand as well as MHC class II (39).

We have recently shown that human iNKT cells enhance antigen specific CTL responses as well. This effect was only dependent on soluble factors and was completely blocked after IFN-γ neutralization (29). These results suggest that the regulation of NK cell activity and antigen specific CTL responses by iNKT cells is mediated by different mechanisms.

Recently it has been demonstrated that adoptive transfer with PBMC enriched for iNKT cells, is well tolerated and resulted in a transient increase in circulating iNKT cells and direct ex vivo IFN-γ production in response to α-GC in an ELISPOT assay (40). Our results provide further evidence that in vitro expanded human iNKT cells can activate NK cells, an effect which depends on α-GC and which is strongly enhanced by IL-21. It may therefore be necessary to combine adoptive iNKT cell therapy with additional IL-21 or α-GC (pulsed DC) treatment to achieve clinical effects in vivo.

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Chapter 5a

High level of MUC1 in serum of ovarian and breast cancer patients inhibits huHMFG-1 dependent cell-mediated cytotoxicity (ADCC)

María Moreno, Hetty J. Bontkes, Rik J. Scheper, Peter Kenemans, René H.M. Verheijen, Silvia von Mensdorff-Pouilly

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High level of MUC1 in serum of ovarian and breast cancer patients inhibits huHMFG-1 dependent cell-mediated cytotoxicity (ADCC)

María Moreno a,*, Hetty J. Bontkes b, Rik J. Scheper b, Peter Kenemans a, René H.M. Verheijen a, Silvia von Mensdorff-Pouilly a

a Department of Obstetrics and Gynaecology, VU Universiteit Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
b Department of Pathology, VU Universiteit Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

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Abstract

The huHMFG-1 (AS1402) antibody is a humanised IgG1 directed against MUC1 and is currently in clinical trials for the treatment of breast carcinoma. Adenocarcinomas over-express and shed MUC1, and high MUC1 serum levels are associated with progressive disease. Here, we have investigated the effects of MUC1 present in sera from breast and ovarian cancer patients and that of NK cells on in vitro huHMFG-1-mediated ADCC, performed with and without the addition of various cytokines. Screening for patients with high levels of NK cells bearing the FcγRIIIa-158V polymorphism, adjusting the dosage to circulating levels of MUC1 and co-administration of NK cell activating cytokines may increase the efficacy of huHMFG-1 treatment.

Keywords: huHMFG-1 monoclonal antibody; MUC1; ADCC; NK cells

1. Introduction

Monoclonal chimeric, humanised and human IgG1 antibody therapies are becoming increasingly available to treat various forms of human malignancies. In a variety of disease entities, such as breast, colon and haematological cancers, antibody therapies have been shown to improve overall survival as well as time to disease progression, particularly when combined with chemotherapy (reviewed in [1]). The mechanism behind anti-tumour activity mediated by monoclonal antibodies depends on their target. Some induce internalisation of growth receptors interfering with signal transduction, such as antibodies directed against Her2/neu and the epidermal growth-factor receptor, but may also induce antibody-dependent cellular cytotoxicity (ADCC) [1–3]. Others, such as antibodies directed against CD20 or the epithelial cel-

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; FCS, foetal calf serum; FcγR, Fc gamma receptor; huHMFG-1, humanized human milk fat globulin 1; IMDM, Iscove’s modified Dulbecco’s medium; mAb, monoclonal antibody; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline.
lular adhesion molecule, primarily act through ADCC [1,4].

MUC1, also known in the clinic as CA15-3 antigen, is a high-molecular-weight transmembrane glycoprotein that is expressed on the ductal cell surface of normal glandular epithelia and is over-expressed in a variety of epithelial cancers [5]. In cancer cells MUC1 is not only over-expressed but also deficiently glycosylated, exposing a variable number of immuno-dominant domains on the MUC1 peptide core, which makes it a good target for immunotherapy [6]. We and others have demonstrated antibody responses to MUC1 in breast, ovarian, colon, lung and pancreatic cancer patients and in healthy women [7–10]. This natural humoral immune response is a favourable prognostic factor [11–14]. Naturally occurring anti-MUC1 antibodies could lead to a capping or clustering of MUC1 on the cell surface, unmasking cell surface molecules involved in cell adhesion and limiting tumour invasion. A redistribution of MUC1 could also be instrumental in exposing cell surface receptors involved in immune recognition processes. This would enable recognition and destruction of the tumour cell by antigen specific T-cells and/or NK-cells.

The humanised MUC1 specific monoclonal antibody huHMFG-1 (AS1402) [15] can mediate in vitro tumour cell killing and is currently evaluated in clinical trials as a potential immunotherapy for breast cancer [16]. MUC1 serum levels correlate with tumour load and progression [17] and could affect huHMFG-1 mediated tumour cell killing. Here we have analysed the effect of MUC1 present in sera of huHMFG-1 mediated ADCC. Approaches to increase the efficacy of huHMFG-1 treatment, such as addition of cytokines and effect of antibody concentration, are investigated and discussed.

2. Materials and methods

2.1. Patients serum samples and MUC1 serum levels

Forty-six serial serum samples obtained from 28 ovarian and breast cancer patients were collected, aliquoted and stored at –80 °C until analysed for MUC1-1 levels and capacity to inhibit huHMFG-1-mediated ADCC. Cancer antigen CA 15-3 (MUC1) serum levels were determined using the ADVIA Centaur CA 15-3 assay (Bayer Corporation, Tarrytown, NY, USA) performed on the fully automated ADVIA Centaur® System. The assay is based on the anti-MUC1 mAb DF3 [18], which is directed to the dominant repeat sequence DTRPAP, to capture antigen and anti-MUC1 mAb 115D8 [19], which is directed to a carbohydrate epitope, as tracer. The cut-off level recommended by the manufacturer (excluding 95% of a normal population) is 30 U/ml [20].

2.2. Isolation of peripheral blood mononuclear cells (PBMCs) and natural killer (NK) cell purification

Peripheral blood mononuclear cells (PBMCs) from 13 different healthy donors were isolated from buffycoats obtained from Sanquin Blood Bank (Amsterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The number of cells was determined and PBMCs were cryopreserved in separate batches until use. NK cell enrichment was performed by negative depletion using the NK cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s recommendations.

2.3. ADCC assay

HuHMFG-1, a humanised IgG1-type MUC1 mAb, was provided by Antisoma plc, London, UK. Highly purified human IgG (Pelicluster-Sanquin, Amsterdam, The Netherlands) was used as control antibody. The breast tumour cell line ZR-75-1 [21] that over-expresses MUC1 was cultured in IMDM plus 10% FCS and was used as target cell line. Donor PBMCs were thawed and cultured overnight in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Gibco, Paisley, Scotland). In some experiments, IL-2 (100 IU/ml), IL-21 (25 ng/ml), IL-15 (10 ng/ml) or IFN-γ (400 IU/ml) were added to the cultures. PBMCs were re-suspended in medium at a concentration of 10^7 cells/ml. Target cells (1 × 10^6 ZR-75-1), were labelled with 100 μCi (3.7 MBq) ⁵¹Cr (Amersham, Buckinghamshire, UK) for 2 h at 37 °C. Cells were washed twice with 10 ml medium and re-suspended in medium at a concentration of 10^⁵ cells/ml. Fifty microlitres of the target cells was seeded in U-bottom 96-well culture plates (Costar, Cambridge, MA, USA). Cells were then incubated with MUC1 mAb or control antibody at a final concentration of 5 μg/ml (unless otherwise specified). Alternatively, serum samples were added. After 30 min incubation at 37 °C, 50 μl of the PBMC were seeded into the culture plates at effector:target cell (E:T) ratios 25:1, 50:1 and 100:1, and the plates were incubated for another 18 h at 37 °C. The release of ⁵¹Cr in the supernatant was determined in an automatic gamma counter. Each measurement was carried out in triplicate. Spontaneous and maximal ⁵¹Cr release was determined in sixfold. The percentage of ⁵¹Cr release and specific release is defined as:

\[
\text{% }^{51}\text{Cr release} = \frac{^{51}\text{Cr release} - ^{51}\text{Cr spontaneous release}}{^{51}\text{Cr maximal release} - ^{51}\text{Cr spontaneous release}} \times 100
\]
%\textsuperscript{51}Cr specific release = %\textsuperscript{51}Cr release with Ab – %\textsuperscript{51}Cr release without Ab

2.4 Flow cytometry

FITC- or PE- labelled mouse anti-human CD3, CD4, CD8, CD14, CD16 (clone CLB-Fc-gran/1), CD19, CD56 and the corresponding murine IgG control antibodies (Pelicluster-Sanquin, Amsterdam, The Netherlands) as well as mouse anti-human CD16 (clone 3G8, Pharmingen, Becton–Dickinson, San Jose, CA, USA) were used for the determination of PBMC surface marker expression by flow cytometry. Expression of NKG2D was analysed on NK cells from healthy donors cultured for 18 h in complete medium with or without 30% patients’ sera with purified anti-NKG2D MAB139 (R&D Systems, Minneapolis, MN, USA), followed by FITC-labelled rabbit anti-mouse immunoglobulins F(ab\textsuperscript{2})\textsubscript{2} (Dako, Glostrup, Denmark). MICA expression on ZR-75-1 was analysed with anti-human MICA (clone AMO1; R&D Systems, Minneapolis, MN, USA), followed by PE-labelled polyclonal goat anti-mouse immunoglobulins F(ab\textsuperscript{2})\textsubscript{2} (Dako, Glostrup, Denmark). Cells were incubated with the antibodies for 30 min at 4°C and analysed using a FACScan; data analysis was performed using CELLQuest software (BD Bioscience, San Jose, CA, USA).

2.5 Fc\textgamma receptor (Fc\gammaR) and NKG2D blocking

Effector cells were pre-treated overnight with monoclonal antibodies against CD16 (clone CLB-Fc-gran/1 and 3G8), CD32 (clone FL18.26), CD64 (clone 10.1) or control mouse IgG (all from Pharmingen, Becton–Dickinson, San Jose, CA, USA). For NKG2D blocking PBMCs were incubated overnight with the antibody (MAB139, 10 \mu g/ml) and the ADCC was performed in the presence of the antibody.

2.6 Statistics

Normality of distribution of the data was confirmed with the Kolmogorov–Smirnov test and Q–Q plots. Pearson correlation and \( \chi^2 \) test were performed to analyse the relation between cell numbers and tumour cell killing and to analyse the influence of MUC1 serum levels on ADCC. A signed-rank test was used to investigate the influence of different cytokines on ability to mediate ADCC. A value of \( p < 0.05 \) was considered significant. All data were analysed using the SPSS 9.0 software.

3. Results

3.1 NK cell numbers and CD16 polymorphism determine the level of ADCC

Based on the developed \textit{in vitro} assay to study whether MUC1 antibodies can mediate ADCC [16], huHMFG-1-mediated ADCC was performed using the human breast cancer cell line ZR-75-1 as target cells and human peripheral blood mononuclear cells as effector cells. Humanised HMFG-1 showed a dose-dependent killing of ZR-75-1 cells, with maximal killing at concentrations ranging between 2.5 and 25 \mu g/ml (not shown). In the presence of 5 \mu g/ml of huHMFG-1, tumour cell killing varied widely, from 43% to 1%, between the 13 healthy donors tested (Fig. 1). Tumour cell killing by low responder donor PBMCs was not improved by increasing the concentration of huHMFG-1 (up to 500 \mu g/ml, data not shown). The percentage of CD56\textsuperscript{+} NK cells within PBMC correlated with the ability to mediate huHMFG-1-mediated ADCC (\( p = 0.023 \), Table 1). There was also a positive correlation with Fc\gammaRIII (CD16) positive cells (\( p = 0.049 \), Table 1). No correlation was found between tumour cell killing and the percentage of CD4\textsuperscript{+} T helper cells, CD8\textsuperscript{+} cytotoxic T lymphocytes, CD19\textsuperscript{+} B cells or CD14\textsuperscript{+} monocytes (not shown).

ADCC is mediated primarily through Fc\gammaRIII (CD16) [22]. Snijdewint et al. [16] have shown that FcR\gammaRIII is involved in huHMFG-1 ADCC. Blocking assays were carried out to confirm this mechanism in our model. Indeed ADCC was blocked after pre-treatment of effector cells with two different monoclonal antibodies against CD16 but not with antibodies against CD32 (Fc\gammaRII) or CD64 (Fc\gammaRI) (Fig. 2).

The proportion of NK cells is not the only component contributing to the observed difference in tumour cell killing by PBMC from different donors. PBMC from high and low responders were enriched to equal numbers of NK cells and analysed in an ADCC assay. Enriched NK cells from a high responder induced 60% specific killing (Fig. 3a), while enriched NK cells from a low responder induced 25% specific killing (Fig. 3b). These results suggest that the observed difference between high and low responders can be attributed to the level of ADCC mediated by the enriched NK cells.

![Fig. 1. Differential huHMFG-1-dependent ADCC mediated by PBMC from 13 healthy donors using 5 \mu g/ml of huHMFG-1.](image-url)
In low responders may also be due to intrinsic characteristics of the NK cells rather than just the number of NK cells. To elucidate this, expression levels of the FcγRIII (CD16) were investigated (Figs. 3c and d). Two different antibodies were used, one of which (clone 3G8) binds with a higher affinity to cells from individuals who are homozygous for the FcγRIIIa 158-Val polymorphism [23,24].

While there was no difference in CD16 expression found with clone CLB-Fc-gran/1 (p = 0.473, Table 1), expression levels determined with clone 3G8 correlated with the capacity to mediate ADCC (p < 0.01, Table 1), suggesting a role for the FcγRIIIa 158-Val polymorphism in huHMFG-1-mediated ADCC.

Next it was investigated whether the addition of the NK cell activating cytokines could enhance huHMFG-1 ADCC mediated by low, and also by high, responders. Previously we demonstrate an increase in ADCC by the addition of IL-2 and IFN-γ [16]. To confirm and extend on these findings, we studied the effect of these cytokines as well as IL-15 and IL-21 on huHMFG-1-mediated ADCC. A 2- to 5-fold enhancement of tumour cell killing was observed when PBMC were pre-treated with IL-2, IL-21 and IL-15, while IFN-γ had differential effects (Fig. 4).

3.2. High MUC1 antigen serum levels inhibit huHMFG-1-ADCC

MUC1 purified from tumour cells can inhibit huHMFG-1-mediated ADCC [16]. MUC1 is frequently elevated in serum of patients with breast and ovarian cancer. As huHMFG-1 is being tested as a tumour immunotherapeutic agent, we analysed the effect of serum MUC1 antigen levels on huHMFG-1-dependent ADCC using high responder PBMC 1 (see Fig. 1). Killing of the ZR-75-1 cell line without antibody was always low (<5%) and was not affected by addition of MUC1 containing patient sera, indicating that natural anti-MUC1 antibodies that may be present in serum were not sufficient to mediate in vitro ADCC. HuHMFG-1 mediated ADCC was however strongly inhibited in the presence of serum containing 1949 U/ml MUC1 (from 40.3% to 8.0% specific lysis). However, addition of serum containing low levels of MUC1 (9 U/ml) had no effect on tumour cell killing (40.3% versus 39.2% specific lysis, Fig. 5a). In 15 out of 20 (75%) serum samples with MUC1 levels below the...
upper limit of normal (30 U/ml) huHMFG-1-dependent ADCC inhibition was less than 20% (Fig. 5b). Inhibition of cytotoxicity was higher than 20% in 22 out of 26 (85%) serum samples with MUC1 levels above 30 U/ml (\( p < 0.001 \), Fig. 5b). Inhibition of tumour cell killing by high levels of serum MUC1 could be overcome with increasing serum dilutions and, thus, decreasing MUC1 concentrations (Fig. 5c). However, other possible inhibiting factors would also be diluted. Therefore, it was investigated whether the inhibiting effect of serum containing high levels of MUC1 could also be resolved by adding increasing levels of huHMFG-1 (1 and 100 \( \mu \)g/ml). 23% killing vs 3% killing in the presence of MUC1). However, in the presence of increasing huHMFG-1 concentrations, inhibition of target cell killing by MUC1 antigen decreased (25 \( \mu \)g/ml) or was completely abrogated (100 \( \mu \)g/ml).

4. Discussion

Clinical successes of monoclonal antibodies specifically targeting tumour cells have created great interest in antibody-based immunotherapy. Trastuzumab is a monoclonal antibody targeting the extracellular domain of the HER2/neu protein. Results of trials that compared adjuvant chemotherapy alone or in combination with trastuzumab in women with surgically removed HER2-positive breast cancer showed a benefit in disease free survival and overall survival in trastuzumab treated patients [25]. Contrary to HER2/neu, which is expressed only in 30% of breast cancers, MUC1 is over-expressed in virtually all breast adenocarcinomas. Due to its tandemly repeated extracellular sequence, MUC1 presents multiple antigenic regions per molecule, ensuring antibody binding even in low expressing tumours. The MUC1 specific antibody huHMFG-1 could therefore be a more widely applicable therapeutic agent [26]. HuHMFG-I leads to a capping or clustering of MUC1 on the surface of the tumour cell, exposing cell surface receptors involved in immune recognition processes. This would enable recognition and
destruction of the tumour cell by antigen specific T-cells and/or NK-cells. Indeed, we have previously shown that huHMFG-1 mediates ADCC of MUC1 positive cell lines [16].

