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

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## **2 Introduction**

### **2.1 The human immune system**

Immunity is the ability of an organism to recognize and eliminate alien bodies like invading microorganisms and endogenous altered or foreign tissues. In the human immune system an innate, unspecific immunity is distinguished from an adaptive, pathogen specific immunity [1].

The innate immune system is responsible for the fast response towards invading pathogens. Certain groups of pathogens express conserved antigens, so called Pathogen Associated Molecular Patterns (PAMPs) which are recognized by cells of innate immunity through Pattern Recognition Receptors (PRRs). Detection of these PAMPs by Toll like receptors (TLRs) or other PRRs results in protective immune responses [2]. Major effector cells are NK cells, neutrophils, macrophages and Dendritic Cells (DC). NK cells possess cytolytic activities [3], neutrophils kill pathogens after phagocytosis and through extracellular release of antimicrobial and cytotoxic substances [4]. Other phagocytosing cells are macrophages which develop from monocytes, and dendritic Cells (DCs). After phagocytosis and intracellular killing, these cells present foreign antigens on their surface via MHC molecules, thus activating cells from the adaptive immune system [1, 5]. The humoral part of innate immunity is the complement system that has lytic properties and opsonizes pathogens to facilitate phagocytosis [1].

The adaptive immune system is responsible for specific secondary immune responses. T lymphocytes and B lymphocytes have highly specific antigen receptors and antigen recognition is followed by selective proliferation of cell clones. CD8<sup>+</sup> cytotoxic T cells are able to induce apoptosis of virus infected cells and tumor cells. CD4<sup>+</sup> T helper (Th) cells recognize antigens presented by APCs resulting in secretion of pro-inflammatory Th1 cytokines like interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) or into a Th2 response, including Interleukin (IL)-4, IL-10, and IL-13. Interaction of T helper cells and antigen presenting B cells via cytokines and cell-cell interactions result in selective expansion of a B cell clone. After maturation into plasma cells, they secrete high numbers of antibodies against their specific antigen. The formation of memory cells

allows for a more rapid, specific immune response in case of repeated contact with the same pathogen [1].

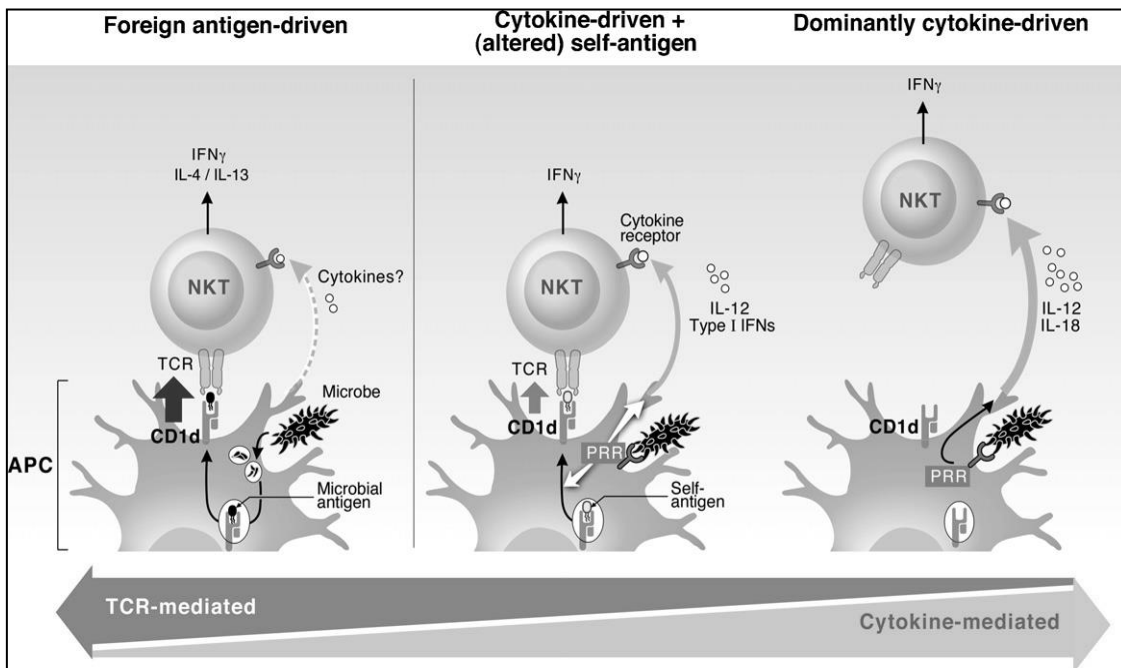
The coordinated transition from innate to adaptive immune mechanisms is essential for an effective defense against invading pathogens.

### **2.1.1 The role of iNKT cells in immunity**

Natural Killer T (NKT) cells compose a low-abundance subset of T lymphocytes that are restricted to the recognition of glycolipids presented by the MHC class I-like molecule CD1d. NKT cells express both, a T cell receptor (TCR) and 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 V $\alpha$ 24-J $\alpha$ 18  $\alpha$ -chain paired with a V $\beta$ 11  $\beta$ -chain [6]. The prototypic antigen for iNKT cells is  $\alpha$ -Galactosylceramide ( $\alpha$ -GalCer), a glycosphingolipid which was originally isolated from the marine sponge *Agelas mauritanus* [7].

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 autoimmune diseases [8], allergic diseases [9], cancer [10], and microbial infections [11]. Mostly due to their constitutive expression of mRNA for immunoregulatory cytokines, iNKT cells are able to release large amounts of cytokines very shortly after antigenic stimulation [12, 13]. iNKT cells produce a variety of cytokines, amongst them the Th1 cytokines IFN- $\gamma$  and TNF- $\alpha$  as well as Th2-type cytokines like IL-4, IL-5, IL-10, and IL-13, chemokines and growth factors [14-16]. This cytokine secretion, as well as direct cytolytic activity of iNKT cells through the secretion of perforin, granzyme B, and FasLigand (FasL) [14, 16, 17] plus direct cell to 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 NK cells [18], differentiation and maturation of DCs, cytokine production by DCs [19-21], promotion of CD4<sup>+</sup> and CD8<sup>+</sup> T cell immunity [21], B cell proliferation, and immunoglobulin production [18, 22, 23]. These characteristics enable iNKT cells to play an important role during infection.

There are different pathways leading to iNKT cell activation [24]. iNKT cells recognize certain microbial lipid antigens presented by CD1d, resulting in iNKT cell activation. Various pathogens expressing a lipid antigen which binds to CD1d have been identified, including *Borrelia burgdorferi* and *Streptococcus pneumonia* [25, 26]. After phagocytosis, microbes are processed, microbial lipid antigens are presented by CD1d and their recognition results in TCR-mediated iNKT cell activation. However, iNKT cell stimulation is not reserved to microbes expressing CD1d binding antigens. In response to foreign antigens, activated APCs present endogenous lipid antigens via CD1d and secrete cytokines. The recognition of these endogenous self-antigens and stimulation with cytokines like IL-12 and IL-18 can lead to a CD1d- and cytokine- dependent iNKT cell activation. This pathway enables iNKT cells to amplify toll-like receptor (TLR) derived signals which makes them responsive to a variety of infectious pathogens. Furthermore, iNKT cells can be stimulated by high amounts of cytokines like IL-12 and IL-18 alone, without the need for CD1d interaction [24] (Figure 1).



**Figure 1: Pathways leading to activation of iNKT cells**

[M. Brigl, M. B. Brenner, Seminars in Immunology 22, 2010, p. 81] [24]

Graph shows different pathways leading to iNKT cell activation. TCR-mediated direct activation of iNKT cells by foreign antigens, cytokine driven activation of iNKT cells dependent on



TCR / CD1d interaction, and an activation pathway dominantly driven by cytokines like IL-12 and IL-18 are displayed.

While there are numerous studies about the function of iNKT cells in bacterial [11] and viral infections [27], knowledge about the iNKT cell response to fungi remains limited.

## **2.2 *Aspergillus fumigatus***

*Aspergillus* species are a ubiquitous genus of filamentous mold whose biological niche is the soil [28]. As saprophytic organisms they cause biodegradation of dead and decayed organic material and thus recycle nutrients for their own supply [28]. For optimal growth, favorable conditions are the presence of water and oxygen, neutral or mildly acidic conditions and a temperature around 37 to 42°C [28, 29].

*Aspergillus spp.* reproduce by producing spores, so-called conidia. Under favorable conditions conidia germinate and become hyphae which branch out during growth, forming a mycelium. On the mycelium's surface, conidiophores produce new conidia which are dispersed through the air. The fungal cell wall functions as an initial barrier against harmful environmental influences and releases hydrolytic and toxic molecules into the direct environment. 90% of the cell wall consists of polysaccharides with an alkali soluble and an alkali insoluble fraction [30]. Cross-linked  $\beta$ 1,3-glucan and chitin form the central core of the cell wall and are covalently bound to other polysaccharides like galactomannan and  $\beta$ 1,3-1,4-glucan [31]. Only few cell wall proteins are covalently bound to the cell wall while most proteins are in transit before being released into the extracellular environment. The outer layer of the cell wall is subject to constant changes depending on morphology, growth stage and nutritional changes. The cell wall of conidia is covered with hydrophobic proteins and melanin which makes them immunologically inert and resistant to environmental damages [32].

The three main PRRs that recognize fungal PAMPs are Dectin-1 and the toll like receptors TLR-2 and TLR-4. Binding of  $\beta$ 1,3-glucan, the specific PAMP of Dectin-1, results in a strong inflammatory response. It is traceable on swollen conidia and germ tubes, but barely detectable on resting conidia and hyphae [29]. TLR-2 and TLR-4 are involved in the recognition of chitin and mannans which are also recognized by the membrane-bound receptors DC-SIGN and mannose receptor [32].

*A. fumigatus* is the clinically most important *Aspergillus spp.* It is responsible for the majority of invasive aspergillosis [33] whereas most of the more than 200 different *Aspergillus spp.* are not pathogenic for humans. Several features of *A. fumigatus* may explain its virulence. To begin with, *A. fumigatus* is thermotolerant growing at temperatures up to 55°C with a temperature optimum ranging from 37°C to 42°C [29] which contrasts the optimal temperature around 25°C of other *Aspergillus* species. In addition, *A. fumigatus* has various characteristics that help evade the human immune system. Resting conidia have an immunologically inert layer of hydrophobic proteins which makes them undetectable for human immune cells [34]. Melanin which is present on the conidial surface provides a protective barrier against UV radiation and decreases susceptibility to reactive oxygen species (ROS) [35]. Glutathione synthesis and oxidoreductase activity increase fungal resistance to oxidative stress [36]. Furthermore, *A. fumigatus* produces gliotoxin which blocks phagocytosis [37], inhibits transcription of inflammatory mediators [38], and induces apoptosis of neutrophils and monocytes [39]. The secretion of catabolic enzymes like proteases, phospholipases, and elastases serves nutrient acquisition and tissue invasion [40].

### **2.2.1 Host defense against *A. fumigatus***

In the immunocompetent host, sufficient innate immune mechanisms exist to eliminate inhaled fungal spores from the lung and thus prevent fungal infection. By virtue of their small size of 2 to 3 µm, inhaled conidia can bypass mucociliary clearance and reach the alveoli [28].

On their way through the bronchial tree, conidia are exposed to soluble collectins like surfactant proteins, mannose-binding lectin (MBL), and complement which all opsonize conidia and thus enhance their phagocytosis.

Alveolar macrophages (AM), the primary resident phagocytes of the lung, recognize conidia via TLR-2, TLR-4 and Dectin-1, resulting in their phagocytosis. After phagocytosis, swollen conidia are killed by reactive oxygen species and acidification of the phagolysosome [40, 41]. Additionally, AM release cytokines and chemokines which attract further immune cells to the lung [29]. Upon phagocytosis, the presence of DHN-melanin on the surface of conidia may inhibit acidification of the phagolysosome. Thus,

conidia avoid killing, start germinating and escape the phagocyte [42]. Corticosteroid therapy impairs the ability of phagocytes to kill *A. fumigatus*.

Neutrophils are essential to inhibit invasive growth of *A. fumigatus*. Mainly attracted by IL-8, neutrophils assemble in the lung and play a key role in the defense against *Aspergillus* hyphae [43]. Recognition of *A. fumigatus* conidia, germlings, and hyphae depends on TLR-2, TLR-4 as well as Dectin-1 and results in distinct defense mechanisms: phagocytosis, degranulation, and formation of neutrophil extracellular traps (NET). While phagocytosis is limited to the morphotype of fungal spores, degranulation and NETosis is effective against fungal hyphae [29]. Neutrophil granules contain numerous molecules with antimicrobial activity like proteases, defensins, pentraxin 3, lysozyme and lactoferrin which can kill fungal hyphae [40]. NETs, which form when neutrophils burst and release their DNA covered with granular proteins, play a role in inhibiting germination and hyphal growth [44]. Underlining the importance of neutrophils in the defense of *A. fumigatus*, neutropenia is the major risk factor for invasive aspergillosis in patients with hematologic malignancies [45]. The aggressive response of neutrophils against *A. fumigatus* is primarily responsible for inflammatory tissue damages in the lung during fungal infection [40].

NK cells which are recruited to the lung [46] contribute to innate antifungal immunity in the lung by secretion of IFN- $\gamma$  which increases the antimicrobial effect of macrophages [47] and displays direct antifungal activity against *A. fumigatus* [48].

These innate immune mechanisms are linked with adaptive immunity via DCs. Multiple PRRs play a role in sensing *A. fumigatus* by DCs, namely TLR 2, 3, 4, 9, Dectin-1, and DC-SIGN. In response, DCs secrete cytokines and chemokines, phagocytose conidia and travel to lymph nodes where they present fungal antigens to T-cells. Depending on fungal morphotypes and the type of PRR which is engaged, DC activation can induce a Th1, Th2 or Th17 response [5]. The balanced Th1 response is associated with fungal clearance [43], whereas Th2 response is rather unfavorable. IL-23 secretion by DCs and stimulation of Th17 cells result in excessive inflammation and tissue destruction [49]. The Th2 cytokines IL-4 and IL-10 have inhibitory properties towards immune responses

and high IL-10 serum levels in patients with invasive aspergillosis correlate with fatal outcome [50].

Once *A. fumigatus* has germinated, the robust architecture of the fungal cell wall and the polarized driving force of hyphal growth enables the penetration of epithelial as well as endothelial barriers [29]. Angioinvasion often results in thrombosis and infarction leading to reduced oxygen supply and tissue necrosis. Dissemination occurs when hyphal fragments break off and breach the endothelial barrier into other organs [40].

In conclusion, a fine coordination of innate and adaptive immune responses and a balanced regulation of protective and destructive inflammation is essential for a favorable outcome of *A. fumigatus* infection.

### **2.2.2 Diseases caused by *A. fumigatus***

Inhalation of *A. fumigatus* spores can result in different disease manifestations.

