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Immune mechanisms in resistance of immunocompetent Dark Agouti and Albino Oxford rats to pulmonary infection with fungi *Aspergillus fumigatus* Fresen.

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Imunski mehanizmi rezistencije imunokompetentnih jedinki Dark Agouti i Albino Oxford pacova na pulmonarnu infekciju izazvanu gljivom *Aspergillus fumigatus* Fresen.

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Defense date .....

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ABSTRACT

Although rat models of pulmonary aspergillosis are used to same extent as mouse models in exploring antifungal therapeutics, the understanding of immunology of *Aspergillus* infection in rats is deficient. In this study pulmonary immune responses to sublethal *A. fumigatus* infection were investigated in two rat strains, Albino Oxford (AO) and Dark Agouti (DA), differently susceptible to some inflammatory diseases.

Slower elimination of fungus following intratracheal (i.t.) application of 10^7 conidia was noted in AO rats (by the day 21 post infection, compared to day 14 in DA rats). In both rat strains elimination of fungi occurs in similar environment: enriched in proinflammatory cytokines interferon-γ (IFN-γ) (level of IFN- γ was slightly higher in infected DA rats at day 3 p.i.) and interleukin-17 (IL-17) and unchanged Th2/anti-inflammatory cytokines (IL-4 and IL-10). Although proinflammatory cytokines were present at similar amount in lung tissue homogenates, in AO rats elimination of fungi from lungs was associated with lower degree of lung leukocyte infiltration and their basic effector activities (i.e. CD11b expression, adhesion, production of reactive oxygen species, intracellular myeloperoxidase content). Generally, similar levels of effector cell-stimulating proinflammatory cytokines IFN-γ and IL -17 production by lung leukocytes during the entire post-infection period (except late in infection) were noted in both strains. In contrast, production of IL-10 was found to be significantly higher in AO rats. In draining lymph nodes (as sites of generation of cytokine-producing cells) cells differentiate toward IFN-γ-producing cells (higher amount of this cytokine detected in infected AO compared to DA rats), IL-17-producing cells (levels of cytokine produced lower in AO compared to DA rats) and IL-10 producing cells (similar levels in both strains). Unchanged (and even decreased antigen specific) IL-4 production was noted as well. $CD4^+$ cells differentiated in draining lymph nodes are the main source of IFN-γ, IL-17 and IL-10. These cells following differentiation migrate to the lung tissue, judging by increased number of $CD4^+$ cells in lung tissue digest and BAL cells.

The knowledge of mechanisms of defense against *A. fumigatus* in rats might be helpful in the future use of rat models of pulmonary aspergillosis in developing and testing immunotherapeutic interventions of fungal diseases.

Keywords:
Aspergillus fumigatus, Pulmonary infection, AO and DA rats, IFN-γ, IL-17, IL-4, IL-10, lung leukocytes effector activities

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Imunski mehanizmi rezistencije imunokompetentnih jedinki Dark Agouti i Albino Oxford pacova na pulmonarnu infekciju izazvanu gljivom *Aspergillus Fumigatus* Fresen.

SAŽETAK

Iako se pacovski model pulmonarne aspergiloze koristi podjednako kao i mišiji modeli u ispitivanju antigljivičnih terapija, slabije su ispitane karakteristike imunskog odgovora na infekciju izazvanu gljivom roda *Aspergillus* kod pacova. U ovom radu ispitan je imunski odgovor u plućima na subletalnu infekciju izazvanu gljivom A. fumigatus kod dva soja pacova, Albino Oxford (AO) i Dark Agouti (DA), za koje je poznato da razvijaju kvalitativno različit odgovor u nekim inflamatornim oboljenjima.

Sporije uklanjanje gljive nakon intratrahealnog (i.t.) ubrizgavanja 10^7 konidija zapaženo je kod jedinki AO soja (do 21. dana nakon infekcije u poređunju sa 14 dana kod DA pacova). Kod oba soja uklanjanje gljive iz pluća se odigrava u sličnoj sredini: povišeni nivoi proinflamatornih citokina interferona -γ (IFN-γ) (nivo IFN-γ je blago viši kod inficiranih jedinki DA soja trećeg dana nakon infekcije) i interleukina-17 (IL-17) dok je nivo Th2/antiinflamatornih citokina (IL-4 i IL-10) bio nepromenjen. Iako je sadržaj proinflamatornih citokina u homogenatu pluća sličan kod oba soja, kod jedinki AO soja uklanjanje gljive iz pluća je povezano sa manjim stepenom infiltracije leukocita u tkivo i nižim osnovnim efektorskim aktivnostima ćelija (ekspresija CD11b molekula, adhezija, produkcija reaktivnih vrsta kiseonika i aktivnost unutarćelijske mijeloperoksidaze). Leukociti izolovani iz pluća jedinki oba soja produkovali su slične količine citokina koji stimulišu efektorske aktivnosti ćelija, IFN-γ i IL-17, u toku infekcije (izuzev u kasnijim terminima nakon infekcije). Nasuprot tome, količina produkovanog IL-10 je bila značajno viša u kulturi leukocita pluća izolovanih iz jedinki AO soja. U regionalnim limfnim čvorovima (mestima gde se nastaju ćelije koje produkuju citokine) zapaža se diferencijacija ćelija u smeru produkcije IFN-γ (veće količine u kulturi ćelija jedinki AO soja), IL-17 (niže količine kod jedinki AO soja) i IL-10. Nepromenjena (smanjena antigen specifična) produkcija IL-4 je takođe zapažena. $CD4^+$ ćelije u regionalnom čvoru su glavni producenti IFN-γ, IL-17 i IL-10. Ove ćelije

nakon diferentovanja migriraju u pluća sudeći po povećanju broja $CD4^+$ ćelija u digestu pluća i bronholaveolarnom ispirku.

Poznavanje mehanizama odbrane u infekciji izazvanoj gljivom *A. fumigatus* kod pacova može da doprinese upotrebi pacovskog modela pulmonarne aspergiloze u razvijanju i testiranju novih terapeutskih modaliteta u tretmanu infekcija izazvanih gljivama.

Ključne reči:

Aspergillus fumigatus, plućna infekcija, AO i DA pacovi, IFN-γ, IL-17, IL-4, IL-10, efektorske aktivnosti ćelija pluća

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
AO	Albino Oxford
BMT	Blood And Marrow Transplantation
CD	Cluster of Differentiation
$CD4^+$ T cells	Helper T lymphocyte
$CD8^+$ T cells	Cytolytic T lymphocyte
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Units
CGD	Chronic Granulatomous Disease
CNPA	Chronic Necrotizing Aspergillosis
COPD	Chronic Obstructive Pulmonary Disease
CR	Complement Receptor
DA	Dark Agouti
DC	Dendritic Cells
dLN	Draining lymph node
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FcR	Fc Receptor
GAS	Gamma-Activated Sequences
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HOCl	Hypochloric Acid
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
IA	Invasive Aspergillosis
IFN-γ	Interferon-γ
IFN-γ-R	Interferon- γ Receptor

Ig	Immunoglobulin
IL	Interleukin
IPA	Invasive Pulmonary Aspergillosis
mAb	Monoclonal Antibody
MAC	Membrane-Attack Complex
MIP-1β (CCL3)	Macrophage Inflammatory Protein 1 beta (CCL3)
MMLV	Moloney Murine Leukemia Virus
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBT	Nitroblue tetrazolium
NETs	Neutrophil Extracellular Traps
NFκB	Nuclear Factor κB
NK cells	Natural Killer cells
NO	Nitric Oxide
p.i.	post infection
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Neutrophils
PMSF	Phenylmethanesulfonyl Fluoride
PRR	Pattern Recognition Receptors
rhSP-D	Recombinant Human Surfactant Proteins
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
SP	Surfactant Protein
TGF-β	Transforming Growth Factor-β
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α
VEGF	Vascular Endothelial Growth Factor

CONTENTS

1. INTRODUCTION

1. Introduction

1.1. Opportunistic fungi

Among described 100,000 species of fungi (Fungi), only a few hundred cause diseases to mammals (Kwon - Chung and Bennett, 1992). The reason for this is the fact that the optimum temperature for growth of most fungi is 25°C and that they can tolerate temperatures ranging from 12 to 30°C (Robert and Casadevall, 2009).

Pathogenic species cause diseases in people with the integrated function of the immune system (immunocompetent organisms). In contrast, opportunistic fungi cause infections in immunocompromised individuals (individuals with weakened or suppressed immune function). The opportunistic species are species of genus *Candida, Aspergillus, Fusarium, Paecilomyces, Trichoderma, Cryptococcus, Mucor, Rhizopus, Alternaria, Saccharomyces* and others (Marr *et al*., 2002; Pappas *et al*., 2003; Hajjeh *et al*., 2004; 2003; Pfaller and Diekema, 2004). Opportunistic fungal infections are associated with excessive morbidity and mortality in susceptible host (Gudlaugsson *et al*., 2003; Lin *et al*., 2001), which includes individuals undergoing solid-organ transplantation, blood and marrow transplantation (BMT), haematological malignancies, particularly hematopoietic stem cell transplantation, leukemia or lymphoma. Prevalence of infections caused by opportunistic fungi increased significantly over the past two decades (Hajjeh *et al*., 2004; Pfaller and Diekema, 2004).

1.2. *Aspergillus fumigatus* (Fresenius, 1863)

Aspergillus fumigatus belongs to the phylum Ascomycota, class Ascomycetes and order Eurotiales. Genus *Aspergillus* (family Trichocomaceae) comprises more than 185 species with a cosmopolitan distribution. A small number of species *(A. fumigatus, A. flavus, A. niger, A. terreus* and *A. nidulans*) causes disease in humans (Denning, 1998; Latge, 1999), with *A. fumigatus* as the most common cause of infection.

Aspergillus fumigatus spores are ubiquitous. However, the organism is more likely to be found in the northern hemisphere during the fall and winter months or in the tropics all year round. Specifically, *A. fumigatus* can be found in soil and soil components (natural habitat) as well as in stored grain, decaying vegetation, or indoor air

environments. This fungus is most commonly found in areas with high temperatures (40^{o}C or higher), high levels of oxygen, and places with reduced water availability. *Aspergillus fumigatus* is a thermotolerant organism: its temperature optimum ranges from 37°C to 42°C (but it can grow at up to 55°C) and thereby approximations the upper temperature limit of eukaryotic organisms. This suggests that *A. fumigatus* evolved distinct mechanisms of stress resistance that might provide the basis of its virulence (Chang *et al*., 2004).

The life cycle of *A. fumigatus* starts with the asexual production of conidia that measure 2-3 μm in diameter and are easily dispersed by air thus contributing to the ubiquitous nature of *A. fumigatus* (Morris *et al*., 2000). The surface of *A. fumigatus* conidia is covered with hydrophobic rodlet (RodAp and RodBp) proteins that increase the buoyancy and distribution of conidia by air currents (Paris *et al*., 2003). Conidia may cut stillness and germinate upon exposure to appropriate nutrients. *A. fumigatus* conidia switch from isotropic to polar growth concurrent with the emergence of germ tubes and septation with mitotic division (Momany and Taylor, 2000).

A. fumigatus produces a variety of secondary metabolites, including gliotoxin (Kosalec and Pepeljnjak, 2005), which has received considerable attention for over 2 decades as a putative virulence factor (Lewis *et al*., 2005). Gliotoxin has been shown to inhibit antigen-mediated lymphocyte stimulation and cytotoxic-T-cell activation *in vitro* (Waring *et al*., 1988), interferon-γ (IFN- γ) production by $CD4^{+}$ lymphocytes (Wichmann *et al*., 2002), tumor necrosis factor (TNF)-α-inducible nuclear binding of NFκB but not constitutively activated NFκB in activated HSCs (Wright, 2001), and promotes apoptosis in professional immune cells such as macrophages and monocytes (Stanzani *et al*., 2005). Further more, this mycotoxin inhibits phagocytosis (Eichner *et al*., 1986) and blocks mast cell degranulation (Niide *et al*., 2006).

Aspergillus fumigatus causes invasive disease in severely immunocompromised patients (Fraser *et al*., 1992).

1.3. Diseases caused by *Aspergillus fumigatus*

There are several form of diseases caused by *A. fumigatus*, including saprophytic colonization (aspergilloma), hypersensitivity pneumonitis (Yocum *et al*., 1976), and

necrotizing pneumonia with angioinvasion (invasive pulmonary aspergillosis) (Gefter, 1992). The disease severity depends on the host's immune status and the presence of essential structural changes in the lungs (Fraser *et al*., 1992).

Pulmonary aspergilloma is a potentially life-threatening disease resulting from the colonization of lung cavities by *Aspergillus fumigatus*. Preexisting cavities, cysts, and other air-contamining spaces predispose the individual to this superinfection (cavities of prior tuberculous infections are the most common spaces, followed by cysts and cavities from sarcoidosis, chronic fungal infections, bronchiectasis, bullae, sites of prior surgery such as lobectomy and pneumonectomies, pulmonary abscesses, and bronchial cysts) (Fraser *et al*., 1989). Aspergillomas consist of intraluminal masses of hyphae, blood clot, and cellular debris. The cavity may be lined by epithelium that often includes foci of squamous metaplasia.

This fungus is causing the group of diseases that are collectively referred as **invasive pulmonary aspergillosis** (IPA) which is evident as a necrotizing pneumonia, hemorrhagic pulmonary infarction, abscess (Bodey and Vartivarian, 1989), or infrequently as a membranous tracheoobronchitis (Clarke *et al*., 1991). Because IPA is angioinvasive, its presentation may simulate thromboembolic disease. The reported mortality varies from 30% to 90%. The fungal hyphae have a propensity to invade blood vessels and to "metastasize" (disseminate by blood stream) to other organs. *Aspergillus spp* can seed abnormal cardiac valves to produce endocarditis, and dissemination to the brain may lead to fatal hemorrhagic infarctions (Richard *et al*., 2008). The thyroid is frequently involved at autopsy as well (Richard *et al*., 2008). Acute invasive pulmonary aspergillosis (IPA) typically occurs in patients with hematologic malignancies, who are treated with intensive cytostatic therapy and have severe neutropenia for prolonged periods of time (Walsh, 1990). IPA has increased in frequency in these patients and can be found in 15 to 40% of patients who die of acute leukemia (Walsh, 1990). Chronic necrotizing aspergillosis (CNPA) is a rare form of pulmonary aspergillosis. That is an indolent focal process caused by the superficial invasion of the lung parenchyma (Gefte, 1992). Necrotizing granulomatous pneumonia is characterized by zones of copulative necrosis with *Aspergillus* invading blood vessels. Surrounding parenchyma is

consolidated by chronic inflammatory cells and fibrosis with scattered giant cells and granulomas with necrotic centers.

The important risk factors for development of invasive aspergillosis include neutropenia, defective neutrophil function, the use of corticosteroids or other immunosuppressive therapies, such as those used to prevent rejection following organ transplantation, late stage human immunodeficiency virus infection (Singh *et al*., 2001), major surgery, neoplastic disease, advanced age, and premature birth (Hajjeh *et al*., 2004).

1.4. Animal models of *A. fumigatus* infection

Animal models of aspergillosis have been used extensively to study various aspects of pathogenesis, innate and adaptive host response, disease transmission and therapy. Originally, the animal models were developed to study the efficacy of antifungal drugs in the treatment of aspergillosis (Patterson *et al*., 1993) or to evaluate diagnostic methods (Patterson *et al*., 1988). Several different animal models of aspergillosis have been developed.

Invasive pulmonary aspergillosis has been established in mice, rabbits, rats, guinea pigs, birds, cows, and monkeys (Polak**,** 1998). Studies done to simulate human disease have relied on models using common laboratory animal species. Guinea pig models have primarily been used in therapy studies of invasive pulmonary aspergillosis (IPA) (Kirkpatrick *et al*., 2002). Rabbits have been used to study IPA and systemic disease (Walsh *et al*., 2003). Birds are more susceptible to the disease compared to humans and mammals (Olias *et al*., 2010). Among the *Aspergillus* species, *Aspergillus fumigatus* accounts for over 95% of the cases of avian infections (Olias *et al*., 2010; Stephen *et al*., 2013). Rodent, particularly mouse, models of aspergillosis prevails as an option for most investigators. Mouse models have been used for a variety of studies on aspergillosis, including examination of the comparative virulence of different isolates of *Aspergillus*, investigation of genes engaged in virulence, comparative susceptibility of different animal strains to infection with *A. fumigatus*, and preclinical antifungal drug efficacy. Due to the different strains of mice available, investigators use both outbred and inbred animals for experiments. The susceptibility of these different strains seems to be

about the same, regardless of genetic background of the mice or of the model of aspergillosis being used (Williams and Drexler, 1981; Dixon *et al*., 1989). Mice with genetic immunological deficiency facilitate studies of the roles that cells, cytokines and chemokines play in host resistance to *Aspergillus.* Characteristics of immune response to *A. fumigatus* were mostly investigated in mice.

There are several routs of infection: inhalation, intranasal (*i.n.*), intratracheal (*i.t.*), intravenous (*i.*v.), intracranial and intraperitoneal (*i.p.*). For most patients the main portal of entry and site of infection with *A. fumigatus* is the respiratory tract. In animal models, infection of the respiratory tract can be evoked by inhalation (in chamber in which mice received aerosols of conidia), direct instilation of conidia in to the nostrils or in to the trachea (Tang *et al*., 1993). Following inhalation of conidia and invasion of the lungs, the fungus diffuses to other organs such as the brain, liver, kidneys and spleen. As in humans, disease develops only if the animal is immunosuppressed. The major deficiency of these models is the highly variable response of animals to a given inoculum and necessitating the use of large groups within each experiment. Inoculation via *i.v.* challenge incites a more uniform pattern of disease, but the mode of infection and the primary infected organ, i.e., kidneys and brain in nonpregnant animals and placenta in pregnant animals, are quite different from the human infection (Jensen *et al*., 1996).

Immunosuppressive treatments significantly increase the susceptibility of animals to pulmonary infection with *A. fumigatus*. Cortisone-acetate and cyclophosphamide are preferred for immunosuppression in IA in mice and in rabbits (Chilvers *et al*., 1989; Spreadbury *et al*., 1989). However, differences in the patterns of infection and inflammation in IA may be related to the type of immunosuppression used (Berenguer *et al*., 1995) as shown in rabbit model where more severe IA in animals suppressed with cytosine arabinoside was noted compared to animals suppressed with cyclosporin A in combination with methylprednisolone (Berenguer *et al*., 1995; Francis *et al*., 1994).

1.5. Immune response to *A. fumigatus* infection

Characteristics of immune response to *Aspergillus* are mostly investigated in mouse models of aspergillosis.

1.5.1. The innate immune response to *A. fumigatus*

The innate defense mechanisms play a major role in the clearance of conidia. Nonspecific immunity includes three major lines of defense: (i) anatomical barriers, (ii) humoral factors such as complement, and (iii) cells and their related antimicrobial products.

1.5.1.1. Anatomical barriers

The majority of the conidia of *A. fumigatus*, like most airborne particles, are excluded from the lungs through the ciliary action of the mucous epithelium. However, the clearance of *A. fumigatus* at this level may be less efficient than with other airborne saprophytic microorganisms, since *A. fumigatus* produce toxic molecules which can inhibit ciliary activity, as well as proteases which can damage the epithelial tissue (Amitani *et al*., 1995; Robinson *et al*., 1990). In addition, epithelial and endothelial cells have been shown to internalize conidia, serving as putative foci of infection (Paris *et al*., 1997; Taramelli *et al*., 1996). *Aspergillus fumigatus* might invade the bronchial mucosa by at least three pathways (Fig. 1): (1) penetration of hyphae through the intercellular spaces in the epithelium; (2) direct penetration of hyphae through epithelial cells; and (3) internalization of conidia within epithelial cells (Amitani and Kawanami, 2009). In animal models of aspergillosis penetration at the epithelial level is common (D'Enfert *et al*., 1996; De Repentigny and Reiss, 1984).