NK cells and the FcγRIII play an important role in mediating huHMFG-1-dependent tumour cell killing. Two different allotypes of the FcγRIIIa have been described (FcγRIIIa-158V and FcγRIIIa-158F) and this polymorphism has been reported to affect IgG1 binding [27]. Donors positive for the FcγRIIIa-158V polymorphism had a better clinical response to treatment with rituximab [28]. This increased clinical response could be explained by the higher affinity of the FcγRIIIa-158V for rituximab, which resulted in an increased NK cell mediated lysis of CD20 positive cells [29]. The FcγRIIIa specific clone 3G8 displays a stronger binding to PBMC from individuals homozygous for the FcγRIIIa 158-Val polymorphism [23,24]. Here we show a correlation between high FcγRIIIa expression using the 3G8 antibody and huHMFG-1 mediated ADCC. This correlation was not observed with the CLB-Fcgrand/1 antibody, which binds equally strong to both FcγRIIIa allotypes. These

Fig. 5. High MUC1 serum levels inhibit huHMFG-1-mediated ADCC, which can be overcome by adding increasing concentrations of the antibody. (a) Example of ADCC of ZR-75-1 by PBMC 1 in the presence of serum with low (9 U/ml) or high (1949 U/ml) levels of MUC1. (b) Correlation between MUC1 levels and percentage of inhibition of huHMFG-1 mediated tumour cell killing ($p = 0.028$). The graph is divided in four quadrants: lower left: normal MUC1 levels ($\leq 30$ U/ml) and less than 20% inhibition ($N = 15$); upper left: normal MUC1 levels and more than 20% inhibition ($N = 5$); lower right: MUC1 above normal levels ($> 30$ U/ml) and less than 20% inhibition ($N = 4$); upper right: MUC1 above normal levels and more than 20% inhibition ($N = 22$). Closed symbols correspond to serum samples with various MUC1 levels taken at different time points from four patients with breast cancer (●, ●, ▲, ▲), and one patient with ovarian cancer (*). Open circles correspond to single samples from individual patients with breast or ovarian cancer. All ADCC assays were performed using 5 µg/ml of huHMFG-1 and high responder PBMC 1 as effector cells. (c) A high MUC1 serum sample (6054 U/ml) obtained from a patient with breast cancer was serially diluted to a MUC1 level of 1 U/ml. Each dilution was tested in an ADCC assay performed using 5 µg/ml of huHMFG-1. One representative experiment of three performed is shown. (d) MUC1 ADCC inhibition can be overcome with increasing concentrations of huHMFG-1. White bars depict huHMFG-1 mediated tumour cell killing in the absence of serum. HuHMFG-1-mediated killing is optimal with mAb concentrations of 1 or 25 µg/ml. Black bars depict tumour cell killing in the presence of a serum sample with high MUC1 levels (6054 U/ml) obtained from a patient with breast cancer. One representative experiment of three performed is shown.
data strongly suggest that the FcγRIIIa polymorphism may also influence huHMFG-1-mediated ADCC. DNA typed analysis should be carried out to confirm this observation.

There are currently numerous efforts ongoing to improve the efficacy of therapeutic IgG1 antibodies. In vitro molecular engineering of the Fc region is one possibility to improve the affinity of mAbs for the FcγRIIIa and thereby to enhance ADCC [30]. Furthermore, combination therapies with NK cell activating cytokines may increase ADCC. It has previously been shown that IL-2, IL-15 and IL-21 can augment ADCC [31–34]. Indeed in our system, stimulation of PBMCs with IL-2, IL-21 and IL-15, but not with IFNγ, increased huHMFG-1-mediated ADCC. Moreover, it has been demonstrated that patients treated with antibodies in combination with IL-2 exhibit an increased in vitro ADCC [32,35]. Gluck et al. showed that the addition of IL-2 to rituximab therapy for the treatment of B-cell non-Hodgkin’s lymphomas results in NK cell expansion that correlates with clinical response. They also observed that ADCC activity was increased or maintained in responding and stable disease patients [36]. However, the in vivo relevance of in vitro results needs further confirmation.

Using different serum samples with varying MUC1 levels, we demonstrated that MUC1 can inhibit huHMFG-1-mediated ADCC. MUC1 serum levels above the upper limit of normal (30 U/ml) inhibited huHMFG-1-mediated ADCC (85%, 22/26 serum samples) and as these levels differ among cancer patients, they have to be considered to define the therapeutic dose of huHMFG-1. One way to confirm the inhibiting effect of MUC1 would be to perform ADCC after removing MUC1 from the sera. Unfortunately, it was not possible to remove MUC1 from patient sera without diluting the sera. However, tumour cell killing could be restored with higher huHMFG-1 concentrations. Antibody concentrations between 25 and 100 µg/ml could overcome in vitro inhibition of tumour cell killing by extremely high MUC1 levels (6054 U/ml), strongly suggesting that MUC1 is indeed inhibiting huHMFG-1-mediated ADCC. These concentrations could be achieved in serum with administration of huHMFG-1 dose levels >3 mg/kg in vivo. This concentration lies well within the tolerable dose, considering that the MTD exceeded 16 mg/kg [26]. Nevertheless, care is needed in extrapolating findings from the in vitro setting to the in vivo situation where dynamic forces in circulating blood may influence antigen–antibody interactions.

Some serum samples (5/20, 25%) inhibited ADCC even though the levels of MUC1 were below the normal limit. Another possible inhibitory factor could be the soluble form of the stress-inducible MHC class I-related chain A (MICA) which is a ligand of the NK cell receptor NKG2D [37]. Soluble MICA is released from tumour cells and inhibits cell-surface NKG2D expression and NKG2D-mediated effector functions of immune cells in patients with progressive malignant tumours [38–41]. MICA was expressed on the target cell we used in the ADCC assays and blocking of NKG2D inhibited killing, suggesting that NKG2D co-stimulation plays a role in huHMFG-1-mediated ADCC. However, we could not demonstrate impairment of NKG2D expression on NK cells after incubation with serum samples (with low MUC1) that inhibited ADCC, suggesting that in these cases soluble NKG2D ligands did not play a role in ADCC inhibition (data not shown).

In conclusion, our in vitro results indicate that the number of NK cells, NK cell activating cytokines, the FcγRIIIa-158 polymorphism and high MUC1 serum levels influence huHMFG-1-mediated ADCC. The use of higher concentrations of huHMFG-1, which lie within the well-tolerated doses recently tested in a phase I clinical trial, can compensate for the elevated MUC1 serum levels. Optimal effects of huHMFG-1 treatment might be seen when the serum MUC1 levels are lower, such as in early stage patients, in contrast to the advanced disease patients. Screening for patients with high levels of NK cells bearing the FcγRIIIa-158V polymorphism, adjusting the dosage to circulating levels of MUC1 and co-administration of NK cell activating cytokines may increase the efficacy of huHMFG-1 treatment.

References

Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells


Chapter 5b

Toll-like receptor agonists and invariant Natural Killer T-cells enhance antibody-dependent cell-mediated cytotoxicity (ADCC)

María Moreno, Berber M. Mol, Silvia von Mensdorff-Pouilly, René H.M. Verheijen, B. Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

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Abstract

huHMFG-1 (AS1402) is a humanised IgG1 against MUC1, which exerts tumour cell killing through antibody-dependent cellular cytotoxicity (ADCC) mediated by Natural Killer (NK) cells. Here we explored the capacity of invariant NK T (iNKT) cells, which are known to activate NK cells, and Toll-like Receptor (TLR) ligands which activate both iNKT and NK cells, to enhance huHMFG-1-ADCC. Addition of iNKT cells, as well as TLR2/6, 7, 8 and 9 agonists to PBMC improved the efficacy of huHMFG-1. These results suggest that transfer of ex vivo expanded iNKT cells or TLR agonist treatment may improve the efficacy of NK cell-mediated antibody-based tumour immunotherapies.

Key words: human, invariant NKT cells, Toll-like receptor, NK cells, ADCC

Abbreviations: α-GC: α-galactosylceramide; ADCC: antibody-dependent cell-mediated cytotoxicity; DC: dendritic cells; huHMFG-1: humanized Human Milk Fat Globulin-1; iNKT: invariant natural killer T-cells; LPS: lipopolysaccharide; mAb: monoclonal antibodies; NK cells: Natural Killer cells; PBMC: peripheral blood mononuclear cells; PGN: peptidoglycan; Poly (I:C): polyionosinic-polyctidylic acid; T-cell receptor (TCR); TLR: Toll-like receptor
Introduction

MUC1, also known as CA15-3 antigen, is a high-molecular-weight transmembrane glycoprotein that is expressed on the ductal cell surface of normal glandular epithelia and is over-expressed in a variety of epithelial cancers [1]. In cancer cells MUC1 is not only over-expressed but also deficiently glycosylated, exposing a variable number of immuno-dominant domains on the MUC1 peptide core, which makes it a good target for immunotherapy [2]. In this context, a humanised MUC1 specific monoclonal antibody (mAb), huHMFG-1 (humanized Human Milk Fat Globulin-1, AS1402), has been developed [3] and is currently being evaluated in clinical trials as a potential immunotherapy for breast cancer. Humanised HMFG-1 can mediate in vitro tumour cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC) [4] through Natural Killer (NK) cells [5], though with limited efficacy. A therapy aiming at activation of NK cells could be of value for huHMFG-1 treatment. In this context, pre-treatment of peripheral blood mononuclear cells (PBMC) with NK cell activating cytokines, such as IL-2, IL-21 and IL-15, promotes an improvement of huHMFG-1-mediated tumour cell killing in vitro [5]. However, cytokine therapies are often highly toxic, limiting their in vivo use. Therefore, alternative strategies should be explored.

CD1d-restricted invariant Natural Killer T (iNKT) cells are T lymphocytes, characterized by an invariant T-cell receptor (TCR) α chain gene rearrangement (Vα24-Jα18 paired with Vβ11 in humans) and co-expression of NK cell receptors [6]. The invariant TCR recognizes glycolipid ligands, such as the artificial ligand α-Galactosylceramide (α-GC), in the context of the monomorphic CD1d antigen-presenting molecule. Upon activation, iNKT cells rapidly secrete cytokines, such as IL-2, IFN-γ, TNF-α and IL-4, leading to the activation of other immune cells, such as dendritic cells (DC) and NK, T and B cells (reviewed in [7]). We have recently demonstrated that in vitro expanded human iNKT cells can enhance antibody-independent NK cell cytotoxicity through the production of cytokines [8]. Therefore, iNKT cells could be exploited as cytokine producing cells to activate NK cells, hence improving huHMFG-1 efficacy in vivo.

Toll-like receptors (TLR) recognize different pathogen-associated molecular patterns (PAMPs) and trigger innate and adaptive immune responses. The TLR-family consists of at least 11 members, although the function of TLR10 is not yet clear and TLR11 appears not to be functional in humans. Bacterial components are mainly recognized by cell surface TLR; for example, bacteria lipopeptides are recognized by the heterodimers TLR1/2 and TLR2/6, lipopolysaccharide (LPS) by TLR4, and flagellin by TLR5. Endosomal-associated receptors are triggered by viral products, i.e. TLR3 is triggered by dsRNA, TLR7 and 8 by ssRNA, and TLR9 by hypomethylated CpG DNA (reviewed in [9]). TLR are mainly expressed by antigen presenting cells, but also by NK cells and cells of the adaptive immune response, such as T and B lymphocytes [10]. NK cell activation by TLR agonists has been extensively reported [11-14]. In addition, we have recently found that PBMC treatment with TLR agonists leads to indirect iNKT cell activation (Moreno et al., submitted).

Based on these results, we investigated whether activated iNKT cells and TLR ligands can enhance the ability of NK cells to mediate huHMFG-1-dependent tumour cell killing in vitro.
Materials and Methods

Media and reagents

IMDM (Cambrex, Verviers, Belgium) was supplemented with 10% Foetal Calf Serum (Perbio, Helsingborg, Sweden) for culture of PBMC, or 8% human pooled serum (Sanquin, Amsterdam, The Netherlands) for the culture of iNKT cells. Both media were supplemented with 100 IU/ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100μg/ml streptomycin sulphate (Radiumfarma-Fisiopharma, Naples, Italy) and 0.01 mM β-mercaptoethanol (Merck, Darmstadt, Germany).

The following TLR agonists were used: peptidoglycan (PGN) from Staphylococcus aureus (10μg/ml), polyionosinic-polycytidylic acid [Poly (I:C), 25μg/ml] and LPS (1μg/ml) (Sigma-Aldrich, Buchs, Germany); flagellin from Salmonella typhimurium (5μg/ml) and CL075 (0.72μg/ml) (InvivoGen, San Diego, CA, USA); and human specific stimulatory CpG ODN type A (ODN A 2216, 5μg/ml) (Coley Pharmaceuticals, Düsseldorf, Germany).

Isolation of peripheral blood mononuclear cells (PBMC) and PBMC stimulation

PBMC were isolated from buffycoats from healthy donors obtained from Sanquin Blood Bank (Amsterdam, The Netherlands) by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The number of cells was determined and PBMC were cryopreserved in separate batches until use.

PBMC cultures containing 4 x10^6 cells/ml were stimulated with different TLR agonists or combination of those for 48h. PBMC were incubated in the absence of any stimulus (only medium) as negative control.

Surface marker expression on iNKT and NK cells

FITC-, PE-, PerCP-Cy5- or APC-labelled isotype controls and mouse anti-human 6B11, CD3, CD4, CD69 (BD Bioscience, San Jose, CA, USA), Vα24, Vβ11 (Immunotech, Marseille, France) and CD56 (IQ products, Groningen, The Netherlands) were used to determine the phenotype of NK and iNKT cells by flow cytometry. Cells were incubated with the antibodies for at least 30 min at 4°C. Flow cytometry was performed on a FACSCALIBUR™ apparatus and data were analyzed using CellQuest™ software (BD Biosciences, San Jose, CA, USA).

iNKT cell expansion

Vα24+ Vβ11+ iNKT cells were enriched from PBMC by positive selection using the iNKT cell specific antibody 6B11 (BD Biosciences, San Jose, CA, USA) and anti-mouse Ig-coated magnetic beads mAb (Milteny Biotec, Bergisch Gladbach, Germany) by MACS sorting. iNKT cells were expanded by weekly stimulation with irradiated autologous PBMC at a 1:1 iNKT : PBMC ratio in the presence of 100ng/ml α-GC (KRN7000, kindly provided by Dr Shigeyuki Yamano, KIRIN Brewery, Gunma, Japan), 40 IU/ml recombinant human (rh) IL-2, 5 ng/ml rhIL-7 and 5 ng/ml rhIL-15 (all purchased from Strathmann Biotech, Hanover, Germany).
Evaluation of NK cell activation by iNKT cells

Donor PBMC (5x10^6 cells/ml) were cultured in the presence or absence of 100 ng/ml α-GC. iNKT cell-depleted PBMC were co-cultured with or without 0.50% in vitro expanded iNKT cells, in the absence or presence of α-GC and cultured for 5 days.

NK cell effector function analysis

Intracellular Granzyme B staining was performed after fixing the cells with 4% paraformaldehyde for 15 minutes at 4°C. PBMC were stained with CD69-FITC, CD3-PerCP and CD56-APC. Subsequently cells were permeabilised using the BD cytofix/cytoperm plus kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer’s instructions, and intracellular staining for Granzyme B was performed with PE-labelled anti-granzyme B (Sanquin, Amsterdam, The Netherlands).

ADCC assay

HuHMFG-1, a humanised IgG1-type MUC1 mAb, was provided by Antisoma plc, London, UK. Highly purified human IgG (Pelicluster-Sanquin, Amsterdam, The Netherlands) was used as control antibody. The breast tumour cell line ZR-75-1 that over-expresses MUC1 was used as target cell line. NK cell tumour cell killing was analysed by huHMFG-1 ADCC assay, performed as previously described [5].

Statistics

Normality of distribution of the data was confirmed with the Kolmogorov-Smirnov test and Q-Q plots. Student T-test for paired samples was performed to analyze the effect of TLR-triggering on NK cells. Pearson correlation test were performed to investigate the relation between Granzyme B production and huHMFG-1 ADCC. A two-tailed p value of < 0.05 was considered significant. All data were analysed using the SPSS 14.0 software.

Results

Addition of in vitro expanded iNKT cells to iNKT-depleted PBMCs enhances huHMFG-1 ADCC in an α-GC dependent manner

We have previously shown that in vitro PBMC stimulation with α-GC, an iNKT cell ligand, induced iNKT cell activation and a limited NK cell activation without enhancing NK cell effector functions [8]. In line with this observation, no enhancement of NK cell mediated huHMFG-1 ADCC was observed when PBMC, containing various numbers of iNKT cells (range 0.01-0.19% iNKT cells among CD3^+ lymphocytes), were pre-treated with α-GC for 2 to 5 days (Figure 1a).

However, in vitro expanded pre-activated human iNKT cells induce NK cell activation and concomitant enhancement of their lytic functions [8]. To test the capacity of these pre-activated iNKT cells to also increase NK cell-mediated huHMFG-1 ADCC, iNKT-depleted PBMC were co-cultured with 0.5% (which is within the physiological proportions) of in vitro expanded iNKT cells (>90% pure). We previously observed a high variation in donor’s ability to mediate huHMFG-1 tumour cell killing [5]. To appreciate the effect of iNKT cells in the
present study, this variability was therefore normalized by calculating the fold-increase (FI) in tumour cell killing. Results are presented as the ratio between tumour cell killing of α-GC- and/or iNKT cell-stimulated and unstimulated PBMC for each donor. iNKT cells induced a mean increase of 73% in huHMFG-1 ADCC at a 25:1 E:T ratio, an increase which depended on α-GC (Figure 1b, Paired Student’s T-test: p=0.029).