Predisposed patients can develop IgE-mediated allergic asthma or rhinosinusitis in response to *A. fumigatus* which involves neither infection nor colonization. Colonization of the airways by *A. fumigatus* followed by IgE-mediated inflammatory responses is the cause for allergic bronchopulmonary aspergillosis (ABPA). Damage of the airway structure through severe asthma, bronchiectasis or cystic fibrosis in atopic immune-competent individuals predispose for this disease. ABPA symptoms comprise reversible bronchospasms and recurrent lung infiltrates [28, 51].

Aspergilloma, which are masses of *Aspergillus* elements and cellular debris, can develop in preexisting lung cavities like tuberculosis caverns, neoplasias, sarcoidosis, and other cave-forming diseases. Most often, aspergillomas are latent and discovered by hazard in chest x-rays. However, sometimes hemoptysis can reveal the disease and constitutes a possibly fatal factor of severity. Alternatively, these fungal balls can form in the sinus resulting in chronic rhinosinusitis [51, 52].

The most severe disease manifestation of *A. fumigatus* infection is invasive aspergillosis (IA) which is a disease occurring almost exclusively in patients with compromised immunity. The most important predisposing factors are prolonged or severe neutropenia, graft versus host disease (GvHD,) and prolonged therapy with corticosteroids, cytotoxic therapy, haematological malignancies, and chronic

granulomatous diseases [51-53]. Depending on the type of immunological impairment, pathological consequences of IA differ. In patients with neutropenia, a side effect of highly cytotoxic therapies, IA is angioinvasive and results in thrombosis and infarction. This is due to the extensive hyphal growth and uncontrolled fungal dissemination via the blood stream which in healthy individuals is limited by neutrophils [40]. On the other hand, patients receiving corticosteroids for prophylaxis and treatment of GvHD develop a type of IA which comes along with excessive inflammation and severe damages of the lung tissue. Corticosteroids impair the function of phagocytes, whereas neutrophils are still recruited to the lung and control hyphal growth. However, the uncontrolled response of neutrophils results in excessive inflammation, pyogranulomatous infiltrates and tissue damages which are eventually considered the cause of death in these patients [40]. Symptoms of invasive pulmonary aspergillosis are similar to those of bronchopneumonia: fever which is unresponsive to antibiotics, dyspnea, cough, sputum production and chest pain [52]. After haematogenic dissemination, parenchymal fungal abscesses can form in nearly every organ of the human body, whereupon invasion of the central nervous system is most severe and often fatal [29].

### **2.2.3 Treatment of invasive aspergillosis**

The two main antifungal agents used today are triazoles and the polyene Amphotericin B.

Triazoles inhibit the lanosterol-14 $\alpha$ -demethylase, an enzyme promoting the conversion of lanosterol into ergosterol. Ergosterol is the main sterol in fungal cell membranes which does not exist in the membrane of human cells. In consequence, accumulation of ergosterol precursors and the lack of ergosterol results in leakage of the cell membrane [54].

The polyene Amphotericin B binds membrane ergosterols through hydrogen bonds and van der Waals forces, bringing forth a formation of pores in the fungal cell membrane and increased permeability for ions. Even if with much lower avidity, Amphotericin B also binds cholesterol, the main sterol in human cell membranes, which explains its toxic side effects and often compels to end therapy or switch the substance

class. Liposomal and lipid-based formulations of Amphotericin B are less toxic while having an equal fungicidal effect [54].

Echinocandins disturb cell wall synthesis by blocking  $\beta$ 1,3-glucan synthesis through inhibition of the enzyme 1,3- $\beta$ -Glucansynthase resulting in a fungistatic effect against *Aspergillus spp.* [55].

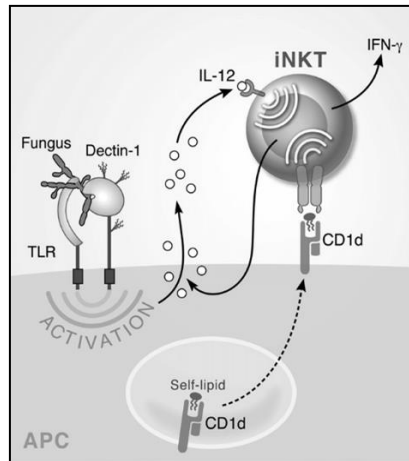
The triazole Voriconazole is the gold standard in treatment of IA. It is distributed well in all body tissues including the brain and its principal side effects are reversible visual disturbances in 40% of patients. Cytochrome P450 metabolism comes with high potential for interactions with other substances. Liposomal Amphotericin B is recommended as primary therapy with less strength or as second-line treatment. The echinocandine Caspofungin is recommended as salvage therapy [56, 57].

Combinations of more than one antifungal substance have not been adequately studied and should only be used in the setting of clinical trials and exceptionally in severely ill patients and refractory disease. Crucial for improved survival is the early administration of antifungal therapy at first signs of infection [57].

### **2.3 Murine iNKT cell response to *A. fumigatus***

Knowledge about iNKT cell response to *A. fumigatus* is so far limited to two studies.

The function of murine iNKT cells during infection with *A. fumigatus* has been first described by Cohen *et al.* [58] who demonstrated iNKT cell activation and accumulation in the airways of mice intratracheally infected with conidia. The stimulation was described to be Th1 polarized with CD69 upregulation and IFN- $\gamma$  production. The iNKT cell activation by *A. fumigatus* hyphae was suggested to be CD1d-dependent and to result from fungal  $\beta$ 1,3-Glucan recognition through Dectin-1 on DCs and subsequent IL-12 secretion (Figure 2).

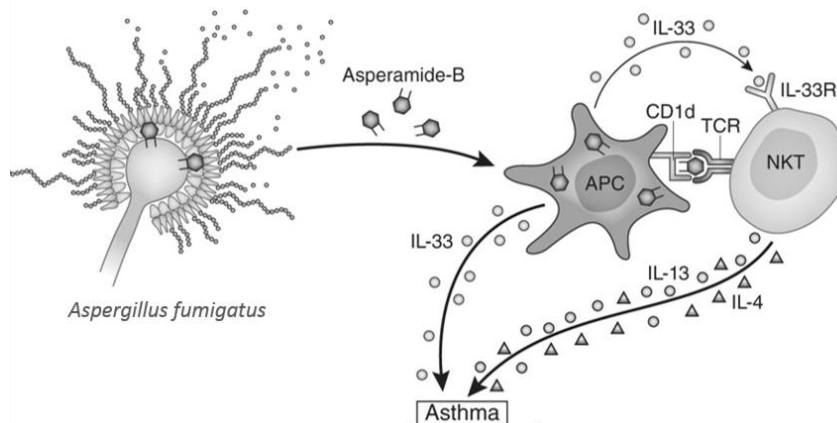


**Figure 2: Indirect iNKT cells activation during *A. fumigatus* infection in mice**

[M. Brigl, M. B. Brenner, Cell Host & Microbe 10, 2011, p. 446] [58]

Graph shows mechanism of iNKT cell activation via IL-12 and self-lipids presented by CD1d in response to *A. fumigatus* in mice.

Albacker *et al.* [59] described a fungal glycolipid extracted from *A. fumigatus*, that could directly activate 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 that could directly stimulate iNKT cells. *In vivo* experiments in mice suggested CD1d, IL-4 and IL-13 dependent induction of airway hyperreactivity by *A. fumigatus* extracts. *In vitro*, Asperamide B was demonstrated to induce production of IL-4 and IFN- $\gamma$  by primary murine iNKT cells. However, for human iNKT cell lines, induction of IL-4 and IL-13 but not IFN- $\gamma$  production was shown *in vitro* [59] (Figure 3).



**Figure 3: Direct activation of iNKT cells by Asperamide B**

[Godfrey D., Pellicci D., Rossjohn J., Nature Medicine, 10/2013, p. 1211] [60]

Graph shows overview of direct iNKT cell activation by *A. fumigatus*. The fungal glycolipid asperamide B is presented by APCs through CD1d and causes iNKT cells to secrete IL-13 and IL-4 as suggested by Albacker *et al.*

## **2.4 Study objective**

Invasive aspergillosis (IA) is the most frequent invasive fungal infection in adult hematopoietic stem cell recipients [61] and mortality of IA is still high at around 30 to 60 % despite adequate antifungal treatment [57]. Availability of antifungal substances has improved in the past years but the need for alternatives is still urgent.

The potential to use the antitumor activity of iNKT cells in cancer therapy is already being investigated in clinical studies. In patients with solid tumors, different approaches of immunotherapy targeting iNKT cells have been tested in phase I studies, comprising direct injection of  $\alpha$ -GalCer, reinfusion of autologous DCs loaded ex vivo with  $\alpha$ -GalCer, and reinfusion of autologous ex vivo expanded iNKT cells. While these therapies were well tolerated, clinical results were rather disappointing [62]. However, experiments in mice, where tumor cells were loaded with  $\alpha$ -GalCer ex vivo before reinfusion, showed promising results, including resistance to the implantation of several tumors [63]. Although much broader knowledge is needed to understand the fine regulation of iNKT cells in order to establish iNKT cell-based immunotherapies, it is a promising approach in the search for alternatives in cancer therapy.

Likewise, iNKT cell targeting for immunotherapy in infectious diseases appears to have great potential. In infectious diseases with such high mortality rates as IA, the search for alternatives to available antimicrobials is of great importance. Vaccination strategies with DCs have already been studied as one type of cell-based therapy in IA [64]. Understanding the role of iNKT cells during *A. fumigatus* infection is fundamental to assess, if the approach of iNKT cell-based immunotherapy is applicable to treatment of IA.

As of today, the very limited knowledge about the role of iNKT cells in host defense against *A. fumigatus* was mainly derived from experiments in mice. The aim of this study was to analyze the response of human iNKT cells towards *A. fumigatus*. A cell culture system was established to expand and incubate iNKT cells and APCs from human peripheral blood with different *A. fumigatus* morphotypes. In order to determine the



response of iNKT cells, the expression of the activation marker CD69 on iNKT cells and the secretion of cytokines was analyzed. This study focused on the influence of three main subpopulations of CD1d<sup>+</sup> cells, namely CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B-cells and CD1c<sup>+</sup> myeloid dendritic cells (mDCs), on the interaction between iNKT cells and *A. fumigatus*.

### 3 Material and Methods

#### 3.1 Material

##### 3.1.1 Laboratory devices and tools

Tools	Name	Company
Incubator	BBD 6220	Heraeus
Flow Cytometer	FACS Calibur	BD Biosciences
Fridge 4°C	Cup 3021	Liebherr
Freezer -80°C	Hera freeze	Heraeus
Liquid nitrogen freezing storage	KSeries KryoStorage System	Taylor Wharton
Magnets for cell isolation	Quadro MACS Mini MACS	Miltenyi Biotec GmbH
Microscopes	Nikon Eclipse TS100 Nikon Eclipse 50i	Nikon
Pipettes	Research Plus	Eppendorf
Shaker	HAT Infors Minitron	Infors AG
Clean bench	MSC Advantage	Thermo Scientific
Vortex shaker	Vortex Genie 2	Scientific Industries
Water bath		Memmert
Centrifuge	Multifuge 3S Multifuge 3S-R	Heraeus
Freezing Container	Mr. Frosty™	ThermoScientific

##### 3.1.2 Consumable supplies

Material	Characteristics	Company
Flow cytometry tubes	5 ml, 12 x 75 mm	BD Biosciences
Falcons	Cellstar Tubes, 50 ml and 15 ml	Greiner bio-one

<b>Material</b>	<b>Characteristics</b>	<b>Company</b>
MACS columns	MS columns LS columns	Miltenyi Biotec GmbH
Canulae	Microlance 0.9 x 40 mm	BD Biosciences
Pasteur pipettes	Packed individually and sterile, 3 ml	BioSigma
Disposable pipette tips	10 µl, 100 µl, 1000 µl	Starlab Group
Cell strainer	40 µm nylon mesh	BD Biosciences
CryoTube Vials	1.0 ml	Thermo Scientific

### 3.1.3 Reagents

<b>Reagent</b>	<b>Characteristics</b>	<b>Company</b>
Ficoll Separating Solution	Density 1,977g/ml	Biochrom GmbH
HANKS's Balanced Salt Solution	With sodium bicarbonate, liquid, sterile-filtered	SIGMA-Aldrich
RPMI Medium	RPMI 1640 + Glutamax + Hepes	Gibco
Fetal Calve Serum	Heat-inactivated at 60°C	SIGMA-Aldrich
Dimethylsulfoxid	C <sub>2</sub> H <sub>6</sub> SO	Carl Roth GmbH
Refobacin	Gentamicin sulfat	Merck
EDTA	0.5 M in H <sub>2</sub> O	SIGMA-Aldrich

### 3.1.4 Microbeads for cell isolation

<b>Microbeads</b>	<b>Company</b>
Anti-PE-Microbeads	Miltenyi Biotec GmbH
CD14 Microbeads	Miltenyi Biotec GmbH
CD19 Microbeads	Miltenyi Biotec GmbH

Microbeads	Company
CD1c <sup>+</sup> (BDCA1)-Isolation Kit	Miltenyi Biotec GmbH
Anti-iNKT Microbeads	Miltenyi Biotec GmbH

### 3.1.5 Cytokines and other stimuli

Stimulus	Characteristics	Company
$\alpha$ -Galactosylceramide	KRN7000 Analogon C5099NO9	Enzo
Interleukin-2	Human IL-2(v126) research grade	Miltenyi Biotec GmbH
Interleukin-12	Human IL-12 Premium Grade	Miltenyi Biotec GmbH

### 3.1.6 Flow cytometry antibodies

Antibodies	Conju- gation	Clone	Isotype	Volume per tube	Producer
CD1c (BDCA-1)	APC	AD5- 8E7	Mouse IgG2a	5 $\mu$ l	Miltenyi Biotec GmbH
CD1d	PE	CD1d42	Mouse IgG1	5 $\mu$ l	BD Biosciences
CD3	PerCP	BW264 /56	Mouse IgG2a	4.5 $\mu$ l	Miltenyi Biotec GmbH
CD14	FITC	MSE2	Mouse IgG2a	2 $\mu$ l	BD Biosciences
CD19	PerCP	HIB19	Mouse IgG1	4.5 $\mu$ l	BioLegend
CD69	APC	FN50	Mouse IgG1	2.5 $\mu$ l	Miltenyi Biotec GmbH
CD69	FITC	FN50	Mouse IgG1	2 $\mu$ l	BD Biosciences
CD137	APC	4B4-1	Mouse IgG1	5 $\mu$ l	Miltenyi Biotec GmbH
iNKT	PE	6B11	Mouse IgG1	10 $\mu$ l	Miltenyi Biotec GmbH
iNKT	PE	6B11	Mouse IgG1	20 $\mu$ l	BD Biosciences

### 3.1.7 Flow cytometry antibodies for isotype

Antibody	Conjugation	Clone	Company
Mouse IgG1	APC	MOPC-21	BD Biosciences
Mouse IgG1	FITC	MOPC-21	BD Biosciences
Mouse IgG1	PE	MOPC-21	BD Biosciences
Mouse IgG1	PerCP	MOPC-21	BD Biosciences
Mouse IgG2a	PE	X39	BD Biosciences
Mouse IgG2a	PerCP	S43.10	Miltenyi Biotec GmbH
Mouse IgG2a	FITC	X39	BD Biosciences

## 3.2 Methods

### 3.2.1 Isolation of mononuclear cells from human peripheral blood

Concentrated peripheral blood mononuclear cells (PBMCs) from healthy volunteer donors were acquired from the department of transfusion medicine of the University Hospital Würzburg. The cell suspension from one donor was diluted with buffer (HBSS with 1 % fetal calve serum (FCS) and 0.4 % EDTA) in a 50 ml falcon. 25 ml diluted cell suspension was then carefully layered onto the same amount of Ficoll in a new 50 ml falcon. Two falcons per donor, with each 25 ml Ficoll and 25 ml diluted cell suspension, were then centrifuged at 2000 revolutions per minute (rpm) for 20 min without brake. Subsequently, the layer of mononuclear cells which had formed at the interphase between Ficoll and plasma was carefully aspirated with a Pasteur pipette and transferred into two new falcons. These falcons were then filled with buffer and centrifuged 15 min at 120 g without break, leading to the formation of a cell pellet at the bottom of the falcon. Following centrifugation, the supernatants were carefully aspirated with a pipette, and the cell pellet was loosened by scratching the falcon at the clean bench's grille. This washing step was repeated twice. PBMCs from one donor were collected in one falcon and the cell number was determined using a counting chamber by Neubauer. For correct counting, collected PBMCs were first diluted in 50 ml buffer and then in trypan blue dye in a ratio of 1:20 in a 96-well-plate, using a 10 µl pipette.