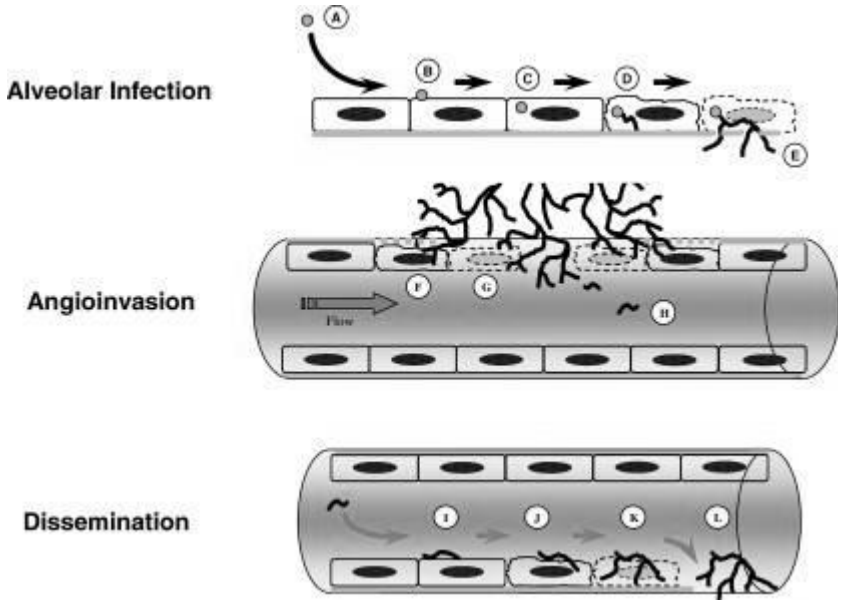

Fig. 1. The process of invasive aspergillosis involves three steps alveolar infection, angioinvasion and dissemination. (A) Alveolar infection includes adhesion of inhaled conidia (B) with pulmonary epithelial cells, (C) endocytosis within the epithelial cells, (D) germination of conidia and hyphal growth within the epithelial cells, and (E) hyphal extrusion from the epithelial cells. Angioinvasion involves (F) penetration of abluminal surface by emergent hyphae, (G) induced cell damage, and (H) spreading of hyphal fragments via blood stream. Finally in dissemination process (I) the hyphal fragments adhere to the cell surface, (J) cells invasion, (K) cell damage and (L) invasion of other deep organs (figure adapted from: Filler and Sheppard, 2006) .

Lung surfactant, produced by epithelial cells, plays a protective role against pathogens as well. Hydrophilic surfactant proteins A (SP-A) and D (SP-D) belong to a family of mammalian C-type lectins (collectins). These molecules are essentially synthesized and secreted into the airspace of the lungs (Crouch *et al*., 1991; Persson *et al*., 1998; Voorhout *et al*., 1992), and bind mannose and glucose residues, which are part of most microbial ligands. Protective role of these molecules in pulmonary aspergillosis has been shown in murine model following administration of human SP-A, SP-D and recombinant human (rh) SP-D (Madan *et al*., 1997a,b; 2001). It was shown that SP-A and SP-D enhance agglutination of *A. fumigatus* conidia as well as phagocytosis and killing of conidia by alveolar macrophages and neutrophils. SP-A and SP-D are also capable of

linking innate immunity with adaptive immunity that includes modulation of dendritic cell function and helper T cell polarization (Madan *et al*., 1997a,b; 2001).

Epithelial cells, beside lung surfactant proteins, produce a variety of antimicrobial molecules essential for anti-*Aspergillus* defence. Among them are lactoferrin (produced by neutrophils as well) and chitinases. Lactoferrin inhibits the growth of *A. fumigatus* by binding the iron which is essential for fungal growth (Zarember *et al*., 2007). Chitinases are endo-b-1,4-N-acetyl glucosaminidases capable of degrading chitin, a component of the cell wall of *A. fumigatus* (Chen *et al*., 2009).

1.5.1.2. Complement system

The complement system is a cascade of sequential activation steps of complement factors, with the final formation of the membrane-attack complex (MAC) that integrates into the membrane of the pathogen forming a pore and thus inducing osmotic lysis. Some studies reported that the absence of complement from the serum in the culture medium facilitated the destruction of spores by macrophages (Robertson *et al*., 1989) which indicate that complement is not important for anti-*Aspergillus* defenses. By contrast Hector *et al*. (Hector *et al*., 1990) showed the importance of complement in anti-*Aspergillus* defenses in an *in vivo* model of pulmonary aspergillosis in mice lacking C5 component of complement. These mice were found to be more susceptible to *A. fumigatus* infection (Hector *et al*., 1990). Activation of complement system occurs in three biochemical pathways: classical pathway, lectin pathway and alternative pathway. Whereas complement activation by resting conidia was mediated by the alternative pathway, there is a progressive involvement of the classical pathway when the conidia begin to swell and transform into hyphae indicating that the way of interaction of fungal particles with the complement system changes with the maturation of resting conidia into hyphae (Kozel *et al*., 1989). Deposition of C3 on the fungal surface results in the degradation of this molecule into bound C3b and CR3-binding molecule iC3b (Latge, 1992; Sturtevant and Latge, 1992a). C3b deposition on *Aspergillus* surface is a central aspect of the interaction between the fungus and the human host and a direct inverse correspondence between C3 deposition and virulence has been proven (Kozel *et al*., 1989; Sturtevant and Latge, 1992a). Opsonization of conidia is a required for the

phagocytosis by monocytes, bronchoalveolar macrophages and polymorphonuclear (PMN) cells (Sturtevant and Latge, 1992b; Bouchara *et al*., 1994).

Inhibition of activation of complement by *A. fumigatus* suggest that this fungus, by binding to factors regulating complement, may inactivate the complement system, thereby escaping from its microbicidal effects (Behnsen *et al*., 2008). *A. fumigatus* produces an inhibitor capable of decreasing the binding of C3b to its surface. This activity is linked to *A. fumigatus* phospholipids (Washburn *et al*., 1990). Furthermore, *A. fumigatus* secretes a soluble factor which inhibits complement activation and opsonization of fungal cells (Washburn *et al*., 1986, 1990).

1.5.1.3. Macrophages

Macrophages, as a first line of defense, have to be able to discriminate between self and foreign structures. Among the different receptors for microbial structure on the surface of macrophages (Pattern Recognition Receptors, PRR), in the response to *A. fumigatus* participate: Dectin-1 and the Toll-like receptors (TLR) 2 and 4. The inflammatory response triggered through TLR2 and TLR4 strongly depends on the *Aspergillus* morphotype. While conidia recognition is mediated by both TLR2 and TLR4, TLR4-mediated signaling is lost in recognition of hyphae (Netea *et al*., 2003). Protective role of dectin-1 was suggested in mice lacking this receptor since they are prone to *Aspergillus* infections (Werner *et al*., 2009). Dectin-1 recognize β-1,3-glucan on the surface of *A. fumigatus*, that triggers a strong inflammatory response and enhances phagocytosis by macrophages (Luther *et al*., 2007). A broader study of the role of TLR4 and TLR2, co-receptor CD14, as well as adapter molecule MyD88, in the activation of murine peritoneal macrophages demonstrated that TLR-2 and MyD88-mediated signals supply an additive contribution to macrophage activation by germinating conidia (Hohl *et al*., 2005). Binding of a pathogen associated molecular pattern (PAMP) to either dectin-1 or TLR activates NF-kB, and thus induce proinflammatory cytokine and chemokine production (Werner *et al*., 2009).

Furthermore, for appropriate activation of macrophages, signal delivered through IFN-γ receptor (IFN-γ-R) following IFN-γ binding is necessary (Huang *et al*., 1993). IFN-γ activates the transcription factors STAT1/2, which bind to gamma-activated

sequences (GAS) in various immune effector genes. These are followed closely by a significant change in cellular morphology and alteration in the secretory profile of the cell. Adversity of chemokines including IL8 (CXCL8), IP-10 (CXCL10), MIP-1β (CCL3), MIP-1β (CCL4), and RANTES (CCL5), are released as chemoattractants for neutrophils, immature dendritic cells, natural killer cells, and activated T cells (Luster, 2002). Additionally several pro-inflammatory cytokines are released, including IL-1β, IL-6 and TNF-α (Duffield, 2003; Gordon, 2003; Mosser, 2003).

The principal role of macrophages and neutrophils is the phagocytosis of microorganisms and the killing of these microorganisms in the phagocyte. Resident alveolar macrophages ingest inhaled conidia very rapidly, destroy them intracellularly through oxidative mechanisms and prevent germination to hyphae (Philippe *et al*., 2003; Aratani *et al*., 2002). Various microbicidal mechanisms come into play during phagocytosis, including oxidative mechanisms operating in the presence of oxygen and destroying microorganisms very efficiently. Role of oxidative mechanism in defense against *A. fumigatus* was suggested in patients with chronic granulatomous disease (CGD) which is characterized by the absence of NADPH oxidase from neutrophils and macrophages, as these patients are susceptible to infection with *A. fumigatus* (IPA is the leading cause of death in patients with chronic granulatomous disease) (Aratani *et al*., 2002). Oxidative mechanisms involve reactive oxygen species and reactive nitrogen species derived from nitric oxide (NO). The enzymes involved in the production of reactive oxygen species during phagocytosis include myeloperoxidase (MPO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, responsible for the production of hypochloric acid (HOCl) and superoxide anions, respectively. These enzymes are essential for the elimination of *A. fumigatus* (Aratani *et al*., 2002), as the absence of MPO and NADPH oxidase is associated with higher mortality rates in mice infected with *A. fumigatus* (Aratani *et al*., 2002). In corroboration, investigation on macrophages isolated from mice lacking NADPH oxidase (p47phox deficient mice) demonstrated that the fungicidal activities of macrophages are associated with the production of reactive oxygen species (Philippe *et al*., 2003).

1.5.1.4. Neutrophils

Polynumorphoclear neutrophils constitute the largest population of intravascular phagocytes and are essential for host defense against microbial infections. The vascular network of the lung, including the capillaries in particular, is a major reservoir of neutrophils, containing 40% of all the body's neutrophils (Reeves *et al*., 2002). During infection, these marginated neutrophils are rapidly recruited to the alveolar spaces to strengthen defenses. When polymorphonuclear neutrophils (PMN) are recruited to the site of infection, they can respond in three ways: phagocytosis, degranulation and production of ROS, or with the release of neutrophil extracellular traps (NETs) (Behnsen *et al*., 2007; Bruns *et al*., 2010).

Neutrophils play an essential role in the elimination of *A. fumigatus*. Indeed, patients with neutropenia have the highest risk of developing IPA. The ability of neutrophils to attack and kill *A. fumigatus* depends on recognition of fungi by TLR2, TLR4 and dectin-1 receptors (Werner *et al*., 2009). Increased expression of genes encoding membrane receptors TLR2, TLR4 and intracellular receptor TLR9 was noted in neutrophils isolated from healthy human and mice following stimulation with conidia and hyphae of *A. fumigates in vitro* (Bellocchio *et al*., 2004a,b). Components of the cell wall of conidia are recognized through TLR2, while recognizing of hyphae by neutrophils occurs *via* TLR4 (Bellocchio *et al*., 2004 a,b). Neutrophils also recognize the DNA of *A. fumigatus* through TLR9 (Bellocchio *et al.*, 2004a). Recognition of conidia and hyphae of *A. fumigatus* by neutrophils leads to a cascade of signal transduction in cells by phosphorylation of p38 MAP kinase which can ultimately activates transcription factor NF- kB and lead to the initiation of transcription of genes for cytokines (Bellocchio *et al*., 2004a).

Neutrophils release a variety of proteins by degranulation. Release of the contents of granules may be stimulated by *A. fumigatus* antigens (Braedel *et al.*, 2004). The recognition of germinated conidia in culture by human neutrophils results in the release of myeloperoxidase (MPO) from the primary granules of neutrophils and lactoferrin from secondary (Levitz and Farrell, 1990; Braedel *et al.*, 2004). Lactoferrin produced by neutrophils, by binding to iron, deprives the fungus of this element essential for its growth and thus contributes to elimination of *A. fumigatus* (Zarember *et al*., 2007).

Myeloperoxidase is important for the elimination of fungi and involved in the formation of reactive oxygen species (Balloy and Chignard, 2009). The relevance of these effector mechanisms in neutrophil is illustrated by the increased sensitivity of patients with MPO and NADPH oxidase (patients with CGD) deficiency to infection with *Aspergillus spp* (Segal *et al.*, 1998). Proinflammatory cytokines, such as G-CSF, IFN-γ and IL-17 may avtivate neutrophils (Roilides *et al.*, 1998a; Zelante *et al.*, 2009). The increased production of reactive oxygen species in neutrophils and consequently increased the damage of hyphae is observed upon treatment of neutrophils with human G-CSF and IFN-γ alone, or in combination (Roilides *et al.*, 1998a). IL-17 stimulate the release of MPO and matrix metalloproteinase-9 by neutrophils (Zelante *et al.*, 2009).

Neutrophil extracellular traps (NETs) are made of processed chromatin bound to granular and selected cytoplasmic proteins. This release of chromatin is the result of a unique form of cell death, dubbed "NETosis" (Fig. 2) (Volker and Arturo, 2012). NETs are believed to function by physically containing the microbe within the sticky mass of chromatin, thus forcing direct contact with, or put it in close proximity to the antimicrobial NETs-associated proteins, including neutrophil elastase (Papayannopoulos *et al*., 2010), calprotectin (Urban *et al*., 2009), lactoferrin, and the long pentraxin 3.

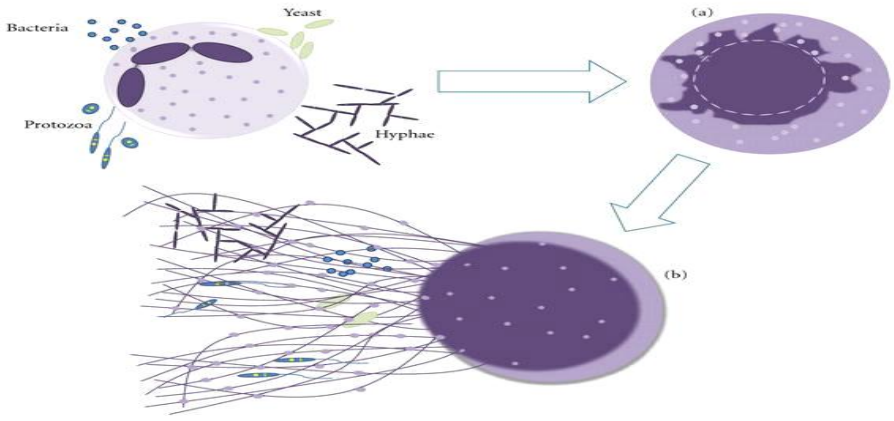

Fig. 2. Mechanism of Neutrophil Extracellular Traps release (figure adapted from: Anderson *et al*., 2012).

1.5.1.5. Dendritic cells

Dendritic cells (DC) have the ability to induce a primary immune response in resting naïve T lymphocytes. To perform this function, DCs are capable of capturing antigens, processing them, and presenting them on the cell surface along with appropriate costimulation molecules. DCs express convocation of pattern recognition receptors on the surface that can specifically interact with PAMPs, including the mannose receptors, c-type lectins and toll-like receptors (TLRs) (Gijzen *et al*., 2006). Receptors on DC are involved in the internalization of unopsonized conidia or hyphae of the fungus. Fungal morphotypes are differentionaly recogniyed by DC. While receptor-mediated internalization of conidia is significantly inhibited by blocking lectin-like receptors, including MR and the DEC-205 (Kan and Bennett, 1988), internalization of hyphae is significantly inhibited by blocking CR3 or FcR II and III with specific-blocking mAbs or specific receptor ligands, such as glucan and laminarin for CR3 (Le Cabec *et al*., 2000).

Recognition of PAMPs leads to maturation of DCs, a step required for optimal activation of T cells (Rutella *et al*., 2006). Mature DCs have ability to promote the generation of Th1, Th2, or other helper T cell subsets and cytotoxic T cells. Pulmonary DC, after phagocytosis of conidia or hyphae, migrate to the draining lymph nodes and spleens, undergo functional maturation, and induce choosy Th priming of $CD4^+$ T lymphocytes (Bozza *et al*., 2002a). Following recognition of fungi, signals from TLR-associated molecule MyD88 can activate recipients TRAF6 that is followed by the activation of NF-κB, JNK and p38 MAPK (Medzhitov, 2001). DCs discriminate between conidia and hyphae of *Aspergillus* in terms of the type of Th cell responses evoked. Recognition of conidia induces IL-12 production, while that of hyphae induces IL-4 and IL-10 *in vivo* (Bozza *et al*., 2002b). Local production of immunoregulatory cytokines (i.e. production of IL-10) may affect the ability of DC to instruct the appropriate T cell responses to the invading pathogens and mediate unresponsiveness to respiratory antigens (Akbari *et al*., 2001).

1.5.1.6. NK cells

NK cells have important role in very early stages of *Aspergillus fumigatus* infections (during the first days of infection) judging by an increased sensitivity of

animals to *A. fumigatus* infection after removal of NK cells (Park *et al.*, 2009). NK cells are the main source of IFN-γ which is required to induce killing of *A. fumigatus* by macrophages (Park *et al*., 2009). However, it is also reported that *A. fumigatus* hyphae modulate NK cell secretion of IFN-γ and GM-CSF (Schmidt *et al*., 2011).

1.5.1.7. The role of cytokines of innate immunity in the defense against *A. fumigatus*

Cytokines are small, soluble and multi functional polypeptides produced by a variety of cells. Recognition of the components of the cell wall of *A. fumigatus* by receptor over the surface of cells of innate immunity initiates a cascade of signaling molecules in these cells, leading to the activation of transcription factors and production of cytokines such as TNF-α, IL-1, IL-6, chemokines, and IL-12 (Wang *et al*., 2001; Dubourdeau *et al.*, 2006). The cytokines are capable of evoking (e.g. TNF-α, IL-1β) or suppressing the inflammation (e.g. IL-10) (Schuh *et al*., 2003).

A. fumigatus conidia or hyphae initiate cytokine or signaling-response mechanisms via IL-1, IL-8, TNF-α and IL-6. These cytokines serve as an attractant to draw neutrophils to the site of infection and initiate phagocytosis of the fungal organisms (Svirshchevskaya and Kurup, 2003).

IL-1 is a proinflammatory cytokine mainly produced by airway macrophages as a part of the non-specific inflammatory response. It stimulates expression of endothelial adhesion molecules and enhances the production of chemokines, NO (Barnes *et al*., 1998; Opal *et al*., 2000) and TNF-α (Yoshimura *et al*., 2003).

TNF-α augments the capacity of neutrophils to damage hyphae, possibly through enhanced oxidative mechanisms (Roilides *et al*., 1998c). Granulocyte colony-stimulating factor (G-CSF), GM-CSF and especially IFN-γ enhance monocyte and neutrophil activity against hyphae (Gaviria *et al*., 1999).

IL-12 inhibits the production of Th2-type cytokines and enhances the Th1-induced differentiation and proliferation (Nutku *et al*., 2001). Increased IL-12 levels have been detected in several respiratory disorders e.g. in virus infections (Ferreira *et al*., 2002) in *A. fumigatus* infection

1.5.2. Adaptive immune response against *A. fumigatus*

1.5.2.1. The role of humoral immunity in the defense against *A. fumigatus*

Little is known about the role of humoral immunity (i.e. B cells and their effector molecules, antibodies) in defense against fungi. Fungus specific antibodies are involved in reduction of biofilm formation (Luis and Arturo, 2005), and also promote fungal ingestion and killing by phagocytes. Some of studies have shown a significant increase in the levels of IgG in the blood of dogs with invasive aspergillosis (Watt *et al*., 1995; Clercx *et al*., 1996). In the other studies insufficiency the synthesis of IgA and IgM in dogs was noted (Berry and Leisewitz, 1996) indicating that these antibodies can be important in the susceptibility against invasive aspergillosis.