![Graph showing the increase in huHMFG-1-dependent tumour cell killing by α-GC-stimulated iNKT cells.](image)

**Figure 1:** Increase in huHMFG-1-dependent tumour cell killing by α-GC-stimulated iNKT cells. The effect of α-GC-stimulated iNKT cells on huHMFG-1-mediated tumour cell killing was studied. a) PBMC were stimulated with α-GC and after 48h NK cell ability to mediate huHMFG-1 ADCC was assessed. One representative experiment out of 7 performed is shown. b) iNKT-depleted PBMC (○) were co-cultured with 0.5% in vitro expanded iNKT cells, in the absence (▲) or presence (●) of iNKT cell ligand, α-GC. After 5 days, NK cell ability to mediate huHMFG-1-dependent tumour cell killing was assessed. Results of all donors (N=5) represented as fold-increase of huHMFG-1-dependent tumour cell killing at 25:1 E:T ratio. The horizontal lines represent the mean. Paired Student’s T-test of percentage of tumour cell killing mediated by α-GC and/or iNKT-stimulated PBMC compared to unstimulated PBMC. p<0.05 for iNKT-depleted PBMC co-cultured with iNKT cells in the presence of α-GC (●).

**PBMC stimulation with different TLR agonists (PGN, CL075 and ODN A) enhances huHMFG-1-dependent tumour cell killing**

TLR agonists can increase NK cell activity [11-14]. Moreover, we (Moreno et al., submitted) and others [15;16] have shown that PGN (a ligand for TLR2/6), CL075 (TLR7 and 8) and CpG (TLR9) promote iNKT cell activation. Since, iNKT cells can enhance NK cell cytotoxicity [8;17], we tested whether TLR-triggering can increase the efficacy of huHMFG-1. PBMC from 6 healthy donors were stimulated with different TLR agonists, Poly (I:C) (a ligand for TLR3), LPS (TLR4) and flagellin (TLR5), and PGN, CL075 and CpG ODN A. After 48h, effects of TLR triggering on NK cell numbers and activation state were analyzed. No significant changes were observed in the proportion of NK cells (8.0 ± 4.5 % of CD3− CD56+ cells among the lymphocyte population, Table I). NK cell activation occurred upon PBMC TLR-triggering. Interestingly, all TLR agonists dramatically induced an up-
regulation of CD69 activation marker expression on NK cells compared to unstimulated PBMC (figure 2 and Table I). PGN, CL075 and ODN A, the same ligands that induced iNKT cell activation, were most effective in inducing CD69 expression (% CD69⁺ NK cells, paired samples t-test p ≤0.001).

Table I. Summary of NK cell characteristics after TLR-stimulation of PBMC.

<table>
<thead>
<tr>
<th>TLR agonist</th>
<th>TLR</th>
<th>% NK cellsᵃᵇ</th>
<th>% CD69⁺ᵇᶜ</th>
<th>% Granzyme B⁺ᵈᵉ</th>
<th>% ADCCᵇᵉ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulus</td>
<td></td>
<td>8.0 ± 4.5 †</td>
<td>22.8 ± 16.9</td>
<td>18.7 ± 17.2</td>
<td>10.1 ± 5.5%</td>
</tr>
<tr>
<td>PGN</td>
<td>2/6</td>
<td>6.3 ± 3.6</td>
<td>74.4 ± 17.5 *</td>
<td>45.7 ± 25.1</td>
<td>14.8 ± 8.1% *</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>3</td>
<td>7.5 ± 4.4</td>
<td>40.0 ± 27.6 *</td>
<td>23.1 ± 15.8</td>
<td>11.7 ± 6.2%</td>
</tr>
<tr>
<td>LPS</td>
<td>4</td>
<td>7.0 ± 4.7</td>
<td>66.4 ± 26.1 *</td>
<td>25.2 ± 21.3</td>
<td>10.5 ± 6.8%</td>
</tr>
<tr>
<td>Flagellin</td>
<td>5</td>
<td>8.0 ± 5.2</td>
<td>51.7 ± 25.7 *</td>
<td>24.1 ± 22.7</td>
<td>14.2 ± 8.3%</td>
</tr>
<tr>
<td>CL075</td>
<td>7 and 8</td>
<td>7.5 ± 4.4</td>
<td>78.7 ± 22.5 *</td>
<td>50.2 ± 29.7</td>
<td>15.0 ± 7.6% *</td>
</tr>
<tr>
<td>ODN A</td>
<td>9</td>
<td>7.2 ± 5.4</td>
<td>90.3 ± 6.5 *</td>
<td>31.6 ± 24.6</td>
<td>13.0 ± 6.4% *</td>
</tr>
</tbody>
</table>

ᵃ among the lymphocytes, ñ N=6, ᵃ among the NK cells (CD3⁻ CD56⁺), ᵇ N=3, ᵈ E:T ratio 25:1, † Mean ± SD, * Paired Student’s T-test, p<0.05

The capacity to enhance huHMFG-1-ADCC was also addressed. A significant increase in huHMFG-1-mediated tumour cell killing was observed when PBMC were stimulated with PGN, CL075 and ODN A (Figure 3a), the same ligands that induced iNKT cell activation and higher NK cell activation, compared to unstimulated PBMC (paired samples t-test p = 0.018, 0.018 and 0.027, respectively) (Table I). PGN, CL075 and ODN A induced 67, 90 and 59% increase in huHMFG-1-mediated tumour cell killing, respectively. Of note, flagellin enhanced huHMFG-1-ADCC, though not statistical significant (p=0.052). Poly (I:C) and LPS had no statistically significant effect on huHMFG-1-ADCC (Figure 3b). Moreover, there is a consistent, though not statistical significant, increase in Granzyme B production by NK cells upon TLR stimulation, in particular with PGN, CL075 and ODN A (Table I). Granzyme B production by NK cells positively correlated with percentage of tumour cell killing mediated by huHMFG-1 (Pearson correlation test, p=0.02).

Figure 2: CD69 expression by NK cells upon PBMC stimulation with different TLR agonists. PBMC were cultured for 48h in medium (no stimulus, □) or in the presence of PGN (a ligand for TLR2/6, ▲), Poly (I:C) (TLR3, ∆), LPS (TLR4, ○), Flagellin (TLR5, ◦), CL075 (TLR7 and 8, ⬤) or ODN A (TLR9, ●). Results of all donors (N=6) represented as percentage CD69⁺ cells among NK cells (defined as CD3⁻ CD56⁺ cells). Horizontal lines represent the mean. Paired Student’s T-test, p<0.05 for Poly (I:C) and p<0.001 for the rest of TLR.
To test whether the combined TLR triggering had synergistic effects, PBMC were stimulated with combinations of the three ligands with strongest effect (i.e. PGN, CLO75 and ODNA). Neither synergistic, nor additive effects on NK cell mediated ADCC were observed (data not shown).

Discussion

There are currently numerous efforts ongoing to improve the efficacy of therapeutic IgG1 monoclonal antibodies (mAb). Combination with NK cell activating therapies may increase ADCC. In this context, we and others have previously shown that NK cell activating cytokines, such as IL-2, IL-15 and IL-21, can augment ADCC, and huHMFG-1-mediated ADCC in particular [5]. However, most cytokine therapies have been shown to be toxic. An alternative approach could be to exploit the cytokine producing ability of iNKT cells. Previously we observed that addition of free $\alpha$-GC to total PBMC did not lead to enhanced NK cell activation, while addition of in vitro expanded and pre-activated iNKT cells to PBMC or isolated NK cells enhanced NK cell activation and cytotoxic effector functions in an $\alpha$-GC-dependent manner [8]. Here we demonstrate that in vitro expanded human iNKT cells, which promote NK cell activation in vitro, can also enhance the efficacy of huHMFG-1-dependent NK cell-mediated ADCC in an $\alpha$-GC dependent fashion.

**Figure 3:** NK cell ability to mediate huHMFG-1-dependent tumour cell killing upon PBMC stimulation with different TLR agonists. PBMC were cultured in medium (no stimulus, □) or in the presence of PGN (a ligand for TLR2/6, ▲), Poly (I:C) (TLR3, ∆), LPS (TLR4, □), Flagellin (TLR5, ○), CL075 (TLR7 and 8, ♦) or ODN A (TLR9, ●). After 48h, NK cell ability to mediate huHMFG-1-ADCC was assessed. a) Results from one representative experiment out of 6 performed are shown. b) Results of all donors (N=6) represented as fold-increase of huHMFG-1-dependent tumour cell killing mediated by TLR-stimulated compared to unstimulated PBMC at 25:1 E:T ratio. Horizontal lines represent the mean. Paired Student’s T-test of percentage of tumour cell killing mediated by TLR-stimulated PBMC compared to unstimulated PBMC. p<0.05 for PGN, CL075 and ODN A (solid symbols).
Another approach to enhance NK cell-mediated cytotoxicity is by triggering TLR. Increase in NK cell lytic activity has been reported upon stimulation of purified NK cells with a broad panel of TLR agonists (in the concentrations used in this study), but co-stimulation by different cytokines was generally required [11;13;14]. TLR-triggering of PBMC by Poly (I:C), TLR7 and 8 agonists, and CpG have been shown to enhance NK cell cytotoxicity [12;18], and up to now, only CpG has been shown to enhance ADCC [18]. In this study, all TLR ligands tested have been shown to activate NK cells but only PGN, CL075 and ODN A had an appreciable effect on huHMFG-1-dependent ADCC. Interestingly, these three TLR agonists also induced the strongest iNKT cell activation (Moreno et al., submitted1). Based on these results and the finding that the increase in huHMFG-1 ADCC by TLR-triggering was comparable to that obtained by the addition of activated iNKT cells (approximately 1.5 fold-increase), it is tempting to speculate that at least part of the TLR effect is achieved through iNKT cell activation. On the other hand, iNKT cell activation by the addition of α-GC to PBMC had no effect. This may be explained by the fact that administration of free α-GC can lead to iNKT cell unresponsiveness [19], due to presentation by non-professional APC, or that the iNKT cell activation by α-GC and TLR ligands induce different cytokine secretion profiles, and therefore lead to a different outcome.

TLR ligands are of great interest in the field of cancer immunotherapy. Their immunostimulatory properties have been exploited to increase the efficacy of various types of immunotherapy. Up to now, only CpG has been reported to modulate ADCC [18]. In animal models, CpG has potent anti-tumour effects when administered in vivo in combination with mAb [20]. Notably, van Ojik et al. have demonstrated that CpG treatment enhances anti-HER2 mAb-dependent NK cell-mediated tumour regression in mice bearing experimental HER2-overexpressing tumours [21]. In the present study, it is demonstrated that next to ODN A, PGN and CL075 are promising in this respect as well, as they have been shown to activate iNKT cells and enhance huHMFG-1-mediated tumour cell killing in vitro. However, they have not yet been tested in humans. Another TLR 7/8 agonist, resiquimod, as well as TLR9 agonists have been proven safe in humans, also in combination with mAb, such as rituximab [22;23].

Although we observed an increase in huHMFG-1-mediated tumour cell killing, the effect was only moderate. It has been implied that huHMFG-1-mediated ADCC could be influenced by Fcγ receptor IIIa (FcγRIIIa) polymorphism [5], which affects IgG1 binding. In the case of huHMFG1, next to the enhancement of NK cell activation by TLR and/or iNKT cells, improvement in antibody affinity is necessary. In vitro molecular engineering of the Fc region can improve the affinity of mAbs for the FcγRIIIa, and thereby enhance ADCC [24].

In conclusion, we showed that NK cell activation and subsequent enhanced ADCC can be achieved by iNKT cells and particular TLR ligands. The mechanism of TLR- triggering is not yet clear but may depend in part on iNKT cell activation. Therefore, our findings suggest that NK cell-mediated mAb-based immunotherapies may benefit from autologous adoptive transfer of ex vivo expanded iNKT cells or administration of TLR agonists which induce iNKT and NK cell activation.

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Footnotes


References


Part II: Invariant NKT cells as enhancers of anti-tumour immunity \textit{in vivo}

Chapter 6a

Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity

Johan W. Molling, María Moreno, Hans J.J. van der Vliet, Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

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Generation and sustained expansion of mouse spleen invariant NKT cell lines with preserved cytokine releasing capacity

Johan W. Molling, Maria Moreno, Hans J.J. van der Vliet, B. Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

VUMC Institute for Cancer and Immunology (V-ICI), Division of Immune Therapy, Department of Medical Oncology, Vrije Universiteit Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
VUMC Institute for Cancer and Immunology (V-ICI), Division of Immune Therapy, Department of Pathology, Vrije Universiteit Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

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Abstract

Invariant Natural Killer T (iNKT) cells are CD1d restricted innate lymphoid cells with an invariant T cell receptor (TCR) α chain gene rearrangement (Vα24-Jα18 in human and Vα14-Jα18 in mouse). iNKT cells play a pivotal role in anti-tumor immune responses via cytokine mediated transactivation of various cells which mediate innate and adaptive immune responses. Here we describe, to our knowledge for the first time, the generation of long-term mouse spleen derived iNKT cell lines. We found that dendritic cells (DC) derived from the D1 line, but not Mφ/4 macrophages, loaded with the artificial iNKT cell ligand α-Galactosylceramide (αGalCer) could be employed to expand iNKT cells in vitro. Furthermore, exogenously added IL-7, but not IL-2 or IL-15 had a pronounced additive 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 helper (Th) 1 and Th2 cytokines upon activation.

In conclusion we have generated iNKT cell lines with high yields that can be maintained for up to 24 months, by repeated stimulation using alpha-GalCer loaded D1 DC and IL-7. These in vitro expanded iNKT cells preserved the capacity to swiftly produce both Th1 and Th2 type cytokines and are currently being utilized in pre-clinical adoptive transfer models to identify and optimize the characteristics of therapeutically effective iNKT cells in an anti-tumor setting.

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Keywords: Mouse; NKT; α-Galactosylceramide; In vitro; Expansion; D1 DC

1. Introduction

Invariant Natural Killer T (iNKT) cells are CD1d restricted innate lymphoid cells characterized by an invariant T cell receptor (TCR) α chain gene rearrangement (Vα24-Jα18 in human and Vα14-Jα18 in mouse) and co-expression of NK cell receptors (Godfrey et al., 2000). The iNKT TCR recognizes glycolipid ligands presented by CD1d (e.g. the putative endogenous ligand...
Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells

In a prospective study we recently observed that a relatively large circulating iNKT cell pool predicts favorable clinical outcome in individuals with squamous cell carcinoma of the head and neck [Molling et al., J Clin Oncol 2007]. Therefore, we hypothesize that residual iNKT cells of cancer patients are still functional is a matter of debate; while we and others have reported a preserved capacity of direct ex vivo IFN-γ secretion upon antigenic triggering by residual circulating iNKT cells in patients with breast cancer, colon cancer and lung cancer while others found a reduction in IFN-γ secretion in e.g. prostate cancer and certain hematological malignancies (Molling et al., 2005; Dhodapkar et al., 2003; Giaccone et al., 2002; Motohashi et al., 2003; Tahir et al., 2001). In a prospective study we recently observed that a relatively large circulating iNKT cell pool predicts favorable clinical outcome in individuals with squamous cell carcinoma of the head and neck [Molling et al., J Clin Oncol 2007]. Therefore, we hypothesize that cancer patients might benefit from immunotherapy aimed at increasing functionally competent peripheral iNKT cell numbers, e.g. by adoptive transfer of in vitro expanded autologous iNKT cells.

To gain more insight in the in vivo effects of adoptively transferred iNKT cells we developed a robust method for the generation of mouse iNKT cell lines that are currently being utilized in pre-clinical transfer models. Mouse cell lines such as Mf4/4 macrophages and D1 derived DC can efficiently present peptide antigens to T cells (Desmedt et al., 1998; Winzler et al., 1997). Here, spleen iNKT cells were co-cultured with αGalCer loaded, CD1d expressing D1 derived DC and Mf4/4 macrophages as antigen presenting cells (APC) based on human iNKT expansion protocols (van der Vliet et al., 2003). Furthermore, the influence of exogenously added homeostatic cytokines IL-2, IL-7 and IL-15 on expansion was determined and the obtained iNKT cells were characterized with regard to cytokine secretion and surface receptor profiles.

2. Materials and methods

2.1. Cell lines and culture media

The following culture media (Cambrex Bio Science, Verviers, Belgium) were used. IMDMc, RPMIc 1640 and DMEMc were supplemented with 10% Foetal Calf Serum (HyClone, Logan, UT, USA), 0.01 mM 2-ME and 50 units/ml penicillin–streptomycin and, in the case of DMEMc 1% Non-Essential Amino Acids (Invitrogen, Carlsbad, CA, USA) (IMDMc, RPMMc and DMEMc respectively). D1, an immature DC line was cultured as described in IMDMc, supplemented with supernatants from the GM-CSF transfected cell line R1 (Foti et al., 1999). J558-CD40L, a CD40L-transfected J558 cell line (gift of Dr. P. Lane, University of Birmingham, Birmingham, UK) and the CD1d-transfected Hela cell line (Brossay et al., 1998) were cultured in IMDMc. M4/4, a macrophage cell line (Desmedt et al., 1998) was cultured in RPMI. DN32.D3, a murine iNKT cell hybridoma line was cultured in DMEMc.