The stained cells were then put onto a Neubauer counting chamber where cells in all quadrants were counted under the light microscope. PBMC number (X) was calculated using following formula, Y being the mean cell number of all quadrants:

$$X = Y \times 10^4 \times 50 \times 20$$

After centrifugation at 1300 rpm for 10 min with brake, followed by decantation of the supernatant, cells were ready to put into culture.

### **3.2.2 Expansion of iNKT cells**

Culture medium for iNKT cell expansion was prepared as followed: RPMI medium was mixed with the antibiotic Refobacin (gentamicin; 60 mg / 500 ml RPMI), 10 % FCS, 100 ng / ml  $\alpha$ -GalCer), and 100 IU / ml human recombinant IL-2. Freshly isolated PBMCs were diluted to a concentration of  $3.3 \times 10^6$  / ml and 3 ml per well were applied onto 6-well plates. During 14 to 15 days of incubation at 37°C and 5 % CO<sub>2</sub>, 1 ml of medium per well was exchanged every three days. To exchange the medium, 1 ml was taken out of each well and collected in a new falcon which was then centrifuged at 1300 rpm for 10 min in order to save aspirated cells. After decanting the supernatant, the cell pellet was resuspended in 20 ml fresh medium and equally distributed back onto the 6-well-plates. Concentrations of RPMI, Refobacin, FCS and IL-2 were as described above. However, no more  $\alpha$ -GalCer was added. Before setting iNKT cells into co-culture, fresh medium was ultimately added on day 12 in order to reduce cytokine exposition prior to the experiments.

To harvest, cells were first loosened from the bottom of the well using a cell scraper. Then, culture medium from one donor was transferred from the well-plates into a 50 ml falcon using 1 ml pipettes with disposable tips. To increase the recovery of cells, each well was rinsed with 1 ml buffer. After collecting all cells in one falcon, cells were centrifuged at 1300 rpm for 10 min at room temperature. Supernatant was decanted, cell pellet was loosened and dissolved in 50 ml buffer. After cell counting, cells were diluted in fresh medium (RPMI with 60 mg / 500 ml Refobacin and 10% FCS) at a concentration of  $1 \times 10^6$  cells / ml.

### **3.2.3 Isolation of APCs**

In order to co-culture expanded iNKT cells with APCs from the same donor, PBMCs were frozen during the time of iNKT cells expansion (14-15 days).  $5 \times 10^8$  freshly isolated PBMCs were diluted in 460  $\mu$ l FCS and transferred into a cryotube. 40  $\mu$ l of dimethyl sulfoxide (DMSO) were added, the cryotubes were well mixed and put in liquid nitrogen at  $-155^\circ\text{C}$ . Before isolation procedures, cell suspension from the cryotubes were transferred into  $37^\circ\text{C}$  warm RPMI and washed three times with 50 ml RPMI at 1300 rpm for 10 min at room temperature in order to eliminate the DMSO. Cells were put through a cell strainer before determination of cell number.

#### **3.2.3.1 CD1d<sup>+</sup>-cell isolation**

To isolate CD1d<sup>+</sup> cells from previously frozen PBMCs,  $1 \times 10^8$  PBMCs were diluted in 1 ml buffer. 110  $\mu$ l Anti-CD1d-PE FACS antibody were added, the suspension was well mixed and incubated at  $4^\circ\text{C}$  for 15 min. 50 ml cold buffer was added to the stained cell suspension, mixed and centrifuged at 300 g for 10 min at  $4^\circ\text{C}$ . This washing step was performed twice. Cells were then resuspended in 800  $\mu$ l buffer per  $1 \times 10^8$  cells, 200  $\mu$ l Anti-PE-Mirobeads per  $1 \times 10^8$  cells were added, well mixed and incubated at  $4^\circ\text{C}$  for 15 min. After another washing step with 50 ml cold buffer, cells were resuspended in  $1 \times 10^8$  cells / 500  $\mu$ l cold buffer. For magnetic separation, LS columns were placed onto a magnet and were rinsed with 3 ml buffer before applying the stained cell suspension. Once the cell suspension had run through the column, 3 ml cold buffer were added three times, always waiting for the buffer to completely pass the column before adding more. Subsequently, the column was put out of the magnet onto a 15 ml falcon and a plunger was used to push 5 ml of buffer through the columns, thus transferring isolated cells from the column into the falcon. To increase purity, the positive selected cells were centrifuged at 1300 rpm for 5 min, resuspended in 1 ml buffer and the described magnetic isolation procedure with a new LS column was repeated. After the second magnetic separation, cell number was determined using a Neubauer counting chamber as described above and cells were put into complete medium (RPMI, Refobacin, FCS) in a dilution of  $1 \times 10^6$  / ml. Purity of selected cells was determined using flow cytometry.

### 3.2.3.2 Isolation of monocytes, B-cells and CD1c<sup>+</sup> mDCs

In order to use different APCs from the same donor, a successive isolation protocol for the three subpopulations was established.

Depending on available PBMC number, the isolation protocol started out with  $3 \times 10^8$  to  $5 \times 10^8$  PBMCs. PBMCs were diluted in a concentration of  $1 \times 10^8$  PBMCs / 400  $\mu$ l buffer, 100  $\mu$ l CD14 Microbeads per  $1 \times 10^8$  cells were added, well mixed and incubated for 15 min at 4°C. The supernatant was decanted and the cell pellet was thoroughly loosened. For magnetic separation,  $1 \times 10^8$  cells were diluted in 800  $\mu$ l buffer and applied to a previously rinsed LS column. After passage of the suspension, the column was rinsed three times with 3 ml buffer while collecting the entire flow through in a 50 ml falcon. CD14<sup>+</sup> selected cells were pushed into a 15 ml falcon using a plunger and 5 ml buffer. Cell number was determined, CD14<sup>+</sup> cells were resuspended in medium in a concentration of  $1 \times 10^6$  / ml and put into the incubator at 37°C and 5 % CO<sub>2</sub>.

After isolation of CD14<sup>+</sup> cells, the CD14<sup>-</sup> flow through was used for further cell isolations. After determining the cell number in the flow through, cells were diluted in 800  $\mu$ l buffer /  $1 \times 10^8$  cells. 200  $\mu$ l CD19 Microbeads per  $1 \times 10^8$  cells were added, well mixed and incubated for 15 min at 4°C. After incubation, cells were washed with 50 ml cold buffer and centrifuged at 300 g for 10 min at 4°C. Washed cells were then resuspended in a concentration of  $1 \times 10^8$  per 500  $\mu$ l and magnetic separation with LS columns was performed as described above. To increase purity, positive selected cells were put through a second LS column. Number of CD19<sup>+</sup> selected cells was determined and cells were incubated at 37°C in a concentration of  $1 \times 10^6$  / ml.

CD14<sup>-</sup> CD19<sup>-</sup> flow through was then used for the final isolation step.  $1 \times 10^8$  cells / 300  $\mu$ l buffer were incubated with 100  $\mu$ l FcR Blocking Reagent and 100  $\mu$ l CD1c Biotin for 15 min at 4°C. After washing with 50 ml cold buffer at 300 g for 10 min at 4°C, Biotin-labeled cells were resuspended in 400  $\mu$ l /  $1 \times 10^8$  cells and incubated with 100  $\mu$ l Anti-Biotin Microbeads for 15 min at 4°C. After another washing step with 50 ml cold buffer at 300 g for 10 min at 4°C, cells were resuspended in 500  $\mu$ l /  $1 \times 10^8$  cells. For CD1c<sup>+</sup>-cell isolation, magnetic separation was performed using MS columns. MS-columns were rinsed with 1 ml buffer, the cell suspension was applied, and the columns were rinsed three times, each with 1 ml of buffer prior to pushing positive selected cells out of the



column using a plunger and 1 ml of buffer. Magnetic separation process was repeated with a second MS column with positive selected cells to increase purity. Purity of positive selected cells was determined using flow cytometry.

### 3.2.4 Preparation of fungal strains

*A. fumigatus* resting conidia (ATCC 46645) were cultivated for 3 days on beer mash plates at 28°C. Conidia were detached from the plate using endotoxin-free sterile water and were filtered through a cell strainer with a 40 µm pore nylon mesh pore membrane to obtain a single-fungal cell suspension. To obtain germ tubes, conidia were cultivated in RPMI at a concentration of  $1 \times 10^6$  / 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 under the light microscope was achieved.

Both germ tubes and conidia were inactivated by 30 to 40 min incubation in 30 ml ethanol. Ethanol was removed and germ tubes and conidia, respectively, were washed five times with buffer at 5000 rpm for 5 min to prevent cell damage by remaining ethanol. Inactivated germ tubes and conidia were diluted at a concentration of  $1 \times 10^8$  / 1 ml buffer and stored at -18°C.

### 3.2.5 *In vitro* co-cultures

For *in vitro* co-cultures, expanded iNKT cells were applied to a 24-well-plate at a density of  $1 \times 10^6$  / ml and 1 ml per well. The following conditions were investigated, each cell combination being combined with each stimuli:

Cells	Stimuli
$1 \times 10^6$ iNKT cells	Unstimulated
$1 \times 10^6$ iNKT cells + $1 \times 10^5$ CD1d <sup>+</sup> cells	100 IU IL-2
$1 \times 10^6$ iNKT cells + $1 \times 10^5$ CD14 <sup>+</sup> cells	10 ng IL-12
$1 \times 10^6$ iNKT cells + $1 \times 10^5$ CD19 <sup>+</sup> cells	100 IU IL-2 + 10 ng IL-12
$1 \times 10^6$ iNKT cells + $1 \times 10^5$ CD1c <sup>+</sup> cells	$2 \times 10^6$ <i>A. fumigatus</i> germ tubes
	$2 \times 10^6$ <i>A. fumigatus</i> conidia
	100 IU IL-2 + 10 ng IL-12 + <i>A. f.</i> germlings

Co-Cultures were incubated for 16 h at 37°C and 5 % CO<sub>2</sub>. After incubation, cells were harvested for flow cytometric analysis and supernatants were collected for ELISA analysis.

### **3.2.6 Flow cytometry**

With flow cytometry, cells can be described according to their volume, their granulation and additionally, using fluorescing antibodies, according to their cell surface marker expression. In a flow cytometry device, cells are pushed through a capillary in such way that a stream of single cells forms and passes a laser beam. Several detectors are pointed at the light beam. One in line with the beam, the forward scatter (FSC), which measures the diffraction of the laser's light and reflects the volume of the cell. Other detectors are pointed perpendicular to the light beam, the side scatter (SSC), which measures the refraction of the laser's light and reflects the inner complexity (granulation, size and shape of nucleus) of cells. Thus, information about volume and granulation can be perceived of more than 1000 cells per second.

Additionally, cells can be stained with fluorescing antibodies that bind to cell surface molecules. The emitted fluorescent is measured by additional fluorescence detectors and reflects the expression of the respective cell surface marker.

Flow cytometry was performed on FACS Calibur (BD Biosciences) and data were analyzed using FlowJo (Tree Star, Ashland, OR, USA).

#### **3.2.6.1 Staining process**

In preparation for flow cytometric analysis, a maximum of  $1 \times 10^5$  cells were put in a FACS tube. First, 2 to 3 ml of buffer were added and tubes were centrifuged at 1800 rpm for 4 min. Supernatant was carefully decanted, leaving the cells suspended in approximately 100  $\mu$ l. Following, 40  $\mu$ l human immunoglobulin solution were added per tube in order to block Fc receptors and prevent unspecific staining. After vortexing and 10 min of incubation at room temperature, tubes were washed as described above (1800 rpm, 4 min). Then, cells were stained by adding the appropriate amount of FACS-antibody per tube. Each tube can be stained with a maximum of four antibodies with different fluorescent dyes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP), and allophycocyanin (APC). For each combination of

staining antibody, one tube with the according isotypes in the same concentrations was prepared.

### 3.2.6.2 Purity of isolated cells

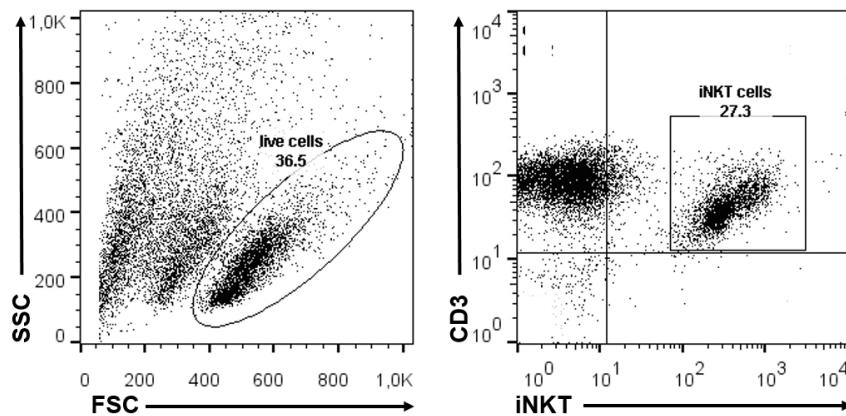
In order to characterize expanded iNKT cells and to define the purity of isolated CD1d<sup>+</sup> cells, flow cytometric analysis was used.

iNKT cells were defined as iNKT<sup>+</sup>CD3<sup>+</sup> cells. The mean amount of iNKT cells in the cultures was 23 % while a total of 93 % of cells were CD3<sup>+</sup>, meaning that other than iNKT cells, there were mainly T cells in the cultures used for experiments.