A number of studies on monoclonal antibodies (MAbs) against *Aspergillus* antigens have been directed towards the specific diagnosis of the disease (Fenelon *et al*., 1999), whereas very limited work has been carried out on the use of MAbs for the treatment of aspergillosis (Cenci *et al*., 2002) compared to candidiasis (Moragues *et al*., 2003). Majority of these antibodies are IgG subclasses. Antibodies against catalase B (MAb-7), actin putative protein (MAb-B), galactomannan (MAb A9) and hypothetical protein of *A. fumigatus* cell wall (MAb-C) have been generated (Ashok *et al*., 2008). Some of antibodies (i.e. MAb A9) are specific for fungal developmental stadium. MAb A9 could prolong the time required for germination, as galactomannan is incorporated into the fungal cell wall during spore germination to hyphae (Fontaine *et al*., 2000). IgG inhibite hyphal development and reduce the duration of spore germination (Matthews and Burnie, 2004). The maximum inhibitory activity shown for MAb-7 could be due to the binding activity of this antibody both with swollen conidia and mycelium (Matthews and Burnie, 2004). MAbs against specific target of the cell wall may have protective value.

1.5.2.2. Cell mediated immunity

The interaction of dendritic cells with fungal pathogens is of major importance for the quality (type) of adaptive T helper-cell response that will subsequently be elicited. This interaction is determined by the ligation of conidia or hyphae of *A. fumigatus* to distinct PRRs on dendritic cells and the mechanism of their phagocytosis (coiling or

zipper-type phagocytosis) by dendritic cells (Mansour and Levitz, 2002; Romani *et al*., 2002). While recognition of *A. fumigatus* conidia through both TLR2 and TLR4 on DC leads to induction of a Th1 adaptive response, recognition of hyphae of *A. fumigatus via* TLR2 leads to Th2 differentiation.

T-lymphocytes are classified to $CD4^+$ and $CD8^+$ cells, and the $CD4^+$ cells are further divided in subsets according to cytokines they produce. Both $CD4^+$ and $CD8^+$ T cells are required for clearance of fungal pathogens. The role of $CD8^+$ T cell in aspergillosis is lyses of infected cells and limitation of tissue damage by reducing inflammatory response. *A fumigatus* extract promote $CD8^+$ T cell activation, proliferation, and differentiation, which may provide limited protection in the absence of $CD4^+$ T cell effector maturation (Tao *et al*., 2006). $CD4^+$ T cells produce essential lymphokines that activate and recruit phagocytic cells to sites of fungal infection. Progressive loss of $CD4^+$ T cells leads to increased susceptibility to *Cryptococcus spp*, *Histoplasma spp*, *Aspergillus spp*, *Candida spp* and *Penumocystis spp* infection (Sarosi *et al*., 1995). Host resistance to *A. fumigatus* infection involves the induction of proinflammatory cytokines, including IFN-γ, IL-1β, IL-12, and TNF-α, while susceptibility to infection is associated with the production of IL-10 and IL-4 (Mencacci *et al*., 2000a).

1.5.2.2.1. Th1 cells

Th1 $CD4^+$ T cells are involved in recruitment and activation of phagocytic cells, generation of cytotoxic T cells, production of opsonizing antibodies and delayed type hypersensitivity. They produce IL-2, IL-12, IL-18, TNF-α and IFN-γ (Mencacci *et al*., 2000b; Huffnagle and Deepe, 2003). $CD4^+$ Th1 type cytokines are produced by cells from immunocompetent mice infected with a virulent strain of *A. fumigatus* and are important for reduction of fungal load (Petri *et al*., 2001).

Production of **IFN-γ** by Th1 cells is fundamental to optimize the microbicidal activity of phagocytes. IFN-γ potentiates the oxidative responses and hyphal damage of human phagocytic cells (Roilides *et al*., 1993), as observed in patients with chronic granulomatous disease treated with IFN-γ (Rex *et al*., 1991). For appropriate activation of macrophages signal delivered through IFN-γ receptor (IFNγ-R) following IFNγ binding is necessary (Huang *et al*., 1993). Involvement of IFN-γ was noted in studies of both

systemic (Cenci *et al*., 1997, Mirkov et al, 2010) as well as in local pulmonary infection (Cenci *et al*, 1998; Centeno-Lima *et al*, 1998; Brieland *et al*, 2001). Kinetic of induction of proinflammatory cytokines in murine models of invasive pulmonary aspergillosis showed temporal induction of IFN-γ and Th1 promoting cytokines (IL-18 and IL-12) (Cenci *et al*, 1998; Brieland *et al*, 2001). Production of Th1 cytokines correlated with improved outcomes in infected mice and it is predominant response of cells from healthy humans to *A. fumigatus* (Grazziutti *et al*., 1997; Chaudhary *et al.*, 2010). There is also agreement that IFN-γ is needed for favorable outcome of aspergillosis in humans (Hebarth *et al*., 2002).

1.5.2.2.2. Th2 cells

Th2 cells contribute to the differentiation of B cells and antibody production (especially IgE antibodies which are involved in allergic reactions) (Mencacci *et al*., 2000b; Huffnagle and Deepe, 2003). These cells produce IL-4, IL-5, IL-13 (Mencacci *et al*., 2000b; Huffnagle and Deepe, 2003). Production of IL-4 by $CD4^+$ T lymphocytes impairs neutrophil antifungal activity (Cenci *et al*., 1999). IL-4 also has proinflammatory effects in fungal infections, such as enhancement of macrophage mannose receptor activity (Stein *et al*., 1992). The involvement of IL-4 was explored in mouse aspergillosis mainly in relation to IFN-γ. Increased levels of IL-4 are considered responsible for susceptibility to invasive aspergiollosis (at low levels of IFN-γ) (Cenci *et al.*, 1998). It seems that there is no IL-4 response in human aspergillosis as no production by peripheral blood mononuclear cells was observed when stimulated with *A. fumigatus* conidia (Grazziutti et al, 1997) or conidia-ladden dendritic cells (Tramsen *et al*., 2002)

1.5.2.2.3. Th 17cells

Th17 cells are subset of $CD4^+$ Th cells which are developed from naïve $CD4^+$ T cells in presence of TGF-β and IL6. Th17 cells mediate protection against fungi (Ye *et al*., 2001; Happel *et al*., 2003). Mouse and human Th17 cells produce **IL-17A** and IL-17F, two members of the IL-17 family (Kolls and Linden, 2004) that induce the mobilization, recruitment and activation of neutrophils and trigger the production of

proinflammatory cytokines and chemokines by a broad range of cellular targets, including epithelial cells, endothelial cells and macrophages (Weaver *et al*., 2007).
Studies have indicated a functional dichotomy between Th1 and Th17, as the Th1-associated cytokines IL-12 and IFN-γ strongly inhibit mouse Th17 cell differentiation (Harrington *et al*., 2005; Park *et al*., 2004).

In contrast to data that unequivocally showed the significance of Th1 (IFN-γ) responses in aspergillosis in mice and humans, there are conflicting data concerning the involvement of IL-17 in defense against the fungus. Production of IL-17 was observed in pulmonary disease in mice (Werner *et al*., 2009, Zelante *et al*, 2009), while antifungal response in humans seems not to rely on this cytokine (Chai et al, 2010; Chaudhary et al, 2010).

1.5.2.2.4. T regulatory cells (Tregs)

Apart from the T cells involved in the above cited types of adaptive immunity (Th1, Th2 and Th17 cells), a role for regulatory T cells (Treg) has been recently demonstrated in the immune response against fungi. Treg cells attenuate Th1 response and decrease inflammation, induce tolerance and mediate resistance to re-infection (Montagnoli *et al*., 2002).

IL-10, a major cytokine produced by $CD4^+$ T cells has an important and rather pleiotropic role in regulating immune response to fungal infections. It inhibits the fungicidal activity of monocytes or neutrophil cells against *C. albicans, A. fumigatus* and *C. neoformans* (Cenci *et al*., 1993; Roilides *et al*., 1997). It has been suggested that IL-10 may have a pathogenetic role in the chronic form of pulmonary aspergillosis, as *A. fumigatus* infection in IL-10-deficient mice is characterized by inflammatory response associated with the up-regulation of innate antifungal Th1 responses, such as the production of IL-12, TNF-α, and IFN-γ (Cortez *et al*., 2006). The suppressive effect of IL-10 on the innate and protective Th1 antifungal responses was demonstrated in mouse models of invasive candidiasis, aspergillosis and histoplasmosis (Del Sero *et al.*, 1999; Vazquez-Torres *et al*., 1999; Clemons *et al.*, 2000; Deepe and Gibbons, 2003). IL-10, more than IL-4, has a suppressive effect on the antifungal activity (i.e. oxidative burst) of mononuclear cells against the fungus (Roilides *et al*., 1997, 1998b). A role for this

cytokine in the optimal development of Th1 responses has been suggested, as endogenous IL-10 is required for optimal costimulation of IL-12-dependent Th1 cells (Mencacci *et al*., 1998). IL-10 was shown to be a part of repertoire of T cell cytokine responses in humans (Hebart *et al.*, 2010), though no production was detected by other authors (Grazziutti *et al.*, 1997; Tramsen *et al*, 2009).

TGF-β, another cytokine produced by regulatory T cells is involved in sapregillosis. Along with IL-10 it is considered as important in prevention allergy to fungus (Montagnoli *et al*., 2006). Studies in humans showed that susceptibility to aspergillosis is associated with lower production of both IL-10 and TGF-β (Sambatakou *et al*., 2006). As these cytokines are antiinflamatory, chronic aspergillosis in these subjects may be a consequence of poor control of inflammatory response.

1.6. Rat models of aspergillosis

Rat models of pulmonary aspergillosis are used in evaluating the usefulness of different methodologies for laboratory diagnosis of infection with the fungus (Aydogan *et al.*, 2010; Khan *et al.*, 2008; McCulloch *et al*., 2012; Scotter and Chambers, 2005; Yu *et al*., 1990; Zhao *et al*., 2010) and in testing the efficacy of antifungal drugs in pulmonary aspergillosis (Cicogna *et al*., 1997; Kurtz *et al*., 1995; Miyazaki *et al*., 1998; Murphy *et al*., 1997; van de Sande *et al*., 2009). Despite the significant use of rat models of pulmonary aspergillosis in therapy and diagnostic studies, immune mechanisms of resistance or sensitivity to the fungus in this species are largely unknown. The involvement of early immune defense was reported in rats, but it is evaluated mainly by histological appearance of lungs (Mahmoud *et al*., 2011; Niki *et al*., 1991; Shibuya *et al*., 1999; Turner *et al*., 1976). In one report, myeloperoxidase (MPO) content in homogenates of lungs was measured and used as marker of the effect of immune augmentation (Lo Giudice *et al*., 2010).

Informations concerning late (adaptive) responses and involvement of cytokines, major activators and regulators of lung immune responses to the fungus are not numerous as well. In one study authors showed that infection with *A. fumigatus* is not associated with changes in the proinflammatory (IFN-γ, TNF-α) or antiinflamatory (IL-4, IL-10) responses in neutropenic RP rats (Becker *et al*., 2003). Another report showed, however,

lack of changes in the levels of IFN-γ, but an increase in IL-10 in lungs of infected Wistar rats immunosuppressed with 5-fluorouracil (Caglar *et al*., 2011). Although differences in the antigenic composition of *A. fumigatus* conidia might have accounted for the observed differences in cytokine responses to the fungus, genetic background, i.e. the strain of the rat employed in above cited studies, might have exerted influence as well. In corroboration, the relevance of genetic variations for the susceptibility to pulmonary aspergillosis was shown in mouse and human studies (Ok *et al.*, 2011; Zaas *et al*., 2008; Svirshchevskaya *et al.*, 2009).

2. OBJECTIVES

2. Aims of the study

Based on the above described background related to scarce data about mechanisms of pulmonary immune defense against *A. fumigatus* in rats and having in mind the relevance of genetic background for immune responses to fungus in mice and humans, **the aim of this study is to investigate pulmonary immune response to *A. fumigatus* infection comparatively in immunocompetent rats of two inbred strains, Albino Oxford (AO) and Dark Agouti (DA) known to differ in the type (Th1 or Th2) and/or in the intensity of response to same immunological insults**.

For that purpose, following specific assignments are given:

1. examination of efficacy of fungal elimination from the lungs
2. determination of basic basic parameters of immune reactivity in lung tissue
3. investigation of activities relevant for fungal elimination of leukocytes recovered from lungs by enzyme digestion (total leukocytes) or by lavage of bronchoalveolar spaces (BAL leukocytes)
4. examination of regional draining lymph node (dLN) responses

To these aims,

1. Dynamics of fungal elimination was monitored during 14 days period (at days 1, 3, 7 and 14 after intratracheal (i.t.) infection with nonlethal inoculum of $1x10^7$ *A. fumigatus* conidia

2. histological appearance of lung tissue and proinflammatory (IFN-γ, IL-17) and Th2/antiinflammatory (IL-4 and IL-10) content in lung tissue homogenates was determined during 14 days period (at days 1, 3, 7 and 14 post infection, p.i.)

3. Leukocyte and T cell subset composition of total as well as BAL leukocytes, effector activities related to direct fungal elimination (i.e. CD11b expression, adhesion, oxidative activity and hyphal damage by cells) of total lung leukocytes, and cytokine production by both total as well BAL leukocytes was assessed.

4. Proliferative activity, basic T cell subset composition, cytokine production and cytokine gene expression by draining lymph node cells was examined. In addition contribution of $CD4^+$ T cells (obtained by immunomagnetic separation) to dLN cytokine production was examined.

It is expected to obtaine data that might be helpful in the future use of rat models of pulmonary aspergillosis and in the exploration of immune-based therapeutic interventions as an adjunct treatment of fungal diseases in particular.

3. MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Animals

Male Dark Agouti (DA) and Albino Oxford (AO) rats, 3-4 months of age were used in the experiments. Rats were conventionally housed at the Institute for Biological Research "Sinisa Stankovic" with temperature 21^{o}C, 12h photoperiod and food and water *ad libitum*. Animals were pre-assigned in the experimental groups (sham infected and infected animals) and each group consisted of four animals. All experiments were carried out in adherence to the guidelines of the Ethical Committee of the Institute for Biological Research "Sinisa Stankovic" (2-31/11, 07.04.2011.).

3.2. Fungal culture conditions

The human isolate of *Aspergillus fumigatus* was obtained from the Institute of Public Health of Serbia"Dr Milan Jovanovic-Batut". The microorganisms were grown on Sabouraud's maltose agar (SMA, Torlak, Belgrade, Serbia) for seven days (Booth, 1971). Conidia were harvested by flooding the surface of agar slants with non-pyrogenic sterile physiological saline and obtained conidial suspension was filtrated throu sterile gauze to remove hyphae and conidiophore. Obtained conidia were counted using improved Neubauer hemocytometer and adjusted to desired concentration.

3.3. Animal infection

Into the trachea of anesthetized rats (Ketamidor, Richter Pharma, Austria) 1×10^7 conidia of *A. fumigatus* in 0.2 ml of pyrogen-free saline were injected. Control rats received the same volume of saline solely.

Animals were euthanized at days 1, 3, 7, 14 and 30 post infection (p.i.) for fungal burden assesment and at days 1, 3, 7 and 14 p.i. for evaluation of immune response.

3.4. Fungal burden assesment

Fungal burden in the lungs was determined using quantitative culture of *A. fumigatus* by counting the number of colony forming units (CFU) on agar slants (Sheppard *et al*., 2006). At selected time points (1. 3, 7, 14 and 30 days) post infection lungs were

aseptically removed and their wet masses weighed using a precision balance (± 0.01 g). Measured left lungs were homogenized with IKA T18 basic homogenizer (IKA Works INC., Wilmington NC) in 10 ml of sterile saline, on ice (primary homogenate). Primary homogenate and two dilutions (1:10 and 1:100) were plated on SMA plates supplemented with streptomycin sulfate (ICN-Galenika, Belgrade, Serbia) and incubated at 37^{o} C for 24 to 48 h. After the period of incubation the number of colonies growing on agar slants was counted and the number of CFU per gram of tissue was determined.

Remaining primary homogenates were frozen and cytokine contents in homogenates were determined by ELISA. Results were expressed as pg per gram of tissue.

3.5. Lung histology

Right lung was used for processing for histology. Lobes were excised immediately fixed in 4% formaldehyde (pH 6.9), and embedded in paraffin wax for sectioning at 5 μm. Hematoxylin and eosin (H&E)-stained histological slides were analyzed subsequently using Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

3.6. Pulmonary cell preparation and culture

Lung leukocytes were obtained by collagenase/DNAse-digestion. Right lung lobes were finely minced and incubated with gentle mixing (magnetic stirer) for half an hour in culture medium supplemented with 0.7 mg/ml collagenase and 30 μg/ml DNase at 37°C in a total volume of 1.5 ml. Digestion was stopped by adding heat-inactivated fetal calf serum (PAA laboratories, Austria) in final concentration of 10%. Cells were resuspended in RPMI-1640 culture medium (Flow, ICN Pharmaceuticals) supplemented with 2 mM glutamine, 20 μg/ml gentamycine (Galenika a.d., Serbia) 5% (v/v) FCS (complete medium) and 5 μg/ml of voriconazole (Pfizer PGM, France) and counted by improved Neubauer hemocytometer. Cell viability exceeded 95% as determined by trypan blue exclusion. Cell concentration was adjusted to 4×10^{6}/ml.

For lung cell composition determination, cytospin preparations of 50.000 lung cells were prepared and stained with May Grünwald - Giemsa protocol. Differential cell counts were determined by differentiating at least 300 cells from air-dried cytospin preparations.

For the cytokine production measurement, cells (0.2×10^6 cells/well, 96 well plate) were cultured for 72 h in culture medium solely (spontaneous production) or with heat-inactivated conidia of *A. fumigatus* at ratio of 2:1 conidia to cells (stimulated production).

3.7. Bronchoalveolar lavage cells isolation and culture

Lung leukocytes were obtained by lavage (twice with 6 ml of saline) of bronchoalveolar spaces (BAL cells).

Cell composition was determined by differentiating at least 300 cells from cytospin preparations of recovered cells stained with May Grünwald - Giemsa.

Concentration of cells was adjusted to 4×10^6/ml in complete medium. For the cytokine production measurement 0.2×10^6 cells/well (96 well plate) were cultured for 72 h in culture medium solely (spontaneous production).

3.8. Draining lymph node (dLN) cell isolation and culture

Thoracic draining lymph nodes were harvested and suspensions of cells prepared by mechanical disintegration of lymph organs. The number of cells and cell viability was determined by Trypan blue exclusion assay using improved Neubauer hemacytometer. Viability always exceeded 95%.

The capacity of dLN cells to proliferate was determined *ex vivo* by culturing 5×10^5 cells/well (96-well plate) for 24 hours, with 0.5 μCi of ^{3}H-thymidine (GE Healthcare, UK) per well during the last eight hours of culture. Incorporation of ^{3}H-thymidine into cellular DNA was measured by liquid scintillation counting (LKB, Wallac).

For cytokine production, cells were cultured at 5×10^6 /ml in 24-well plates (Sarsted, Nümbrecht, Germany) for 72 hours in the culture medium solely (spontaneous production) or in the presence of *A. fumigatus* conidia at ratio of 2:1 conidia to cells (stimulated production).

3.9. Immunomagnetic separation

Immunomagnetic separation set (anti rat CD4 particles, BD IMag, BD Bioscience, San Jose, California, USA) was used for isolation of $CD4^+$ cells according to manufacturer instructions. The purity of cells was checked by flow cytometry and was >92%. For

cytokine production, $CD4^+$ and $CD4^-$ cells were cultured at 5×10^6/ml in 96-well plates (Sarsted, Nümbrecht, Germany) for 72 hours in the medium (spontaneous production) or in the presence of *A. fumigatus* conidia at ratio of 2:1 conidia to cells (stimulated production).

3.10. Flow cytometry analysis

Cells (10^6) were incubated on ice in a volume of 100 μl with FITC-conjugated mouse mAbs to rat CD3 (eBioscience Inc., San Diego, CA, USA) (lung cells), FITC-conjugated mouse mAbs to CD4 and PE-conjugated rat CD8 (eBioscience Inc., San Diego, CA, USA) (lung, BAL and dLN cells) or with mAb OX-42 (mouse anti rat CD11b/CD11c, IgG2a) followed by incubation with FITC-conjugated F(ab)2 goat anti mouse IgG (Serotec Ltd., Bicester, UK) (lung cells). After washing, the cells were fixed (in 1% paraformaldehid) and assayed for fluorescence intensity on a FACSCalibur cytometer (BD Biosciences, Heidelberg, Germany).

