Original Article

Human Invariant Natural Killer T cells possess immune-modulating functions during Aspergillus infection

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Abstract

Aspergillus fumigatus is the most common cause for invasive fungal infections, a disease associated with high mortality in immune-compromised patients. CD1d-restricted invariant natural killer T (iNKT) cells compose a small subset of T cells known to impact the immune response toward various infectious pathogens. To investigate the role of human iNKT cells during A. fumigatus infection, we studied their activation as determined by CD69 expression and cytokine production in response to distinct fungal morphotypes in the presence of different CD1d+ antigen presenting cells using flow cytometry and multiplex enzyme-linked immunosorbent assay (ELISA). Among CD1d+ subpopulations, CD1d+CD1c+ mDCs showed the highest potential to activate iNKT cells on a per cell basis. The presence of A. fumigatus decreased this effect of CD1d+CD1c+ mDCs on iNKT cells and led to reduced secretion of TNF-α, G-CSF and RANTES. Production of other Th1 and Th2 cytokines was not affected by the fungus, suggesting an immune-modulating function for human iNKT cells during A. fumigatus infection.

Key words: invariant natural killer T (iNKT) cells, Aspergillus fumigatus, CD1d+ cells, mDCs.

Introduction

Natural killer T (NKT) cells compose a small subset of T lymphocytes that are restricted to recognition of glycolipids presented by the MHC class I-like molecule CD1d and express a T cell receptor (TCR) as well as NK cell markers (CD161/CD56). The human, predominantly studied type I NKT cells, also called invariant natural killer T (iNKT) cells, express an invariant TCR consisting of a Va24-Jα18 α-chain paired with a Vβ11 β-chain.1

Their unique effector functions predispose iNKT cells to be an important factor in linking innate and adaptive immunity and to impact a wide range of diseases including auto-immune diseases,2 allergic diseases,3 cancer,4 and microbial infections.5 Mostly due to their constitutive...
expression of mRNA for immune-regulatory cytokines, iNKT cells are able to release high quantities of cytokines very shortly after antigenic stimulation.6,7 iNKT cells produce a variety of cytokines, among them the Th1 cytokines interferon (IFN)–γ and tumor necrosis factor (TNF)–α as well as Th2-type cytokines like interleukin (IL)-4, IL-5, IL-10, and IL-13, chemokines and growth factors.8,9 This cytokine secretion, as well as direct cytolytic activity of iNKT cells through the expression of perforin, granzyme B, and FasL10 plus direct cell interactions through costimulatory molecules like CD40/CD40L and CD80/86 entail a high potential for iNKT cells to modulate immune responses. The diverse effects of iNKT cells include maturation and increased cytotoxicity of natural killer (NK) cells,11 differentiation and maturation of dendritic cells (DCs), cytokine production by DCs,12–14 promotion of CD4+ and CD8+ immunity14 and B cell proliferation and immunoglobulin production.11,15,16 These characteristics enable iNKT cells to play an important role during infection.

iNKT cell activation results either from direct recognition of microbial lipid antigens presented by the MHC-I-like protein CD1d on antigen presenting cells (APCs), or from recognition of endogenous self-antigens presented by APCs in combination with stimulation by cytokines.17 The last-mentioned pathway enables iNKT cells to amplify toll-like receptor (TLR) derived signals which makes them responsive to a variety of pathogens. While there are numerous studies about the function of iNKT cells in bacterial5 and viral infections,18 knowledge about the iNKT cell response to fungi remains limited.

The function of murine iNKT cells in infection with A. fumigatus has been first described by Cohen et al.19 who showed that CD1d-dependent iNKT cell activation played a role in early pulmonary clearance of A. fumigatus. This iNKT cell activation was postulated to be induced by the recognition of fungal β1,3-Glucans through Dectin-1 on DCs and subsequent IL-12 secretion which then stimulated iNKT cells. Albacker et al.20 described a glycolipid extracted from A. fumigatus that directly activated iNKT cells via a CD1d dependent, Dectin-1 independent mechanism. This glycosphingolipid, asperamide B, was the first fungal antigen shown to be presented by CD1d and to directly stimulate iNKT cells.