2.2. Generation of APC

Mf4/4 macrophages were cultured for 24 h in the presence of LPS (1 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) and IFN-γ (400 IU/ml; R&D systems, Minneapolis, MN, USA). Immature D1 DC were cultured for 4 or 48 h in the presence of LPS (100 ng/ml), and IFN-γ or LPS and PGE2 (10−7 M; Sigma-Aldrich) to generate D1.DC1 or D1.DC2 respectively. These maturation procedures were performed in the presence of αGalCer (100 ng/ml) or vehicle control (Kalinski et al., 1999; Langenkamp et al., 2000; van der Vliet et al., 2003). APC cytokine profiles were analyzed after a 48 h stimulation of 4 × 10^4 APC with 8 × 10^4 J558-CD40L in 200 μl IMDMc in 96 well round bottom plates (Nunc, Wiesbaden, Germany). Supernatants were analyzed for TNF-α, IL-6, IL-10 and IL-12p70 using flow cytometry beads (BD Biosciences, San Diego, CA, USA). In other experiments 1 × 10^4 APC were used to activate 5 × 10^4 DN32.D3 in 200 μl IMDM 18 h in 96 well round bottom plates. The level of αGalCer specific DN32.D3 activation was determined by IL-2 production quantified by IL-2 ELISA (BD Biosciences). Auto-presentation of αGalCer by DN32.D3 was prevented via
the following approach: first, the maximum density of DN32.D3 to prevent IL-2 secretion due to auto-
presentation was determined in pilot experiments (not shown); second, all APC were pulsed with vehicle or αGalCer during maturation and next extensively washed before co-culturing with DN32.D3. Specific IL-2 production was calculated by subtracting IL-2 levels from DN32.D3 stimulated with vehicle treated APC from IL-2 levels obtained with DN32.D3 and αGalCer loaded APC. Mature APC, J558-CD40L and DN32.D3 were irradiated at 5000 Rad before all co-
culture experiments.

2.3. Mice

Donor mice used were 6–10 week old female C57Bl/6 (Harlan Netherlands B.V., Horst, The Netherlands). Mouse care was in line with the guidelines provided by the VU Animal Ethical Committee, who approved the study.

2.4. T lymphocyte isolation from mouse spleen

Single cell suspensions were obtained by gently pressing mouse spleens through 100 μm filters. Cells were then centrifuged at 600 ×g and cells were re-suspended with PBS/0.1 M EDTA/0.2 M NH₄Cl/0.01 M KHCO₃. Cells were left on ice for 5 min allowing lysis of erythrocytes and then centrifuged at 240 ×g. Non-T cells were labeled with a mixture of rat monoclonal antibodies directed against F4/80, B220, MHC class II and CD11b (hybridoma derived clones F4/80, 6B2, M5/114 and Mac-1 respectively; kindly provided by Dr. Janneke N Samsom, Erasmus MC, Rotterdam) and subsequently labeled with magnetic goat-anti-rat IgG beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). T lymphocytes were then obtained by negative selection using the MiniMACS system (Miltenyi Biotec). In each individual expansion experiment (e.g. comparing the effects of different APC or of different cytokines on iNKT cell expansion) paired data were obtained by using T cells isolated from one spleen (depicted as single donor T cells).

2.5. Expansion of iNKT cells

For evaluation of iNKT cell expansion, 4× 10⁵ T lymphocytes were co-cultured with 4× 10⁴ irradiated APC (5000 Rad) per well for 7 days in 48 well plates in IMDMc. Cultures were weekly re-stimulated with fresh APC in a T cell/APC ratio of 5 to 1. During the first week no cytokines were added, to reduce growth of non-αGalCer-stimulated (i.e. non-iNKT cells), and in the following weeks mIL-2 (10 IU/ml), mIL-7 (10 ng/ml) and/or mIL-15 (10 ng/ml) (R&D Systems) were added as indicated. In some experiments, dose related effects of these cytokines were evaluated as indicated. iNKT cell purity was analyzed by detection of TCR mediated binding to αGalCer loaded CD1d:IgG dimers (BD Biosciences) by flow cytometry.

2.6. Evaluation of iNKT cell cytokine production

The intracellular cytokine profile of iNKT lines was determined when purity was above 95%, using the Cytofix/Cytoperm™ kit (BD Biosciences). iNKT cells were harvested, washed and 1 × 10⁶ cells were stimulated 4h with 1 × 10⁵ Hela-CD1d in the presence of 0.66 μl/ml monensin (BD Biosciences) and 100 ng/ml αGalCer or vehicle in 96 well round bottom plates (total volume 200 μl). The following antibodies were used: Cy5 labeled hamster-anti-mouse TCR-β-chain (clone H57-597), FITC-labeled rat-anti-mouse IFN-γ (clone 25723.11), APC-labeled rat-anti-mouse IL-4 (clone 3010.211) or FITC and APC-labeled rat IgG1 (clone A110-1) and Cy5 labeled hamster IgG2 (clone Ha4/8) isotype control. For determination of cytokine secretion supernatants were harvested after a 24 h co-culture of 1 × 10⁶ iNKT cells and 1 × 10⁵ Hela-CD1d cells in the presence of 100 ng/ml αGalCer or vehicle in 96 well round bottom plates (total volume 200 μl). Supernatants were analyzed for IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, GM-CSF, IFN-γ and TNF-α using flow cytometry beads (Bender Medsystems, Vienna, Austria) and analyzed for IL-21 by ELISA (R&D systems).

2.7. Surface marker expression on APC and iNKT cells

For evaluation of APC surface receptors, the following monoclonal antibodies were used: rat-anti-mouse CD40 or CD86 (clones 3/23 and GL-1 respectively, provided by Dr. Janneke N Samsom), followed by PE-labeled donkey-anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, US). FITC-labeled anti-mouse CD1d (clone 1B1) (BD Biosciences), PE-labeled anti-mouse CD80 (clone 1G10) (Southern Biotech, Birmingham, AL, US), PE-labeled anti-mouse MHC class II (clone M5/114.15.2) (eBioscience, San Diego, CA, US) and FITC or PE-labeled rat IgG2b isotype control (clone A95-1) (BD Biosciences).

For evaluation of iNKT cell purity, cells were incubated for 10 min at 37 °C with αGalCer or vehicle loaded CD1d:IgG dimers, washed twice and subsequently stained with PE-labeled rat-anti-mouse IgG1
(clone A85-1) and APC-labeled anti-CD3ε (clone 145-2C11) or Armenian hamster IgG1 isotype control (clone A19-3) (BD Biosciences).

For evaluation of the expression of other surface markers the following reagents were used: biotin conjugated rat-anti-mouse TCR-Vβ2, Vβ7 and Vβ8.2 (clones B20.6, TR310 and MR5-2 respectively) (BD Biosciences) combined with FITC-labeled streptavidin (Dako Cytomation, Glostrup, DK), polyclonal goat-anti-mouse IL-15Rα (R&D) combined with FITC-labeled polyclonal rabbit-anti-goat Ig (Dako), FITC-labeled rat-anti-mouse Ly-49A, Ly-49C+I, Ly-49D and Ly49-G2 (clones A1, 5E6, 4E5, 4D11 respectively), FITC-labeled rat-anti-mouse CD4 (clone GK1.5), PE-labeled mouse-anti-mouse NK1.1 (clone PK136), rat-anti-mouse CD8β (clone H35-17.2), CD49b (clone DX5), CD127 (IL-7Rα) (clone SB/199), APC-labeled rat-anti-mouse CD25 (IL-2Rα) (clone 1B11) and rat-anti-mouse CD44 (clone IM7) (BD Biosciences) and the appropriate isotype controls. Expression levels were determined by Mean Fluorescence Index (MFIndex=mean fluorescence marker/mean fluorescence isotype). Propidium iodide was added prior to analysis to exclude dead cells and flow cytometry was performed on a FACSCALIBUR apparatus (BD Biosciences).

2.8. Statistical analyses

Depending on whether variables fitted a Gaussian distribution or not, statistical analyses performed were Student’s T test, Wilcoxon signed ranks test or variance analysis (ANOVA and Tukey–Kramer). P values below 0.05 were considered significant.

3. Results

3.1. Characterization of APC

In order to obtain insight in their potential to stimulate the expansion of iNKT cells, the different APC were first characterized with regard to surface expression of CD1d, co-stimulatory molecules, MHC class II and their capacity to secrete inflammatory mediators.

We found that CD1d expression was most prominent on D1.DC2 and low to undetectable on D1.DC1 and MF4/4. Expression of MHC class II and the co-stimulatory molecules CD40, CD80 and CD86 were the highest on D1.DC2 (Fig. 1). The secretion of inflammatory mediators was determined after 48 h of stimulation with CD40-L transfected J558 cells. Overall MF4/4, D1.DC1 and D1.DC2 did not significantly differ in the secreted amounts of TNF-α (mean±SEM=7207±4412, 3100±1102 and 3431±972 pg/ml respectively; p=0.3794, repeated measures ANOVA), IL-6 (mean±SEM=2949±2126, 3785±976 and 7782±3607 pg/ml respectively) (p=0.1012, repeated measures ANOVA) and IL-10 (mean±SEM=12785±8444, 7705±4995 and 18246±15641 pg/ml respectively; p=0.4805, repeated measures ANOVA). IL-12p70 was not detected in any of the samples.

Fig. 1. Surface receptor expression on the different APC. (A) D1.DC1, D1.DC2 and MF4/4 were analyzed for surface expression of CD1d, CD40, CD80, CD86 and MHC class II (open histograms). The grey histograms represent the isotype controls. The data in the upper right corners indicate the Mean Fluorescence Index (MFI) values (MFI=mean fluorescence marker/mean fluorescence isotype control). Data are from one experiment representative of four.
Finally we determined whether all APC were able to present αGalCer to the iNKT cell hybridoma DN32.D3, which secretes IL-2 upon TCR triggering (Brutkiewicz et al., 2007).

Fig. 2. D1.DC1 are more potent than D1.DC2 in mediating iNKT cell expansion. iNKT cell expansion and purity was assessed after three weekly stimulations with αGalCer loaded D1.DC1 (n=24), D1.DC2 (n=24) or Mφ4/4 (n=8) in the presence of different combinations of IL-2, IL-7 and/or IL-15. (A) Fold expansion of αGalCerCD1d dimer+ iNKT cells (p<0.0001 Kruskal–Wallis); (B) Percentage of iNKT cells of total cells in culture (p<0.0001 Kruskal–Wallis). Dots represent individual cultures, bold lines represent medians.

Fig. 3. Different effects of IL-2, IL-7 and IL-15 on iNKT cell expansion. iNKT cells were expanded by co-culturing with D1.DC1 in medium supplemented with IL-2, IL-7 and/or IL-15. After three weekly stimulation rounds, cells were analyzed by FACS (CD3+/αGalCer: CD1d dimer+) and counted. (A) Fold expansion of iNKT cells using IL-7 and IL-15, IL-2 and IL-15, IL-2 and IL-7 (white, grey or black bar respectively) or all three combined (hatched bar) in paired experiments. Bars represent means, error bars represent SEM (n=5). (B) Fold expansion of iNKT cells using escalating doses of IL-2 and/or IL-7. Markers represent means, error bars represent SEM (n=3). Data are from three donors in one experiment, representative of three. (C) Several iNKT lines were cultured for a prolonged period. Lines indicate growth kinetics of the individual iNKT lines, percentages indicate iNKT cell purity determined by CD3/αGal-CerCD1d dimer staining at the time of last analysis.⁎: maintained in culture for up to 24 months.
et al., 1995). D1.DC1 and D1.DC2 were significantly more potent in inducing αGalCer specific IL-2 release by DN32.D3 than Mf4/4 (mean±SEM = 25867±12878, 25707±12568 and 11721±9454 pg/ml, \( p<0.001 \) repeated measures ANOVA; \( n=5 \)). These findings indicate that, despite the low level of expression of CD1d, all APC could functionally present αGalCer to iNKT cells and that the intensity of CD1d expression was not predictive of DN32.D3 activation.

### 3.2. D1.DC but not Mf4/4 can induce iNKT cell expansion

Next, we evaluated whether D1.DC1, D1.DC2 and Mf4/4 were able to induce the *in vitro* expansion of iNKT cells. Spleen T cells (~95% pure) were weekly re-stimulated using αGalCer loaded Mf4/4, D1.DC1 or D1.DC2 in the presence of matched combinations of IL-2, IL-7 and IL-15. At the start of culture the median [inter quartile range (IQR)] iNKT cell purity was 1.7 [1.3–1.9] % and after three stimulation rounds all cultures consisted solely of T cells (not shown). At this point iNKT cell expansion and purity were most pronounced using D1.DC1 compared to D1.DC2, whereas no iNKT cell expansion was observed when using Mf4/4 (median expansion [IQR]=24 [11–51], 1 [0–5] and 0 [0–0.5] fold respectively, \( p<0.0001 \) Kruskal–Wallis) (median purity [IQR]=50 [27–66], 6 [1–28] and 0.1 [0–0.3] % respectively, \( p<0.0001 \) Kruskal–Wallis) (Fig. 2). Of note, although both D1.DC1 and D1.DC2 could be applied to generate iNKT bulk cultures, repeated *in vitro* stimulation with D1.DC2 resulted in overall cell death after 4 to 5 total re-stimulation rounds (not shown). These data indicate that, of the three APC that we compared, D1.DC1 provide the best tool for expanding iNKT cells from mouse spleen T cells.

### 3.3. IL-2, IL-7 and IL-15 have different effects on iNKT cell expansion

Next we investigated the influence of IL-2, IL-7 and IL-15 on the *in vitro* iNKT cell expansion from single donor T cells after three stimulation rounds with D1.DC1 (\( n=5 \)). Combining equal units of IL-7 and IL-15 resulted in a mean±SEM = 21±8 fold expansion (Fig. 3A). When IL-7 was replaced by IL-2, no remarkable difference was observed (mean±SEM =17±9 fold). However, when IL-2 and IL-7 were combined a striking increase in iNKT cell expansion occurred (mean±SEM =46±21 fold).

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**Fig. 4. CD1d dimer staining and intracellular staining for IFN-γ and IL-4 production.** Four iNKT lines were selected on the basis of their high purity and used for further characterization (depicted above plots). A) Histogram plots indicate staining with vehicle (grey histograms) or αGalCer (open histograms) loaded CD1d:IgG dimers. Numbers in upper right indicate percentage of αGalCerCD1d dimer positive cells. B) Dot plots indicate intracellular staining for IFN-γ and IL-4 after 4 h stimulation with Hela-CD1d in the presence of vehicle (upper plots) or αGalCer (100 ng/ml) (lower plots). Numbers in upper right indicate percentage of cells in the corresponding quadrants.
These findings indicate that IL-2 and IL-7 can collectively support iNKT cell expansion, whereas IL-15 hampers the effects of the former two. This was underlined by the fact that the mean ± SEM iNKT cell expansion returned to 22 ± 8 fold when IL-15 was added to the combination of IL-2 and IL-7.

To further elucidate whether IL-2 and IL-7 contributed synergistically or cumulatively to the mixture, the two cytokines were combined in a dose response assay. Addition of IL-2 did not clearly affect the strong dose dependent increase in iNKT cell expansion caused by IL-7 (n = 3). Furthermore, in the dose range tested IL-2 alone did not support iNKT cell expansion in our system (Fig. 3B). Altogether the above data demonstrate that IL-7 was most effective in supporting iNKT cell expansion driven by D1.DC1, while IL-15 hampered the effect of IL-7.

Fig. 3C shows data from long-term cultures of several iNKT lines that were initially generated using D1.DC1 and combinations of the above cytokines and were maintained by weekly re-stimulation with exogenously added D1.DC1 and IL-7.

3.4. iNKT cell lines obtained with D1.DC1 and IL-7 secrete both Th1 and Th2 cytokines

The most notable characteristic of iNKT cells is their capacity to rapidly secrete large amounts of IFN-γ and IL-4 upon activation. We investigated here whether this trait was retained after in vitro culture with D1.DC1 and exogenous IL-7. First, intracellular expression of IFN-γ and IL-4 was assessed in four iNKT cell lines (>95% purity, Fig. 4A) after 4 h of αGalCer stimulation using flow-cytometry. All cell lines displayed αGalCer reactivity as shown by αGalCer-CD1d dimer reactivity and by an increased percentage of IL-4 and/or IFN-y positive cells (Fig. 4) when cells were treated with αGalCer (bottom) as compared to vehicle (top). Next, we investigated the T helper (Th)1/Th2 cytokine secretion profile after 24 h of αGalCer stimulation.

![Cytokine secretion profiles of iNKT cell lines.](image-url)
In addition to IFN-γ and IL-4, all iNKT cell lines secreted GM-CSF, IL-5, IL-6 and IL-10. TNF-α, IL-1α, IL-2, IL-17 and IL-21 were not detectable in the supernatants. These data indicate that all four iNKT cell lines had retained the capacity to swiftly secrete both Th1 and Th2 cytokines.