3.11. Reverse transcription- real time polymerase chain reaction (RT-PCR)

Total RNA was isolated from the draining lymph node cells with mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) following the manufacturer's instructions. The isolated RNA (1μg) was reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, according to manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) according to the recommendations of the manufacturer in a total volume of 20 μl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions comprised an initial step at 50°C for 5 minutes, followed by a step at 95°C for 10 minutes and subsequent 2-step PCR program at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. The PCR primers used in the study were: **β-actin**: forward 5'- GCT TCT TTG CAG CTC CTT CGT -3', reverse 5'- CCA GCG CAG CGA TAT CG -3'; **IFN-γ**: forward 5'- TGG CAT AGA TGT GGA AGA AAA GAG -3', reverse 5'- TGC AGG ATT TTC ATG TCA CCA T -3'; **IL-17**: forward 5'- ATC AGG ACG CGC AAA CAT G -3', reverse 5'- TGA TCG CTG CTG CCT TCA C -3'; **IL-4**: forward 5'- CGG

TGA ACT GAG GAA ACT CTG TAG A -3', reverse 5'- TCA GTG TTG TGA GCG TGG ACT C -3'; **IL-10**: forward 5'- GAA GAC CCT CTG GAT ACA GCT GC -3', reverse 5'- TGC TCC ACT GCC TGG CTT TT -3'. Accumulation of PCR products was detected in real time and the results were analyzed with 7500 System Software (AB) and calculated as 2^{-dCt}, where dCt was the difference between Ct values of specific gene and endogenous control (β-actin).

3.12. Enzyme-linked immunosorbent assays (ELISA)

Cytokine content in lung homogenates and media conditioned by lung, BAL or dLN cells was determined using commercially available ELISA sets for rat IFN-γ, IL-4 and IL-10 (R&D Systems, Minneapolis, USA) and mouse IL-17 cross-reactive with rat IL-17 (eBioscience Inc., San Diego, CA, USA). Tests were done according to manufacturer's recommendations. Cytokine titer was calculated by a reference to a standard curve constructed using the known amounts of recombinant IFN-γ, IL-4, IL-17 or IL-10.

3.13. Lung cell myeloperoxidase (MPO) activity

Myeloperoxidase activity was assessed on the basis of the oxidation of *o*-dianisidine dihydrochloride in the presence of H_2O_2 (Bozeman *et al.*, 1990) in lung cells lysates. Activity of MPO was evaluated by addition of 967 µl of substrate solution (0.147 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0) to 33 µl cells lysate (4×10^6 cells resuspended in 33 µl 0.5% HTAB). Absorbance was read at 450 nm after 10 min and activity of MPO was calculated against the standard of myeloperoxidase. Values are expressed as MPO units/10^6 cells.

3.14. Lung cell adhesion

The capacity of lung cells to adhere to acellular (plastic) substrate was employed for cell adhesion evaluation and performed as described by Oez (Oez *et al.*, 1990). Briefly, isolated cells (5×10^5/well in flat-bottomed 96-well plates) were cultured without (spontaneous) or with (100 ng/ml) PMA (stimulated cell adhesion) for 60 minutes. After removal of nonadherent cells, adhesion was evaluated by staining of adherent cells with

0.1% methylene blue followed by measurement of dissolved dye (0.1 N HCl) spectrophotometrically (A670nm/540nm) by an ELISA 96-well plate reader.

3.15. Cytochemical assay for the respiratory burst (NBT test)

The activation of lung cells was evaluated by the quantitative cytochemical assay for the respiratory burst, based upon cell's spontaneous or PMA stimulated capacity to reduce tetrazolium salt nitroblue tetrazolium (NBT) to the colored end-product formazan. Nitroblue tetrazolium (NBT, ICN Pharmaceutical, Costa Mesa, CA, USA) reduction by neutrophils and monocytes, as a measure of spontaneous cell-derived superoxide formation, e.g. cell activation, was determined in lung cells according to a modification of a method described (S h e n *et al*., 1995). Briefly, lung cells (0.2×10^6) were incubated with 0.5 mg/ml of NBT at 37°C for 20 minutes, followed by a 10-minute incubation at room temperature. Enumeration of cells with intracellular dots of formazan (NBT-positive cells) was performed by optical microscopy on cytospin preparations stained by May Grünwald-Giemsa. Rasults were exppresed as ralative number of NBT^+ cells on caunted 100 cells.

3.16. Phagocytosis

Capacity of lung leukocytes to engulf conidia was determined following incubation of cells (10^6 cells in 0.5ml of culture medium placed on 24 × 24 mm sterile glass coverslips) with 10^6 viable conidia of *A. fumigatus* for 1h on 37^oC. Following incubation, coverslips were washed, stained with May Grünwald-Giemsa and the number of cells which internalized conidia were determined by counting them under immersion oil. Phagocytosis was expressed as percent of cells with engulf conidia.

3.17. Data presentation and statistics

Results were pooled from at least two independent experiments (4-6 animals per group in each experiment) and expressed as mean values ± S.D. Statistical analysis of data was performed using a Mann-Whitney U test. *p*-values less than 0.05 were considered significant.

4. RESULTS

4. Results

4.1. Characteristics of lung response following administration of *A. fumigatus* conidia to AO and DA rats

4.1.1. Lung fungal burden following administration of *A. fumigatus* conidia to AO and DA rats

Animals were infected and monitored for 30 days. There were no signs of disease or deaths during observation period.

Progressive decrease in the lung fungal burden was observed in infected rats of both strains, but much efficient in DA rats, which eliminated infection by the day 14 compared to AO rats which removed fungus from the lungs by the day 21 (Fig. 3).

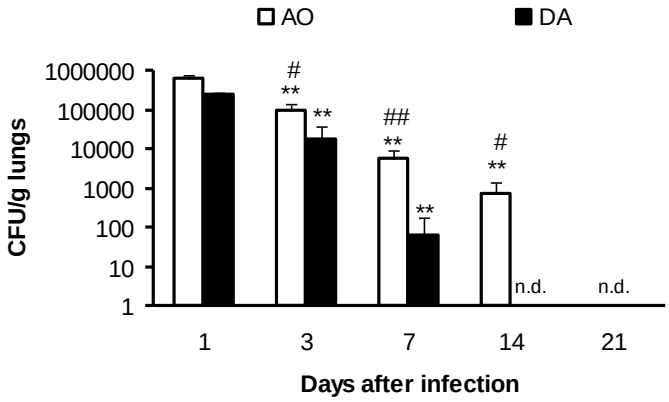

Fig. 3. Time course of fungal elimination from lungs following *A. fumigatus* conidia administration to AO and DA rats. Fungal burden was determined using a colony forming unit (CFU) assay in lung tissue homogenates and expressed as number of formed colonies per gram of tissue. Results are expressed as mean ± S.D. of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: ** $p < 0.01$ from day 1 post infection of respective strain and $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ *vs* DA rats. n.d. – not detectable.

4.1.2. Basic parameters of immune reactivity in lung tissue following administration of *A. fumigatus* conidia to DA and AO rats

Administration of conidia resulted in no changes in wet lung mass of infected AO rats (3.4 ± 1.2 *vs* 2.3 ± 0.8, 2.8 ± 0.7 *vs* 2.7 ± 0.7, 2.6 ± 0.6 *vs* 3.0 ± 0.9, 3.3 ± 1.2 *vs* 3.0 ± 0.8 for sham infected and infected animals at days 1, 3, 7 and 14 p.i., respectively) and DA rats (1.7 ± 0.3, 1.9 ± 0.4, 2.0 ± 0.4 and 2.0 ± 0.5 at days 1, 3, 7 and 14 p.i., respectively) compared to sham infected animals (2.2 ± 0.5, 1.7 ± 0.4, 1.9 ± 0.6 and 2.0 ± 0.5). Progressive decrease in lung fungal burden was observed in both rat strains showing that triggering of immune response to fungus occurred. However, fungal elimination was slower in AO compared to DA rats (Fig. 3).

4.1.3. Lung histology

Histological analysis of lung tissue of infected animals of both strains revealed pulmonary inflammation characterized by interstitial widening and perivascular leukocyte infiltration (Fig. 4). Semi-quantitative analysis of lung tissue specimens revealed no strain differences in the intensity of cell infiltration at day 1 p.i., while, judging by cell infiltration score values, in DA compared to AO rats more intense polymorphonuclear cell infiltration at day 3 p.i. (1.6 ± 1.3 in DA *vs* 0.6 ± 0.4 in AO rats, $p = 0.045$) and mononuclear cells infiltration at day 7 p.i. (1.8 ± 0.8 in DA *vs* 0.3 ± 0.4 in AO rats, $p = 0.0043$) were noted.

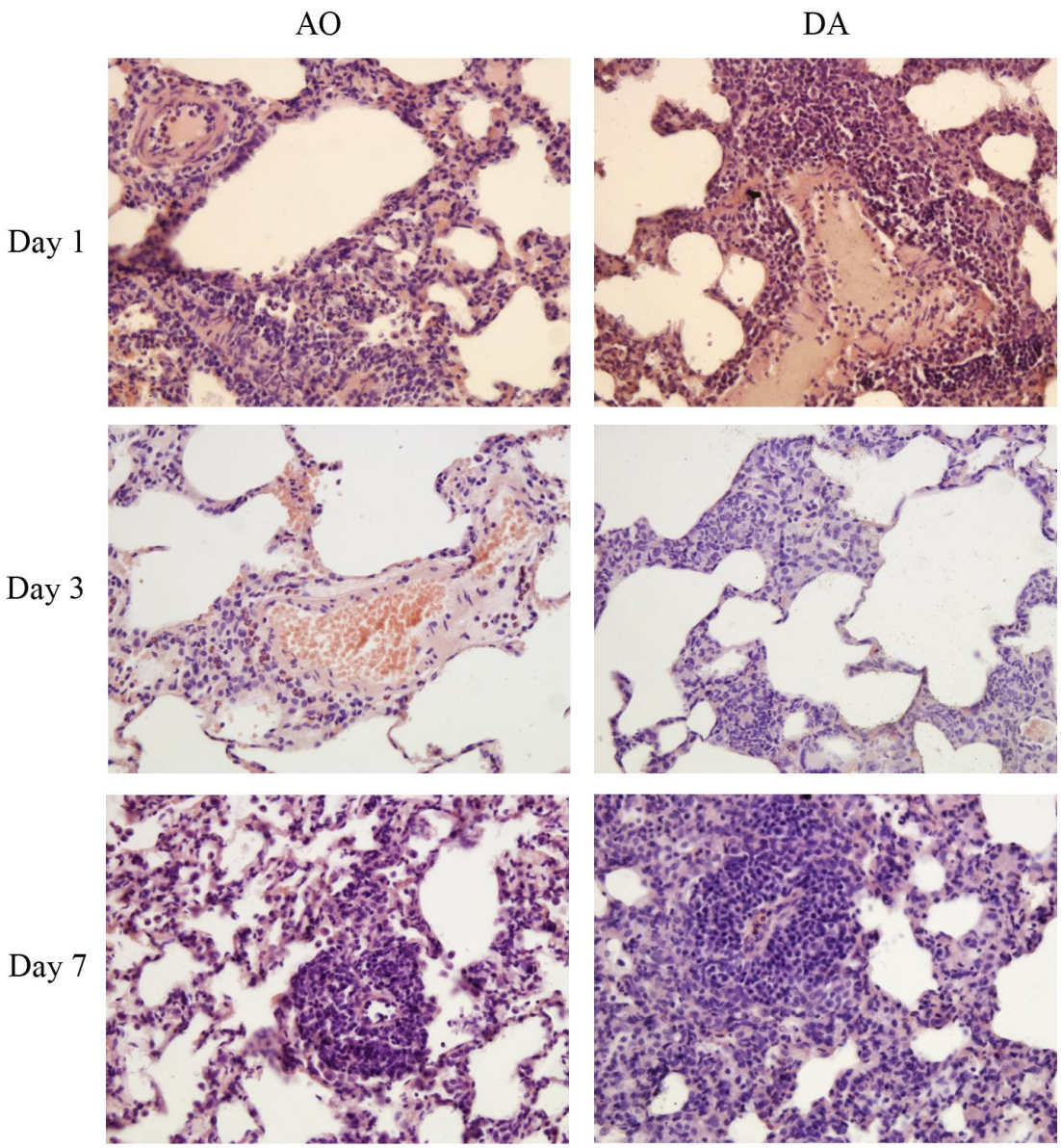

Fig. 4. Lung tissue histology following intratracheal *Aspergillus fumigatus* administration in rats. Lung tissue histology from infected AO and DA rats at days 1, 3 and 7 p.i.. Pulmonary inflammation characterized by interstitial widening and perivascular leukocyte infiltration (H&E; original magnification ×200).

4.1.4. Cytokine *milieu* in lung tissue following administration of *A. fumigatus* conidia to AO and DA rats

In order to examine in which environment elimination of fungus occurred lung tissue content of proinflammatory (IFN-γ and IL-17) and Th2/anti-inflammatory (IL-4 and IL-10) cytokines was measured next.

Infection resulted in an increase in amounts of **INF-γ** in lung tissue homogenates of infected (compared to respective sham infected controls) AO rats at day 3 p.i. and in DA rats at days 3 and 7 p.i. (higher at day 3 p.i. $p = 0.013$) (Fig. 5). Level of IFN-γ in lung tissue homogenates in infected DA rats was higher compared to infected AO rats at day 3 p.i..

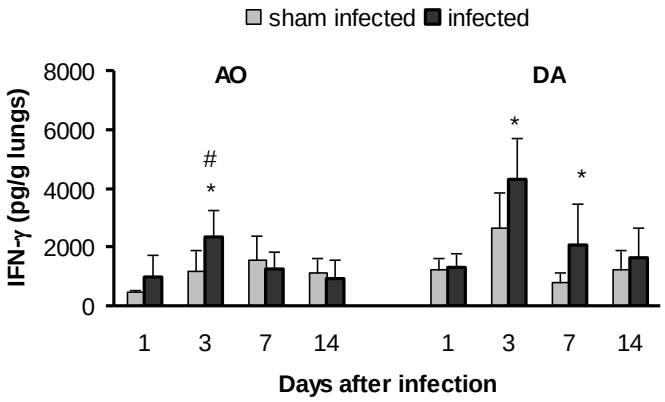

Fig. 5. IFN-γ content in lung tissue homogenates following *A. fumigatus* conidia administration to AO and DA rats. Cytokine content in homogenates of infected and sham-infected rats was determined by ELISA and expressed as pg of cytokine per gram of lung tissue. Results are presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ *vs* sham-infected animals of the respective strain and $^{\#}$ $p < 0.05$ *vs* DA rats.

A. fumigatus administration resulted in increased **IL-17** content at days 1 and 3 p.i. in AO rats, and from day 1 p.i. to day 7 p.i. in infected DA rats (Fig. 6).

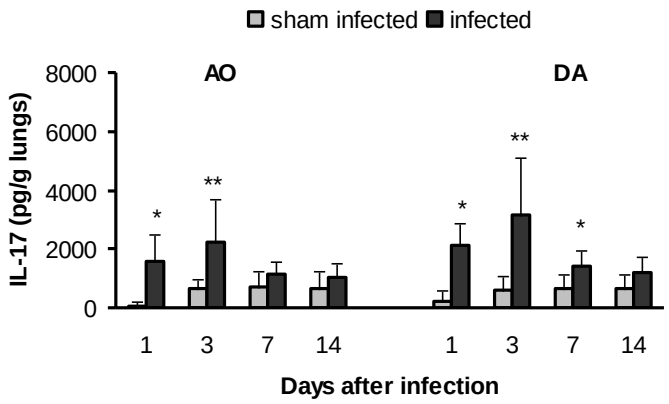

Fig. 6. IL-17 content in lung tissue homogenates following *A. fumigatus* conidia administration to AO and DA rats. Cytokine content in lung homogenates of infected and sham-infected rats was determined by ELISA and expressed as pg of cytokine per gram of lung tissue. Results are presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain.

No changes were observed in **IL-4** content in lungs of infected compared to sham-infected animals in both strains (Fig. 7).

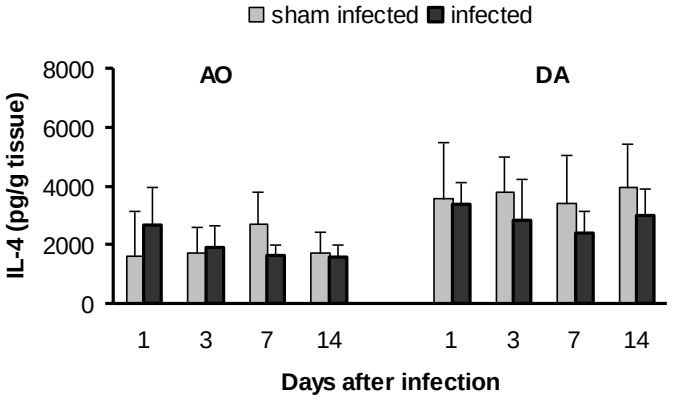

Fig. 7. IL-4 content in lung tissue homogenates following *A. fumigatus* conidia administration to AO and DA rats. Cytokine content in lung homogenates of infected and sham-infected rats were determined by ELISA and expressed as pg of cytokine per gram of lung tissue. Results are presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment.

Unchanged **IL-10** lung tissue content was observed in infected compared to sham-infected animals of both strains (Fig. 8).

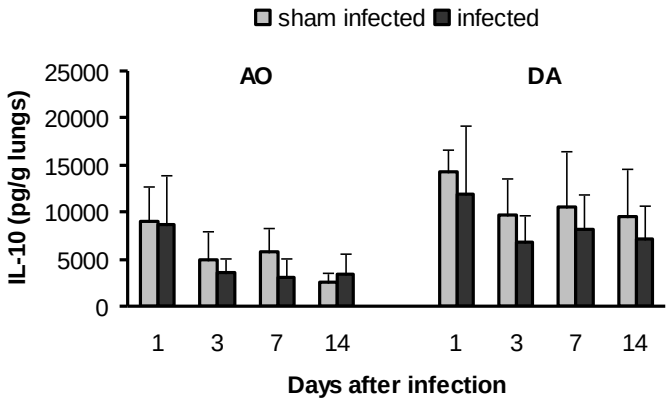

Fig. 8. IL-10 content in lung tissue homogenates following *A. fumigatus* conidia administration to AO and DA rats. Cytokine content in lung homogenates of infected and sham-infected rats were determined by ELISA and expressed as pg of cytokine per gram of lung tissue. Results are presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment.

4.2. Activity of leukocytes obtained from lungs

4.2.1. Lung cells recovery, leukocyte and T cell subset composition following administration of *A. fumigatus* conidia to AO and DA rats

In infected AO rats infection resulted in a significantly increased (compared to sham-infected individuals) numbers of lung cells recovered at day 1 p.i. and numerically increased number at day 3 p.i. (p = 0.073) (Table 1). No differences were observed in numbers of lymphocytes or macrophages in lung digest cell preparation of infected compared to sham-infected animals. Number of neutrophils in infected AO rats (Table 1) rose at days 3 (significantly) and 7 p.i. (numerically, p = 0.067), and was higher at day 3 p.i. compared to day 7 (p = 0.019). Increased number of $CD3^+$ cells at days 7 and 14 p.i. was noted in infected compared to sham-infected AO rats. Number of $CD3^+$ cells at day 14 following infection was lower in AO compared to DA rats. Infection resulted in increased number of $CD4^+$ cells at day 14 p.i. and $CD8^+$ cells at day 3 p.i.. This dynamic of changes resulted in increase (compared to sham-infected animals) in $CD4^+$ to $CD8^+$ cells ratio at day 14 p.i..