CD1d<sup>+</sup> subpopulations were defined as CD1d<sup>+</sup>CD14<sup>+</sup>, CD1d<sup>+</sup>CD19<sup>+</sup>, and CD1d<sup>+</sup>CD1c<sup>+</sup> cells. Purity of CD1d<sup>+</sup> cells was over 99 %. Purity of CDd<sup>+</sup>CD14<sup>+</sup> was 86.2 %, purity of CD1d<sup>+</sup>CD19<sup>+</sup> 85.2 % and isolated CD1d<sup>+</sup>CD1c<sup>+</sup> had a mean purity of 92.9 %.

### 3.2.6.3 CD69 expression on iNKT cell surface

To evaluate co-cultures, cells were stained with CD69 FITC, iNKT PE, CD3 PerCP and CD137 APC antibodies. The live cells were gated on CD3<sup>+</sup>iNKT<sup>+</sup> cells for which the CD69 expression was determined (Figure 4).



**Figure 4: Flow cytometric analysis of expanded iNKT cells**

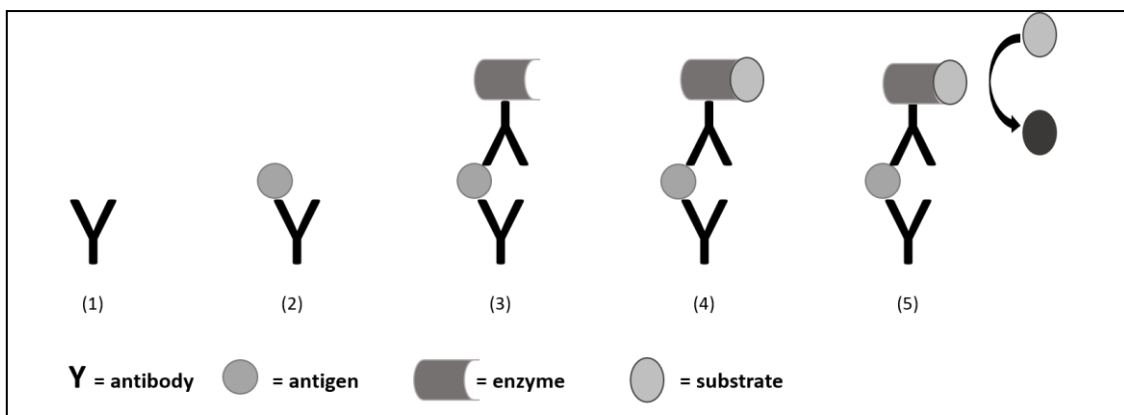
Graph shows gating procedure during flow cytometric analysis of expanded iNKT cells. A first gate was set on all live cells. Live cells were then gated on CD3<sup>+</sup>iNKT<sup>+</sup> cells.

## 3.2.7 Detection of cytokines and growth factors in cell culture supernatants

### 3.2.7.1 ELISA

Enzyme Linked Immunosorbent Assay (ELISA) is an antibody-based method to detect proteins in a liquid sample. A microtiter plate coated with specific antibodies is used. If

the specific antigen is present in the sample, it binds to the antibodies on the titer surface. The sample is then washed away prior to adding a second enzyme-linked antibody which then binds to antigens which are already captured by the first antigen. Another washing step removes unbound detection antibodies. Now the enzyme's substrate is added and its conversion results in a color change. The amount of antigen in the sample predicts the amount of enzyme-linked antibodies which in turn predicts the amount of converted substrate. Conclusively, the color change reflects the protein concentration of the sample. Fluorescence is measured by a photometer and comparison to a standard curve with known concentrations makes it possible to determine the exact protein concentration of the detected protein in the sample.



**Figure 5: ELISA**

Graph shows main principle of ELISA. Antigens from a liquid sample bind to titer plate coated antibodies (2). A second antibody specific to the same antigen and conjugated to an enzyme is added (3). Redundant antibodies are washed away and the enzyme's substrate is added (4), whose conversion results in a color change (5).

### 3.2.7.2 Multiplex Immunoassay

Multiplex Assays are very similar to ELISA and enable the simultaneous detection of different target molecules in one sample.

In this study, cytokine concentrations within supernatants were determined using multiplex technology (Bio-Plex Pro Human Cytokine 27-plex Assay, Bio-Rad). This multiplexing technology uses color-coded beads which are conjugated with reactants that are specific to different target molecules. Identification and quantification of target molecule concentrations are based on bead-color and fluorescence.

Multiplex assays were performed according to the manufacturer's instructions in the laboratory of Prof. Dr. med. O. Kurzai, Septomics Research Center, Hans-Knoell Institute Jena.

### **3.2.8 Ethics Statement**

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

### **3.2.9 Statistical Analysis**

Data was analyzed with Graph Pad Prism 5. Wilcoxon signed-ranked test was used for ELISA data (n = 3 for CD14<sup>+</sup> and CD19<sup>+</sup> co-cultures and n = 7 for CD1c<sup>+</sup> co-cultures). Paired t-test was used for the analysis of FACS data.

## **4 Results**

### **4.1 Establishing a co-culture system of iNKT cells and APCs**

#### **4.1.1 iNKT cell expansion**

The iNKT cell expansion was based on an existing protocol previously established in the laboratory of Professor Löffler. In the context of this study, further experiments were performed to maximize iNKT cell outcome and improve iNKT cell responsiveness to external stimuli.

iNKT cells have been described to internalize their invariant TCR (iTCR) and become anergic upon single stimulation with  $\alpha$ -GalCer, with IL-2 being able to break this state of unresponsiveness [65]. Therefore, the existing protocol was changed insofar, as  $\alpha$ -GalCer was solely added to the medium at day 1 of cell culture and for continuous medium changes only IL-2 was applied to stimulate iNKT cell proliferation.

Furthermore, complete elimination of  $\alpha$ -GalCer was tried to be achieved by exchanging the entire medium at day 7. However, this measure did not result in higher iTCR expression by iNKT cells and was hence neglected.

#### **4.1.2 Isolation of iNKT cells**

Isolation of iNKT cells was attempted using NKT Microbeads (Miltenyi Biotec GmbH). The most successful tries to isolate iNKT cells from fresh PBMCs provided a positive selected cell population ranging between 50 and 82 % CD3<sup>+</sup>iNKT<sup>+</sup> cells. However, the very low cell recovery with major amounts of dead cells was insufficient for further co-culture experiments.

In order to increase cell outcome, the same isolation protocol was performed with expanded iNKT cells. However, this was also unsuccessful with poor to no viability of positive selected cells.

Therefore, the entire culture of expanded cells was used for co-culture experiments, containing iNKT and T cells.

#### **4.1.3 Isolation of CD1d<sup>+</sup> cell subpopulations**

The protocol for isolation of CD14<sup>+</sup>, CD19<sup>+</sup> and CD1c<sup>+</sup> cells from PBMCs using MACS positive selection procedures was established by combining and adjusting the manufacturer's protocols for the according Microbeads. Due to their high number in

peripheral blood, CD14<sup>+</sup> cells were isolated first. A small subset of B cells is CD1c<sup>+</sup>, hence, CD19<sup>+</sup> B cells were isolated next, followed by isolation of CD1c<sup>+</sup> mDCs. Elimination of CD3<sup>+</sup> cells using CD3 Microbeads was performed preceding CD14<sup>+</sup> cell isolation to increase purity of CD1d<sup>+</sup> subpopulations but did not improve the results. In order to maximize purity, each positive selected cell population was run through a second magnetic column.

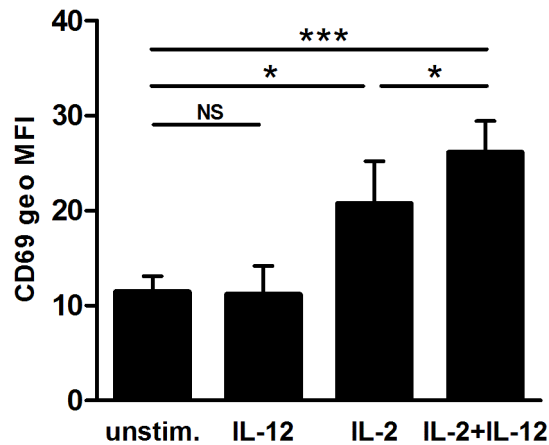
Isolation of CD1d<sup>+</sup> subpopulations was performed with fresh PBMCs after two weeks of storage in liquid nitrogen. Isolation of frozen PBMCs promised higher outcome of cells compared to the isolation of subpopulations from fresh PBMCs with subsequent freezing.

The final protocol for the isolation of CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B cells and CD1c<sup>+</sup> mDCs is described in the methods section.

## **4.2 Direct interaction studies of iNKT cells and *A. fumigatus***

### **4.2.1 Cytokines activate iNKT cells**

At first, the responsiveness of expanded iNKT cells to T cell stimulating cytokines IL-2 and IL-12 was investigated. IL-12, which was added in concentrations of 10 ng / ml and 100 ng / ml, did not upregulate CD69 expression on iNKT cells. In contrast, IL-2 in a concentration of 100 IU / ml did stimulate iNKT cells ( $p < 0.05$ ). IL-12 further increased the activating effect of IL-2 when a cocktail of both cytokines was added ( $p < 0.05$ ). Thus, both cytokines have a stimulating effect on iNKT cells whereupon IL-12 only comes to an effect in the presence of IL-2 (Figure 6). Expression of CD137, a member of the TNF receptor superfamily, was not observed in any experiment.



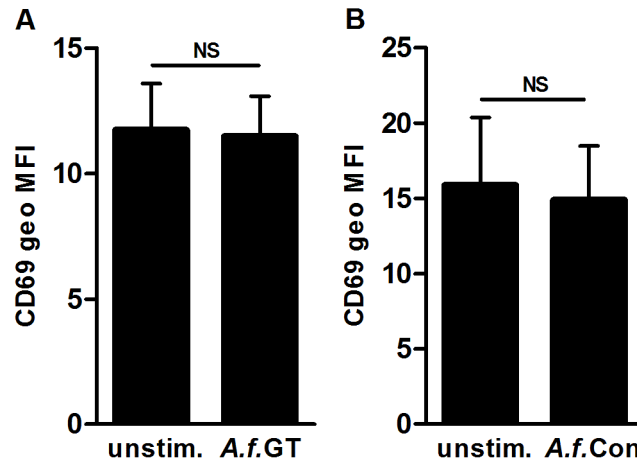
**Figure 6: Cytokines stimulate iNKT cells**

Graph shows CD69 expression on iNKT cells. iNKT cells were cultured alone (unstim.) or with IL-12 (10 ng, n = 7), IL-2 (500 IU, n = 8) or a cytokine cocktail of IL-2 (10 ng) and IL-12 (500 IU, n = 12). CD69 upregulation was seen upon stimulation with IL-2 but not IL-12. The cytokine cocktail of IL-2 and IL-12 led to significantly higher stimulation of iNKT cells than IL-2 alone. CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. Mean and standard error of the mean (SEM) of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \* (p < 0.05), \*\*\* (p < 0.001) and NS (not significant).

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

To explore the influence of *A. fumigatus* on iNKT cells, the first approach was to investigate the direct interaction of cells and fungus. iNKT cell incubation with live *A. fumigatus* for 6 and 12 h was not analyzable due to excessive hyphal growth with subsequent cell loss during staining procedures for flow cytometric analysis. Consequently, experiments were continued with inactivated fungus. A significant change in CD69 expression on the iNKT cell surface was neither observed for germ tubes (Figure 7A) nor for conidia (Figure 7B).





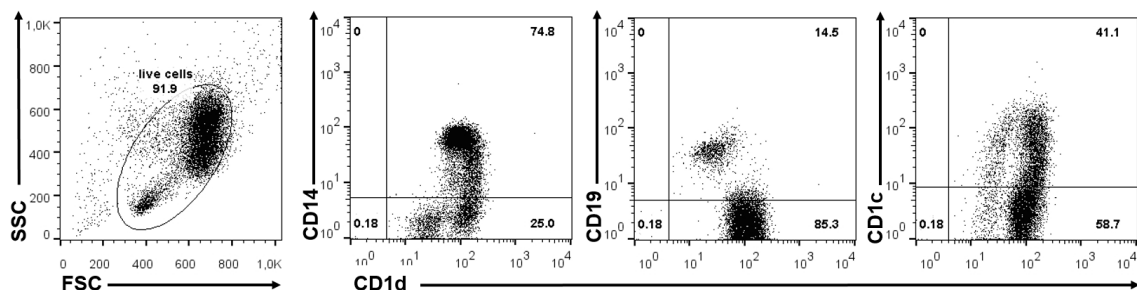
**Figure 7: Direct effect of *A. fumigatus* on iNKT cells**

Graph shows CD69 expression on iNKT cells. iNKT cells were cultured alone (unstim.) or with (A) *A. fumigatus* germ tubes (*A.f.* GT, MOI = 2, n = 16) or (B) conidia (*A.f.* Con., MOI = 2, n = 5). CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Not significant differences are indicated by NS.

### 4.3 iNKT cell co-culture with CD1d<sup>+</sup> cells

CD1d<sup>+</sup> cells comprise three main subpopulations, monocytes, B cells and dendritic cells. Peripheral blood dendritic cells compose CD11c<sup>-</sup> plasmacytoid (p)DCs and CD11c<sup>+</sup> myeloid (m)DCs which are further subdivided in CD1c (BDCA-1)<sup>+</sup>, CD141 (BDCA-3)<sup>+</sup> and CD16<sup>+</sup> mDCs [66]. Other CD1d expressing cells are CD56<sup>+</sup> NK cells and CD303<sup>+</sup> pDCs.

CD1d<sup>+</sup> cells that were isolated after having been frozen in liquid nitrogen for 15 days composed on average 61.85 % CD14<sup>+</sup> cells, 17.7 % CD19<sup>+</sup> B cells and 37.94% CD1c<sup>+</sup> mDCs. A subset of B-cells also expresses CD1c. The percentage of CD19<sup>-</sup>CD1c<sup>+</sup> cells in CD1d<sup>+</sup> selected cells averaged 29.21 % (n = 7; Figure 8), relating to mDCs.