3.5. The IFN-γ/IL-4 balance is reflected in the surface marker expression profile

Next, we analyzed TCR-Vβ chain usage and surface receptor expression on the selected iNKT cell lines (Fig. 6). The iNKT lines predominantly expressed the

![Image](image_url)
TCR-Vβ8.2 or -Vβ7 chain, indicating that iNKT lines primarily using the low avidity TCR-Vβ2 chain most likely can not be obtained using the method described here. At present it is believed that the developmental stage during iNKT cell maturation is reflected by a gradual shift in their cytokine profile upon activation, from the predominant production of IL-4 towards IFN-γ. Throughout their development iNKT cells sequentially enhance CD44 expression, acquire CD49b and NK.1.1 and presumably finally lose CD4 expression (Benlagha et al., 2002, 2005; Coles and Raulet, 2000; Gadue and Stein, 2002; Pellicci et al., 2002). We therefore assessed expression of these markers on the four iNKT cell lines selected above to determine whether their surface marker phenotypes fitted their IFN-γ/IL-4 balance. All lines consisted of a high number of CD44high cells (mean [SD] = 89 [3], 81 [3], 75 [3] and 84 [5] %) (n=4), similar to the levels observed in wildtype (Wt) spleen (Matsuda et al., 2000). However, the lines differed in the expression of CD49b (mean [SD] = 67 [27], 66 [17], 33 [13] and 34 [7] %) and CD4 (mean [SD] = 1 [0,4], 2 [2], 50 [24] and 59 [3] %) (n=3). Expression of NK.1.1 was very rarely found (mean [SD] = 2 [1], 3 [3], 0 [0] and 0 [0] %) (n=3), most likely due to activation induced down-regulation of this receptor. The presence of other NK markers was also investigated. All iNKT cell lines were negative for the activating NK receptor Ly-49D and the inhibitory NK receptors Ly-49A, Ly-49C+I and Ly-49G2 (not shown). Based on the differential expression of CD49b and CD4, these data suggest that iNKT cell lines #1 and #2, with a higher IFN-γ/IL-4 ratio (Fig. 4), indeed have a more mature surface receptor phenotype. However, we did not observe any clear cut differences in IFN-γ or IL-4 production between the CD4+ and CD4- subsets within individual iNKT cell lines as determined by intracellular cytokine staining (not shown).

4. Discussion

Although other studies have demonstrated that it is possible to generate from mice either short lived cell cultures containing bona fide iNKT cells (Maeda et al., 2001; Chamoto et al., 2004; Ikarashi et al., 2005) or long lived clone derived iNKT cell hybridomas (Lantz and Bendelac, 1994; Gumperz et al., 2000; Behar et al., 1999), this is the first study to make available large scale, highly pure long-term oligocolonal murine iNKT cell lines representative of in vivo iNKT cells. The strategy was to investigate whether αGalCer loaded Mf4/4 or D1.DC could be used to generate iNKT cell lines from mouse spleen T cells. Mf4/4 macrophages were treated with αGalCer, LPS and IFN-γ, since this combination has previously been described to support their maturation into Th1-oriented APC (Desmedt et al., 1998). D1 were treated with αGalCer, LPS and IFN-γ or αGalCer, LPS and PGE2 to mature them into D1.DC1 or D1.DC2 respectively (Kalinski et al., 1999; Langenkamp et al., 2000). Preceding experiments with similar treatment of human moDC revealed that using DC1 we could generate more Th1-oriented iNKT cell lines, whereas using DC2 induced more potent expansion of iNKT cells (van der Vliet et al., 2003). All APC expressed co-stimulatory molecules and did not remarkably differ in their capacity to secrete TNF-α, IL-6 and IL-10. Surprisingly, no IL-12p70 was detected in supernatants of CD40 triggered APC. IL-12p70 secretion might depend on additional signaling and thus be more potent during co-culture with iNKT cells, as e.g. in vitro CD1d triggering of human monocytes also induces IL-12 (Yue SC et al. PNAS 102(33): 11811, 2005) and in vitro IL-12p70 secretion by TNF-α matured D1 cells, also required antigen presentation to antigen specific T cell clones (Winzler et al., 1997).

Expression of CD1d on the investigated APC was low to undetectable using our staining method. However, surface levels might increase during co-culture with iNKT cells allowing D1.DC2 and Mf4/4 to present αGalCer, since it has been reported that GM-CSF and IL-4 (both secreted swiftly by iNKT cells) can increase mouse CD1d expression (Mandal et al., 1998). In line with this, here, all APC induced an αGalCer dependent activation of the iNKT cell hybridoma DN32.D3. Still, only D1.DC1 and D1.DC2 could be used to generate iNKT cell lines from mouse spleen. The different efficacy of the APC in inducing iNKT cell activation and expansion might be related to the limited in vitro life span of Mf4/4 upon maturation (not shown), which might not be sufficiently long to allow the proper activation of iNKT cells. Furthermore, D1.DC1 were superior to D1.DC2 in mediating in vitro iNKT cell expansion and were the only APC that allowed long-term cultures, as the iNKT cell lines generated using D1.DC2 could not be maintained for more than four weeks (not shown). One could speculate that the relatively strong expression of CD1d on D1.DC2 compared to D1.DC1 induces in vitro contraction of the iNKT cell response to αGalCer as described for the repeated in vivo stimulation of iNKT cells with this ligand (Uldrich et al., 2005). However, iNKT cells have been shown to be relatively resistant to apoptosis as a result of intrinsically elevated levels of anti-apoptotic proteins (Harada et al., 2004; Seino et al., 2004). An evaluation of the expression profiles of pro- and anti-apoptotic genes in
iNKT cells stimulated with D1.DC1 or D1.DC2 might lead to further clarification.

IL-7 and IL-15 were reported to play a role in the in vivo homeostasis of iNKT cells, whereas IL-2 was redundant. Indeed, IL-7 was more effective than IL-2 in enhancing iNKT cell expansion. Remarkably, IL-15 hampered the effects of IL-7. This negative effect of IL-15 was unforeseen since this cytokine has been described to be the predominant mediator of in vivo (invariant) NKT cell homeostasis (Golden-Mason et al., 2004; Matsuda et al., 2002). IL-15 can perform its functions by direct binding to the IL-15 receptor (IL-15R) complex on T cells, which consists of IL-15Rα, IL-2/IL-15Rβ chain and the common-γ-chain (γC). Alternatively, and likely to be physiologically more important, IL-15RαγC can “transpresent” surface bound IL-15 to the IL-15R complex on T, NK and (i)NKT cells (Dubois et al., 2002; Schluns et al., 2004). The iNKT cell lines are expected to be susceptible to IL-15 signaling because they expressed IL-15Rα (not shown). We found no IL-15Rα expression by D1.DC1 (not shown), suggesting that the antagonizing effect of IL-15 on IL-7 in our system was caused by competition for γC. iNKT cell expansion experiments comparing the effects of IL-7 and IL-15 using IL-15Rα and IL-15RαγC APC might provide confirmation of this hypothesis.

The most notable characteristic of iNKT cells in vivo is their capacity to rapidly secrete large amounts of IFN-γ and IL-4 upon activation (Matsuda et al., 2000; Nakagawa et al., 2001; Crowe et al., 2003). As earlier described for short term cultured iNKT cells (Ikarashi et al., 2005), we demonstrate here that this trait was retained after repeated stimulation with D1.DC1 and exogenous IL-7. After 4 h of stimulation with αGalCer loaded Hela-CD1d the iNKT cell lines produced both IFN-γ and IL-4. Other Th1 and Th2 type cytokines (GM-CSF, IL-5, IL-6 and IL-10) were also produced, indicating that all lines fitted a Th0 profile. The limited amount of cells producing both IFN-γ and IL-4 in the intracellular cytokine staining experiments was somewhat surprising. One might argue that the human CD1d-transfected APC did not provide the proper antigenic and co-stimulatory signals. However, the relative amount of iNKT cells producing both IFN-γ and IL-4 was not increased by stimulating with PMA/ionomycin (not shown). Furthermore, we found no clear cut differences in the IFN-γ/IL-4 balance between the CD4+ and CD4- subsets within iNKT cell cultures in contrast to the finding that freshly isolated human CD4+ iNKT are the predominant source of IL-4 (Gumperz et al., 2002; Lee et al., 2002). These results indicate that, at least for mouse iNKT cells, additional markers distin-

guishing the IFN-γ and IL-4 secreting subsets still need to be determined. Still, we found that iNKT cell lines with a theoretically more mature surface receptor phenotype tended to have a more prominent Th1 profile, which fits with the proposed in vivo developmental stages of iNKT cells (Benlagha et al., 2002, 2005; Coles and Raulet, 2000; Gadue and Stein, 2002; Pellicci et al., 2002). These findings correlated with the period these lines had been maintained in culture at the time of phenotypical and functional analysis (10, 6, 4 and 3 months respectively). The very low numbers of NK1.1+ cells in the CD4+ iNKT cell lines is in contrast to the in vivo development of iNKT cells, since acquisition of this molecule has been postulated to precede loss of CD4 expression during thymic development (Pellicci et al., 2002). However, the iNKT cell lines presented here were chronically activated by weekly re-stimulation with αGalCer loaded DC and thus might have down-modulated NK1.1 expression (Chen et al., 1997; Wilson et al., 2003). This lack of NK1.1 was also observed recently by Ikarashi et al. (2005) after 6 days of in vitro iNKT cell stimulation with αGalCer. Alternatively, one can not exclude the possibility that loss of NK1.1+ cells results from the preferential in vivo expansion of the NK1.1- subset which lacks NK receptors of the Ly-49 family, since Ly-49/HMC class I interactions were reported to inhibit iNKT proliferation in vitro (Maeda et al., 2001)

In conclusion, iNKT cell lines can be efficiently generated from mouse spleen T-cells by stimulation with αGalCer loaded D1.DC1 in the presence of IL-7. Using this method up to 10^8 iNKT cells can be obtained from one spleen within 12 to 14 weeks, and cell lines can be continued for up to 24 months, without losing the capacity to swiftly secrete cytokines upon activation and thus have retained their strong capacity to strengthen immune responses. These iNKT cells are currently being used for adoptive transfer experiments to investigate their biology in immune responses and to translate into a potentially successful immunotherapeutic strategy.

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Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells


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Chapter 6b

Chronically stimulated mouse invariant NKT cell lines have a preserved capacity to enhance protection against experimental tumor metastases

Johan W. Molling, María Moreno, Jan de Groot, Hans J.J. van der Vliet, B. Mary E. von Blomberg, Alfons J.M. van den Eertwegh, Rik J. Scheper, Hetty J. Bontkes

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Abstract

In pre-clinical models, CD1d restricted invariant Natural Killer T (iNKT) cells play a pivotal role in natural anti-tumor immune responses, mainly by trans-activating cells of both the innate and adaptive arms via swift and potent cytokine secretion. We have previously reported that patients with a severely reduced circulating iNKT cell pool have a poor clinical response to radiotherapy of head and neck squamous cell carcinoma. Therefore, these patients might benefit from an immunotherapeutic approach aimed at the increase of circulating levels of iNKT cells. Furthermore, we have generated both human and mouse iNKT cell lines, and demonstrated that they had retained the capacity to release both Th1 and Th2 type cytokines even after long-term in vitro expansion using α-galactosylceramide (αGalCer) pulsed dendritic cells (DC). Here, we establish, in a pre-clinical tumor model that the large scale long lived polyclonal iNKT cell lines we generated have a preserved capacity to evoke an in vivo cytokine storm upon adoptive transfer, independently of supplemental αGalCer administration. This results in an augmented NK cell mediated protection against B16.F10 experimental lung metastases in vivo. These findings underscore the potential of autologous adoptive transfer of ex vivo expanded iNKT cells as a strategy to enhance immunotherapeutic modalities for the treatment of cancer patients.

1. Introduction

iNKT cells are T lymphocytes, characterized by an invariant TCR α chain gene rearrangement (Vα24-Jα18 in human and Vα14-Jα18 in mouse) and co-expression of NK cell receptors [1]. The iNKT cell TCR recognizes glycolipid ligands presented by the MHC class-I homologue CD1d such as the putative endogenous ligand isoglobotrihexosylceramide (iGb3), bacterial glycosphingolipids or the artificial ligand αGalCer [2–7].

In vivo activation of iNKT cells induces a cytokine storm in which iNKT cells rapidly produce large amounts of e.g. IFN-γ and IL-4 [1], leading to the trans-activation of other immune cells (e.g. NK, T, B cells and DC) [8–11]. Hence, the therapeutic potential of iNKT cells as powerful regulators of immune responses in a broad variety of diseases is under thorough investigation [12,13]. In pre-clinical cancer models these cells appear to play a pivotal role in natural tumor immune surveillance [11,14–19]. Furthermore, activation of resident iNKT cells by treatment of mice with αGalCer or αGalCer loaded DC induces potent anti-tumor immune responses in various tumor models [20–24].

In humans, we and others have demonstrated that cancer patients have reduced circulating iNKT cell levels compared to age and gender matched healthy individuals [25–28]. In addition, in a prospective study we recently observed that reduced numbers of circulating iNKT cells predict poor clinical outcome in individuals with squamous cell carcinoma of the head and neck [29]. These findings suggest that cancer patients might benefit from immunotherapy aimed at the expansion of their peripheral blood iNKT cell pool.

Clinical phase I studies in cancer patients have revealed that administration of αGalCer, αGalCer pulsed DC or autologous
peripheral blood lymphocyte cultures enriched for iNKT cells can be performed safely, leading to limited iNKT cell activation but no consistent and robust anti-tumor effects [30–34]. Patients might benefit more from adoptive transfer of well defined high purity pro-inflammatory autologous iNKT cell lines. However, in order to obtain therapeutically sufficient amounts of iNKT cells repeated in vitro re-stimulation, potentially altering their in vivo functionality, is necessary. We previously described the in vitro generation of high purity pro-inflammatory iNKT cell lines from cancer patients using αGalCer loaded DC [35].

In addition, we recently developed a method for the generation and sustained in vitro expansion of iNKT cell lines from mouse spleen using αGalCer loaded DC. These chronically stimulated cells preserved their capacity to readily secrete a variety of cytokines in vitro [36].

Here we show that upon adoptive transfer, chronically stimulated iNKT cell lines mice have retained the intrinsic capacity to enhance the innate immune response against experimental B16.F10 lung metastases. Notably, the anti-tumor response, though initiated by the adoptively transferred iNKT cells, was predominantly carried out by secondarily activated NK cells and was independent of supplemental systemic αGalCer administration. These findings add to the view that autologous adoptive transfer of ex vivo expanded iNKT cells might exert beneficial effects in cancer patients.

2. Materials and methods

2.1. Mice

6–10 week old female C57BL/6 mice were obtained from Harlan (Harlan Netherlands B.V., Horst, The Netherlands). Mouse care was in line with the guidelines provided by the VU Animal Ethical Committee, who approved the study.

2.2. Cell lines and culture media

IMDM (Cambrex Bio Science, Verviers, Belgium) was supplemented with 10% Foetal Calf Serum (Hyclone, Logan, UT, USA), 0.01 mM β-mercaptoethanol and 50 units/ml penicillin-streptomycin (IMDMc) and used to culture B16.F10 melanoma cells or CD1d transfected HeLa cells (HeLa-CD1d) [44]. D1, an immature DC line was cultured as described in IMDMc supplemented with supernatants from the GM-CSF transfectected cell line R1 as described previously [36].

2.3. iNKT cell expansion from mouse spleen

3–4 days prior to isolation of splenocytes, mice received 100 μg/kg αGalCer by way of intra-peritoneal injection, to increase the binding to αGalCer loaded CD1d:mouse-IgG1 dimers (BD Biosciences). When spleens were harvested, single cell suspensions were obtained by gently pressing the spleens through 100 μm filters. Mononuclear cells were then obtained by Lympholyte®-M density gradient centrifugation for 15 min at 1000 × g (Cedarlane, Burlington, NC, USA). Cells were incubated for 5 min at 4°C with Fcy-R II/III blocking antibody (clone 2.4G2) and subsequently incubated for 10 min at 37°C with saturating amounts of αGalCer loaded CD1d:mouse-IgG1 dimers (BD Biosciences). Next, cells were washed and labeled with magnetic goat-anti-mouse-IgG1 beads. Finally, iNKT cells were isolated by positive selection using the MiniMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany). The isolated iNKT cells were directly co-cultured and weekly re-stimulated at a 10:1 iNKT/DC ratio in the presence of 10–20 ng/ml mouse IL-7 (R&D Systems, Minneapolis, MN, USA), with D1 DC that were first matured for 3–6 h in the presence of LPS (100 ng/ml), αGalCer (100 ng/ml; provided by Kirin Breweries Pharmaceutical Research Laboratories, Gunma, Japan) and IFN-γ (400 U/ml) as described previously [36].

2.4. Evaluation of cytokine secretion

For determination of cytokine secretion by iNKT cell lines, supernatants of co-cultures of 4 × 10^5 iNKT cells and 4 × 10^5 D1 DC per ml were harvested 24 h after re-stimulation. For determination of serum cytokines upon iNKT cell transfer, mice received i.v. injections of PBS or 1 × 10^6 B16.F10 cells and, 3 h later, of 10^6 cultured iNKT cells or PBS as indicated. Samples were collected by orbita-punction under a light isoflurane anesthesia. For determination of cytokine secretion by splenocytes of tumor inoculated mice, single cell suspensions were obtained by gently pressing mouse spleens through 100 μm filters (BD FALCON™; BD Biosciences). Mononuclear cells were then obtained by Lympholyte®-M density gradient centrifugation for 15 min at 1000 × g (Cedarlane). Next, 2 × 10^6 cells/well were incubated for 48 h at 37°C in 500 μl IMDMc in 48 well plates (triplicates for each spleen) before harvesting the supernatants. All samples were stored at −20°C before analyzing for the presence of IL-4 and IFN-γ by ELISA according to the manufacturer’s protocol (BD Biosciences).