Infection resulted in a significantly increased (compared to sham-infected individuals) numbers of lung cells recovered in DA rats at all time points examined (Table 1). Similar relative number of lymphocytes, as well as macrophages, was observed between infected and sham-infected animals. Number of neutrophils in infected (compared to sham-infected) animals was increased at all time points examined (Table 1). Although no significant changes were noted in number of $CD4^+$ or $CD8^+$ cells in infected compared to sham-infected animals, increased number of $CD3^+$ T cells at days 7 and 14 p.i. was noted in infected DA rats.

Table 1. Total lung cells recovery and composition following *A. fumigatus* conidia administration to AO and DA rats.

	Day 1		Day 3		Day 7		Day 14	
	Sham	**infected**	**sham**	**infected**	**sham**	**infected**	**sham**	**Infected**
AO								
Cell number (x10^6/g tissue)	16.4 ± 5.1	25.8 ± 10.3*	16.2 ± 4.1	26.6 ± 14.0	14.8 ± 7.0	21.0 ± 11.3	21.4 ± 7.3	24.2 ± 7.1
Lymphocyte (%)	83.7 ± 5.1	84.2 ± 5.3	62.7 ± 14.2	75.0 ± 12.5	59.9 ± 11.2	64.9 ± 11.2	80.0 ± 7.0	63.7 ± 11.5
Macrophage (%)	14.3 ± 4.6	14.0 ± 5.1	31.4 ± 15.7	21.9 ± 11.2	39.0 ± 11.5	33.8 ± 11.2	32.2 ± 5.7	35.5 ± 11.4
Neutrophils (%)	0.9 ± 1.0	0.4 ± 0.5	1.6 ± 1.7	3.2 ± 2.0*	0.5 ± 0.4	1.3 ± 1.0	0.5 ± 0.7	1.0 ± 0.7
$CD3^+$ (%)	21.6 ± 3.9	20.6 ± 2.7	19.1 ± 5.2	25.0 ± 10.0	22.1 ± 5.9	29.7 ± 7.3**	20.3 ± 7.6	26.6 ± 2.7* $^{\#\#}$
$CD4^+$ (%)	19.1 ± 2.5	17.3 ± 1.7	16.5 ± 3.2	19.1 ± 4.0	17.6 ± 2.4	17.9 ± 2.5	15.2 ± 5.1	21.4 ± 1.8*
$CD8^+$ (%)	7.0 ± 1.1	6.7 ± 0.6	5.7 ± 1.7	8.0 ± 1.5**	6.8 ± 2.8	7.0 ± 1.0	6.3 ± 2.8	5.0 ± 1.1
$CD4^+/CD8^+$	2.8 ± 0.1	2.6 ± 0.1	2.3 ± 0.3	2.3 ± 0.7	2.6 ± 0.7	2.7 ± 0.6	2.6 ± 0.6	4.5 ± 1.0***
DA								
Cell number (x10^6/g tissue)	9.3 ± 5.2	20.7 ± 5.3*	6.4 ± 1.3	30.3 ± 27.6**	15.5 ± 8.9	28.5 ± 12.2**	10.2 ± 8.5	14.1 ± 6.5*
Lymphocyte (%)	86.4 ± 2.5	86.8 ± 2.3	62.7 ± 8.6	60.0 ± 10.0	65.4 ± 7.2	65.4 ± 7.2	48.7 ± 8.6	57.4 ± 15.4
Macrophage (%)	12.9 ± 2.6	11.3 ± 3.3	36.6 ± 9.3	37.1 ± 10.9	32.0 ± 7.6	41.1 ± 9.3	50.2 ± 7.8	43.0 ± 12.8
Neutrophils (%)	0.3 ± 0.4	2.6 ± 2.2*	0.5 ± 0.6	3.3 ± 1.6*	1.1 ± 0.8	2.3 ± 1.4*	0.7 ± 0.6	2.6 ± 1.2 *
$CD3^+$ (%)	22.8 ± 2.4	25.8 ± 1.8	21.5 ± 4.7	20.8 ± 7.6	25.8 ± 5.9	33.6 ± 6.9*	23.0 ± 9.1	37.5 ± 2.0**
$CD4^+$ (%)	20.2 ± 1.3	19.8 ± 2.1	13.8 ± 4.7	15.0 ± 7.6	18.2 ± 2.6	21.4 ± 7.2	24.9 ± 3.3	26.4 ± 3.1
$CD8^+$ (%)	10.1 ± 3.9	12.6 ± 1.1	8.7 ± 2.9	7.7 ± 2.0	8.3 ± 1.5	8.1 ± 2.3	9.4 ± 4.6	10.9 ± 2.1
$CD4^+/CD8^+$	1.1 ± 0.0	1.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.6	2.2 ± 0.2	2.5 ± 0.5	2.4 ± 0.6	2.5 ± 0.3

Results are pooled from at least two independent experiments with 4-6 animals per group per experiment and expressed as mean values ± S.D. Significance at *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham infected animals of respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

4.2.2. Effector activities of total lung leukocytes related to direct fungal elimination in AO and DA rats

Activities of lung leukocytes that contribute to direct fungal elimination (i.e. CD11b expression, adhesion, oxidative activity and phagocytosis) were investigated next.

4.2.2.1. Adhesion of total lung leukocytes following administration of *A. fumigatus* conidia to AO and DA rats

Capacity of lung cell to spontaneously adhere to noncellular matrix was increased in DA rats solely, at all time points following infection (Fig. 9a). Following stimulation of lung cells from infected DA rats with PMA, adhesion increased (compared to spontaneous adhesion) and it was higher compared to control at all post-infection time points, while lung cells isolated from infected AO animals at day 14 solely responded to PMA-stimulation and that adhesion was increased compared to control (Fig. 9b).

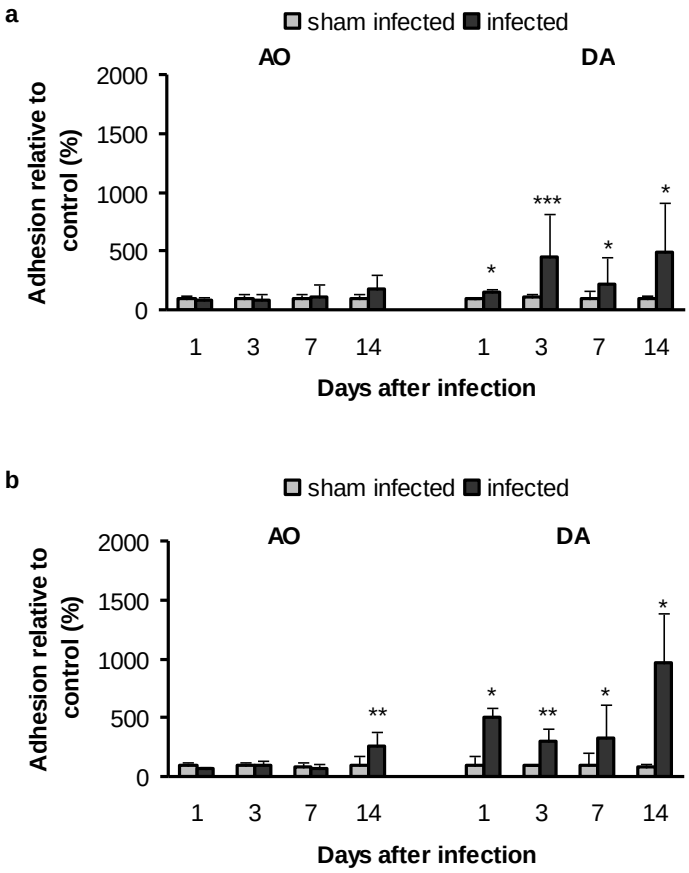

Fig. 9. Adhesion of total lung leukocytes from AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Lung cells of sham-infected and infected AO and DA rats were isolated by enzyme digestion at the indicated time-points and spontaneous (a) and PMA-stimulated (b) adhesion of cells to noncellular matrix was measured. Results are expressed as percentage of changes relative to control values and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain.

4.2.2.2. CD11b expression on lung lukocytes following administration of *A. fumigatus* conidia to AO and DA rats

Increased number of total, as well as granular, cells that express CD11b integrin molecule was noted in infected DA and AO rats at day 1 following infection (Table 2). Number of $CD11b^+$ cells was lower in AO compared to DA rats. Surface density (mean fluorescence density, MFI) of CD11b molecule was increased (compared to sham infected animals) on total and granular cells from AO rats at day 1 p.i., and on total (at days 1 and 3 p.i.) and granular cells (at day 1 p.i.) from lungs of DA rats.

Table 2. CD11b^{+} expression in total lung leukocytes following *A. fumigatus* conidia administration to AO and DA rats.

		Day 1		Day 3		Day 7		Day 14	
		sham	**infected**	**sham**	**infected**	**sham**	**infected**	**sham**	**infected**
AO									
total cells	(%)	25.7 ± 0.8	32.7 ± 6.8* ##	32.6 ± 14.6	26.2 ± 5.5	31.7 ± 6.5	37.5 ± 7.9	16.4 ± 2.3	20.6 ± 3.9
	MFI	242 ± 10	364 ± 37*	129 ± 81.1	93 ± 11	331 ± 13	331 ± 21	152 ± 13	166 ± 16
granular cells	(%)	49.8 ± 10.7	74.2 ± 6.2* ##	57.8 ± 14.4	37.4 ± 18.3	65.8 ± 19.5	58.1 ± 14.3	27.4 ± 5.2	42.8 ± 5.8
	MFI	360 ± 16	563 ± 40 *	393 ± 57	288 ± 197	480 ± 55	545 ± 170	310 ± 86	231 ± 36
mononuclear cells	(%)	15.2 ± 2.4	16.3 ± 4.3	30.4 ± 17.8	17.6 ± 4.2	23.7 ± 5.4	28.0 ± 9.9	7.9 ± 0.3	10.6 ± 1.7
	MFI	103 ± 2	110 ± 12	89 ± 10	92 ± 24	136 ± 25	136 ± 31	62 ± 3	67 ± 6
DA									
total cells	(%)	41.1 ± 7.5	50.7 ± 1.9*	47.1 ± 7.5	39.6 ± 8.9	39.7 ± 5.6	35.7 ± 5.1	21.6 ± 1.2	26.2 ± 3.6
	MFI	259 ± 30	392 ± 8*	276 ± 27	418 ± 54*	336 ± 57	401 ± 57	151 ± 8	173 ± 36
granular cells	(%)	66.9 ± 6.1	85.8 ± 2.6*	53.1 ± 14.1	42.8 ± 12.0	56.5 ± 11.5	54.1 ± 9.5	41.1 ± 0.8	56.5 ± 11.2
	MFI	446 ± 42	547 ± 23*	406 ± 77	487 ± 113	430 ± 125	422 ± 69	221 ± 26	229 ± 19
mononuclear cells	(%)	27.7 ± 6.6	24.6 ± 1.1	35.1 ± 4.3	26.1 ± 2.8	29.9 ± 4.5	23.5 ± 3.7	12.8 ± 2.2	15.1 ± 2.0
	MFI	88 ± 14	98 ± 6	110 ± 33	118 ± 35	128 ± 25	128 ± 10	56 ± 1	52 ± 5

Results are pooled from at least two independent experiments with 4-6 animals per group per experiment and expressed as mean values ± S.D. Significance at *$p < 0.05$ *vs* sham infected animals of respective strain and at ## $p < 0.01$ *vs* DA rats.

4.2.2.3. NBT reduction by total lung leukocytes following administration of *A. fumigatus* conidia to AO and DA rats

Transient (only at day 7 p.i., and lower compared to DA rats) increase in numbers of NBT^+ cells was noted in infected AO rats, while it was noted at day 3 p.i. in DA rats as well (Fig. 10).

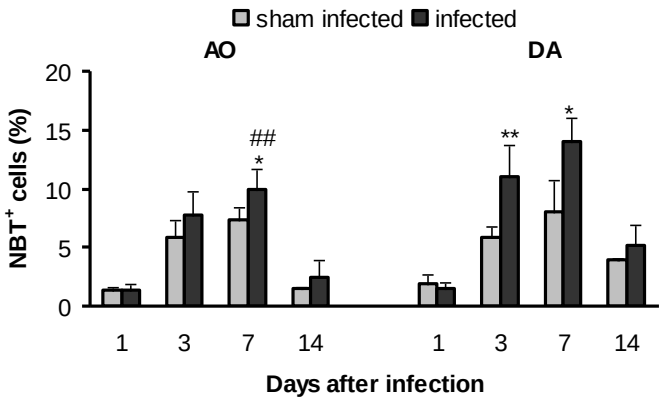

Fig. 10. Number of NBT^+ cells in population of total lung leukocytes from AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Lung cells of sham-infected and infected AO and DA rats were isolated by enzyme digestion at the indicated time-points and the activation of lung cells was evaluated by the quantitative cytochemical assay for the respiratory burst. Results are expressed as percentage cells with intracellular dots of formazan and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

4.2.2.4. Intracellular MPO activity in total lung leukocytes following administration of *A. fumigatus* conidia to AO and DA rats

Intracellular MPO lung cell content was increased in infected compared to sham infected animals of both strains at days 3 and 7 p.i., but it was numerically ($p = 0.092$) higher and significantly higher in DA compared to AO rats at days 3 and 7 p.i., respectively (Fig. 11).

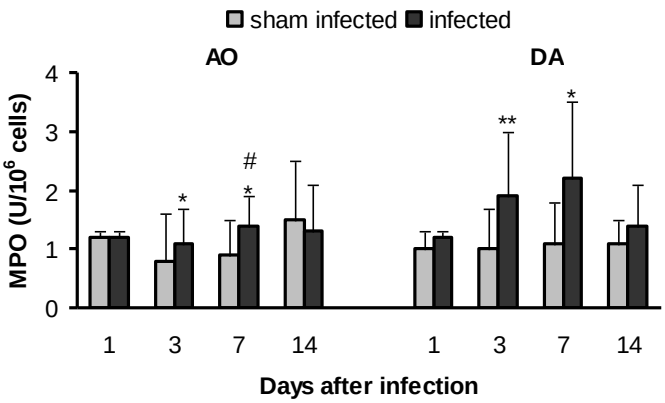

Fig. 11. Intracellular myeloperoxidase activity in total lung leukocytes from AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Lung cells of sham-infected and infected AO and DA rats were isolated by enzyme digestion at the indicated time-points and intracellular MPO activity in lung leukocytes was determined. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and $^{\#}$ $p < 0.05$ *vs* DA rats.

4.2.2.5. *In vitro* phagocytosis of conidia by total lung leukocytes following administration of *A. fumigatus* conidia to AO and DA rats

Infection resulted in an increase in numbers of cells with phagocytosed conidia in both strains early (day 1 p.i.) in infection. At day 14 p.i., even a decrease in numbers of cells that engulfed conidia was observed in infected DA rats (Fig. 12).

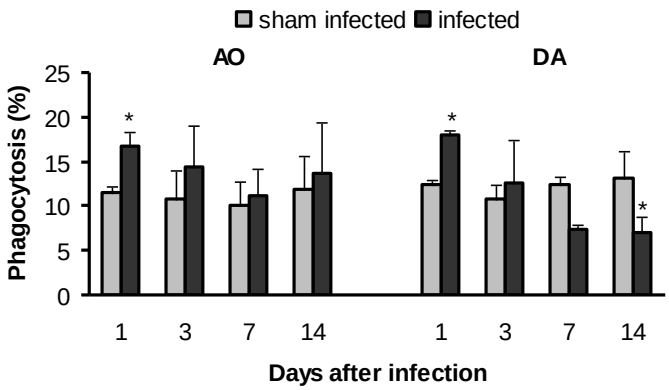

Fig. 12. *In vitro* phagocytosis of *A. fumigatus* conidia by total lung leukocytes from AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Lung cells of sham-infected and infected AO and DA rats were isolated by enzyme digestion at the indicated time-points and the capacity of cells to engoulf conidia *in vitro* was determined. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ *vs* sham-infected animals of the respective strain.

4.2.3. Cytokine production in culture of total lung leukocytes

Lung leukocytes from infected animals of both strains produce higher amount of **IFN-γ** compared to sham infected animals at all time points post infection (numerically in DA rats at days 3 and 7 p.i., $p = 0.097$ and $p = 0.100$, respectively) (Fig. 13). Numerically lower IFN-γ production was noted in infected AO compared to DA rats at day 1 p.i. ($p = 0.093$). Similar pattern of cytokine production following stimulation with *A. fumigatus* conidia *in vitro* was observed.

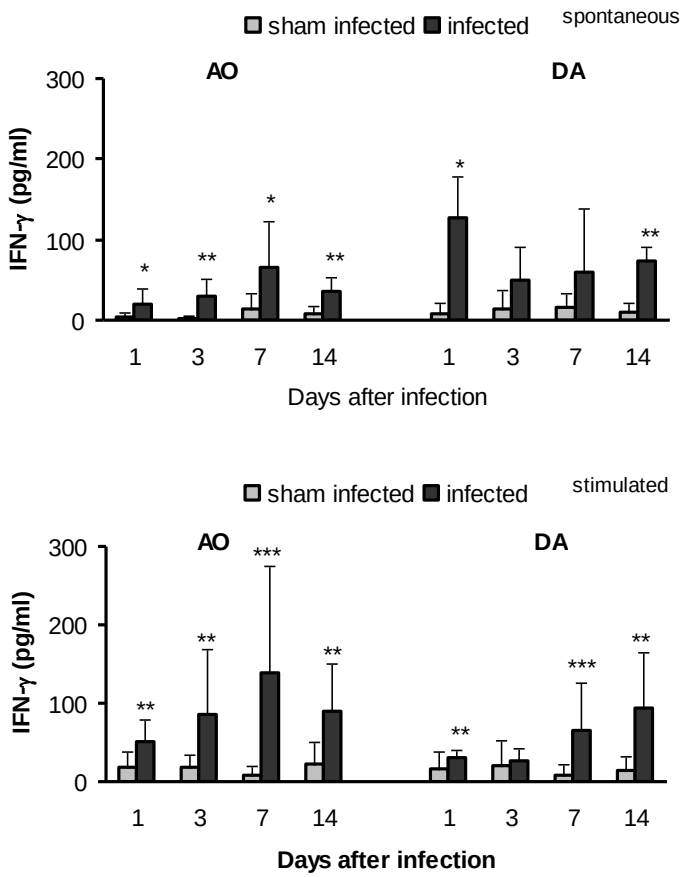

Fig. 13. Production of IFN-γ by total lung leukocytes in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from lungs of infected AO and DA rats and from sham-infected animals were isolated by enzyme digestion at the indicated time-points and cultured for 72 h in medium only (spontaneous production) or with heat-inactivated conidia of *A. fumigatus* (stimulated production) for the measurement of IFN-γ production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain.

Lung cells start to produce **IL-17** at day 3 p.i. (tendency of increase $p = 0.056$ in AO rats and $p = 0.062$ in DA rats) (Fig. 14). Amount of IL-17 rose further by the day 7 p.i. ($p = 0.007$ and $p = 0.050$ for day 3 *vs* day 7 p.i. in AO and DA rats, respectively) in both strains. Amount of IL-17 in DA rats returned to control level by the day 14 p.i., while in AO rats it stayed high at day 14 p.i. as well. Following stimulation higher (compared to sham infected animals) production of IL-17 was noted from day 3 to day 14 in both rat strains.