**Figure 8: Subpopulations of positive selected CD1d<sup>+</sup> cells**

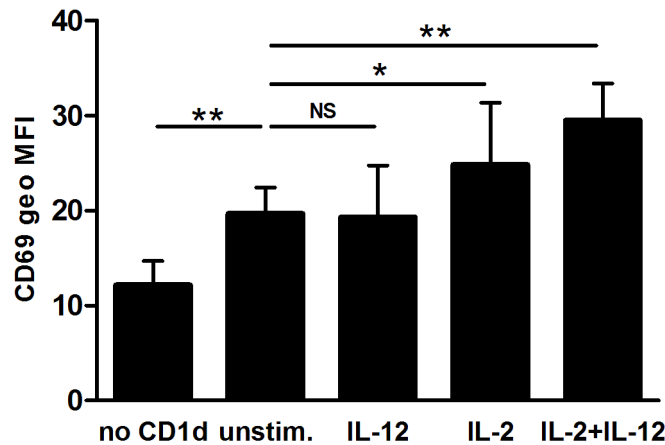
Graph exemplarily shows flow cytometric analysis of isolated CD1d<sup>+</sup> selected cells of one donor, gated on live cells. Purity of isolated cells is 99.8 %, 74.8 % of isolated CD1d<sup>+</sup> cells are CD14<sup>+</sup> monocytes, 14.5 % are CD19<sup>+</sup> B cells and 41.2 % are CD1c<sup>+</sup> cells (including CD1c<sup>+</sup> B cells and CD1c<sup>+</sup> mDCs).

#### 4.3.1 CD1d<sup>+</sup> cells stimulate iNKT cells

After investigating the direct effect of cytokines and fungus on iNKT cells, the effect of CD1d<sup>+</sup> cells on iNKT cells was analyzed. CD1d<sup>+</sup> cells were isolated from PBMCs and co-cultured with expanded iNKT cells from the same donor. The presence of CD1d<sup>+</sup> cells resulted in upregulated CD69 expression on iNKT cells in the absence of further added stimuli ( $p < 0.005$ ) (Figure 9).

#### 4.3.2 Cytokines further stimulate iNKT cells co-cultured with CD1d<sup>+</sup> cells

To assess if cytokines further increase CD69 expression on iNKT cells in the presence of CD1d<sup>+</sup> cells, IL-12 (10 ng) and IL-2 (100 IU) were added separately and in combination. Similar to the results for iNKT cells alone, IL-12 did not stimulate iNKT cells whereas IL-2 ( $p < 0.05$ ) and the cytokine cocktail ( $p < 0.01$ ) increased the activating effect of CD1d<sup>+</sup> cells (Figure 9).



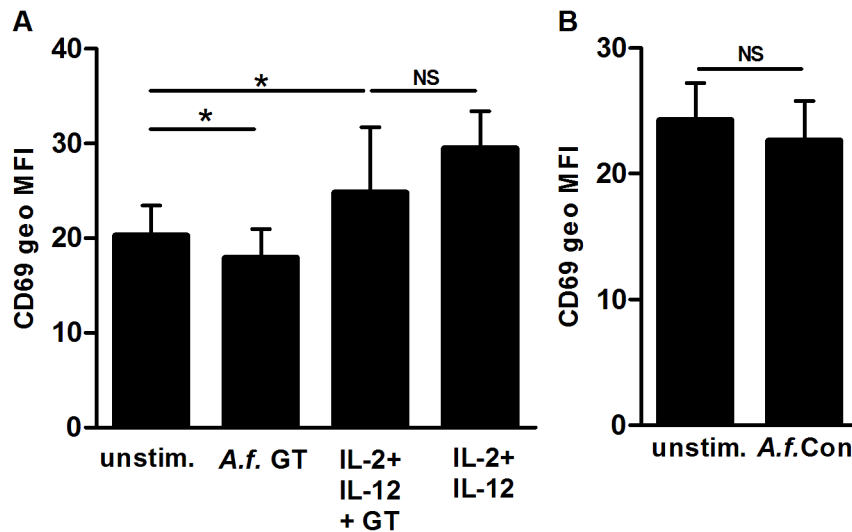
**Figure 9: Effect of cytokines on iNKT cells co-cultured with CD1d<sup>+</sup> cells**

Graph shows CD69 expression on iNKT cells. iNKT cells were cultured alone (no CD1d,  $n = 9$ ) and with CD1d<sup>+</sup> selected cells (unstim.,  $n = 9$ ). iNKT / CD1d<sup>+</sup> cell co-culture was performed with the addition of IL-12 (10 ng,  $n = 4$ ), IL-2 (500 IU,  $n = 5$ ) or a cytokine cocktail of IL-2 (10 ng) and IL-12 (500 IU,  $n = 8$ ). CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. CD69 expression on iNKT cells increased in co-culture with

CD1d<sup>+</sup> cells. IL-2 and the cytokine cocktail of IL-2 and IL-12 further activated iNKT cells in CD1d<sup>+</sup> cell co-culture. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \*\* ( $p < 0.01$ ), \* ( $p < 0.05$ ) and NS (not significant).

#### 4.3.3 *A. fumigatus* reduces activating effect of CD1d<sup>+</sup> cells on iNKT cells

After showing that iNKT cells did not directly interact with *A. fumigatus*, the potential of APCs to promote iNKT cell responses to the fungus was assessed. Experiments showed that CD1d<sup>+</sup> cells co-cultured with *A. fumigatus* germ tubes induced higher CD69 expression on iNKT cells compared to iNKT cells alone, however, to a lower extent than CD1d<sup>+</sup> cells alone in the absence of fungal germlings. In other words, *A. fumigatus* germlings decreased the stimulating effect of CD1d<sup>+</sup> cells on iNKT cells regarding CD69 expression (Figure 10A). In contrast, *A. fumigatus* conidia did not influence the effect of CD1d<sup>+</sup> cells on iNKT cells (Figure 10). This effect of germ tubes to reduce stimulation of iNKT cells in the presence of CD1d<sup>+</sup> cells was reversed by IL-2 and IL-12 ( $p < 0.005$ ) (Figure 10A).



**Figure 10: Effect of *A. fumigatus* on iNKT cells co-cultured with CD1d<sup>+</sup> cells**

Graph shows CD69 expression on iNKT cells. iNKT cells were cultured with CD1d<sup>+</sup> selected cells adding *A. fumigatus* germ tubes (*A.f.* GT; MOI = 2), *A. fumigatus* conidia (*A.f.* Con; MOI = 2); or a cytokine cocktail of IL-12 (10 ng) and IL-2 (500 IU) with (IL-2+IL-12+GT) or without (IL-2+IL-12) *A. fumigatus* germ tubes. CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. In co-culture with CD1d<sup>+</sup> cells, iNKT cells showed

reduced CD69 expression in response to *A. fumigatus* germ tubes. This effect was not seen with *A. fumigatus* conidia. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \* ( $p < 0.05$ ) and NS (not significant).

#### 4.4 iNKT cell co-culture with different CD1d<sup>+</sup> subpopulations

One aim of this work was to further differentiate the effects of different CD1d<sup>+</sup> cell subpopulations on iNKT cells. CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B-cells and CD1c<sup>+</sup> mDCs were isolated and co-cultured with expanded iNKT cells.

##### 4.4.1 CD1d expression on subpopulation is highest on CD1c<sup>+</sup> mDCs

The expression of CD1d on the surface of the different subpopulations was determined using flow cytometric analysis. Isolated CD1c<sup>+</sup> mDCs expressed significantly higher CD1d than isolated CD14<sup>+</sup> cells ( $p < 0.01$ ) (Figure 11). Because staining with Anti-CD19 antibodies of isolated CD19<sup>+</sup> B cells was merely successful in two donors, possibly due to previous isolation with CD19 Microbeads, CD1d expression of CD19<sup>+</sup> B cells was measured on the CD19<sup>+</sup> subpopulation of CD1d<sup>+</sup> isolated cells. CD1d expression on CD19<sup>+</sup> cells was significantly lower than on CD14<sup>+</sup> monocytes as well as on the CD1c<sup>+</sup> subpopulation of CD1d<sup>+</sup> selected cells. So, CD1d expression was highest on CD1c<sup>+</sup> mDCs, lower on monocytes and lowest on B-cells (Figure 11).

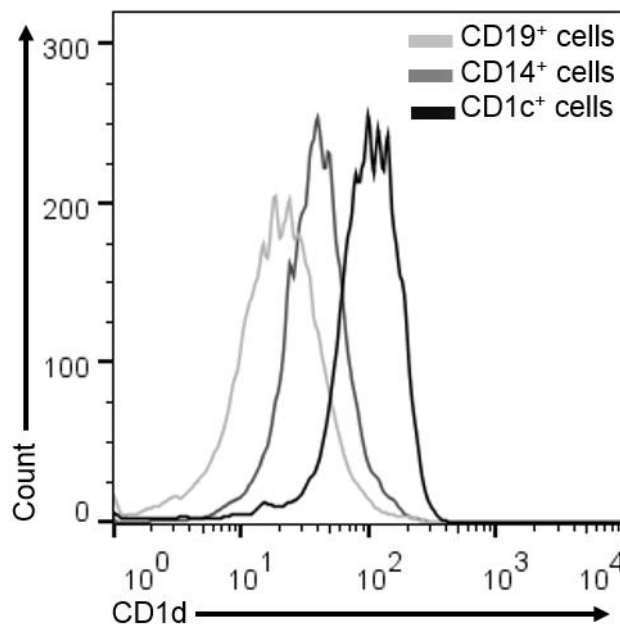
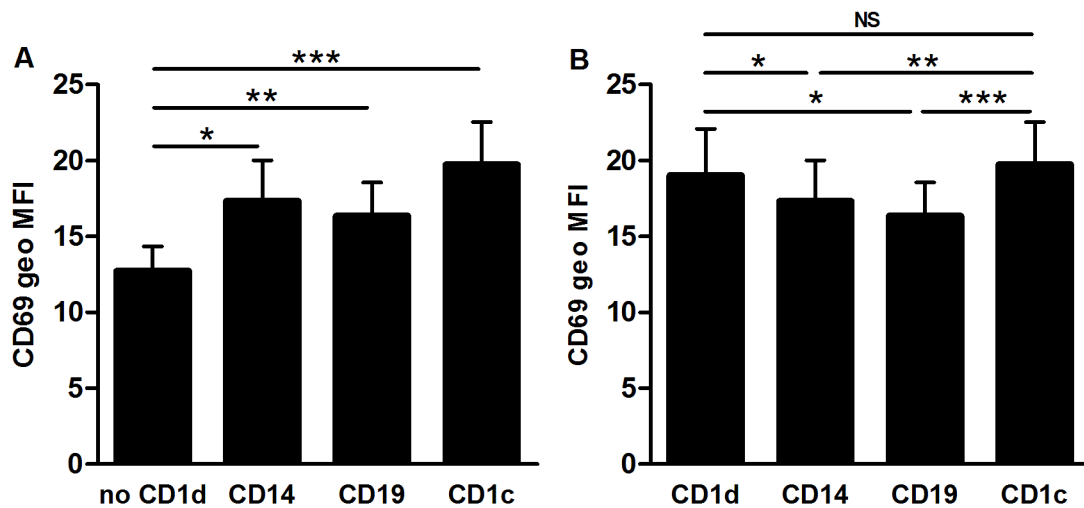


Figure 11: CD1d expression on CD19<sup>+</sup>, CD14<sup>+</sup> and CD1c<sup>+</sup> cells

Graph exemplarily shows CD1d expression on CD19<sup>+</sup>, CD14<sup>+</sup> and CD1c<sup>+</sup> selected cells from one donor. CD1d expression was highest on CD1c<sup>+</sup> mDCs, lower on CD19<sup>+</sup> cells and lowest on CD14<sup>+</sup> cells.

#### 4.4.2 Monocytes, B-cells and CD1c<sup>+</sup> mDCs stimulate iNKT cells

All of the three subpopulations by themselves had a stimulating effect on iNKT cells (monocytes  $p < 0.05$ ; B-cells  $p < 0.05$ ; CD1c<sup>+</sup> mDCs  $p < 0.001$ ), whereupon the effect of monocytes and B-cells was significantly lower than the effect of the complete CD1d<sup>+</sup> cell population ( $p < 0.05$ ) (Figure 12A). Moreover, CD69 upregulation on iNKT cells was significantly higher when incubated with CD1c<sup>+</sup> mDCs than with monocytes ( $p < 0.01$ ) and B-cells ( $p < 0.001$ ) (Figure 12B). In summary, the iNKT cell activation by CD1d<sup>+</sup> cells in the absence of further stimuli was, on a per cell basis, highest in co-culture with the CD1c<sup>+</sup> mDC subpopulation.



**Figure 12: CD1d<sup>+</sup> subpopulations induce iNKT cell activation**

Graph shows CD69 expression on iNKT cells. iNKT cells were co-cultured with CD1d<sup>+</sup> cells, CD14<sup>+</sup> cells, CD19<sup>+</sup> cells and CD1c<sup>+</sup> cells. CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. All subpopulations showed activating effect on iNKT cells. Increase in CD69 expression on iNKT cells was highest when co-cultured with CD1c<sup>+</sup> mDCs. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \*\*\* ( $p < 0.001$ ), \*\* ( $p < 0.01$ ), \* ( $p < 0.05$ ) and NS (not significant).

#### 4.4.3 Cytokines stimulate iNKT cells co-cultured with different CD1d<sup>+</sup> subpopulations

The cytokine effects on iNKT cells in combination with isolated CD1d subsets was assessed with consistent concentrations of IL-2 and IL-12. In co-culture of iNKT cells and monocytes, neither IL-2 nor IL-12 had a further activating effect on iNKT cells, whereas the combination of both resulted in upregulated CD69 expression ( $p < 0.05$ ) (Figure 13A). Incubation of iNKT cells and B-cells with IL-12 neither increased CD69 expression on iNKT cells, but, IL-2 ( $p < 0.05$ ) had an activating effect on iNKT cells as well as the cytokine cocktail ( $p < 0.01$ ) (Figure 13B).

Contrasting, in the presence of CD1c<sup>+</sup> mDCs, IL-12 alone was capable of inducing a CD69 upregulation on iNKT cells ( $p < 0.01$ ), likewise IL-2 alone and the combination of both (each  $p < 0.05$ ) (Figure 13C).