2.5. Intra-dermal B16.F10 melanoma assay

To determine the direct effects of iNKT cells on B16.F10 melanoma in vivo, B16.F10 target cells (10^6 cells/injection) and cultured iNKT cells or naive spleen lymphocytes (effectors) were mixed at the indicated E/T ratios and injected i.d. in 100 μl PBS in the flanks of mice. Prior to injection all cells were extensively washed (3 times) with PBS and reconstituted in PBS. The tumor diameter was measured daily, starting at day 6 after injection, to determine tumor growth kinetics.


To determine the effect of cultured iNKT cells on the systemic immune response against tumors, the B16.F10 lung metastasis model was used. Prior to injection all cells were extensively washed (3 times) with PBS and reconstituted in PBS. Mice were injected i.v. with B16.F10 cells and, 3 h later, with cultured iNKT cells as indicated. In some cases, 30–60 min after iNKT transfer mice received 100 μg/kg αGalCer or vehicle control by way of i.p. injection. Transfer of iNKT cells was performed 4 days after in vitro re-stimulation with αGalCer loaded DC. TCR expression, known to be down-regulated upon activation [38], was restored at this time and the injected cells contained no contaminating DC (not shown). Mice were sacrificed on day 11; lungs were harvested, bleached in Fekete’s fixative and subsequently fixed for 2 days in formaldehyde. Next, all lobes were dissected, digitally photographed and the amount of surface metastases was determined by counting or by determining the relative tumor load through a point to point method, using Leica Qprodit software (Leica Microsystems, Rijswijk, The Netherlands). Data were recorded as the mean number of tumor colonies or % of tumor positive lung surface ± S.E.M. of 5 lobes.

2.7. In vivo depletion of CD8+ T cells or NK cells

In some cases in the B16.F10 lung metastasis model, mice were depleted from CD8+ T cells or NK cells by i.p. injection of 200 μg
anti-CD8β hybridoma supernatant (clone 2.43) or i.v. injection of 50 μl of polyclonal anti-asialo-GM1 (Wako Pure Chemicals GmbH, Neuß, Germany), respectively, 2 days prior to tumor inoculation. PBS control injections were given i.p. Depletion of CD8+ T cells or NK cells was confirmed by flowcytometric analysis (antibody clones H35-17.2 and PK136, respectively) of peripheral blood samples and was shown to exceed 90% for up to 11 days (not shown).

2.8. Flowcytometry

For evaluation of iNKT cell purity, cells were incubated for 10 min at 37°C with αGalCer or vehicle loaded CD1d mouse IgG1 dimers (BD Biosciences), washed twice and subsequently stained with PE labeled rat-anti-mouse IgG1 (clone A85-1) and APC labeled anti CD3ε (clone 145-2C11) or Armenian hamster IgG1 isotype control (clone A19-3) (BD Biosciences).

For evaluation of TCR-Vβ-chain expression and for the confirmation that no contaminating non-Vα/Vβ (BD Biosciences), washed twice and subsequently stained with PE labeled rat-anti-mouse CD8 (clone H35-17.2) or CD49b (clone DX5) to identify CD3+ T cells or CD3-CD49b+ NK cells, respectively. Cells were identified as viable lymphocytes on basis of light scatter field gating. Flowcytometry was performed on a FACSCALIBUR™ apparatus and data were analyzed using CellQuest™ software (BD Biosciences).

For evaluation of CD69 expression on peripheral blood lymphocytes, blood samples were acquired by puncturing the orbital venous plexus. Samples were incubated for 5 min in ice cold isotypic NH4Cl solution (0.155 M NH4Cl, 0.01 M KHCO3, 0.1 mM EDTA; pH 7.4) to lyse erythrocytes and granulocytes. Next, cells were washed in IMDM and subsequently stained in flowcytometry buffer (PBS, 0.1% BSA, 0.05% NaN3) for >15 min on ice with a combination of allophycocyanin (APC)-labeled Armenian hamster-anti-mouse CD3ε (clone 145-2C11) and phycoerythrin (PE)-labeled rat-anti-mouse CD8β (clone H35-17.2) or CD49b (clone DX5) to identify CD3+CD8β+ T cells or CD3-CD49b+ NK cells, respectively.

For each iNKT cell line, we monitored IFN-γ production by T cells co-cultured with 4 × 104 melanoma cells (Fig. 1). The delay was not associated with any apparent direct cytolytic activity of the iNKT cell lines against B16.F10 in vitro (not shown).

2.9. Statistical analyses

Variables fitted a Gaussian distribution. Statistical analyses performed were Student’s T-test, linear regression and Log Rank statistics. P-values below 0.05 were considered significant.

3. Results

3.1. Limited direct effects of cultured iNKT cells on B16.F10 melanoma

To study direct in vivo anti-tumor activity of long-term stimulated iNKT cell lines, mice received i.d. injections of three different iNKT cell lines (characterized in Table 1) and B16.F10 melanoma cells mixed at two different E/T ratios (30:1 and 5:1) (Fig. 1). One out of three iNKT cell lines tested (iNKT #II), significantly delayed the average time to detection of palpable tumors at the highest E/T ratio (mean ± standard error = 10 ± 1 days) compared to the control (6 ± 0 days) (p = 0.0082 Log Rank test) (n = 4 per group). Although tumor outgrowth was delayed, the tumors did progressively grow (not shown).

Table 1

<p>| Characteristics of the iNKT lines investigated at time of in vivo application |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>In vivo model</th>
<th>Cultured in vitro</th>
<th>TCR-Vβ usage</th>
<th>CD3ε/CD8β (% cells)</th>
<th>IFN-γ (pg/ml ± S.D.)</th>
<th>IL-4 (pg/ml ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNKT #I</td>
<td>i.d. B16.F1O</td>
<td>12 monthsa</td>
<td>Vβ7</td>
<td>0/100</td>
<td>2572 ± 1284b</td>
</tr>
<tr>
<td>iNKT #II</td>
<td>i.d. B16.F1O</td>
<td>3 months</td>
<td>Vβ8.1/8.2</td>
<td>50/50</td>
<td>17,815 ± 8279</td>
</tr>
<tr>
<td>iNKT #III</td>
<td>i.d. B16.F1O</td>
<td>4 months</td>
<td>Vβ8.1/8.2</td>
<td>59/41</td>
<td>9791 ± 5202</td>
</tr>
<tr>
<td>iNKT #IV</td>
<td>i.d. B16.F1O</td>
<td>24 months</td>
<td>Vβ7</td>
<td>0/100</td>
<td>12,913 ± 6974b</td>
</tr>
<tr>
<td>iNKT #V</td>
<td>i.v. B16.F1O</td>
<td>20 months</td>
<td>Vβ8.1/8.2</td>
<td>2/98</td>
<td>5317 ± 1957</td>
</tr>
<tr>
<td>iNKT #VI</td>
<td>i.v. B16.F1O</td>
<td>1 month</td>
<td>Vβ8.3</td>
<td>0/100</td>
<td>12,345 ± 3699</td>
</tr>
<tr>
<td>iNKT #VII</td>
<td>i.v. B16.F1O</td>
<td>2 months</td>
<td>Vβ2</td>
<td>46/54</td>
<td>8496 ± 5539</td>
</tr>
</tbody>
</table>

Prior to their application in anti-tumor models in vivo, the iNKT cell lines were characterized regarding phenotypical and functional aspects. All lines used were established to be bona fide >99% pure iNKT cell lines by flowcytometry using αGalCer loaded mouse IgG1-CD1d dimers.

a Monitoring tumor growth kinetics after i.d. B16.F10 melanoma with or without co-injection of iNKT cells as indicated in manuscript.
b Duration of weekly in vitro stimulation with αGalCer pulsed mature D1 dendritic cells in the presence of mIL-7 at time of first in vivo application.

c Cytokines in supernatant after 24 h stimulation of 1 × 106 iNKT with 1 × 106 αGalCer pulsed CD1d transferred HEL.A1 cells/ml (n = 4).

d Determined tumor load in lungs 11 days after i.v. injection of B16.F10 melanoma, followed by i.v. PBS or iNKT cells as indicated in manuscript.

e Cytokines in supernatant of 24 h co-cultures of 4 × 105 iNKT + 4 × 104 αGalCer pulsed mature D1 dendritic cells/ml in the presence of mIL-7 (n = 2).
Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells

117

3.3. Intravenous injection of chronically stimulated iNKT cell lines induces a cytokine storm

The hallmark of iNKT cells is their capacity to readily release cytokines upon activation including IL-4 and IFN-γ, hereby swiftly trans-activating other immune cells leading to a systemic cytokine storm. Adoptive transfer of chronically stimulated iNKT cell lines resulted in similar events. 24 h after adoptive transfer of iNKT #II into naïve or B16.F10 inoculated mice IFN-γ, but not IL-4, was detectable in the serum (mean serum IFN-γ ± S.D.; naïve mice = 790 ± 312 pg/ml and tumor bearing mice = 766 ± 444 pg/ml) (n = 6 per group) (Fig. 3A). Subsequently, IFN-γ levels dropped to undetectable within the next 24 h (not shown). However, splenocytes taken at day 11 after tumor inoculation, from mice that received iNKT cells (iNKT #I, #II, #IV or #VI) (n = 22) 3 h after tumor inoculation, but not from PBS treated mice (n = 6), were secreting some IL-4 and substantial amounts of IFN-γ in vitro without exogenous activation (median range); IL-4 = 15 [0–47] pg/ml and IFN-γ = 891 [124–8536] pg/ml (Fig. 3B). These results indicate that adoptive transfer of chronically stimulated iNKT cells into tumor bearing mice induces a donor iNKT cell dependent cytokine storm lasting up to 11 days.

3.4. NK cells are required for the protective anti-tumor effects of iNKT cell transfer

Next, we determined the contribution of CD8+ T cells and NK cells to the inhibition of tumor metastases after adoptive transfer of chronically stimulated iNKT cells (Fig. 4). Transfer of iNKT cells into control mice (mean tumor load ± S.D.; PBS control = 39 ± 10%, i.e. IFN-γ = 19 ± 7%) (p = 0.0037, Student’s T) or CD8+ T cell depleted mice (mean tumor load ± S.D.; PBS control = 31 ± 11%, i.e. IFN-γ = 17 ± 11%) (p = 0.0497, Student’s T) significantly hampered lung metastasis development. However, treatment of NK depleted mice had no apparent effect on B16.F10 lung metastases (mean tumor load ± S.D.; PBS control = 69 ± 14%, i.e. IFN-γ = 57 ± 11%) (p = 0.146, Student’s T) (n = 6 per group). In accordance, we observed an up-regulation of the activation marker CD69 on both circulating CD8+ T and especially NK cells within 24 h of iNKT cell transfer, which was more pronounced on the latter. (Supplementary Fig. 1). The high tumor load in NK depleted mice underscores their importance in the first line of defense against B16.F10. These findings were accentuated in a follow up experiment (Fig. 5). Herein iNKT treated mice (n = 14) had a larger fraction of NK cells within lymphocytes, compared to PBS treated mice (n = 7) at day 4 after adoptive transfer (mean% of NK cells ± S.D.; PBS control = 8.8 ± 5.6%, i.e. IFN-γ = 17 ± 7.7%) (p = 0.0235, Student’s T). This was not the case for CD8+ T cells (mean% of CD8+ T cells ± S.D.;
4. Discussion

Depending on the model, resident iNKT cells can augment innate as well as adaptive anti-tumor immune responses upon systemic treatment with αGalCer or αGalCer loaded DC in C57Bl/6 mice. For instance, a single i.v. injection of dying hematologic tumor cells together with αGalCer induces long lasting protective immunity, depending on both CD4+ and CD8+ T cells. This as a result of enhanced maturation of cross priming DC by resident iNKT cells [39]. On the other hand, treatment with αGalCer or αGalCer loaded DC can partially protect mice against experimental metastases of B16.F10 melanoma as a result of NK cell trans-activation by iNKT cells [11,21,22].

Here, we first investigated the direct effect of chronically stimulated iNKT cell lines on intra-dermal B16.F10 tumors. In two independent experiments, only 1 out of 3 lines investigated was capable of significantly inhibiting tumor outgrowth for up to 12 days after injection compared to control mice, when mixed with B16.F10 melanoma cells at a high effector/target ratio prior to i.d. inoculation. We did not observe any potent direct in vitro cytotoxicity against B16.F10 by these 3 iNKT cell lines (not shown). As shown in Table 1, the iNKT cell line that delayed tumor development (iNKT #III) was capable of secreting substantially more IFN-γ upon activation than the other 2 lines tested here (iNKT #I and #IV). The transient inhibition might therefore be caused by an anti-angiogenic effect of locally released IFN-γ by the injected iNKT cells, as has been described for mice systemically treated with αGalCer [40], and not by direct cytotoxicity against the tumor. Alternatively, iNKT derived cytokines might have a direct inhibitory effect on tumor outgrowth and/or the intra-dermal co-injection with iNKT cells might cause a transient influx of e.g. NK cells. In contrast to our results, Chamoto et al. demonstrated that CD3+CD1+ T cells do acquire direct NK like cytotoxic ability in vitro after 5 days of culture with αGalCer loaded DC in the presence of exogenously added IL-2 [15]. This discrepancy could be explained by the use of IL-7 in our culture system, although both IL-2 and IL-7 have been shown to enhance the NK like cytotoxic capacity of in vitro CD3 activated killer cells [41]. Furthermore, one cannot exclude that the actual killing in the study of Chamoto et al. was performed by non-CD1d restricted instead of invariant NKT cells, since no distinction was made between the two. Alternatively, the acquired cytotoxicity of iNKT cells might again be lost after chronic re-stimulation in vitro. However, in that same study Chamoto et al. demonstrated that despite the in vitro cytolytic activity of short-term cultured iNKT cells, in vivo activated iNKT cells predominantly enhanced NK cell mediated killing of tumors rather than they exerted direct tumor cell killing [15].

Secondly, we demonstrate here that long-term chronically in vitro stimulated iNKT cell lines retain the capacity to augment anti-tumor immune responses. Crowe et al. previously reported the partial inhibition of B16.F10 lung metastases via adoptive transfer of freshly isolated splenic iNKT cells in combination with systemic
Fig. 3. Cytokine secretion by splenocytes of tumor bearing mice. (A) On day 0 mice received i.v. injections as indicated with PBS or B16.F10 melanoma and 3 h later with PBS or 10^6 iNKT cells. Serum samples were collected prior to the first injection and on days 1, 2 and 4. Bars indicate mean serum IFN-γ level at day 1; error bars indicate standard error of mean (n = 3 per group). No IFN-γ was detected on the other time points. (B) On day 0 mice received i.v. injections with B16.F10 melanoma and 3 h later with PBS (n = 6) or 10^6 iNKT cells (n = 22). On day 11 splenocytes were isolated and cultured for 48 h in IMDM. The amount of IL-4 and IFN-γ in the cell supernatants was determined by ELISA. Dots indicate individual mice, horizontal lines indicate median. Data are from 1 experiment representative of 2.

Fig. 4. Anti-tumor effects of adoptive iNKT cell transfer in CD8^+ T or NK cell depleted mice. On day 0 mice received i.v. injections with PBS or B16.F10 melanoma and 3 h later with PBS (n = 7) or 10^6 iNKT cells (either iNKT #II or #V) (n = 14). On day 4 lung lymphocytes were analyzed for the percentage of CD8^+ T cells (A) or NK cells (B) by flow cytometry. Bars indicate mean; error bars indicate standard error of mean. Data are pooled from two independent experiments. *p < 0.05, Student's T.

GalCer treatment [42]. In addition, Shin et al. successfully inhibited B16.F10 liver metastases when transferring iNKT cells that were pre-activated with IL-12 in vivo into recipient mice in the absence of GalCer [43]. Here, in multiple independent experiments, 4 out of 4 iNKT cell lines investigated were able to significantly hamper B16.F10 lung metastasis development compared to control mice when administered shortly after tumor cell inoculation (50–60% reduction in tumor load). In our hands, additional GalCer treatment did not enhance the anti-tumor effects observed. Transfer of these pre-activated iNKT cells was performed 4 days after in vitro re-stimulation with GalCer loaded DC. As day 4 pre-activated iNKT can display substantial IFN-γ production without GalCer triggering ([36] and unpublished results), the finding here might reflect that the injected iNKT cells were already sufficiently activated to trans-activate immune effector cells, which is in line with the results by Shin et al. Interestingly, they also established that iNKT cell transfer therapy was far superior to injecting high dose IL-2, IL-4 or IFN-γ. Even though there was no direct correlation between the IFN-γ levels in the tumor bearing animals after iNKT
cell adoptive transfer (either in serum on day 1 or by splenocytes on day 11) and anti-tumor effects, the fact that the low IFN\(\gamma\) producing iNKT cell line \#II was only effective at the higher dose level, while the high IFN\(\gamma\) producing iNKT cell line \#IV was already effective at a 5 times lower dose suggests a role for iNKT cell derived soluble factors in the observed protective effects.