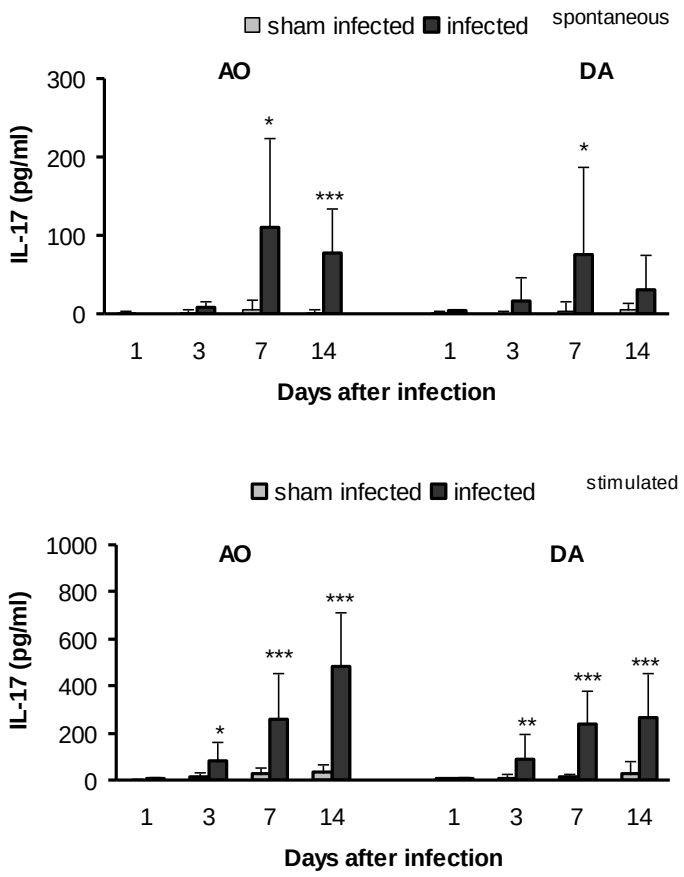

Fig. 14. Production of IL-17 by total lung leukocytes in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from lungs of infected AO and DA rats and from sham-infected animals were isolated by enzyme digestion at the indicated time-points and cultured for 72 h in medium only (spontaneous production) or with heat-inactivated conidia of *A. fumigatus* (stimulated production) for the measurement of IL-17 production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: $*p < 0.05$ and $**p < 0.01$ *vs* sham-infected animals of the respective strain and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* DA rats.

Low **IL-4** production was observed in both strains in all time points post infection. Significantly higher amount of this cytokine in infected compared to sham infected animals was noted only in AO rats at day 7 p.i. (Fig. 15). No differences between sham infected and infected animals were noted in IL-4 production following stimulation.

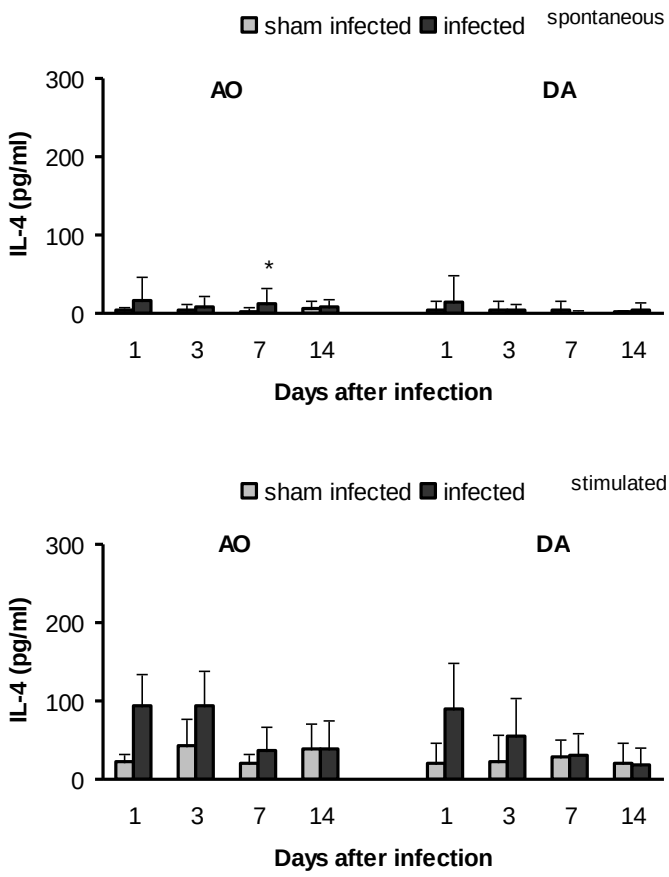

Fig. 15. Production of IL-4 by total lung leukocytes in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from lungs of infected AO and DA rats and from sham-infected animals were isolated by enzyme digestion at the indicated time-points and cultured for 72 h in medium only (spontaneous production) or with heat-inactivated conidia of *A. fumigatus* (stimulated production) for the measurement of IL-4 production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ *vs* sham-infected animals of the respective strain.

Progressive increase of **IL-10** level was noted in AO rats from day 3 to day 14 p.i. (p = 0.052 for day 3 *vs* day 7, and p = 0.036 for day 7 *vs* day 14 p.i.) (Fig. 16). In infected DA rats increased level of IL-10 was noted at days 3, 7 and 14 p.i. (increase from day 3 to day 7, p = 0.048 for day 3 *vs* day 7, and maintained at that level by the day 14 p.i.). Lung cells from infected AO rats produce more IL-10 compared to DA rats at days 3, 7 and 14 p.i.. Following

stimulation with *A. fumigatus in vitro*, higher amounts (compared to sham infected animals) of IL-10 were noted in AO rats at day 7, and in DA rats at days 3, 7 and 14 p.i..

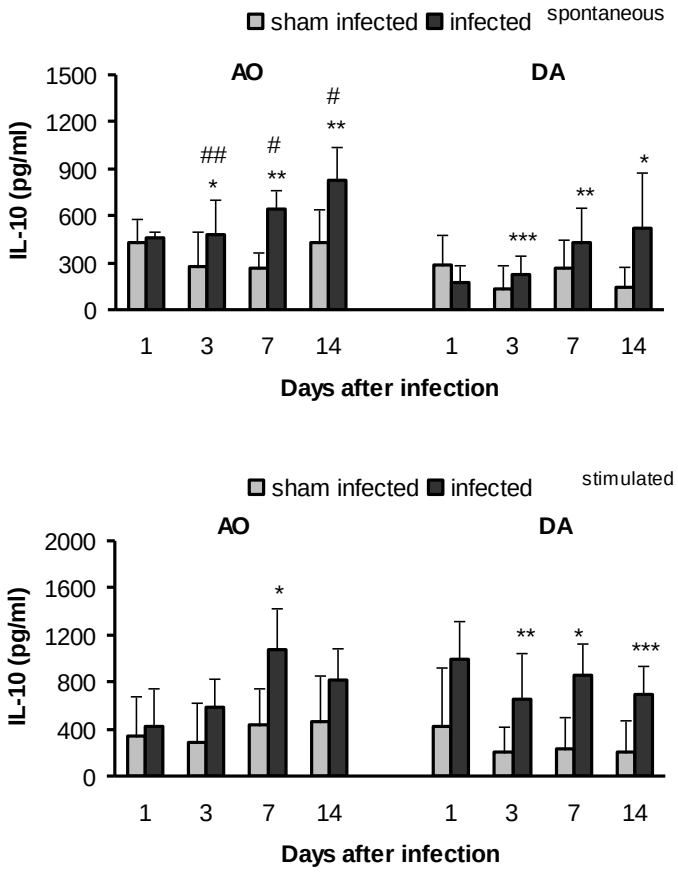

Fig. 16. Production of IL-10 by total lung leukocytes in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from lungs of infected and sham-infected AO and DA rats were isolated by enzyme digestion at the indicated time-points and cultured for 72 h in medium only (spontaneous production) or with heat-inactivated conidia of *A. fumigatus* (stimulated production) for the measurement of IL-10 production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* DA rats.

4.2.4. BAL cells recovery, leukocyte and T cell subset composition production following administration of *A. fumigatus* conidia to AO and DA rats

Increased number of total leukocytes recovered from BAL species of infected compared to sham infected DA rats was observed at day 3 p.i. (Table 3). In contrast, decreased number of cells (compared to sham-infected animals) was recovered from infected AO rats at days 1 and 3 p.i.. Similar relative number of macrophages (as well as lymphocytes) was observed in infected and sham infected animals of both strains, while the number of neutrophils was increased at day 3 p.i. in infected DA rats solely.

In infected AO rats numerical increase in $CD4^+$ cells (p = 0.078) at day 7p.i. and significantly increased number at day 14 p.i. was observed. Number of $CD8^+$ cells was higher in infected compared to sham infected AO rats at day 1 p.i.. Higher $CD4^+$ to $CD8^+$ cells ratio was noted in infected AO rats (compared to sham infected animals) at days 7 and 14 p.i.. Infection resulted in increase of number of $CD4^+$ cells in DA rats (compared to sham infected animals) at days 3, 7 and 14p.i. and in unchanged number of $CD8^+$ cells. This pattern of changes resulted in increase in $CD4^+$ to $CD8^+$ cells ratio at dyas 7 and 14 p.i..

Table 3. BAL cells recovery, leukocyte and T cell subset composition following *A. fumigatus* conidia administration to AO and DA rats.

	Day 1		Day 3		Day 7		Day 14	
	Sham	**infected**	**sham**	**infected**	**sham**	**infected**	**sham**	**Infected**
AO								
Cell number ($x10^6$)	2.0 ± 1.4	1.0 ± 0.2*	3.0 ± 1.3	2.2 ± 2.8*	4.7 ± 5.0	3.1 ± 2.6	3.0 ± 1.6	3.6 ± 1.8
Lymphocyte (%)	81.6 ± 6.1	85.5 ± 3.9	77.7 ± 6.0	82.3 ± 6.3	87.6 ± 5.9	89.5 ± 4.4	70.2 ± 2.0	79.5 ± 4.4
Macrophage (%)	18.3 ± 6.0	15.5 ± 2.9	21.0 ± 5.8	15.8 ± 5.3	10.9 ± 5.4	9.5 ± 4.2	18.3 ± 8.1	20.0 ± 4.4
Neutrophils (%)	0.1 ± 0.1	0.4 ± 0.5	0.8 ± 0.6	1.0 ± 1.1	1.0 ± 1.2	1.1 ± 1.2	0.5 ± 0.5	0.4 ± 0.6
$CD4^+$ (%)	2.9 ± 2.3	6.8 ± 1.2	9.7 ± 3.4	12.9 ± 6.9	12.7 ± 9.8	27.2 ± 14.9	12.7 ± 9.7	26.1 ± 11.8*
$CD8^+$ (%)	1.1 ± 0.8	4.2 ± 1.4*	5.7 ± 3.6	7.2 ± 4.4	6.4 ± 6.8	6.6 ± 3.3	4.2 ± 2.2	5.4 ± 3.5
$CD4^+/CD8^+$	2.0 ± 0.8	1.7 ± 0.2	1.7 ± 0.7	1.6 ± 0.4	1.7 ± 0.5	4.2 ± 0.7**	2.2 ± 1.1	5.0 ± 2.8**
DA								
Cell number ($x10^6$)	2.4 ± 1.1	3.3 ± 1.1	2.2 ± 1.9	3.9 ± 3.3*	1.4 ± 0.9	2.0 ± 1.2	4.2 ± 2.6	4.1 ± 2.9
Lymphocyte (%)	78.9 ± 2.4	77.5 ± 11.1	74.0 ± 8.2	77.7 ± 7.7	84.2 ± 6.0	85.9 ± 7.2	85.5 ± 4.5	77.9 ± 8.2
Macrophage (%)	21.1 ± 2.3	22.0 ± 11.5	25.1 ± 8.3	20.4 ± 8.2	14.9 ± 5.7	13.0 ± 6.5	15.7 ± 4.1	21.5 ± 8.0
Neutrophils (%)	0.2 ± 0.2	0.3 ± 0.3	0.5 ± 0.6	3.2 ± 1.2***	0.5 ± 0.6	0.1 ± 0.3	0.4 ± 0.4	0.6 ± 0.5
$CD4^+$ (%)	3.0 ± 1.8	6.2 ± 7.8	10.5 ± 6.2	30.7 ± 15.3**	31.5 ± 5.4	45.9 ± 9.7*	31.1 ± 5.5	54.8 ± 8.1*
$CD8^+$ (%)	0.7 ± 0.7	0.6 ± 0.2	3.3 ± 1.4	7.9 ± 4.9	6.7 ± 3.9	6.7 ± 3.3	8.5 ± 2.5	9.4 ± 4.3
$CD4^+/CD8^+$	3.8 ± 1.5	3.3 ± 2.3	2.6 ± 0.6	3.8 ± 1.7	3.5 ± 0.8	5.5 ± 1.4*	4.6 ± 1.7	7.3 ± 3.4*

Results are pooled from at least two independent experiments with 4-6 animals per group per experiment and expressed as mean values ± S.D. Significance at *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham infected animals of respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

4.2.5. Cytokine production by BAL leukocytes

BAL cells recovered from infected animals of both strains released significantly higher amounts of **IFN-γ** compared to sham infected controls at days 3, 7 and 14 p.i. (Fig.17). Higher level of IFN-γ was observed in infected AO rats as compared to DA rats at day 14 p.i..

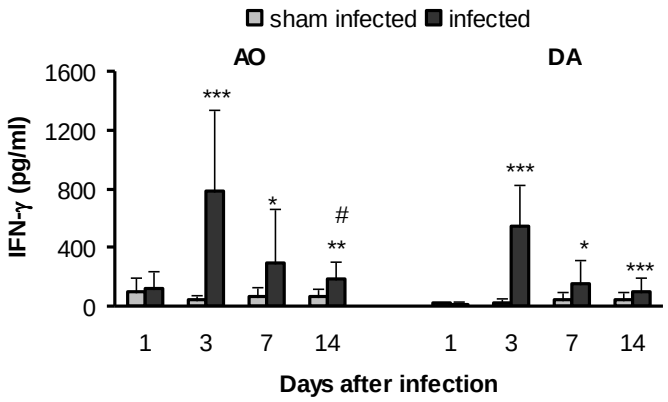

Fig. 17. Production of IFN-γ in culture of BAL leukocytes from infected and sham-infected AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were obtained by lavage of bronchoalveolar spaces at the indicated time-points and cultured for 72 h in medium only for the measurement of IFN-γ production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and $^{\#}$ $p < 0.05$ *vs* DA rats.

Increased (compared to sham infected animals) **IL-17** production in culture of BAL cells was observed in infected DA rats at days 7 and 14p.i. and in AO rats from day 3 to day 14 p.i. (Fig. 18). Amount of IL-17 produced was higher in infected AO compared to DA rats at day 14 p.i..

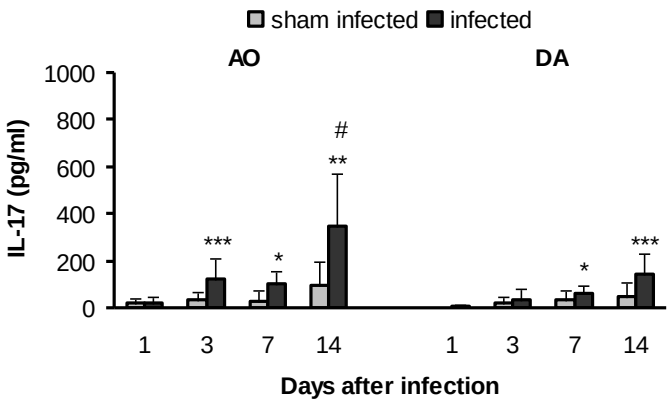

Fig. 18. Production of IL-17 in culture of BAL leukocytes from infected and sham-infected AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were obtained by lavage of bronchoalveolar spaces at the indicated time-points and cultured for 72 h in medium only for the measurement of IL-17 production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and $^{\#}$ $p < 0.05$ *vs* DA rats.

No changes were observed in **IL-4** production by BAL cells of infected animals of both strains (Fig. 19).

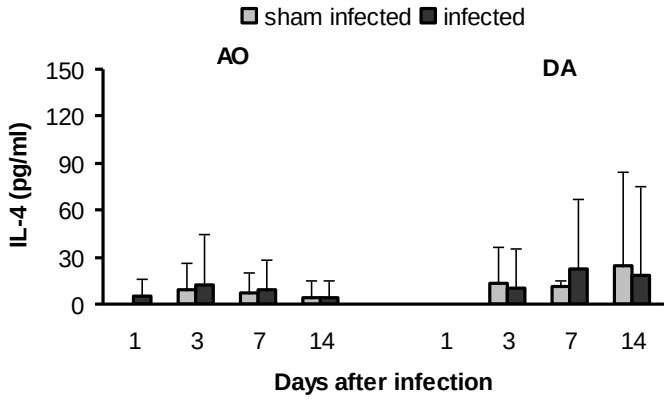

Fig. 19. Production of IL-4 in culture of BAL leukocytes from infected and sham-infected AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were obtained by lavage of bronchoalveolar spaces at the indicated time-points and cultured for 72 h in medium only for the measurement of IL-4 production. Cytokine levels were determined in cell culture supernatants by ELISA.

Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment.

Infection resulted in increased **IL-10** production in culture of BAL cells of infected DA rats at day 7 p.i. and AO rats at day 3 p.i. (Fig. 20).

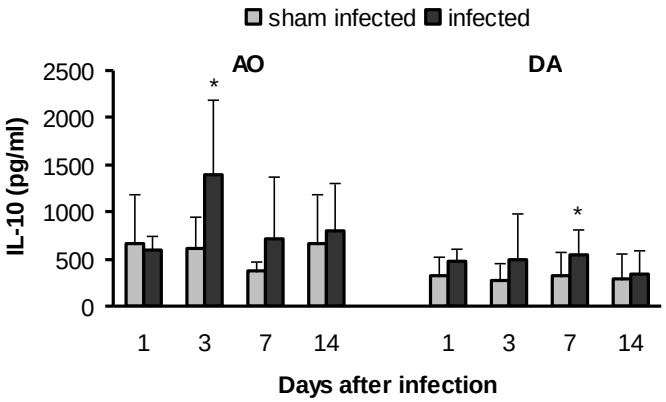

Fig. 20. Production of IL-10 in culture of BAL leukocytes from infected and sham-infected AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were obtained by lavage of bronchoalveolar spaces at the indicated time-points and cultured for 72 h in medium only for the measurement of IL-10 production. Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ *vs* sham-infected animals of the respective strain.

4.3. Draining lymph node cell response following administration of *A. fumigatus* conidia to AO and DA rats

Lymph nodes are places of generation of cytokine-producing cells. Thus cellularity, proliferative activity and cytokine production were determined next.

4.3.1. Draining lymph node cell number and proliferation following administration of *A. fumigatus* conidia to AO and DA rats

Increased number of cells was observed in infected *vs* sham infected DA rats at all time points post infection examined and in AO rats at days 3 and 7 p.i. (Fig. 21a). Higher number of DLN cells was noted in infected AO compared to DA rats at day 3 p.i.. Changes in DLN cell numbers in infected animals of both strains coincided with increased proliferation of cells (Fig. 21b).

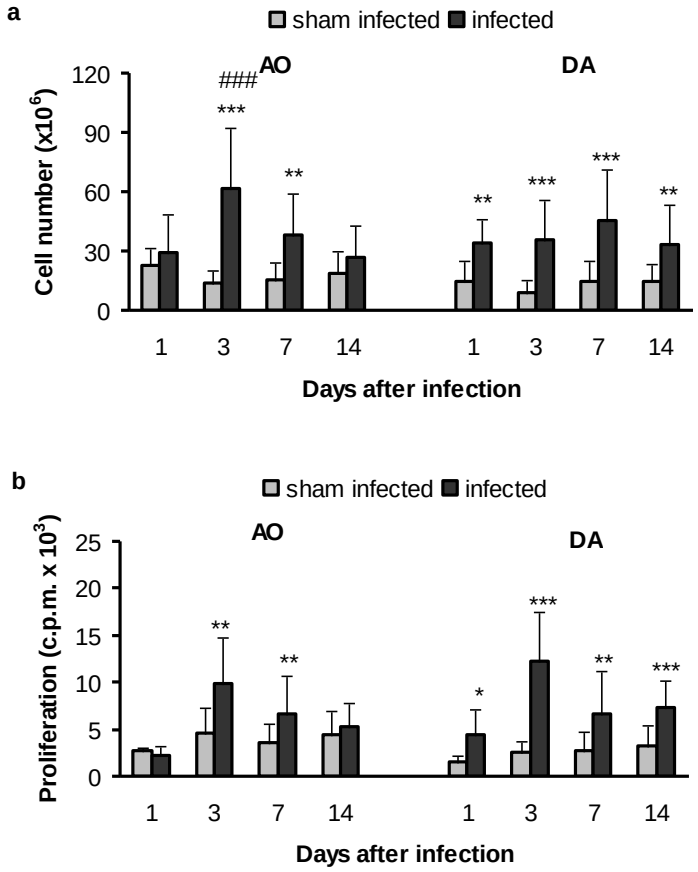

Fig. 21. DLN cell number (a) and proliferation (b) following administration of *A. fumigatus* conidia to AO and DA rats. Results are presented as mean ± SD of individual samples from at least two

independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ *vs* DA rats.