However, there is very limited data about the interaction of human iNKT cells with A. fumigatus. Moreover, it is unclear whether all different CD1d expressing APCs interact equally with iNKT cells. Thus, the aim of our study was to analyze the in vitro interaction of human iNKT cells with A. fumigatus. We established a co-culture system to incubate expanded iNKT cells with different types of CD1d+ APCs from human peripheral blood with distinct A. fumigatus morphotypes. For the first time, this study presents data on the influence of three main subpopulations of CD1d+ cells, namely CD14+ monocytes, CD19+ B-cells and CD1c+ myeloid dendritic cells (mDCs), on the interaction of iNKT cells and A. fumigatus.

**Material and methods**

**Cells**

Mononuclear cells from peripheral blood (PBMCs) were obtained from buffy coats of healthy volunteer donors by Ficoll (Biochrom GmbH) density gradient centrifugation. iNKT cells were expanded from PBMCs as previously described.21 CD1d+ cells were isolated from PBMCs, which were frozen in liquid nitrogen during expansion of iNKT cells, by MACS positive selection procedures using anti-CD1d-PE antibodies coupled with PE Microbeads, CD14 Microbeads (100 μl Microbeads / 1 × 10^6 PBMCs), CD19 Microbeads, and CD1c Dendritic Cell Isolation Kit (all Miltenyi Biotec, concentrations according to manufacturer’s protocol).

**Fungal strains**

A. fumigatus conidia and germ tubes were prepared as previously described.22 Briefly, to obtain germ tubes, conidia were cultivated in RPMI at a concentration of 1 × 10^6 / ml by room temperature overnight under continuous shaking at 200 rpm. Temperature was then upregulated to 37°C until an even appearance of germ tubes with an approximate length of 20 μm was achieved. Germ tubes and conidia were inactivated using 100% ethanol.

**In vitro co-cultures**

For co-culture experiments, 1 × 10^6 expanded iNKT cells were cultured with 1 × 10^5 CD1d+ cells in 1 ml medium plus stimuli in 24-well-plates. Different stimuli per well were 10 ng IL-12, 500 IU IL-2, 2 × 10^6 A. fumigatus germ tubes and 2 × 10^6 A. fumigatus conidia (MOI = 2). Cells were harvested after 16 hours at 37°C and analyzed by flow cytometry, supernatants were analyzed by Multiplex ELISA.

**Flow cytometry**

Purity of isolated CD1d+ cells was assessed by flow cytometry analysis. Purity of CD1d+ cells was >99% (CD14 FITC, CD1d PE [BD Pharmingen], CD3 PerCP, CD1c APC [Miltenyi Biotec], CD19 PerCP [Biolegend]). For flow cytometric analysis of co-cultured iNKT cells, cells were stained with CD69 FITC (BD Pharmingen), iNKT PE and CD3 PerCP (both Miltenyi Biotec) following a FcR blocking step.
For evaluation of CD69 expression on iNKT cells, cells were gated on live, iNKT⁺CD3⁺ cells, with an anti-iNKT antibody reacting with the T-cell receptor Vα24-Jα18 combined with Vβ11. Flow cytometry analysis was conducted using FACS Calibur (BD Biosciences), data analysis was performed using FlowJo 7.6.5.

**Multiplex ELISA Assays**

The concentrations of cytokines within supernatants were determined using multiplex technology (Bio-Plex Pro Human Cytokine 27-plex Assay, Bio-Rad). The analyses were performed according to the manufacturer’s instructions.

**Statistical analysis**

Data were analyzed with Graph Pad Prism 5. Wilcoxon matched pairs signed-ranked test was used for analysis of ELISA data (n = 3 individual donors for CD14⁺ and CD19⁺, n = 7 individual donors for CD1c⁺ co-cultures) and paired t-test was used for analysis of FACS data. Significant differences are indicated by * (P < .05), ** (P < .01), and *** (P < .001).

**Ethics statement**

Research with blood samples from healthy volunteer donors was approved by the Ethics Committee of the University of Wuerzburg. Participants provided their written consent to participate in this study, and the Wuerzburg Ethics Committee approved this consent procedure.