We next set out to investigate how downstream anti-tumor immunity might commence upon transfer of chronically stimulated iNKT cell lines. Within 24 h after iNKT cell transfer we could detect serum IFN\(\gamma\) which was already undetectable at 48 h after transfer. This relatively short-term peak suggests that the cytokine was a donor iNKT cell product and that the transferred cells quickly disappeared from circulation. Splenocytes from iNKT cell treated mice (but not from control mice) were still secreting IFN\(\gamma\) and also IL-4 at day 11 after transfer without an additional stimulus. Four days after transfer the injected iNKT cells were not back traceable in lungs, livers or spleens after labeling with carboxy fluorescein diacetate succinimidyl ester (CFSE) or 5-(and-6)-(((4-chloromethyl)benzoyl) amino) tetramethyl rhodamine (CMTMR) (not shown) suggesting that the cytokines secreted at day 11 were produced by resident cells, activated by the transferred iNKT cells. In line with previous reports, we established that NK cells but not CD8\(^+\) T cells were required for the anti-tumor effects of adoptive iNKT cell transfer \[11,18,22\]. In addition, we found that lung lymphocytes of iNKT recipient mice (both tumor inoculated and naive mice) were enriched for NK cells but not CD8\(^+\) T cells at day 4 after transfer. It remains to be elucidated whether this recruitment of NK cells is mediated by direct local chemokine release from the injected iNKT cells or indirectly via their secretion of inflammatory mediators.

While the present results are promising, no total protection against B16.F10 was established and the outgrowth of established metastases was not inhibited (not shown). Crowe et al. have previously demonstrated that, in contrast to adoptive transfer of spleen derived iNKT cells which leads to a partial protection similar to as we have described here, adoptive transfer with liver derived iNKT cells leads to a full protection against B16 tumors. Thus, the origin of iNKT cells clearly affects their activity in anti-tumor responses. Since it will be difficult to obtain liver derived iNKT cells in a human setting we have focused on spleen derived iNKT cells which have the same origin as circulating iNKT cells. Our results show that further optimization of iNKT cell mediated treatment is warranted. Smyth et al. demonstrated that moderate iNKT cell driven innate immunity against B16.F10 can be dramatically enhanced when aGalCer loaded DC are combined with systemic IL-21 treatment \[11\]. We have recently demonstrated in a human setting that iNKT cells expanded with DC over-expressing IL-12 produce increased levels of IFN\(\gamma\). These iNKT cells drive the expansion and activation of tumor antigen specific CTL in an IFN\(\gamma\) dependent fashion when tumor antigen was presented (Moreno et al., submitted for publication). Thus, the efficacy of iNKT cell adoptive transfer may be strongly increased if combined with additional IL-21 treatment or tumor antigen vaccination and/or aGalCer-pulsed DC.

In summary, we describe here that the unique large scale long lived polyclonal iNKT cell lines, which our generation have described previously \[36\], have a preserved capacity to evoke an vivo cytokine storm upon adoptive transfer, which is measurable in the spleen up to 11 days after iNKT cell injection. This results in an augmented, NK cell mediated, partial protection against B16.F10 experimental lung metastases. These combined findings underscore the potential of autologous adoptive transfer of ex vivo expanded iNKT cells to enhance immunotherapeutic strategies for the treatment of cancer patients.

Conflict of interest

The authors declare no conflict of interest or financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2008.02.007.

References


Innate and adaptive tumour immunity: Role of invariant Natural Killer T-cells


Chapter 7

Discussion
Harnessing invariant Natural Killer T cells for the treatment of cancer
Pre-clinical studies illustrate the potential of invariant Natural Killer T (iNKT) cells as regulators of immune responses to tumours [reviewed in chapter 1] and suggest the benefit of exploiting iNKT cells for the treatment of cancer. Analyses in patients with malignant melanoma, primary lung cancer, advanced prostate cancer and other progressing solid tumours demonstrated a numerical deficiency of iNKT cells in the peripheral blood of these patients when compared with healthy volunteers [1-5]. Whether this deficiency represents a cause or an effect of the disease is not yet clear. Although iNKT cells from cancer patients have functional defects, i.e. impaired proliferation and cytokine secretion [4-6], they still possess the capacity to proliferate and to secrete IFN-γ when properly stimulated in vitro [7]. This suggests that iNKT cells of cancer patients, though reduced in number, may still be capable of enhancing anti-tumour responses, particularly after therapies aiming at their expansion and activation. Here, different therapeutic approaches to exploit human iNKT cells to improve the anti-tumour immunity are discussed.

1 In vivo activation of human iNKT cells

α-Galactosylceramide injection

Recognizing the promise of iNKT cell activation in immunotherapy of cancer, attempts have been made to target them in vivo in clinical phase I studies, based on the use of α-Galactosylceramide (α-GC) as a stimulatory ligand. In murine models, resident iNKT cells can augment anti-tumour immune responses upon systemic treatment with the strong agonist α-GC, mainly as a result of Natural Killer (NK) cell trans-activation and IFN-γ production [8-10].

In humans, in vitro stimulation of peripheral blood mononuclear cells (PBMC) with α-GC strongly promoted iNKT cell activation, as evidenced by an increased proportion of iNKT cells expressing CD69 and CD25. In contrast, α-GC induced only a modest increase in IFN-γ production. Although NK cells became phenotypically activated, functional activity of NK cells was not enhanced by this approach. No increase in NK cell-mediated cytotoxicity against K562, a NK-sensitive cell line, was detected (chapter 4). Likewise, no enhancement of NK cell-mediated antibody dependent cell-mediated cytotoxicity (ADCC) could be observed (chapter 5b). These results indicate that iNKT cell stimulation with free antigen is not sufficient to translate phenotypical into functional activation, in vitro.

In vivo, weekly intravenous (i.v.) injections of α-GC (KRN7000, a pharmaceutical-grade synthetic analogue of α-GC) have been shown to be safe in clinical phase I studies [11;12]. In parallel to the in vitro observations, administration of α-GC to cancer patients with advanced disease induced a transient and limited increase in serum levels of immunostimulatory cytokines (IL-12, IFN-γ, TNF-α and GM-CSF) only in patients with relatively high iNKT cell levels, without enhancing NK cell cytolytic activity [12]. Moreover, rapid loss of detectable iNKT cells from the circulation was observed within 24 h after α-GC administration and iNKT cell levels remained low for at least 3 weeks after the first administration. The lack of detectable iNKT cells shortly after i.v. injection of the glycolipid may reflect their antigen-specific activation by α-GC, leading to T-cell receptor (TCR) internalization, as it has been previously demonstrated in mice [13;14]. In line with the decline in iNKT cell levels, no increase in serum cytokines was observed after a second and third weekly injection with α-GC [12]. Similarly, systemic treatment with free α-GC in mice induced iNKT cell anergy, rendering iNKT cells unresponsive to subsequent α-GC treatment [15]. This anergy may be related to the relatively high dose of the glycolipid injected and the short interval between repeated injections. In a clinical phase I/II trial in chronic Hepatitis C...
patients, re-appearance, close to baseline levels, of circulating iNKT cells was observed within 2 weeks after i.v. injection of low dose α-GC. In this trial, α-GC exerted moderate immunomodulatory effects, i.e. cytokine responses (TNF-α and IFN-γ), again only in patients with relatively high iNKT cell levels [16]. Of note, no α-GC-induced clinical responses were observed in either study [12;16].

Taken together, these results suggest that inappropriate scheduling of α-GC administration may prohibit effective immunotherapy. Indeed, chronic activation of iNKT cells in mice by repetitive treatment with α-GC resulted in the generation of anergic iNKT cells with a defective cytokine production [17-19]. This poses a problem when considering repeated iNKT cell activation as a therapeutic approach. Furthermore, it has been reported that previous exposure to α-GC favours a type 2 immune response [20;21], which can be counterproductive for the development of a successful anti-tumour response. Therefore, the clinical use of α-GC needs to be carefully evaluated with respect to achieving maximal iNKT cell activation without inducing tolerance or an undesirable cytokine secretion profiles.

**IL-2 administration**

IL-2, in combination with α-GC, induces *in vitro* expansion of peripheral blood iNKT cells of healthy controls, as well as advanced cancer patients. As such, it could be considered a putative adjuvant to promote *in vivo* iNKT cell proliferation.

In a clinical study in HIV-1-infected patients, who have a numerical deficiency of iNKT cells, subcutaneous administration of IL-2 in addition to the standard antiretroviral treatment, led to an expansion of iNKT cells, as well as other CD4+ T cells [22]. No significant change in the CD4+/CD4- ratio within the iNKT cell population was observed, indicating an expansion of both subsets. Sustained IL-2 treatment was necessary in order to maintain the increased iNKT cell levels.

In patients with advanced melanoma and renal cell cancer, i.v. injection of high-dose of IL-2 led to a transient decrease in the frequency and absolute number of iNKT cells. This decline was accompanied by an increase in the proportion of potentially pro-inflammatory double-negative iNKT cells, though without detectable augment in IFN-γ secretion, and was followed by an increase in the total number of circulating iNKT cells [23].

Interestingly, iNKT cell expansion occurred in the absence of iNKT cell antigen. Standard protocols for *in vitro* expansion of peripheral blood iNKT cells of healthy controls, as well as advanced cancer patients, include iNKT cell antigen, in addition to IL-2. In view of this, IL-2 therapy aiming at restoring and maintaining the iNKT cell population *in vivo* could benefit from the combination with α-GC treatment. The effect of this approach has not yet been addressed in cancer patients.

**TLR stimulation**

Because iNKT cell - dendritic cell (DC) cross-talk is an important step in linking and regulating the innate and adaptive immune responses, understanding the molecules capable of fine-tuning this interaction is of great importance for harnessing iNKT cells for the treatment of cancer. Microbial-derived glycolipid antigens other than α-GC presented by CD1d on DC have been shown to directly modulate iNKT cell activation [24-27]. Furthermore, other microbial-derived molecules, such as Toll-Like Receptor (TLR) ligands, can induce iNKT cell activation through IL-12 production by TLR-stimulated DC in the presence of a weak
CD1d/TCR signal [24;28]. TLR agonists are of great interest in the field of cancer immunotherapy due to their immunostimulatory properties. Particularly, their capacity to promote iNKT cell-DC interaction could be exploited to increase the efficacy of iNKT cell-based immunotherapy. However, it is still not clear whether iNKT cells can be directly regulated by TLR ligands.

We determined that human iNKT cells from healthy donors expressed TLR1, 2, 4, 6, 7 and 9 mRNA, whereas TLR3, 5 and 10 mRNA expression was low and TLR8 mRNA was absent (chapter 2). Although iNKT cells express most TLR at the mRNA level, iNKT cells do not respond directly to TLR agonists [29] (and chapter 2). Upon PBMC stimulation with the TLR ligands PGN, CL075 and ODN A (ligands for TLR2, TLR7 and 8, and TLR9, respectively), iNKT cell activation, as evidenced by up-regulation of CD69 expression and IFN-γ production, was induced as a result from cross-talk between iNKT cells and DC (chapter 2). Interestingly, in contrast to the results from Mattner and co-workers [24], who demonstrated iNKT cell activation upon DC stimulation with LPS, we did not observe iNKT cell activation after PBMC stimulation with LPS. This discrepancy may be explained by the complex network of activating and inhibiting signals induced by TLR agonists on different immune cells, such as activation of Tregs that may modulate the activation of iNKT cells [30], influencing the net effect. All TLR agonists tested have been shown to activate NK cells upon PBMC stimulation. However, only PGN, CL075 and ODN A, the three TLR agonists that induced the strongest iNKT cell activation, have an appreciable effect on NK cell-mediated cytotoxic function, determined as antibody-dependent NK cell-mediated ADCC (chapter 5b). These observations highlight the relevance of defining and considering the in vivo effect of TLR ligands not only on iNKT cells but also on the final desirable outcome, when exploiting TLR ligands for iNKT cell activation as a therapeutic approach.

Our in vitro results demonstrate an indirect activation of iNKT cells by TLR2/6, 7, 8 or 9 rather than TLR3, 4 or 5 agonists. Combinations of these TLR ligands with α-GC may act as strong adjuvants for cancer immunotherapy, as they have been shown to enhance iNKT cell activation and type 1 IFN-γ-mediated immune responses, such as NK cell activation and cytolytic activity in vitro. However, their ability to activate iNKT cells in vivo has not yet been tested in humans.

These data, demonstrating a limited effect on iNKT cell numbers and effector functions upon stimulation with free α-GC, IL-2 or TLR agonists even of PBMC derived from healthy donors, with relatively high numbers of iNKT cells, exemplify that alternative treatments to expand and activate iNKT cells in vivo or in vitro are necessary. Vaccination with α-GC-pulsed mature monocyte-derived DC (moDC) or adoptive transfer of in vitro expanded iNKT cells are possible alternative methods to increase iNKT cell numbers in vivo.

**DC activation**

The relatively low in vivo responsiveness of circulating iNKT cells upon i.v. injection of free α-GC parallels the reduced in vitro responsiveness of cancer patient derived iNKT cells when stimulated with autologous α-GC-loaded PBMC [4-6]. Both phenomena may be explained by the impaired function of antigen presenting cells (APC) in cancer patients. On the other hand, α-GC-loaded, in vitro generated, fully functional mature DC restore iNKT cell proliferation in vitro [31]. Consequently, the injection of α-GC-pulsed DC is expected to induce a more effective in vivo expansion of iNKT cells.

Three independent groups have now shown that this approach is well tolerated in humans. In two consecutive studies in Australia, a total of 16 patients with metastatic
malignancies were treated with autologous α-GC-pulsed immature moDC. The treatment resulted in a mild, albeit not sustained, increase in circulating iNKT cell numbers [32;33]. In the second study, transient but potent pro-inflammatory effects were observed in peripheral blood samples after DC treatment, consisting of elevated levels of IL-12 and IFN-γ, in contrast to reduced levels of IL-4, and activation of T cells. Moreover, they found a transient increase in NK cell number, activation and cytotoxicity [33]. A second injection of immature moDC pulsed with α-GC produced the same immunological effects. Further, it was found that serum tumour markers were significantly decreased in two patients with adenocarcinoma, indicating some anti-tumour effect of α-GC-pulsed DC.

Mature DC are far more efficient in activating T cells and inducing their proliferation than immature DC [34]. Thus, in vivo activation of iNKT cells with properly matured DC is expected to be a more appropriate approach. Indeed, Chang and co-workers were able to induce dramatic expansion of circulating iNKT cells in all 5 advanced cancer patients who received i.v. injections with pure, properly matured, α-GC-pulsed autologous moDC (>100 fold expansion at peak level) [35]. Strikingly, despite having undetectable peripheral blood iNKT cell counts at the time of enrolment, the iNKT cell level remained above baseline after DC treatment for more than 85 days in all patients and for up to 6 months in two patients with longer follow up. In addition, indirect proof that iNKT cell activation was followed by myeloid DC activation (elevated serum levels of myeloid DC associated soluble factors IL-12p40, MIP-1β and IP-10) was observed, and an expansion of CD8⁺ memory T cells specific for viral antigens was measured. These findings strongly suggest an adjuvant effect of the treatment on existing adaptive immunity. On the other hand, DC treatment was not sufficient to overcome the impaired iNKT cell IFN-γ secretion. In parallel with the absence of IFN-γ response, no NK cell activation was observed. Moreover, there was a transient decline in NK cell numbers after DC treatment, either with unloaded or α-GC-loaded moDC.

Ishikawa and co-workers also reported activation and transient expansion of resident iNKT cells in 3 out of 12 advanced lung cancer patients upon injection of α-GC-pulsed autologous PBMC cultured for 7 to 14 days in the presence of GM-CSF, IL-2 and α-GC [36].

These clinical studies show that vaccination with α-GC-pulsed DC results in an increase in circulating iNKT cell numbers and IFN-γ producing PBMC however, hardly any clinical responses were observed. The α-GC-induced anti-tumour effect has been shown to be primarily dependent on IL-12 in various tumour models. For that reason, the efficacy of DC treatment may be enhanced by using α-GC loaded, IL-12 over-expressing DC. We demonstrated that PBMC cultures stimulated with α-GC-loaded moDC transfected with IL-12 contained significantly higher numbers of iNKT cells. As previously described, stimulation with IL-12 over-expressing moDC leads to an increased tumour specific cytotoxic T lymphocyte (CTL) response [37]. IL-12 over-expressing DC pulsed with α-GC induced significantly more tumour specific CTL as compared to either IL-12 over-expressing DC or α-GC-pulsed DC (chapter 3). This approach is expected to lead to an increased anti-tumour immune response mediated by tumour specific CTL, as well as IFN-γ producing iNKT cells.

From these findings it can be concluded that injection of preparations containing α-GC-pulsed autologous DC is feasible, as shown in phase I studies, and results in distinct activation of iNKT and downstream effector cells. The anti-tumour efficacy could be improved by combination with IL-12 treatment, although the safety of this latter approach still has to be explored. An alternative approach is adoptive transfer of autologous iNKT cells, which have been previously expanded and activated in vitro.
2 iNKT cell autologous adoptive transfer

The numerical and functional defects of iNKT cells in cancer patients [4;6], combined with the observed poor clinical outcome of radiotherapy in head and neck squamous cell carcinoma patients with low numbers of circulating iNKT cells [7], suggests that cancer patients may benefit from reconstitution of the iNKT cell pool. A controlled way of increasing the number of systemic iNKT cells, as well as restoring their function, is by the direct transfer of ex vivo expanded autologous iNKT cells.