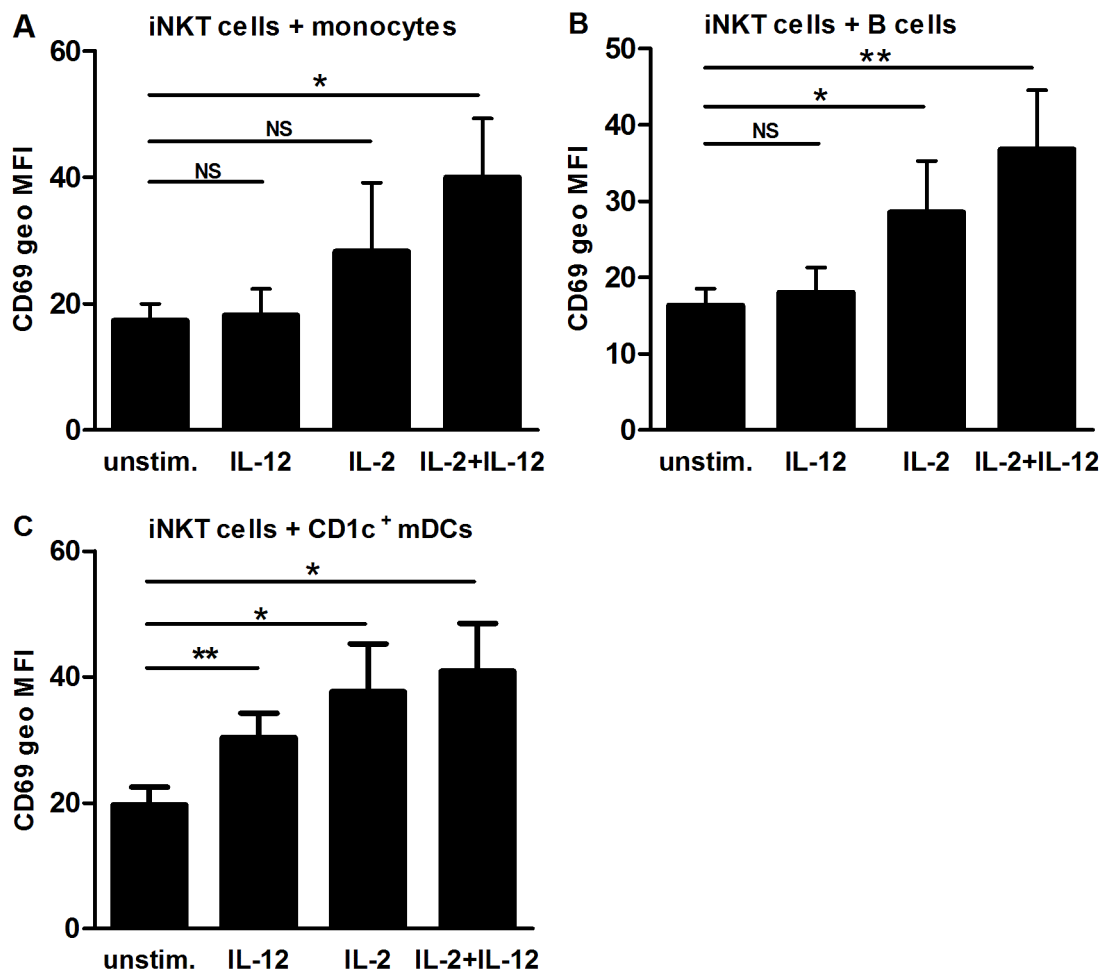
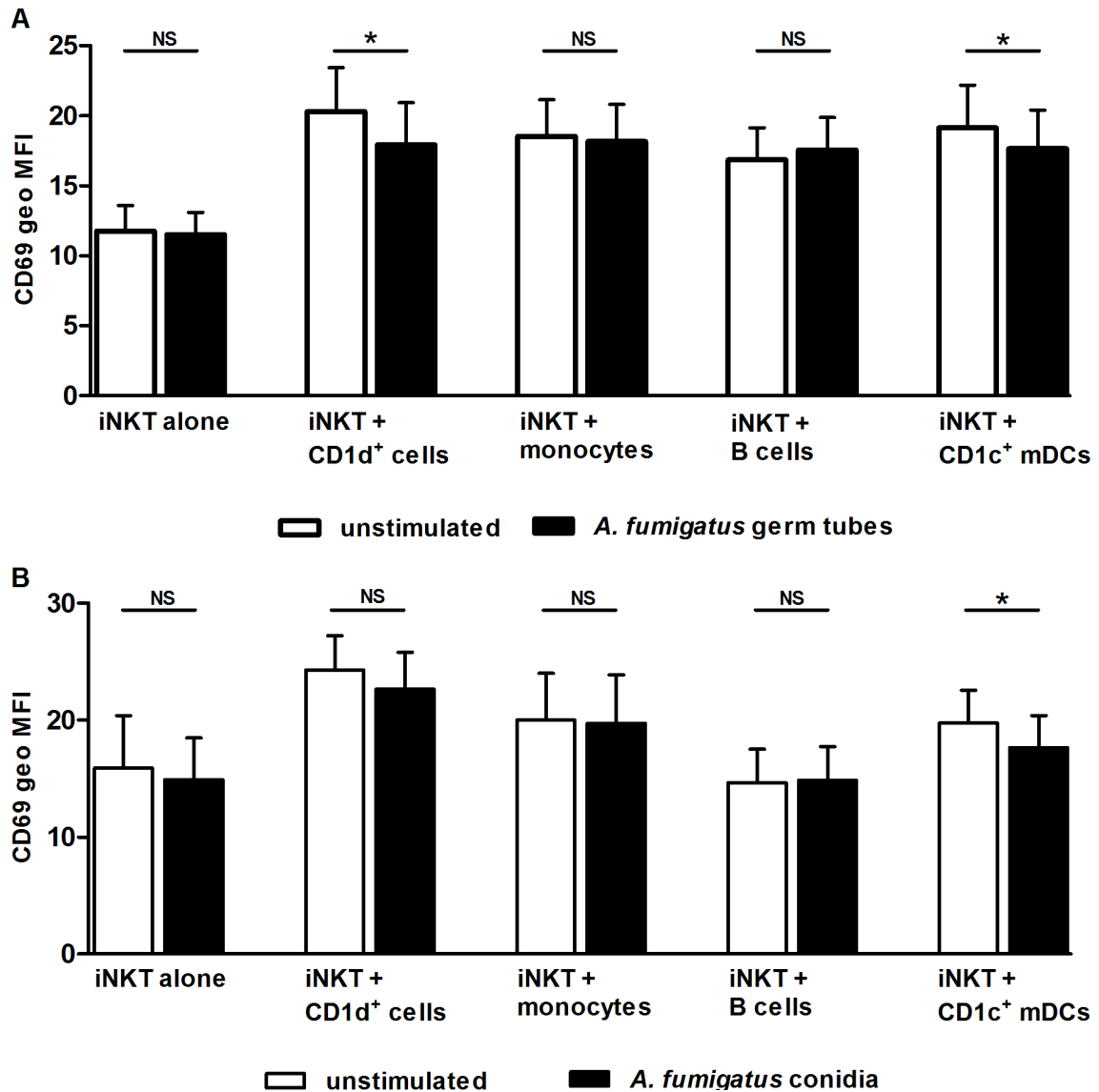


Figure 13: Effect of cytokines on iNKT cells co-cultured with CD1d<sup>+</sup> subpopulations

Graph shows CD69 expression on iNKT cells. iNKT cells were co-cultured with (A) CD14<sup>+</sup> cells, (B) CD19<sup>+</sup> cells and (C) CD1c<sup>+</sup> mDCs. Each cell co-culture was incubated with IL-12 (10 ng), IL-2 (500 IU) and a cytokine cocktail of IL-12 (10 ng) and IL-2 (500 IU). CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. (A) In Co-culture with CD14<sup>+</sup> monocytes, iNKT cells showed increased CD69 expression in response to IL-2 and IL-12. (B) iNKT cells co-cultured with B cells were activated by IL-2 alone and the cocktail of IL-2 and IL-12. (C) In co-culture with CD1c<sup>+</sup> mDCs IL-2 and the cocktail of IL-2 and IL-12 further activated iNKT cells. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \*\*\* ( $p < 0.001$ ), \*\* ( $p < 0.01$ ), \* ( $p < 0.05$ ) and NS (not significant).

#### **4.4.4 *A. fumigatus* reduces stimulating effect of CD1c<sup>+</sup> mDCs on iNKT cells**

The same co-cultures were run with *A. fumigatus* germlings and conidia. As in the co-culture system with the entire CD1d<sup>+</sup> population, *A. fumigatus* germ tubes reduced the stimulating effect of CD1c<sup>+</sup> mDCs on iNKT cells (Figure 14A). In contrast to the experiments with all CD1d<sup>+</sup> cells, in combination with CD1c<sup>+</sup> mDCs, this inhibitory effect was also observed with *A. fumigatus* conidia (Figure 14B). No effect of *A. fumigatus* was seen on the interaction of monocytes or B-cells with iNKT cells.



**Figure 14: Effect of *A. fumigatus* on iNKT cells co-cultured with CD1d<sup>+</sup> subpopulations**

Graph shows CD69 expression on iNKT cells. iNKT cells were co-cultured alone, with CD1d<sup>+</sup> cells, CD14<sup>+</sup> cells, CD19<sup>+</sup> cells, and CD1c<sup>+</sup> mDCs. *A. fumigatus* (A) germ tubes (MOI = 2) or (B) conidia (MOI = 2) were added and compared to unstimulated co-cultures. CD69 expression on iNKT cells was determined after 16 h of co-culture by flow cytometry, gating on iNKT<sup>+</sup>CD3<sup>+</sup> cells. In the presence of CD1d<sup>+</sup> cells, *A. fumigatus* germ tubes had an inhibitory effect on iNKT cells. In the presence of CD1c<sup>+</sup> mDCs, *A. fumigatus* germ tubes and conidia caused reduced CD69 expression on iNKT cells. This effect was neither observed in co-culture with B-cells nor monocytes. Mean and SEM of the geometric mean fluorescence intensity (geo MFI) from independent experiments are shown. Significant differences are indicated by \*\*\* (p < 0.001), \*\* (p < 0.01), \* (p < 0.05) and NS (not significant).



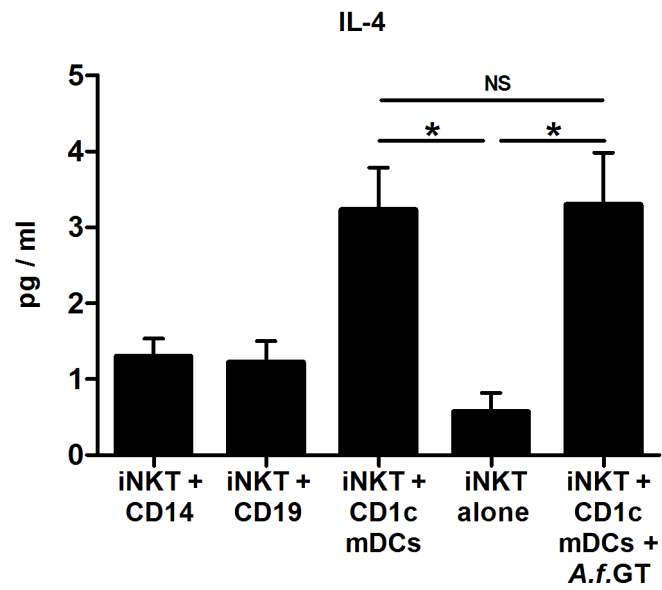
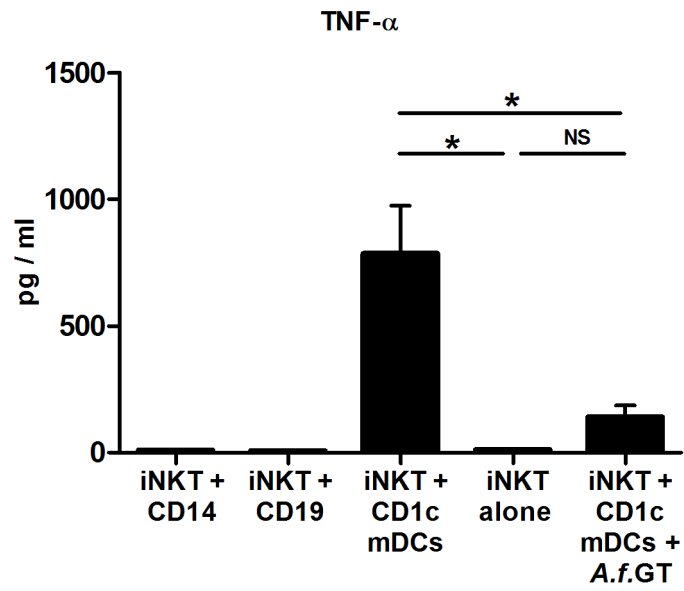
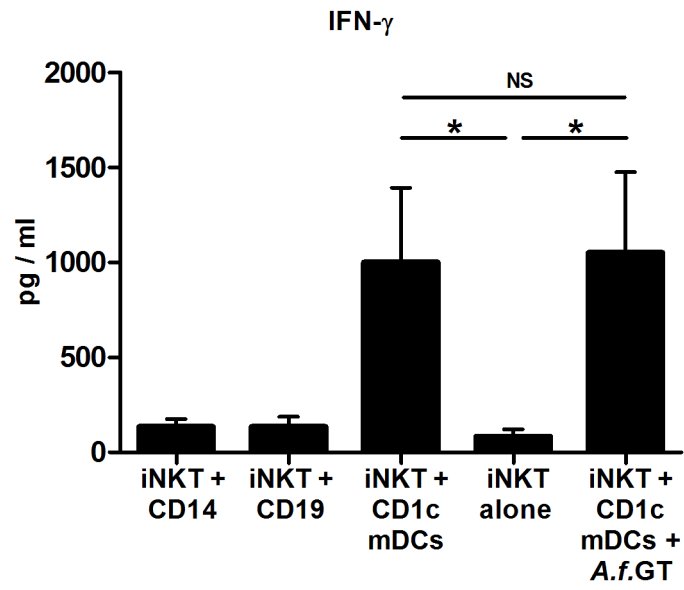
## **4.5 Cytokine production in co-culture supernatants**