**Results**

**No direct activation of iNKT cells by *A. fumigatus***

To explore the influence of *A. fumigatus* on iNKT cells, we first investigated their direct interaction by incubating iNKT cells with different fungal morphotypes. No activation of iNKT cells as determined by the upregulation of the activation marker CD69 on the iNKT cell surface was observed for germ tubes (Fig. 1b) nor for conidia (Fig. 1c) whereas the addition of cytokines (IL-2 and IL-12) resulted in upregulated CD69 expression on iNKT cells. This stimulating effect of cytokines was observed for IL-2 (P < .05) and the combination of IL-2 and IL-12 (P < .001), whereas the cytokine cocktail led to a significantly higher activation than IL-2 alone (P < .05). IL-12 alone had no stimulating effect on iNKT cells (Fig. 1a).

The activating effect of CD1d⁺ cells on iNKT cells is reduced by *A. fumigatus*

After analyzing the direct effect of cytokines and fungus, we investigated the effect of APCs on iNKT cells. The presence of CD1d⁺ cells induced an activation of iNKT cells in the absence of any further stimuli (P < .005). The presence of IL-2 and the cocktail of IL-2 and IL-12 induced further stimulation of iNKT co-cultured with CD1d⁺ cells (Fig. 2a).

In the presence of *A. fumigatus* germ tubes, CD1d⁺ cells stimulated iNKT cells, however to a lesser extent than CD1d⁺ cells in the absence of fungal germ tubes (Fig. 2b). *A. fumigatus* germ tubes decreased the stimulating effect of CD1d⁺ cells on iNKT cells regarding their CD69 expression (Fig. 2b). In contrast, *A. fumigatus* conidia did not
CD1c⁺ subpopulation with highest potential for activating iNKT cells

The three main subpopulations of CD1d⁺ cells are monocytes, B-cells, and myeloid dendritic cells. Peripheral blood dendritic cells are composed of CD11c⁻ plasmacytoid DCs and CD11c⁺ myeloid DCs which are further subdivided in CD1c⁺ (BDCA-1)⁺, CD141⁺ (BDCA-3)⁺ and CD16⁺ myeloid DCs.²³

To differentiate which subgroup of CD1d⁺ cells is accountable for the stimulating effects on iNKT cells, co-cultures were set up with CD14⁺ monocytes, CD19⁺ B-cells and CD1c⁺ mDCs. Although all three subpopulations alone had a stimulating effect on iNKT cells as determined by CD69 expression (Fig. 3a), the effect of monocytes and B-cells was significantly lower than the effect of mDCs (P < .05) (Fig. 3b). In conclusion, the mDC subpopulation shows the highest potential for activating iNKT cells on a per-cell basis.

The same co-cultures were run with A. fumigatus germ tubes and conidia. As in the co-culture system with the entire CD1d⁺-population, A. fumigatus germ tubes reduced the stimulating effect of CD1c⁺ mDCs on iNKT cells (Fig. 4a). The same effect toward iNKT cells was mediated when CD1c⁺ mDCs were co-cultured with A. fumigatus conidia (Fig. 4b). Neither germ tubes nor conidia in combination with monocytes or B-cells induced a similar effect (data not shown).

CD1c⁺ mDCs increase cytokine production in the absence of further stimuli

Multiplex ELISA analysis was performed with supernatants from iNKT cell co-cultures with APCs and A. fumigatus germ tubes. The co-incubation of CD14⁺ monocytes or CD19⁺ B-cells with iNKT cells without further stimuli did not cause production of any of the tested cytokines (data not shown). Differently, the interaction of CD1c⁺ mDCs with iNKT cells resulted in increased production of the Th1 cytokines IFN-γ, TNF-α, and IL-2, the Th2 cytokines IL-4, IL-5, IL-6, IL-10, and IL-13, IL-1β, IL-1ra, the growth...
mDC subpopulation

A. fumigatus

The higher stimulating effect of CD1c

cells on iNKT cells. CD69 expression on iNKT cells is

mean fluorescence intensity (geo MFI) from independent experiments

mDC / iNKT cell co-culture with CD1c

Reduction of TNF-α and G-CSF production in CD1c

mDC / iNKT cell co-culture with A. fumigatus

The presence of A. fumigatus did not affect the secretion of IFN-γ, IL-1β, IL-1ra, IL-4, IL-5, IL-6, IL-8, IL-13, and VEGF in iNKT cell co-culture with CD1c

mDCs. However, the comparison of CD1c

mDCs with and without A. fumigatus germ tubes showed a reduced production of TNF-α, G-CSF, and RANTES when fungal germ tubes were present (Fig. 5).