4.3.2. Basic T cell subset composition of DLN cells following administration of *A. fumigatus* conidia to AO and DA rats

No differences in relative number of $CD3^+$, $CD4^+$ or $CD8^+$ cells were observed between sham infected and infected animals of both strains, except increase in $CD3^+$ and $CD4^+$ cells in infected AO rats at day 14 p.i. (Table 4).

Table 4. Basic T cell subset composition of draining lymph node cells following *A. fumigatus* conidia administration to AO and DA rats.

	Day 1		Day 3		Day 7		Day 14	
	Sham	**infected**	**sham**	**infected**	**sham**	**infected**	**sham**	**Infected**
AO								
$CD3^+$ (%)	78.8 ± 7.1	80.3 ± 2.1	82.8 ± 4.7	69.8 ± 7.6	75.4 ± 1.9	74.1 ± 10.4	72.3 ± 3.8	80.3 ± 5.3*
$CD4^+$ (%)	40.5 ± 1.1	42.4 ± 5.0	45.8 ± 8.1	36.9 ± 7.1	47.9 ± 8.0	42.5 ± 8.4#	45.1 ± 4.2	52.7 ± 2.6*
$CD8^+$ (%)	24.5 ± 3.5	23.9 ± 4.5	28.9 ± 2.0	27.2 ± 5.9	22.3 ± 1.9	26.2 ± 5.4	23.5 ± 2.0	24.9 ± 3.7
$CD4^+/CD8^+$	1.7 ± 0.2	1.8 ± 0.5	1.7 ± 0.3	1.6 ± 0.4	2.2 ± 0.3	1.7 ± 0.3	2.0 ± 0.2	2.1 ± 0.3
DA								
$CD3^+$ (%)	84.3 ± 2.4	81.9 ± 2.0	73.3 ± 9.0	75.3 ± 7.3	76.5 ± 3.3	76.0 ± 2.5	73.9 ± 5.6	75.9 ± 4.6
$CD4^+$ (%)	50.5 ± 4.5	49.3 ± 1.3	49.6 ± 5.6	45.3 ± 4.3	49.2 ± 6.7	54.1 ± 3.7	56.1 ± 2.4	57.3 ± 3.3
$CD8^+$ (%)	23.1 ± 4.0	21.6 ± 1.9	17.2 ± 4.3	20.9 ± 2.8	19.1 ± 1.2	21.1 ± 2.6	19.0 ± 1.6	18.0 ± 1.4
$CD4^+/CD8^+$	2.3 ± 0.5	2.3 ± 0.2	2.9 ± 0.5	2.4 ± 0.6	2.8 ± 0.5	2.5 ± 0.5	3.0 ± 0.2	3.2 ± 0.3

Results are pooled from at least two independent experiments with 4-6 animals per group per experiment and expressed as mean values ± S.D. Significance at $*p < 0.05$ *vs* sham infected animals of respective strain.

4.3.3. Draining lymph node cell cytokine response following administration of *A. fumigatus* conidia to AO and DA rats

4.3.3.1. IFN-γ response

Significant increase in spontaneous IFN-γ production was observed in infected as compared to sham infected AO and DA rats at all time points examined, though due to high variation in cytokine values only numerical rise ($p = 0.100$) was observed at day 7 p.i. in DA rats (Fig. 22a). Stimulation (Fig. 22b) resulted in significantly higher (compared to spontaneous) IFN-γ production by dLN cells of infected animals in both strains, and the levels of IFN-γ were higher in infected compared to sham infected AO and DA rats at all time point following infection. Higher amount of this cytokine was detected in infected AO rats compared to DA rats at days 1 and 7 p.i., both in spontaneous and stimulated production.

Increased levels of IFN-γ mRNA (Fig. 23) was observed in infected as compared to sham infected AO rats at days 1 and 3 p.i., but it decreased at days 7 and 14 p.i.. Numerical increase ($p = 0.07$) and a tendency of an increase ($p = 0.051$) of IFN-γ mRNA was noted at days 3 and 7 p.i. respectively, in dLN cells from infected DA animals.

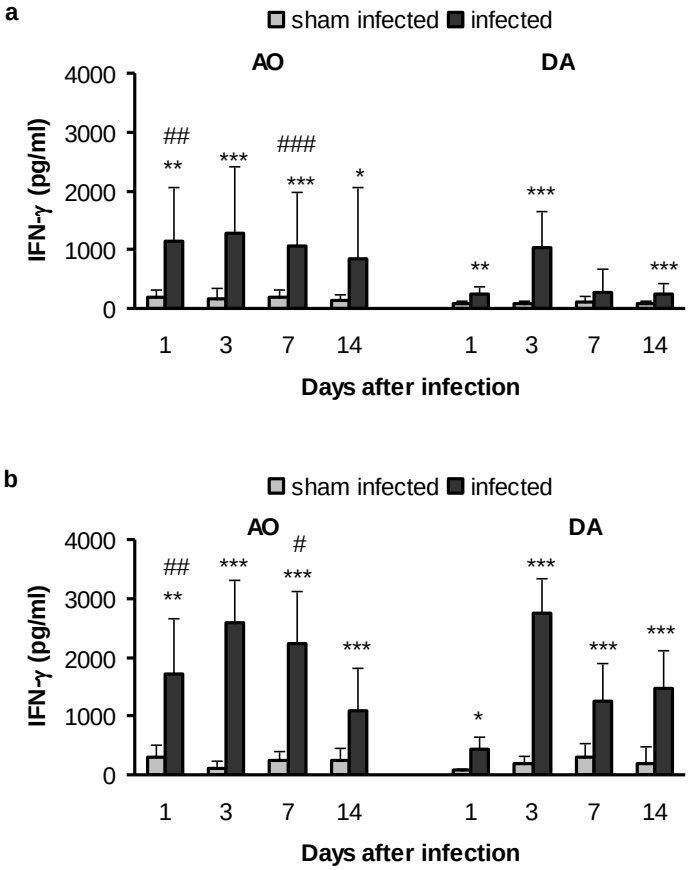

Fig. 22. Draining lymph node IFN-γ response in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from dLN of infected AO and DA rats, and from sham-infected animals were isolated and cultured for 72 h in medium only (spontaneous production) (a) or with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production) (b). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain, and at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* DA rats.

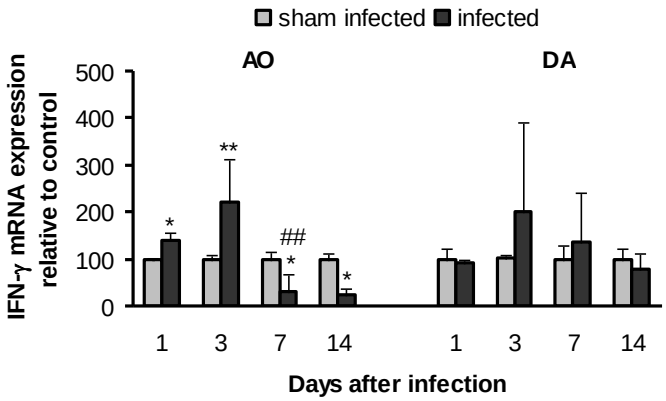

Fig. 23. Cytokine mRNA expression in dLN cells in DA and AO rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cell RNA from dLN of infected or sham-infected animals was extracted, reverse transcribed and a relative expression of IFN-γ genes were analyzed usig RT-PCR. Results are expressed as percentages of mRNA expression in dLN cells from infected animals relative to cells from respective sham infected individuals and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and $^{\#\#}$ $p < 0.01$ *vs* DA rats.

Examination of relative contribution of $CD4^+$ and $CD4^-$ cells to IFN-γ production revealed that $CD4^+$ cells are the main cytokine producers in both strains (Fig. 24). Significantly increased levels of IFN-γ produced by $CD4^+$ cells following stimulation with *A. fumigatus* conidia *in vitro* were observed in infected as compared to sham infected animals of both strains at days 3, 7 and 14 p.i.. No changes were noted in IFN-γ production by $CD4^-$ cells (Fig. 24).

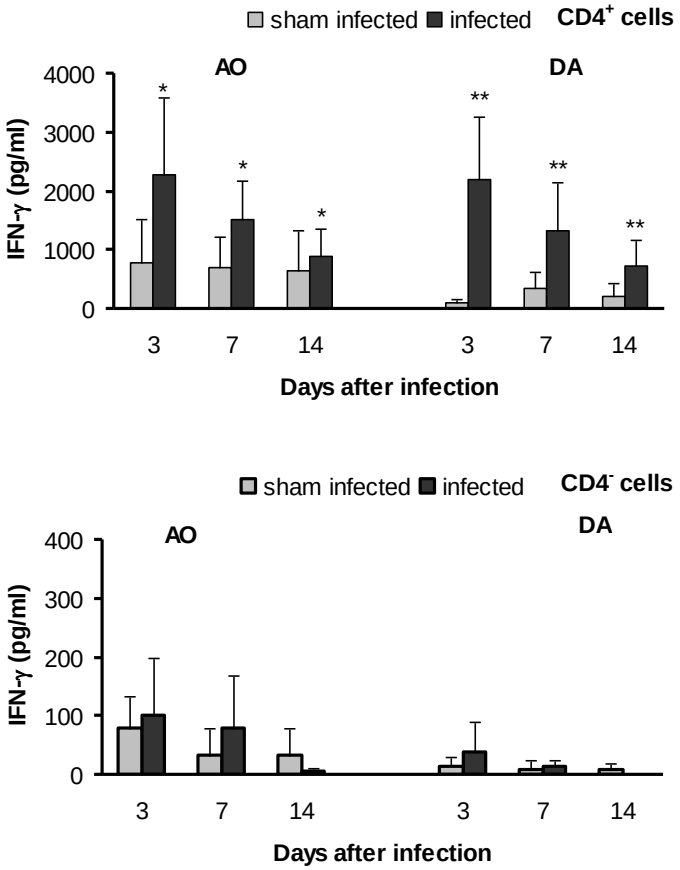

Fig. 24. Production of IFN-γ in CD4$^+$ and CD4$^-$ cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were isolated by immunomagnetic separation and cultured for 72 h with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain.

4.3.3.2. IL-17 response

Increased IL-17 production by dLN cells in infected AO rats (compared to sham infected animals) was observed at days 7 and 14 p.i., and in DA rats at days 3, 7 and 14 p.i. (Fig. 25a). Stimulation with *A. fumigatus* conidia *in vitro* resulted in increased IL-17 production (compared to spontaneous) that was higher in infected compared to sham infected AO rats at days 3, 7 and 14, and in DA rats at all time points post infection

(Fig. 25b). Levels of IL-17 produced following stimulation were lower in AO compared to DA rats.

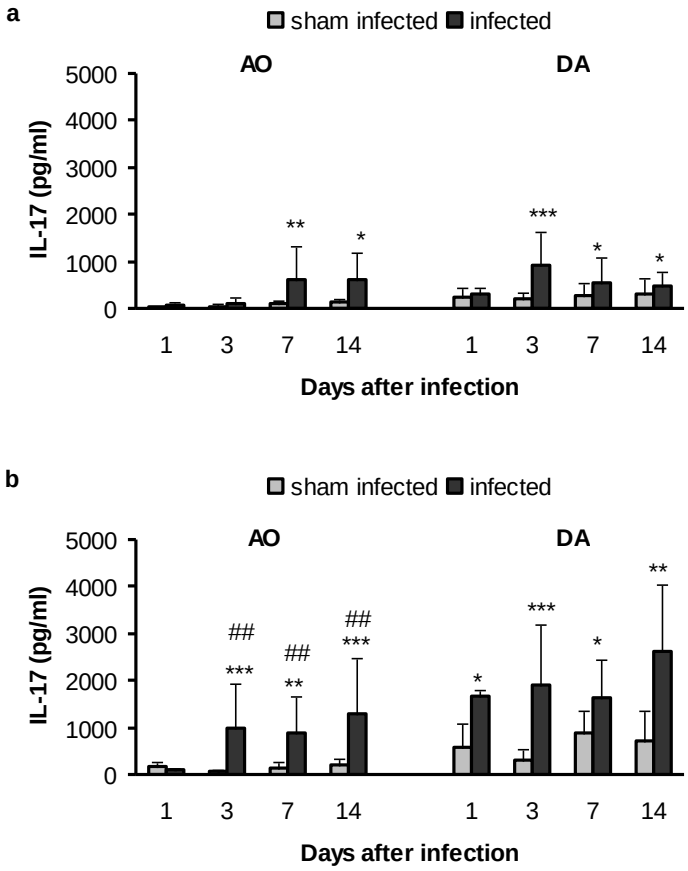

Fig. 25. Draining lymph node IL-17 response in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from dLN of infected AO and DA rats, and from sham-infected animals were isolated and cultured for 72 h in medium only (spontaneous production) (a) or with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production) (b). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

Increase in IL-17 mRNA level in infected AO rats (compared to sham infected) was noted at day 1 p.i. solely. Significantly increased IL-17 mRNA levels at days 1, 3

and 14 p.i. (and a tendency of an increase, $p = 0.050$ at day 7 p.i.) were noted in DA rats (Fig. 26).

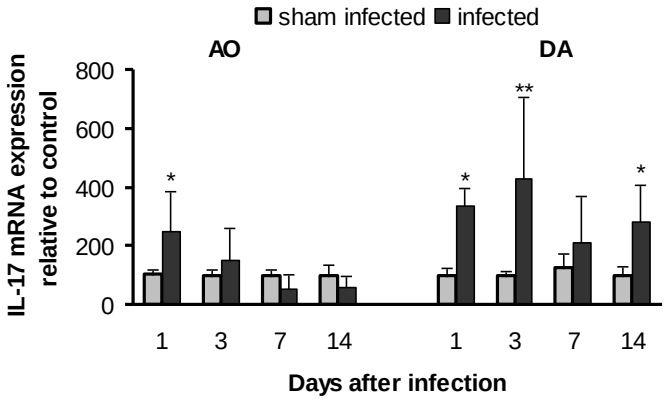

Fig. 26. Cytokine mRNA expression in dLN cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cell RNA from dLN of infected or sham-infected animals was extracted, reverse transcribed and a relative expression of IL-17 genes were analyzed usig RT-PCR. Results are expressed as percentages of mRNA expression in dLN cells from infected animals relative to cells from respective sham infected individuals and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.05$ *vs* sham-infected animals of the respective strain.

$CD4^+$ cells are the main cell source of IL-17 in both strains (Fig. 27). Increased levels (compared to sham infected animals) of this cytokine were noted in both strains at days 3, 7 and 14 p.i. following stimulation with *A. fumigatus* conidia. Cells isolated from dLN of AO rats produced less IL-17 compared to cells from DA rats at days 3 and 7 p.i.. Low and unchanged IL-17 production was detected in culture of $CD4^-$ cells (Fig. 27).

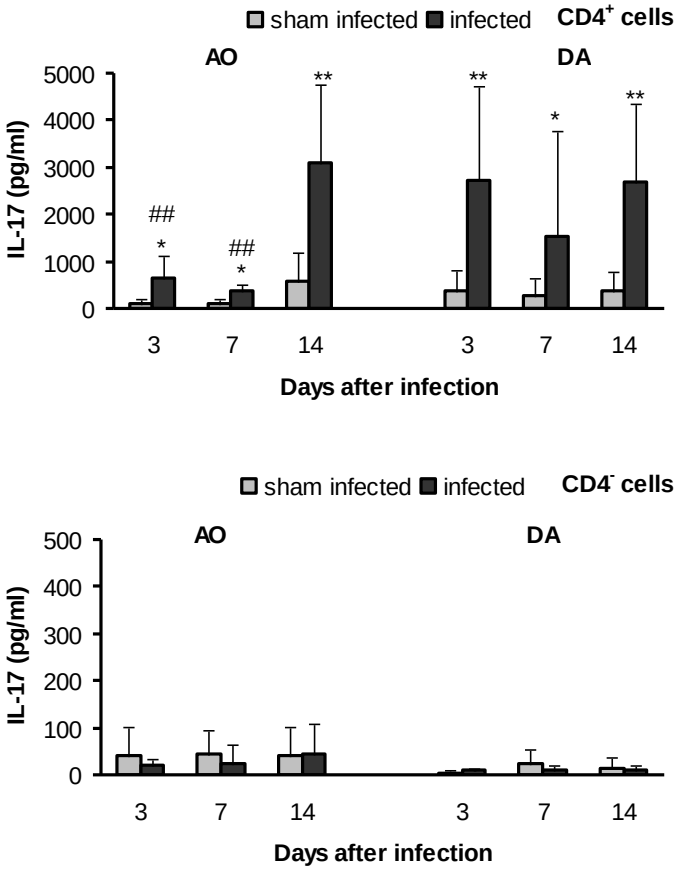

Fig. 27. **Production of IL-17 in CD4$^+$ and CD4$^-$ cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia.** Cells were isolated by immunomagnetic separation and cultured for 72 h with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

4.3.3.3. IL-4 response

Similar amounts of IL-4 were produced by dLN of sham infected and infected animals of both strains, except at day 7 p.i. in AO and at 3 p.i. in infected DA rats when a decrease was observed (Fig. 28a). Following stimulation with *A. fumigatus* conidia, the amount of IL-4 produced by dLN cells from infected AO rats at day 1 p.i. was lower in infected compared to sham infected animals, while infected DA animals produced

significantly lower amounts of this cytokine during infection (with a tendency to be lower $p = 0.052$, at day 7 p.i.) (Fig. 28b).

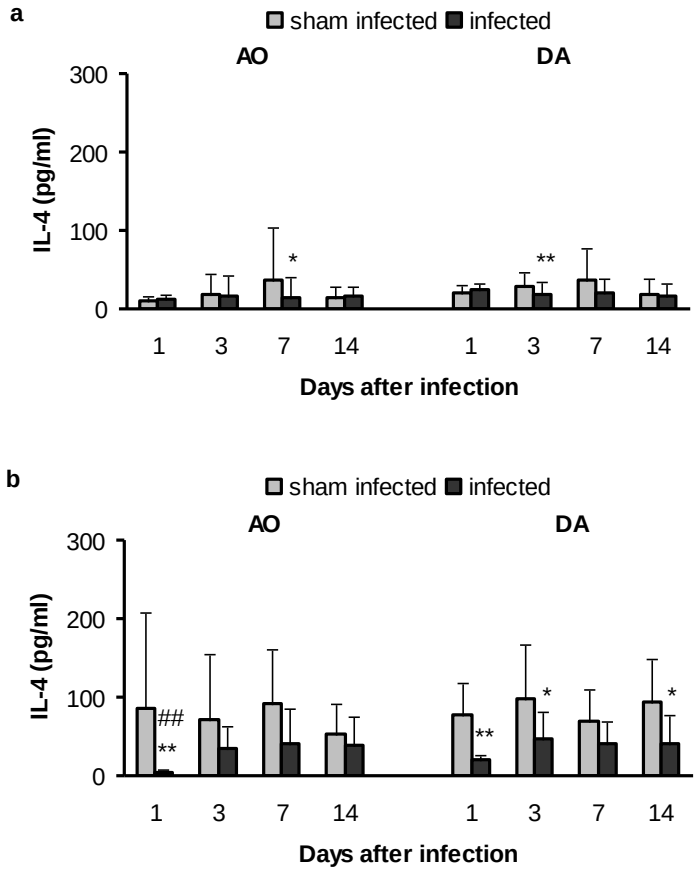

Fig. 28. Draining lymph node IL-4 response in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from dLN of infected AO and DA rats, and from sham-infected animals were isolated and cultured for 72 h in medium only (spontaneous production) (a) or with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production) (b). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and at $^{\#\#}$ $p < 0.01$ *vs* DA rats.