We have developed a method to expand peripheral blood iNKT cells of healthy controls as well as advanced cancer patients in vitro, and to polarize them towards a type 1 cytokine profile by stimulation of isolated TCR-Vα24+ T cells with mature α-GC-pulsed moDC in the presence of IL-15 [38]. Using an adapted version of this method, we expanded iNKT cells from healthy donor PBMC to investigate their capacity to activate NK cells. In contrast to addition of free α-GC, addition of these in vitro expanded human iNKT cells to PBMC cultures promoted NK cell phenotypical and functional activation in vitro (chapter 4). Furthermore, addition of expanded iNKT cells enhanced the efficacy of antibody-dependent NK cell-mediated ADCC (chapter 5b). The enhancing effects of expanded iNKT cells were dependent on additional α-GC stimulation. These results suggest that additional activation of the previously expanded and activated iNKT cells is a prerequisite for the effect on downstream NK cell activation.

Motohashi and co-workers prepared autologous PBMC cultures for adoptive transfer: PBMC derived from 6 non-small cell lung cancer patients were cultured in the presence of IL-2 and α-GC, resulting in cell populations that were enriched for iNKT cells [39]. This approach was well tolerated and resulted in a transient increase in circulating iNKT cells and elevation of IFN-γ producing cells in response to α-GC. The fold-increase in the iNKT cell number varied among the patients (range: 50 – 5250-fold), and seemed to be partially dependent on the initial frequency of iNKT cells. In addition to numerical deficiency, iNKT cell functional defects could also affect expansion. In this regard, Yanagisawa and co-workers have shown that granulocyte colony stimulating factor (G-CSF) can partly restore iNKT cell function repressed in cancer patients, promoting proliferation [6].

Since CD1d is monomorphic, DC for in vitro expansion of iNKT cells are not required to be autologous. Therefore, DC cell lines could be used as a standardized unlimited source of in vitro APC, leading to defined iNKT cell populations for adoptive transfer. It would also facilitate repeated stimulation of cultured iNKT cells in vitro, as it eliminates the need of additional blood sampling to generate sufficient amounts of DC. We have previously demonstrated that human iNKT cells can be expanded using α-GC-pulsed DC derived from the CD34+ human acute myeloid leukaemia derived cell line, MUTZ-3 (M3) [40]. Analogous to the moDC approach discussed above, iNKT cells successively stimulated with M3 over-expressing CD1d (M3-CD1d) and M3 over-expressing CD1d plus IL-12 (M312-CD1d) were highly activated, were capable of producing high levels of IFN-γ, and enhanced tumour specific CTL priming in vitro (chapter 3). Thus, M3-CD1d and M312-CD1d are attractive sources of DC for large scale expansion and activation of functional iNKT cells from cancer patients.

In conclusion, injection of preparations containing iNKT cells is feasible, since it can be performed safely and results in distinct activation of iNKT and downstream effector cells. This promising strategy requires long-term in vitro culture in order to obtain sufficient numbers to repopulate patients with iNKT cells up to healthy control levels, particularly in those individuals who would probably benefit most from this therapy, namely those with a severe deficiency in circulating iNKT cells and/or a poor in vitro proliferative response.
towards α-GC. 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.

We therefore developed a method to generate long-term high purity oligoclonal mouse iNKT cell cultures representative of in vivo iNKT cells. D1 DC cell line [41] was shortly treated with LPS and IFN-γ, and pulsed with α-GC to generate mature DC, capable of facilitating the in vitro expansion of iNKT cells isolated from mouse spleen (chapter 6a) [42]. In successive experiments we weekly re-stimulated iNKT cells with α-GC-loaded mature D1 cells in the presence of IL-7, which supports iNKT cell expansion. Previously, either short lived iNKT cell cultures [43-45] or long lived clone derived iNKT cell hybridomas [46-48] were described. Our study was the first to make highly pure, long-term oligoclonal mouse iNKT cell lines representative of in vivo iNKT cells available on a large scale. These iNKT cell lines were used in different experiments to determine whether their in vitro functionality and, more importantly, their capacity to enhance anti-tumour responses in vivo remained unaffected.

Our iNKT cell cultures consisted of both CD4⁺ and CD4⁻ cells and retained their most distinctive characteristics, i.e. recognition of α-GC/CD1d complexes and capacity to 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 (chapter 6a and 6b), reflecting the dichotomous regulatory capacity of in vivo iNKT cells [49-51]. Intravenous injection of iNKT cell lines evoked systemic cytokine production, i.e. elevated IFN-γ levels in serum and induction of splenocyte capacity to secrete IFN-γ and IL-4 that lasted for up to 11 days. Furthermore, iNKT cell transfer resulted in a preferential influx of NK cells into the lungs of these mice. Most importantly, all four iNKT cell lines investigated, enhanced NK cell mediated protection against B16.F10 experimental lung metastases upon adoptive transfer into wild-type mice shortly after tumour injection (chapter 6b) [52].

Subsequent studies have shown anti-tumour effects of adoptively transferred iNKT cells into tumour inoculated iNKT cell deficient recipients [53-55]. Crowe and co-workers investigated the effect of transfer of freshly isolated iNKT cells on B16.F10 lung metastases in iNKT cell deficient mice. The anti-tumour effects depended on the iNKT cell subtype in combination with systemic α-GC treatment [54]. We demonstrated that additional α-GC injection was not required when iNKT cells had been extensively activated in vitro with α-GC-pulsed D1 DC. This is in line with the findings by Shin and co-workers, who observed inhibition of experimental B16.F10 liver metastases upon adoptive transfer of in vitro IL-12 pre-activated iNKT cells into iNKT deficient mice without additional α-GC treatment [55].

In summary, we demonstrated that adoptive transfer of long-term high purity oligoclonal mouse iNKT cells with proven maintained in vitro functionality lead to enhanced immune protection against an experimental tumour. In the B16.F10 lung metastasis model we used, protection was achieved via the trans-activation of NK cells. The potential of autologous adoptive transfer of ex vivo expanded iNKT cells as an immunotherapeutic strategy for the treatment of cancer patients is illustrated by these pre-clinical studies.

Conclusion

Clinical phase I studies showed that injection of preparations containing α-GC-pulsed DC and/or iNKT cells is feasible and safe. Both approaches result in an increase in circulating iNKT cell numbers and a limited activation of downstream effector cells. Sporadic clinical responses were observed in these phase I trials in cancer patients with advanced disease. We
have demonstrated that the use of IL-12-transduced DC may enhance the efficacy of both treatment modalities. In vitro expansion of iNKT cells using IL-12 over-expressing (allogeneic) DC leads to an iNKT cell population for adoptive T-cell transfer, which is superior in providing help for antigen specific CTL, and NK cells. However, additional treatment with α-GC, presented by DC, may be needed to re-activate the iNKT cells in vivo. However, a combination of iNKT cell adoptive transfer with α-GC pulsed DC may become even more effective when these DC are loaded with tumour associated antigen and engineered to over-express IL-12. 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.

References


Summary
&
Nederlandse Samenvatting
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.
Nederlandse Samenvatting

Kanker is na hart en vaatziekten de tweede doodsoorzaak door ziekte in de westere wereld. Er is in de laatste jaren veel vooruitgang geboekt in de behandeling van kanker met behulp van o.a. chemotherapie en bestraling. Bij deze vormen van kankertherapie ontstaan echter veel bijwerkingen omdat niet alleen kankercellen worden gedood, maar ook normale gezonde cellen. Een andere veelbelovende therapie is immuuntherapie. Het doel van immuuntherapie is het induceren en/of activeren van afweercellen zodat zij tumorecellen kunnen doden. De zogenaamde killer T-cel (CTL) kan stukjes eiwit die alleen, of in sterk verhoogde hoeveelheden in tumorecellen voorkomen en niet, of slechts in lage hoeveelheden aanwezig zijn in gezonde cellen [tumor geassocieerde antigenen (TAA)] herkennen als zij in de context van de zogenaamde transplantatie antigenen (HLA) gepresenteerd worden. HLA antigenen zitten op het oppervlak van alle normale kernhoudende cellen. Nadat de CTL TAA gepresenteerd door HLA herkent, kan deze de tumor cel doden. Echter in veel gevallen verdwijnt het HLA van het oppervlak van tumor cellen en kan de CTL deze niet meer herkennen. Op dit moment wordt een tweede type “killer” cel van belang, de zogenaamde natural killer (NK) cel. Dit type killer cel laat alle normale cellen met HLA op het oppervlak met rust, maar zal cellen die geen HLA op het oppervlak meer hebben, bijvoorbeeld door ontstaarding als kankercel, juist actief doden. Dit kunnen zij op twee manieren, direct of via herkenning van antistoffen die gebonden zijn aan de tumor cel. Het laatste mechanisme wordt ook wel “antibody dependent cellular cytotoxicity” of ADCC genoemd. ADCC is één van de belangrijkste mechanismen van tumor specifieke monoklonale antistof therapie. Antistoffen bestaan uit een constant gedeelte en een variabel gedeelte, het variabele gedeelte is voor elke antistof anders en bepaalt waar deze aan zal binden, bijvoorbeeld een bepaald eiwit wat alleen op het oppervlak van tumor cellen voorkomt maar niet op normale cellen. Een bekend voorbeeld van zo’n eiwit is MUC1, wat veel voorkomt op borst- en eierstokkanker cellen. NK cellen hebben een receptor voor het constante gedeelte van de antistof. Na binding van een antistof die met het variabele deel vast zit aan de tumor cel en met het constante deel aan de NK cel, wordt de NK cel aangezet tot het doden van de tumor cel.

Bij de zogenaamde niet-specifieke immuuntherapie wordt de patiënt behandeld met stoffen die een algemene activering van het afweer systeem veroorzaken (cytokinen). Omdat bij deze vorm van immuuntherapie het gehele immuunsysteem sterk wordt geactiveerd, gaat deze behandeling ook gepaard met relatief veel bijwerkingen en daarnaast zijn de resultaten helaas teleurstellend. Bij specifieke immuuntherapie wordt het afweersysteem gestimuleerd door middel van vaccinatie met TAA om TAA specifieke CTL te induceren die vervolgens tumor cellen kunnen doden maar gezonde cellen met rust laten. Deze vorm van immuuntherapie heeft dan ook relatief weinig bijwerkingen, en de eerste resultaten zijn veelbelovend. Om voldoende en effectieve tumorcel herkenden CTL te induceren is het noodzakelijk om de TAA aan te bieden op het HLA van een gespecialiseerd celtype; de dendritische cel (DC). Na activering is de DC het enige celtype wat in staat is primaire TAA specifieke CTL te induceren. Huidige vaccinatie strategieën maken dan ook vaak gebruik van geactiveerde DC tegenover het bloed van patiënten die met TAA eiwit zijn beladen en vervolgens teruggegeven worden aan de patiënt. Naast DC zijn ook bepaalde cytokinen nodig om een effectieve antitumor CTL respons op te wekken. Echter, zoals eerder genoemd, systemische behandeling met cytokinen leidt tot ernstige bijwerkingen.

Invariante Natural killer T (iNKT) cellen zijn afweer cellen die kort na activering zelf relatief grote hoeveelheden cytokinen kunnen uitscheiden. Door deze eigenschap spelen zij een belangrijke rol in de regulatie van afweerreacties, o.a. de versterking van de afweerreactie gericht tegen tumoren. iNKT cellen worden, net als CTL, geactiveerd door DC. Zij herkennen echter geen eiwitten maar bepaalde suikervetten (o.a. het uit een zeespons geïsoleerde α-
Galactosylceramide (α-GalCer)) gepresenteerd door het CD1d molecul. Studies in muizen tumormodellen hebben aangetoond dat na toediening van α-GalCer het aantal iNKT cellen toeneemt en het ontstaan van uitzettingen wordt geremd met name door activering van o.a. CTL en NK cellen. Wij hebben eerder aangetoond dat het aantal circulerende iNKT cellen in het perifere bloed verlaagd is in patiënten met kanker. Daarnaast hebben wij ook in een groep van patiënten met hoofd-hals tumoren, die met radiotherapie worden behandeld, aangetoond dat patiënten met een relatief laag aantal circulerende iNKT cellen een slechtere prognose hebben dan patiënten met een relatief hoog aantal circulerende iNKT cellen. Deze data suggereren dat kankerpatiënten baat zouden kunnen hebben bij behandeling die er op gericht is het aantal circulerende iNKT te verhogen. Echter, anders dan bij de muis, is bij de mens is gebleken dat toediening van vrij α-GalCer leidt tot een tijdelijke afname van NKT cellen. Activering van het immuunsysteem werd alleen gezien als het aantal iNKT cellen voor behandeling relatief hoog was. In tegenstelling tot de behandeling met vrij α-GalCer, had de behandeling met DC beladen met α-GalCer wel effect: het aantal circulerende iNKT cellen nam toe. Een alternatieve methode om de aantallen circulerende iNKT cellen te verhogen is door middel van toediening van iNKT cellen van de patiënt, welke in het laboratorium vermeerderd zijn met behulp van bepaalde kweektechnieken.

Het onderzoek beschreven in dit proefschrift is gericht op het analyseren van de effectiviteit van geactiveerde iNKT cellen in de antitumor immuunreactie. Hiertoe hebben wij in deel 1 de effecten onderzocht op NK cel en/of CTL: na iNKT cel activering door middel van een aantal bekende immuun potentie rende reagentia (TLR liganden) en na toevoegen van gekweekte humane iNKT cellen. In dit deel van het onderzoek hebben wij gebruik gemaakt van perifere witte bloed cellen van voornamelijk gezonde donoren. In deel 2 hebben wij in een muizenmodel onderzocht of de toediening van gekweekte iNKT cellen ook daadwerkelijk leidt tot verminderde uitgroei van tumoren.

Uit onze resultaten beschreven in deel 1 is gebleken dat bepaalde TLR liganden in staat waren iNKT cellen indirect te activeren, waarna de actieve iNKT cellen vervolgens in staat waren DC te activeren. Deze DC activering is noodzakelijk voor het opwekken van effectieve CTL. Vervolgens hebben wij aangetoond dat TAA specifieke CTL met veel grotere efficiëntie geïnduceerd kunnen worden door TAA beladen DC wanneer gekweekte iNKT cellen werden toegevoegd. Dit effect was afhankelijk van de aanwezigheid van het iNKT cel activerende ligand α-GalCer op de DC. Een vergelijkbaar effect werd geobserveerd met betrekking tot NK cel activiteit. Gekweekte iNKT cellen induceerden een toegenomen functionele activiteit van NK cellen. Deze bleken na een kortdurende incubatie in de aanwezigheid van iNKT cellen en α-GalCer beter in staat tumorcellen te doden, zowel via de directe methode alsook via monoklonale antistoffen.

In deel 2 beschrijven wij een methode voor het langdurig kwaken van muizen iNKT cellen. Deze chronisch gestimuleerde iNKT cellen bleken dezelfde kenmerken te hebben als hen direct vers geïsoleerde tegenhangers. Vervolgens hebben wij bij 4 verschillende batches van iNKT cellen getest met betrekking tot antitumor effectiviteit in vivo. Onze resultaten tonen aan dat gekweekte iNKT cellen inderdaad in staat zijn tumorgroei te remmen en in het model dat wij hebben bestudeerd deden zij dat met name via NK cel activering.

Concluderend: toediening van gekweekte iNKT cellen bij kanker patiënten is een potentieel effectieve manier om het immuunsysteem te activeren. Combinatie met α-GalCer en TAA beladen DC vaccinatie zal de effectiviteit nog verder verhogen en leiden tot een sterke CTL en NK cel gemedieerde antitumor afweer reactie. De resultaten van dit onderzoek wijzen op nieuwe methodes om de aspecifieke afweer ("innate immunity") in tezetten om de specifieke afweer ("adaptive immunity") tegen tumoren te versterken.
Curriculum Vitae
&
List of Publications
Curriculum Vitae

María Moreno Jauge was born in Montevideo, Uruguay, on February 9th, 1976. She followed her basic education at Colegio Latinoamericano and Liceo San Juan Bautista, in Montevideo. In 1994 she started her studies in Biochemistry at the Facultad de Ciencias, Universidad de la República, in Uruguay. Once obtained her degree in Biochemistry on 2000, she was awarded with two years grant from the University Scientific Research Council (CSIC, Comisión Sectorial de Investigación Científica) from Uruguay.

She moved to Amsterdam on September 2002 to start her PhD project that is described in this thesis at the Department of Obstetrics and Gynaecology in close collaboration with the Department of Pathology, at the VU University Medical Center, under the supervision of Prof. Dr. René Verheijen, Prof. Dr. Rik Scheper, Dr. Silvia von Mensdorff-Pouilly and Dr. Hetty Bontkes.
List of Publications


Molling JW, **Moreno M**, de Groot J, van der Vliet HJ, von Blomberg BME, van den Eertwegh AJM, Scheper RJ, Bontkes HJ. 2008. Chronically stimulated mouse invariant NKT cell lines have a preserved capacity to enhance protection against experimental tumour metastases. *Immunology Letters 118* (1): 36-43


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