Surface expression of CD1d is highest on CD1c

mDC

The upregulation of CD69 expression on iNKT cells correlates positively with the CD1d expression on co-incubated APCs (R square = 0.935, slope = 0.1026 ± 0.01104, P < .0001; n = 8 [Fig. 6]). Differences in CD1d expression on the surface of different subpopulations of CD1d

cells may explain the dominating effect of CD1c

mDCs on iNKT cells. On CD1c

mDCs, surface expression of CD1d was significantly higher than on CD14

cells (P < .01; n = 6) and CD19

cells (P < .001). This entails a higher potential for interaction with iNKT cells which may be one reason for the greater impact of CD1c

mDCs on iNKT cells when compared to CD1d

cells and B-cells (Fig. 7).

Aspergillus species are a ubiquitous genus of mold with the clinically most important species A. fumigatus, the most frequent cause for invasive aspergillosis. Taking the high mortality rate of immune-suppressed patients with invasive aspergillosis, further characterization of distinct mechanisms of the innate and adaptive immune responses is essential to develop new prevention strategies and treatment options.

Interaction with CD1d

cells is essential for the activation of iNKT cells. Our findings show that while the three main subpopulations of CD1d

cells all induce a stimulation of iNKT cells, it is the CD1c

mDC subpopulation which shows the highest potential for activating iNKT cells on a per-cell basis. These results from flow cytometric analysis were confirmed by analysis of cytokine concentrations in co-culture supernatants where B-cells and monocytes did not cause an increase in cytokine production in iNKT cell co-culture. In contrast, co-culture with CD1c

mDCs resulted in augmented secretion of Th1 cytokines like IFN-γ and TNF-α as well as various Th2 cytokines, growth factors and chemokines. Although Th1 as well as Th2 cytokines were produced, the considerably higher concentrations of IFN-γ and TNF-α imply a predominance of Th1 cytokines in the given context. However, considering the experimental setting and the bidirectional interaction between iNKT and CD1d

cells, it remains unclear whether cytokines were secreted by iNKT cells or CD1d

cells.

In previous studies, certain glycolipids that bind to CD1d have been shown to also be presented by CD1c with the capability to directly interact with NKT cells. Furthermore, co-expression of CD1c on CD1d

APCs has been demonstrated to enhance the stimulation of iNKT cells by α-GalCer. The higher stimulating effect of CD1c

mDCs in this study might result from a direct interaction or co-stimulating effect of CD1c molecules and iNKT cells. Furthermore, a significant positive correlation of the CD69 upregulation on iNKT cells and the CD1d expression on co-incubated APCs was observed. This suggests that the potential of APCs to activate iNKT cells increases with the level of CD1d expression. This study showed highest CD1d surface expression on CD1c

mDCs compared to other CD1d

APCs, resulting in the highest interaction potential with iNKT cells on a per-cell basis. A role of cytokine secretion, further co-stimulatory molecules, or other factors cannot be excluded at this point.

Previous studies have investigated the role of iNKT cells during A. fumigatus infection in mice. Cohen et al. suggested a defensive role of iNKT cells during pulmonary infection with A. fumigatus conidia. Analysis of
A. fumigatus reduces secretion of G-CSF, TNF-α and RANTES in iNKT cell/CD1c⁺ mDC co-culture. Graph shows concentrations of (a) G-CSF, (b) TNF-α, and (c) RANTES in culture supernatants. iNKT cells were co-cultured with CD1c⁺ mDCs with and without A. fumigatus germ tubes. Supernatants were collected after 16 h of co-culture and cytokine concentrations were determined by ELISA. Mean and SEM of five independent donors are shown. Significant differences are indicated by * (P < .05), ** (P < 0.01) and NS (not significant).