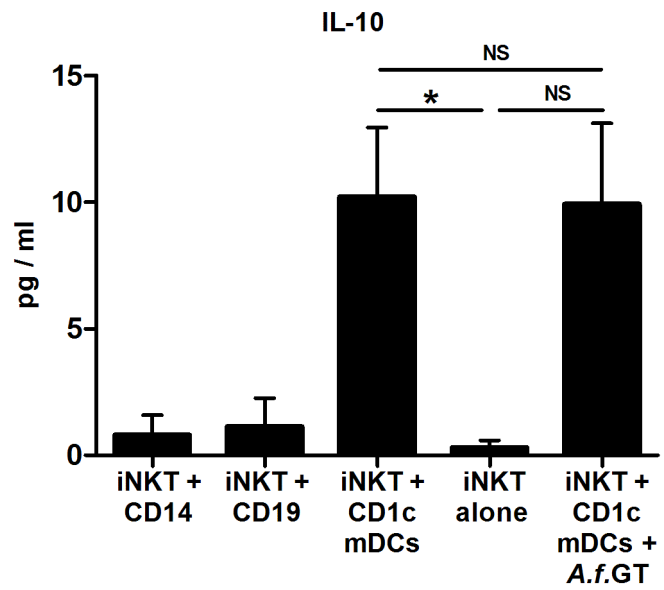
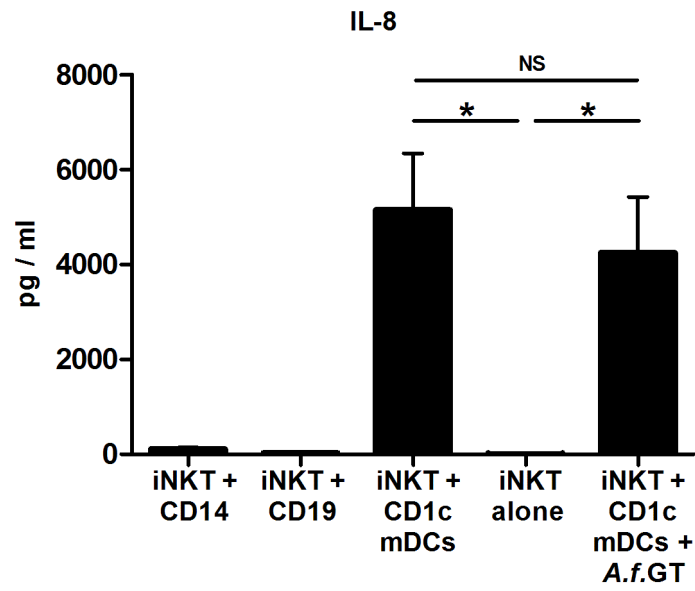
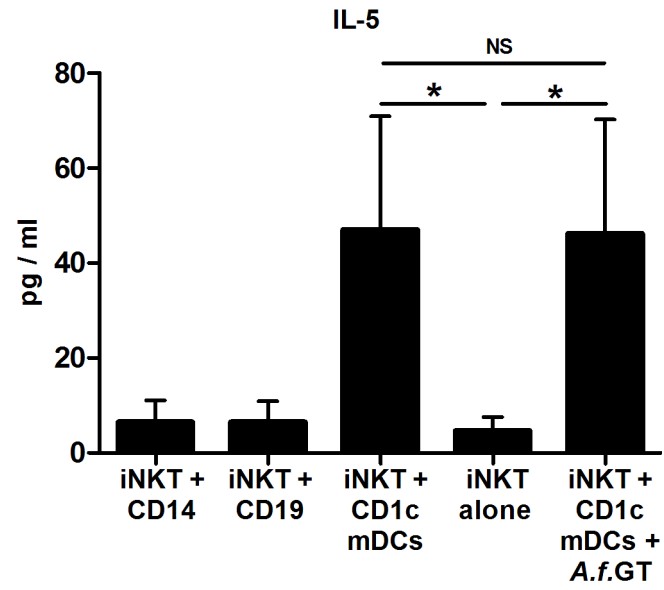
### **4.5.1 CD1c<sup>+</sup> mDCs stimulate cytokine production in iNKT cell co-culture**

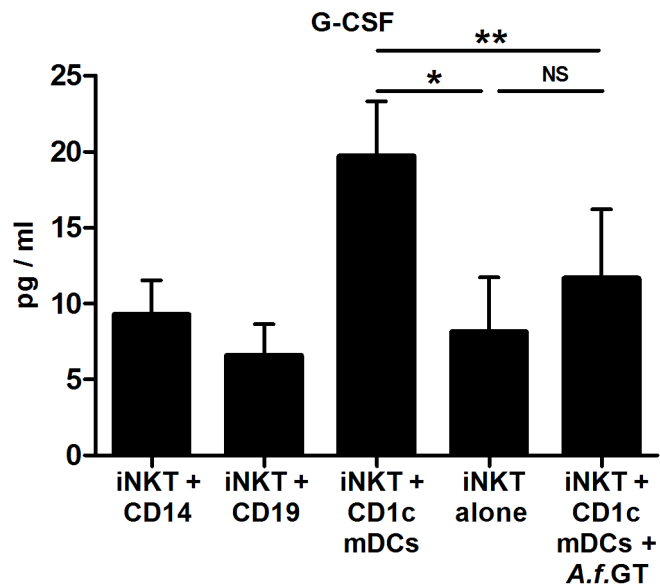
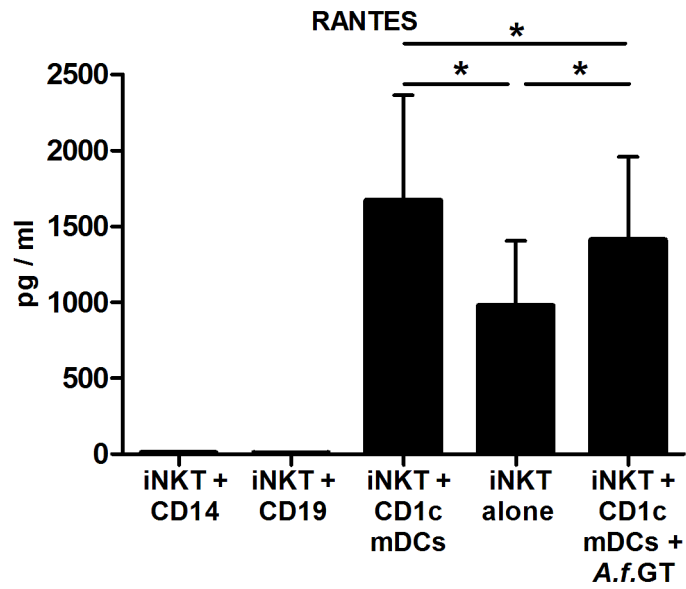
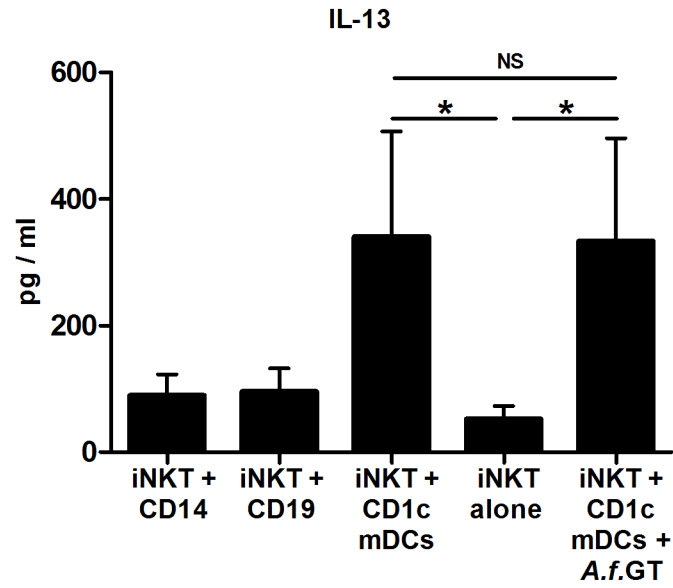
Multiplex ELISA analysis was performed with supernatants from iNKT cell co-cultures with different APCs and *A. fumigatus* germ tubes. The co-incubation of CD14<sup>+</sup> monocytes or CD19<sup>+</sup> B-cells with iNKT cells without further stimuli did not induce a production of any of the tested cytokines. Differently, the interaction of CD1c<sup>+</sup> mDCs with iNKT cells resulted in increased production of the Th1 cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-2, the Th2 cytokines IL-4, IL-5, IL-6, IL-10, and IL-13, IL-1 $\beta$ , IL-1ra, the growth factors FGF, G-CSF, GM-CSF, and VEGF as well as the chemokines IL-8 and RANTES (Figure 15).

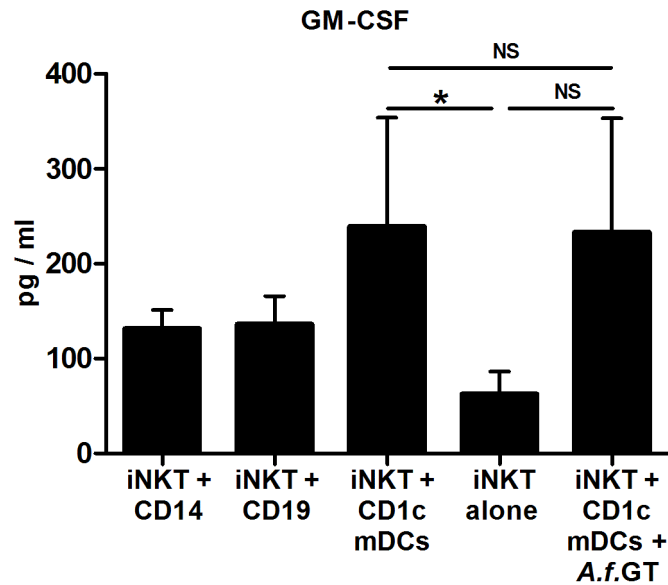
### **4.5.2 *A. fumigatus* reduces cytokine release in CD1c<sup>+</sup> mDC / iNKT cell co-culture**

As in co-cultures in the absence of fungus, concentrations of IFN- $\gamma$ , IL-1 $\beta$ , IL-1ra, IL-4, IL-5, IL-6, IL-8, IL-13 and VEGF were significantly upregulated in co-cultures of *A. fumigatus* germ tubes, CD1c<sup>+</sup> mDCs and iNKT cells compared to iNKT cells alone. But, compared to the co-culture system with iNKT cells and CD1c<sup>+</sup> mDCs without further stimuli, no differences were observed. In contrast, the comparison of CD1c<sup>+</sup> mDC / iNKT cell co-culture with and without *A. fumigatus* germ tubes showed reduced production of TNF- $\alpha$ , G-CSF and RANTES when fungal germ tubes were present (Figure 15).









**Figure 15: Cytokine release in iNKT cell co-cultures with CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B-cells, CD1c<sup>+</sup> mDCs, and CD1c<sup>+</sup> mDCs + *A. fumigatus* germ tubes**

Graph shows concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-5, IL-10, IL-13, IL-8, RANTES, G-CSF and GM-CSF in culture supernatants. iNKT cells were co-cultured with CD14<sup>+</sup> monocytes, CD19<sup>+</sup> B cells or CD1c<sup>+</sup> mDCs. iNKT cell co-culture with CD1c<sup>+</sup> mDCs was performed with and without *A. fumigatus* germ tubes. Supernatants were collected after 16 h of co-culture and cytokine concentrations were determined by ELISA. No significant cytokine release was observed in co-cultures of iNKT cells and monocytes or iNKT cells and B cells. In iNKT cell co-culture with CD1c<sup>+</sup> mDCs, production of IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-5, IL-10, IL-13, IL-8, RANTES, G-CSF and GM-CSF was observed. When *A. fumigatus* germ tubes were added to iNKT / CD1c<sup>+</sup> mDC co-culture, production of TNF- $\alpha$ , G-CSF and RANTES was reduced. Mean and SEM of five independent donors are shown. Significant differences are indicated by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and NS (not significant).

## 5 Discussion

### 5.1 Experimental setup

A major challenge when working with human iNKT cells is their very small population size of 0.01 to 0.5 % in human peripheral blood [67], requiring iNKT cell expansion prior to co-culture experiments. An existing protocol to expand iNKT cells was altered as described in the results section.

Since isolation of iNKT cells with magnetic isolation procedures failed, contaminating T cells could not be removed from iNKT cell culture prior to co-culture experiments. A new CD3<sup>+</sup>CD56<sup>+</sup> cell isolation kit might be more successful than the Anti iNKT Microbeads (both Miltenyi Biotec GmbH) tried in this work. But, purification of iNKT cells with iTCR specific reagents, like Anti iNKT Microbeads, is critical since it may cause iNKT cell activation and falsify experiments [68].

Protocols for iNKT cell culture and expansions have been published [67-69] and state FACS sorting to be the ideal method to isolate iNKT cells. However, this method was not available for this study. Apart from FACS sorting, other studies with human iNKT cells used iNKT cell lines [59, 70].

In this study, work was continued with expanded iNKT cells from peripheral blood of individual donors and contaminating T cells need to be considered when interpreting this study's results. Gating on live, CD3<sup>+</sup>iNKT<sup>+</sup> cells during analysis of CD69 expression on iNKT cells allowed specific evaluation of CD69 regulation on iNKT cells exclusively.

In pre-experiments, intracellular FACS staining was performed to measure intracellular IFN- $\gamma$  production. Cells were pre-treated with Brefeldin A to prohibit release of cytokines followed by a fixation and permeabilization treatment to allow intracellular staining. The cytotoxic effects of this procedure further reduced already low numbers of live iNKT cells for flow cytometric analysis. Thus, intracellular FACS staining was discontinued. In consequence, cytokine production was analyzed by ELISA, using co-culture supernatants, so T-cell contamination needs to be considered. However, a key feature of iNKT cells is their ability to rapidly secrete cytokines shortly after antigenic stimulation, in contrast to naïve T cells which undergo a phase of proliferation before acquiring their ability to secrete cytokines [71]. Thus, the incubation period of 16 h was

in favor of rather measuring the response of iNKT cells as opposed to an eventual T cell response with a later onset.

## **5.2 No direct interaction between *A. fumigatus* and iNKT cells**

To begin with, a direct interaction of iNKT cells with *A. fumigatus* germ tubes and conidia was excluded. This finding was not surprising, considering the well-established knowledge about the essential role of CD1d<sup>+</sup> APCs during iNKT cell activation. The stimulation of iNKT cells through IL-2 and IL-12 constituted a positive control to show responsiveness of expanded iNKT cells.

## **5.3 CD1d<sup>+</sup> cells stimulate iNKT cells**

Co-culture of iNKT cells with CD1d<sup>+</sup> cells revealed an unequal potential for the different CD1d<sup>+</sup> subpopulations to activate iNKT cells.

The results showed that while the three main subpopulations of CD1d<sup>+</sup> cells all induced a stimulation of iNKT cells, it was the CD1c<sup>+</sup> mDC subpopulation which showed the highest potential to activate iNKT cells on a per-cell basis. Considering the different cell numbers of different APCs in peripheral blood, a projection to the total potential of an entire cell population is not feasible at this point.

The stimulating potential of different CD1d<sup>+</sup> cells differed between the individual subpopulations with CD1c<sup>+</sup> mDCs having the strongest effect followed by monocytes and B cells. 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 by iNKT cells. In contrast, co-culture with CD1c<sup>+</sup> mDCs resulted in augmented secretion of Th1 cytokines like IFN- $\gamma$  and TNF- $\alpha$  as well as various Th2 cytokines, growth factors and chemokines.

### **5.3.1 CD1d surface expression: a predictor of excitatory potential**

Differences in CD1d expression on the surface of different subpopulations may explain the dominating effect of CD1c<sup>+</sup> mDCs on iNKT cells. On CD1c<sup>+</sup> mDCs, surface expression of CD1d was significantly higher than on monocytes and B cells. This entails a higher potential for interaction with iNKT cells which may be one reason for the greater impact of CD1c<sup>+</sup> mDCs on iNKT cells compared to monocytes and B-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 [72, 73]. Furthermore, co-expression of CD1c on CD1d<sup>+</sup> APCs has been demonstrated to enhance the stimulation of iNKT cells by  $\alpha$ -GalCer [73]. Concluding, the higher stimulating effect of CD1c<sup>+</sup> mDCs in this study might result from a direct interaction or co-stimulating effect of CD1c molecules and iNKT cells. A role of cytokine secretion, further co-stimulatory molecules, or other factors cannot be excluded at this point.