Bronchoalveolar lavage fluid of infected mice showed augmented iNKT cell numbers with increased CD69 surface expression and IFN-γ production. Albacker et al. described IL-4 and IFN-γ secretion by murine iNKT cells in response to the fungal lipid asperamide B. However, for human iNKT cell lines, induction of IL-4 and IL-13 but not IFN-γ was shown.

As to the role of iNKT cells in infection with A. fumigatus in humans, our data suggest that the fungus has an impact on iNKT cells, which is mediated by CD1c⁺ mDCs. This effect of fungal germ tubes and conidia on iNKT cells...
was inhibitory, relating to a decrease in CD69 surface expression on iNKT cells and a reduction of TNF-α, G-CSF, and RANTES concentrations in co-culture supernatants. At the same time, secretion of IFN-γ and Th2 cytokines did not change in iNKT cell co-cultures with CD1c+ mDCs and A. fumigatus germ tubes. Since all experiments were performed with ethanol-inactivated fungal morphologies, this APC dependent suppressive effect of A. fumigatus on iNKT cells was due to direct contact rather than soluble A. fumigatus moiety.

CD69 has long been known as an early leucocyte activation marker. Recently, lack of CD69 in knockout mice has been described to enhance Th17 pro-inflammatory responses. TNF-α, along with IFN-γ, has been ascribed a protective role in A. fumigatus infection and neutralization of TNF-α by specific antibodies in a mouse model has been shown to reduce neutrophil numbers in the lung while mortality increased. In parallel, G-CSF is an inducer of granulopoiesis and enhances the oxidative burst of neutrophils, thus increasing the damaging effect of neutrophils against A. fumigatus hyphae. The decreased CD69 expression and the inhibited secretion of TNF-α and G-CSF by iNKT cells in the presence of A. fumigatus germ tubes might eventually reflect an immunoregulatory function of iNKT cells during infection with A. fumigatus. This confirms the multiply described role of iNKT cells as a fine-tuner of immune responses.

These findings differ from the results of studies in mice. Murine immune cells differ in their number and function from human immune cell populations, especially neutrophils which compose 50–70% of peripheral blood cells in humans compared to only 10–25% in mice. Neutrophils play an essential role in the first line defense against A. fumigatus and neutropenia is one of the most important risk factors for invasive aspergillosis. Furthermore, humans express five different CD1 genes corresponding to five different CD1 proteins, CD1a, CD1b, CD1c, CD1d, and CD1e, whereas mice express only one CD1 protein which is an ortholog to CD1d. The results of this study as well as previous studies with human cells have predicted an influential role for all CD1 proteins, especially CD1c, in iNKT cell function. Taking these aspects, differences in human and murine immune response to fungi appear quite likely.

Analysis of cytokine concentrations in culture supernatants did not detect considerable amounts of IL-12. However, it is well known that cytokines like IL-12 and IL-18 play a major role in iNKT cell activation. Freshly isolated CD1c+ mDCs, as used in our experiments, have previously been ascribed a minimal to low capacity of IL-12 production, as well as poor production of IL-18. This low ability of immature mDCs to produce IL-12 leaves room for speculations about a different response of iNKT cells to A. fumigatus if more potent IL-12 producers were used as APCs.

It remains to be investigated if the effect of CD1d+ cells on iNKT cells is mediated by CD1d molecule or by co-stimulatory molecules or cytokines. Further experiments with mature CD1c+ mDCs will be essential to fully understand the potential of human mDCs to interact with iNKT cells during A. fumigatus infection. Moreover, the investigation of CD1c- DC subsets in this context will be an interesting topic for further research.

In summary, our experiments do not demonstrate a direct interaction between A. fumigatus and human iNKT cells. We provide first insights into the impact peripheral blood CD1d+ cells have on iNKT cells in the presence of A. fumigatus conidia and germ tubes by showing that in this infectious context, the stimulating effect of peripheral blood mDCs on iNKT cells is decreased, whereas iNKT cell response to monocytes and B-cells is not affected by A. fumigatus.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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