On B cells, CD1d expression varies on different B cell types with lowest CD1d levels on memory B cells and highest on marginal zone-like B cells. Activation of B cells results in downregulation of CD1d expression, thus leaving only a short time frame for B cells to interact with NKT cells via CD1d after B cell activation [70].

CD1c expression on B cells shows similar regulation [74]. Marginal zone-like B cells show highest levels of CD1c expression amongst B cells. Memory B cells, in parallel to their low CD1d expression, show lowest CD1c levels, whereas naïve B cells show high CD1d but low CD1c levels [70]. However, in this study, B cell subtypes were not differentiated and experiments were performed with the entire CD19<sup>+</sup> isolated B cell population from peripheral blood.

This variation in CD1d expression does not exist for monocytes. Freshly isolated CD14<sup>+</sup> monocytes from peripheral blood constitutively express CD1d with no upregulation after stimulation [74].

### **5.3.2 iNKT cells co-cultured with CD1d<sup>+</sup> cells produce Th1 and Th2 cytokines**

Co-culture of expanded iNKT cells with monocytes or B cells showed CD69 upregulation on iNKT cells but no significant cytokine production. The weak stimulus that monocytes and B cells displayed towards iNKT cells, which was reflected by a comparatively low upregulation of CD69 on iNKT cells, seemed insufficient to cause cytokine secretion in iNKT cell co-culture.

However, in co-cultures of iNKT cells with CD1c<sup>+</sup> mDCs, Th1 cytokines like IFN- $\gamma$  and TNF- $\alpha$  as well as various Th2 cytokines, growth factors and chemokines were released. Although Th1 as well as Th2 cytokines were produced, the considerably higher



concentrations of IFN- $\gamma$  and TNF- $\alpha$  imply a predominance of Th1 cytokines in the given context.

Since cytokine production was analyzed in co-culture supernatants, the detected cytokines cannot exclusively be ascribed to the iNKT cells but might also come from APCs. Interpretation of the study's results is still valid, as it is the bidirectional interaction of iNKT cells with APCs which needs to be considered when evaluating the interplay and response of these cells during *A. fumigatus* infection. In this study, cytokine concentrations in co-cultures were compared to cell cultures of iNKT cells alone. For further experiments, an additional comparison with cell culture of CD1c<sup>+</sup> mDCs alone would increase the result's value. Due to low outcome of CD1c<sup>+</sup> mDCs after isolation, this second negative control was not realizable in this study.

A state of low activation through CD1d interaction in the absence of foreign stimuli is reflected in these results. Also, the ability of the expanded iNKT cells to produce Th1 and Th2 cytokines at the same time is demonstrated, a particular feature of iNKT cells previously described in the literature [17, 71].

Given the results from flow cytometry, where an effect of *A. fumigatus* was only seen in iNKT cell co-culture with CD1c<sup>+</sup> mDCs, analysis of cytokine production in the presence of fungus was limited to co-cultures with CD1c<sup>+</sup> mDCs. Consistent with the results from flow cytometry, where CD69 on iNKT cells was shown to be downregulated in this co-culture setting, supernatant analysis showed a reduction of the pro-inflammatory cytokine TNF- $\alpha$ , the growth factor G-CSF, and the chemokine RANTES.

Thus, cytokine analysis confirmed the interaction of iNKT cells with CD1c<sup>+</sup> mDCs and strengthened the results from flow cytometry, suggesting a CD1c mediated immune-regulating function of iNKT cells during fungal infection.

#### **5.4 Human iNKT cell response to *A. fumigatus* infection**

As to the role of iNKT cells in infection with *A. fumigatus*, this study suggests an impact of *A. fumigatus* on human iNKT cells which is restricted to the presence of CD1c<sup>+</sup> mDCs. This effect of fungal germlings and conidia on iNKT cells was inhibitory, relating to decreased CD69 surface expression on iNKT cells and reduction of TNF- $\alpha$ , G-CSF and RANTES concentrations in co-culture supernatants. At the same time, secretion of IFN- $\gamma$

and Th2 cytokines did not change in iNKT co-cultures with CD1c<sup>+</sup> mDCs and *A. fumigatus* germ tubes.

TNF- $\alpha$ , along with IFN- $\gamma$ , was ascribed a protective role in *A. fumigatus* infection [75], and neutralization of TNF- $\alpha$  by specific antibodies in a mouse model was shown to reduce neutrophil numbers in the lung while mortality increased [76]. In parallel, G-CSF is an inducer of granulopoiesis and enhances the oxidative burst of neutrophils, thus increasing the damage of *A. fumigatus* hyphae caused by neutrophils [77]. The inhibited secretion of TNF- $\alpha$  and G-CSF by iNKT cells in the presence of *A. fumigatus* germ tubes may reflect an immune-regulatory function of iNKT cells during infection with *A. fumigatus*.

While neutrophils are essential for clearance of fungal hyphae from the lung, their aggressive response is cause for inflammatory tissue damages during fungal infection which, when exacerbated, can be fatal [40]. Hence, a fine tuning of immune responses against *A. fumigatus* is pivotal for favorable outcomes of invasive aspergillosis. The immune-regulatory function of iNKT cells in response to *A. fumigatus* described in this study may relate to an inhibition of neutrophil recruitment and functionality, which is, however, a highly speculative hypothesis at this point.

#### **5.4.1 The role of different DC subtypes during *A. fumigatus* infection**

Human CD11c<sup>+</sup> myeloid DCs can be subdivided into CD1c (BDCA-1)<sup>+</sup>, CD141 (BDCA-3)<sup>+</sup> and CD16<sup>+</sup> subsets [78]. They have been described to internalize *A. fumigatus* conidia as well as hyphae and to respond with upregulated expression of co-stimulatory surface molecules and IL-12p70 as well as IL-10 production [79] which is a disagreement to the results of this study. However, there are no investigations explicitly addressing the CD1c (BDCA-1)<sup>+</sup> cell subset. An immunoregulatory phenotype and function of CD1c<sup>+</sup> mDCs, as this study proposes for the interplay of iNKT cells and CD1c<sup>+</sup> mDCs during *A. fumigatus* infection, has been described in response to *Escherichia coli* [80].

Analysis of cytokine concentrations in culture supernatants did not detect considerable amounts of IL-12, although it is well known that cytokines like IL-12 and IL-18 play a major role in iNKT cell activation [24]. An essential role of IL-12 has also been described in the activation of iNKT cells during *A. fumigatus* infection [58]. Freshly isolated CD1c<sup>+</sup>

mDCs, as used in our experiments, have previously been ascribed a minimal to low capacity to produce IL-12 [81-83], as well as IL-18 [82]. However, following DC maturation during 24 to 48 h of stimulatory *in vitro* cultivation, CD1c<sup>+</sup> mDCs acquired the ability to produce IL-12 upon antigenic stimulation, even though produced amounts were still lower than those of other DC subsets, such as monocyte derived DCs [82, 84]. This low capability of immature mDCs to produce the Th1 cytokine 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.

### **5.5 Results in the context of existing studies**

The current knowledge about the iNKT cell function during *A. fumigatus* infection originates from studies in mice.

Albacker *et al.* described direct stimulation of iNKT cells through a fungal lipid antigen presented by CD1d [59]. In contrast, Cohen *et al.* suggested indirect stimulation of iNKT cells in a CD1d and cytokine dependent manner leading to a Th1 polarized cytokine response [58]. In Albacker's study, the response of human iNKT cells towards *A. fumigatus* seemed to be Th2 polarized.

The results of this study with human iNKT cells from peripheral blood showed decreased cytokine secretion in response to *A. fumigatus*, in particular a decrease of the Th1 cytokine TNF- $\alpha$ . There was no increased IFN- $\gamma$  production detected, which is consistent with results from human iNKT cell line experiments by Albacker *et al.* and contrasts findings from murine studies [58, 59]. Even though coming from different experimental results, Albacker *et al.* as well as this study concluded an immune-modulating function of iNKT cells during *A. fumigatus* infection.

The differing outcomes of the presented studies reflect the challenge of working with iNKT cells and the complexity of iNKT cell function.

The fundamental difference to be considered when comparing the existing data with this study is interspecies variability, a topic which is addressed in a separate section. Further differences to take into account are the source and treatment of cells, as cytokine treated bone marrow derived DCs and iNKT cell lines were used in murine studies, while this study worked with untreated cells from peripheral blood.

Besides, pre-treatment of the fungus may cause variation of antigenic properties. Cohen *et al.* used live conidia for *in vivo* experiments and heat-killed *A. fumigatus* hyphae for *in vitro* experiments, Albacker *et al.* worked with *A. fumigatus* extracts and the experiments in this study were conducted with ethanol inactivated *A. fumigatus* conidia and germ tubes. A change in fungal morphotype comes along with a change in antigen display and inactivation procedures may cause further alterations.

#### **5.5.1 Limitations in the comparison of murine and human data**

Murine immune cells differ in their number and function from human immune cell populations, especially neutrophils which compose 50 to 70 % of peripheral blood cells in humans, compared to 10 to 25 % in mice [85]. Neutrophils play an essential role in the first line defense against *A. fumigatus* [43, 86, 87] and neutropenia is one of the most important risk factors for IA. Besides, the habitat of mice near the ground brings forth a permanent inhalation of soil and fungal spores, hence a much higher antigen exposition for mice compared to humans. Indicating the relevance of the different habitats between mice and humans, bronchus-associated lymphoid tissue is found in mice but is absent in humans [88].

Furthermore, interspecies differences of APCs, which play an essential role in iNKT cell activation, need to be considered. While humans express five different CD1 genes corresponding to five different CD1 proteins, CD1a, CD1b, CD1c, CD1d, and CD1e, mice only express one CD1 protein which is an ortholog to CD1d [71, 89]. 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 [72, 73].

Taking these aspects, differences in human and murine immune response to fungi appear most likely.

#### **5.6 Outlook**

This study is one of the first to focus on the response of human iNKT cells towards *A. fumigatus*. The results indicate an immune-regulating function for iNKT cells during fungal infection with *A. fumigatus*. Further research is essential to confirm these findings and to ascertain the complex behavior of iNKT cells and interacting APCs in this infectious context.

This study showed an effect of *A. fumigatus* on iNKT cells that was restricted to the presence of CD1c<sup>+</sup> mDCs. In order to confirm that this effect is dependent on the CD1c molecule itself, experiments with CD1c blocking antibodies will be revealing.

Further experiments with mature CD1c<sup>+</sup> mDCs will be essential to fully understand the potential of human mDCs to interact with iNKT cells during *A. fumigatus* infection. A stimulatory effect of mature CD1c<sup>+</sup> mDCs on iNKT cells would consolidate the essential role of IL-12 for iNKT cell response towards *A. fumigatus*. Moreover, the investigation of other DC subsets in this context will be an interesting topic for further research. Especially monocyte derived DCs appear promising for interesting results as they are known to phagocytose *A. fumigatus* conidia [43], express CD1d [90, 91], and are potent producers of IL-12 [80, 82].

The experiments of this study were all performed with an incubation time of 16 h and an MOI of 2. It may be that the single concentration and single time did not produce the maximum response. Analysis of co-cultures at multiple times would consolidate the findings of this study. Also, other effects might be seen at different times or different concentrations.

Great potential remains in the investigation of human iNKT cell function in *A. fumigatus* infection.

## 6 Conclusion

*Aspergillus* species are a ubiquitous genus of mold with the clinically most important species *A. fumigatus* which is responsible for the majority of cases of invasive aspergillosis [33]. Taking the high mortality rate of immune-suppressed patients with invasive aspergillosis, further characterization of distinct mechanisms of the innate and adaptive immune responses are essential to develop new prevention strategies and treatment options.

As to our knowledge, this study is the first to focus on the interaction of human iNKT cells with *A. fumigatus*, an up to date missing aspect in the understanding of host immunity against *A. fumigatus*. The results show that there is no direct interaction between *A. fumigatus* and human iNKT cells.

While the three main subpopulations of CD1d<sup>+</sup> cells all induced a stimulation of iNKT cells, it is the CD1c<sup>+</sup> mDC subpopulation which was primarily responsible for the activating effect of CD1d<sup>+</sup> cells on iNKT cells. Co-culture of iNKT cells with CD1c<sup>+</sup> mDCs resulted in augmented secretion of Th1 cytokines like IFN- $\gamma$  and TNF- $\alpha$  as well as various Th2 cytokines, growth factors and chemokines, with a predominance for Th1 cytokines. Furthermore, this study provides first insights into the impact peripheral blood CD1d<sup>+</sup> cells have on iNKT cells in the presence of *A. fumigatus* conidia and germ tubes. We show that, in this infectious context, the stimulating effect of peripheral blood CD1c<sup>+</sup> mDCs on iNKT cells is decreased, including lower concentrations of pro-inflammatory cytokines (TNF- $\alpha$ ), growth factors (G-CSF) and chemokines (RANTES) in culture supernatants. This inhibition of iNKT cells by *A. fumigatus* was restricted to CD1c<sup>+</sup> mDCs and did neither occur when iNKT cells were directly exposed to *A. fumigatus* nor in iNKT cell co-cultures with monocytes or B-cells.

Concluding, this study proposes a CD1c mediated immune-modulating role of human iNKT cells during infection with *A. fumigatus*. Thus, iNKT cells may act as an important counterplayer to pro-inflammatory fungicidal responses and contribute to establish a balanced immune response towards *A. fumigatus*, preventing excessive and possibly fatal tissue destruction. To fully understand the complex responses of iNKT cells during fungal infection in humans, further investigations will be needed.

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## 9 List of abbreviations

ABPA	Allergic Bronchopulmonary aspergillosis
<i>A. f.</i>	<i>Aspergillus fumigatus</i>
$\alpha$ -GalCer	$\alpha$ -Galactosyl-Ceramide
AM	alveolar macrophages
APC	antigen presenting cell
CD	cluster of differentiation
DHN	dihydroxynaphtalene
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FCS	fetal calve serum
FGF	fibroblast growth factor
FSC	forward scatter
G-CSF	granulocyte colony stimulating factor
geo MFI	geometric mean fluorescence intensity
GM-CSF	granulocyte macrophage colony stimulating factor
GvHD	graft versus host disease
h	hours
IA	invasive aspergillosis
IFN- $\gamma$	interferon $\gamma$
Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T
IU	international units
mDC	myeloid dendritic cells
MHC	major histocompatibility complex

min	minutes
mRNA	messenger ribonucleic acid
NET	neutrophil extracellular traps
NK cell	natural killer cell
NKT cell	natural killer T cell
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PRRs	pattern recognition receptor
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
rpm	revolutions per minute
SEM	standard error of the mean
SSC	side scatter
TCR	T cell receptor
Th cell	T helper cell
TLR	toll like receptor
TNF- $\alpha$	tumor necrosis factor $\alpha$
VEGF	vascular endothelial growth factor

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