A decrease (compared to sham infected animals) of IL-4 mRNA was observed at days 3 and 14 p.i. in AO rats and at all points post infection, and DA rats (except at day 7 p.i., when only numerically ($p = 0.08$) lower values were observed) (Fig. 29). Higher

IL-4 mRNA levels at day 3 p.i. and lover at day 14 was noted in AO compared to DA rats.

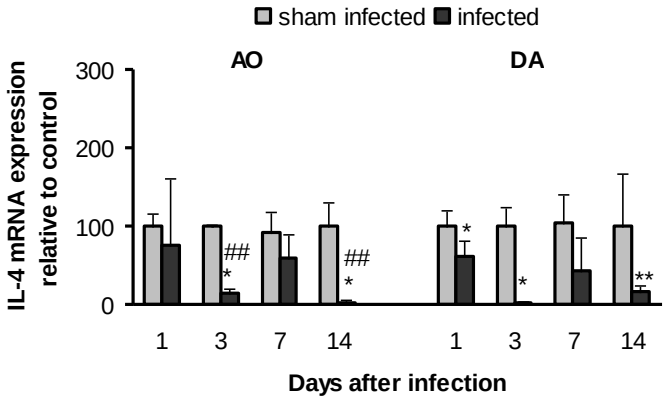

Fig. 29. Cytokine mRNA expression in dLN cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cell RNA from dLN of infected or sham-infected animals was extracted, reverse transcribed and a relative expression of IL-4 genes were analyzed usig RT-PCR. Results are expressed as percentages of mRNA expression in dLN cells from infected animals relative to cells from respective sham infected individuals and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain and $^{\#\#}$ $p < 0.01$ *vs* DA rats.

No changes (compared to sham infected animals) were noted in IL-4 production by $CD4^+$ or $CD4^-$ cells in both strains (Fig. 30).

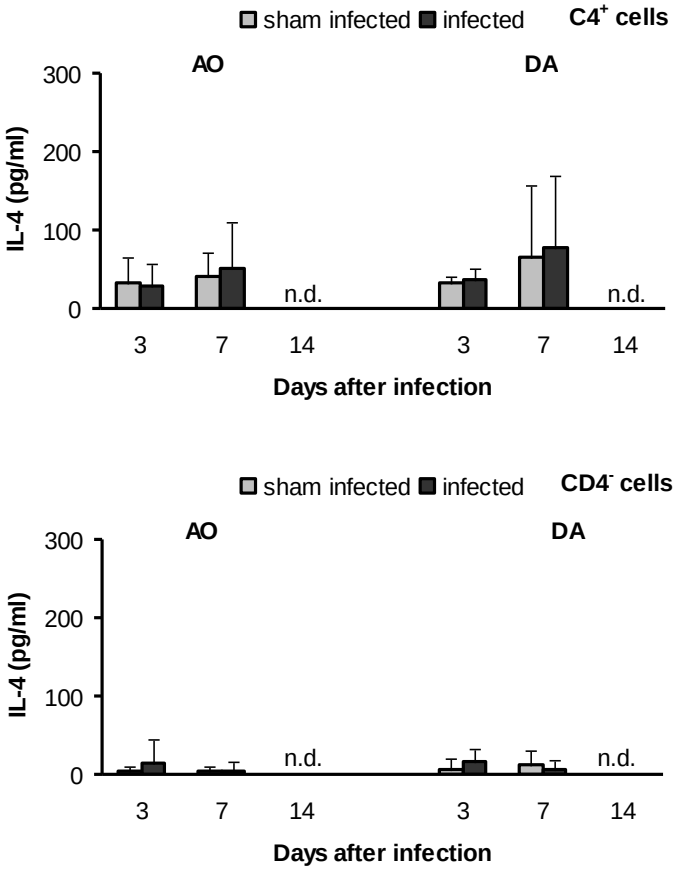

Fig. 30. Production of IL-4 in $CD4^+$ and $CD4^-$ cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were isolated by immunomagnetic separation and cultured for 72 h in with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. n.d. – not detected.

4.3.3.4. IL-10 response

Infection resulted in increased spontaneous IL-10 production (compared to sham infected animals) by dLN cells from both strains at days 3 and 7 p.i. (Fig. 31a). Following stimulation higher prodution of IL-10 in infected compared to sham infected AO rats was observed at days 3 and 7 p.i.. dLN cells isolated from infected DA rats following stimulation produced higher amounts of IL-10 compared to cells from sham

infected animals at all time points post infection (Fig. 31b). Level of IL-10 produced in AO rats at day 7 p.i. was lower compared to DA rats.

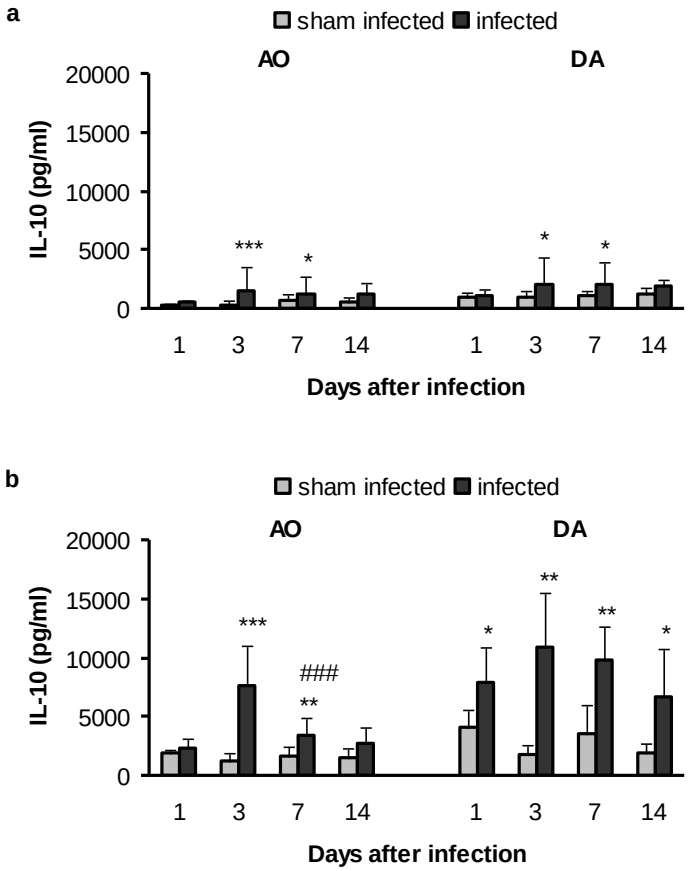

Fig. 31. Draining lymph node IL-10 response in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells from dLN of infected AO and DA rats, and from sham-infected animals were isolated and cultured for 72 h in medium only (spontaneous production) (a) or with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production) (b). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs* sham-infected animals of the respective strain and at $^{###}$ $p < 0.001$ *vs* DA rats.

IL-10 mRNA expression was increased in infected AO rats at day 1 p.i., and decreased at days 7 and 14 p.i., while in DA rats it remain at the control level (Fig. 32).

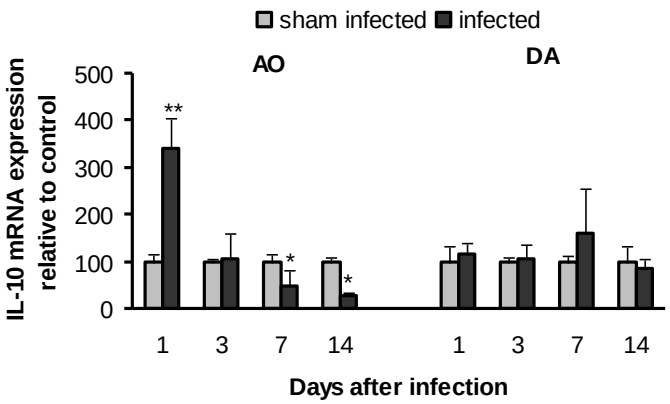

Fig. 32. Cytokine mRNA expression in dLN cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cell RNA from dLN of infected or sham-infected animals was extracted, reverse transcribed and a relative expression of IL-10 genes were analyzed usig RT-PCR. Results are expressed as percentages of mRNA expression in dLN cells from infected animals relative to cells from respective sham infected individuals and presented as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ and ** $p < 0.01$ *vs* sham-infected animals of the respective strain.

Following stimulation $CD4^+$ cells from both rat strains produced higher levels of IL-10 (compared to sham infected animals) at days 3 (numerically higher in AO rats, $p = 0.076$) and 7 p.i. (Fig. 33). No differences in IL-10 production between sham infected and infected animals were noted following stimulation of $CD4^-$ cells in both strains.

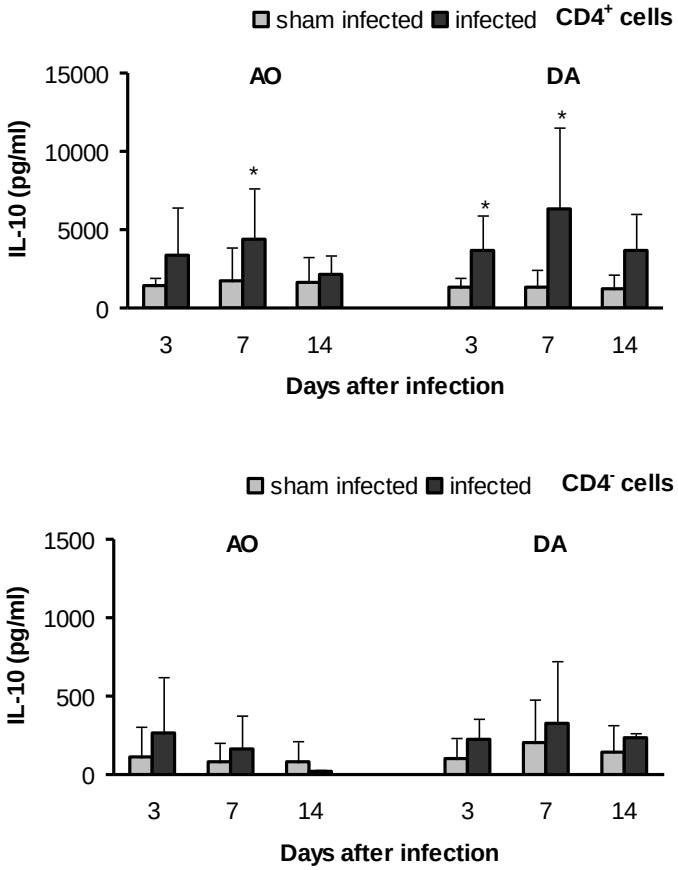

Fig. 33. Production of IL-10 in CD4$^+$ and CD4$^-$ cells in AO and DA rats following intratracheal administration of *Aspergillus fumigatus* conidia. Cells were isolated by immunomagnetic separation and cultured for 72 h with heat-inactivated conidia of *Aspergillus fumigatus* (restimulated production). Cytokine levels were determined in cell culture supernatants by ELISA. Results are expressed as mean ± SD of individual samples from at least two independent experiments with 4-6 animals for each post infection time point per experiment. Significance at: * $p < 0.05$ *vs* sham-infected animals of the respective strain.

5. DISCUSSION

5. Discussion

Characteristics of immune responses to pulmonary infection with *A. fumigatus* in two inbred rat strains, AO and DA, were analyzed in the present study. These two strains were chosen as they differ in responsiveness to inflammatory stimuli in various tissues including nervous system (Mostarica-Stojković *et al*., 1982; Dimitrijević *et al*., 2001) and pancreas (Pravica *et al*., 1993). Data presented in this study showed that these two strains differ in the responsiveness to infection as well. Lower degree of lung cell infiltration and their basic effector activities along with differences in the pattern of proinflammatory (IFN-γ and IL-17) and Th2/anti-inflammatory (IL-4 and IL-10) cytokine responses in lungs and draining lymph nodes might have accounted for less efficient elimination of fungus in AO rats. Beside differences in the intensity of inflammatory responses, disparate dynamics (shorter duration and/or daleyed start of responses might have contributed to slower elimination of fungus from AO rats.

Rats successfully control the infection with *A. fumigatus* in lungs judging by the complete removal of fungus by the day 14 p.i. in DA rats and day 21 p.i. in AO rats and by the lack of fungal growth up to the day 30 post infection.

Antifungal response was associated with mild inflammation in both strains, judging on similar wet lung mass in sham and infected animals. These findings are in the line with studies in mice (Cenci *et al*., 1998). Absence of symptoms of respiratory problems were noted even in immunosuppressed Wistar rats in which histological signs of immune reactivity to *A. fumigatus* in lungs at day 1 following infection were demonstrated (Mahmoud *et al*., 2011). In contrast, changes in clinical parameters (i.e. animal's activity, body weight changes, presence of secretion on rat's snouts (Becker *et al*., 1999), as well as pulmonary lesions (some of them haemorrhagic) were reported in immunosuppressed RP albino rats inoculated with *A. fumigatus* (Becker *et al*., 1999, 2006). The use of immunocompetent (non-immunosuppressed) animals that efficiently remove *A. fumigatus* conidia from lungs in the present study might be one of the factors contributing to the lack of clinical signs of infection. The strain of *A. fumigatus* used for infection might be responsible as well. In corroboration, mild or moderate effects on respiratory function (dyspnea and tachypnea) in immunocompetent rats have been noted following infection with 10^9 conidia of some *A. fumigatus* strains, while the other

strains cause more severe respiratory problems at the same dose in same rat strain (Land *et al*., 1989).

Aspergillus fumigatus infection resulted in leukocyte infiltration in lungs in both rat strain judjing by histological appearance of lung tissue and the number of cells recovered following enzyme digestion and bronchoalveolar lavage. These data are in line with data obtained in mouse model of pulmonary aspergillosis (Cenci *et al*., 1998). Lower degree and transient leukocyte infiltration was noted in lungs of infected AO rats compared to infected DA rats. These findings might imply lower propensity of leukocytes from AO rats to infiltrate lung tissue. In corroboration, our data demonstrated significantly lower propensity of lung cells from AO rats to adhere to noncellular matrix, as well as lower numbers of lung leukocytes that express CD11b integrin molecules in AO rats compared to DA rats early (day 1 p.i.) following infection. These integrin molecules (such as CD11b/CD18) are present on macrophages and neutrophils and are important for their migration (Arnaout, 1990) and functional activation of infiltrated phagocytosis cells in the environment of infected lungs (Abram and Lowell, 2009). Although lower numbers of $CD11b^+$ cells in infected AO compared to infected DA rats at day 1 p.i. might rely on lower numbers in sham infected animals, this difference might have exerted influence on the efficacy of elimination of fungus. Presence of neutrophils in the lungs during the entire post infection period in DA rats reflects the absolute need for these cells, most probably because of their multiple importances in elimination of fungus (Balloy and Chignard, 2009). Variety of neutrophil antifungal activities (Dagenais and Keller, 2009) are expected to be induced, as conjoint antifungal activities of alveolar macrophages (Philippe *et al*., 2003) and neutrophils (Dagenais and Keller, 2009) are necessary for killing of *A. fumigatus*. A decrease in numbers of macrophages at day 3 p.i. in infected compared to sham infected AO rats might have resulted from destroying of these cells *in situ*, as phagocytes are expendable at sites of inflammation. *A. fumigatus* produces a variety of secondary metabolites, including gliotoxin (Kosalec and Pepeljnjak, 2005) which is known to cause apoptotic cell death in professional immune cells such as macrophages and monocytes (Stanzani *et al*., 2005).

Fungal elimination in rats took place in *milieu* enriched in proinflammatory (IFN-γ and IL-17) cytokines, but with unchanged IL-4. In the line with data from mouse

models of pulmonary aspergillosis (Brieland *et al*., 2001), presence of IFN-γ in homogenates of lungs from infected rats was shown but, in contrast to mouse data (that showed maximal amounts of cytokine at day 2 p.i., followed by a sharp drop to low/negligible concentrations at day 4 p.i. and disappearance at day 7 p.i.), our results demonstrated presence of high amounts of this cytokine in lung tissue homogenates at days 3 and 7 p.i.. Comparable levels of IFN-γ production by lung cells and BAL cells of both strains, as opposed to higher IFN-γ levels produced by dLN cells of AO rats compared to DA rats, suggest negative regulation of cytokine producing cells in lung microenvironment. It might have resulted from higher levels of IL-10 produced by lung cells of AO rats compared to DA rats (Bruin *et al*., 2000). The late production of IFN-γ (days 7 and 14 p.i) suggests T cells as cellular sources of this cytokine in lung digest cell preparation of infected rats. This is in line with data from mice that showed production of this cytokine at day 7 p.i. by bronchoalveolar $CD4^+$ T cells which are induced in draining lymph nodes (Rivera *et al*., 2005). In addition to those data, we showed that development of IFN-γ response in draining lymph nodes started early after infection and that it was present at the time when infection was eliminated as well. Early production of antigen-specific IFN-γ producing cells in draining lymph nodes might be explained by the need of innate (TLR-mediated) signals from antigen-presenting cells as described in mice (Rivera *et al*., 2006). Production of IFN-γ by day 14 p.i. in both strains might reflect the need for the presence of cytokine as a mean to preclude re-establishing of infection. Occurrence of IFN-γ production at day 14 p.i. without mRNA induction might be explained by influence of tissue culture conditions *in vitro* during the 3-day culture period.

Late (day 7 p.i.) increase in numbers of $CD3^+$ (T cells) imply existence of adaptive immune responses. T cells are of utmost importance in antifungal responses being producers of cytokines, mediators and regulators of immune response, increase in number of $CD3^+$ cells might be responsible for higher lung IFN-γ content in DA compared to AO rats. However, increase in $CD3^+$ cells was similar in both strains, except day 14 when higher numbers of $CD3^+$ T Cells in DA rats compared to AO rats was noted (this might be one of the factors which caused the rise level IFN-γ at day 14 p.i.), what imply other sources of this cytokine. In this regard, IFN-γ production by neutrophils was reported in some infections in mice (Elis and Beaman, 2002) and

increased numbers of these cells were found at day 7 p.i. in DA rats (unchanged in AO rats). Along with neutrophils, lung NK cells were shown to be the largest cell population that produces IFN-γ in early postinfection period in mice with aspergillosis (Park *et al*., 2009).

In mouse model of pulmonary aspergillosis IFN-γ production by lung $CD4^+$ cells (at day 7 p.i. when it was measured) was shown (Cenci *et al*., 1998; Rivera *et al*., 2005). Our data suggest that $CD4^+$ cells are the main cytokine source in rats as well, judjing by increased antigen-specific IFN-γ production by $CD4^+$ cells in draining lymph nodes. In corroboration, highest production of IFN-γ by BAL cells was detected from days 3 to day 14 p.i., when accumulation of $CD4^+$ lymphocytes was observed. Beside $CD4^+$ T cells, $CD8^+$ cells, which numbers (numerically) increased in BAL cell population at day 3 p.i., might have contributed to production of this cytokine as well. Responsiveness of $CD8^+$ cells to *A. fumigatus* antigens was reported in mice (Tao *et al*., 2006) and the significant increase in IFN-producing $CD8^+$ cells in BAL fluid of mice infected with pulmonary aspergillosis was observed recently (Templeton *et al*., 2011). The observed reduction of IFN-γ gene expression was likely related to the effect of induced IL-10 on APCs and/or T cell functions, as suppression of proinflammatory cytokine production is major activity of IL-10 in dampening of inflammation (Trinchieri, 2007).

Higher levels of IFN-γ in lung tissue in infected DA rats compared to infected AO rats might be responsible for efficient elimination of fungus from lungs of DA rats. The importance of IFN-γ in phagocytosis-mediated effector responses to *A. fumigatus* is demonstrated and included stimulation of reactive oxygen production in neutrophils and the hyphal damage by these cells (Roilides *et al*., 1993) as well as enhanced killing of ingested *A. fumigatus* by macrophages (Shao *et al*., 2005).

Presence of IL-17 in lung tissue homogenates early (day 1 p.i.) following infection in rats is in line with recently published data that showed presence of this cytokine in lung tissue of infected immunocompetent mice at day 1 p.i. (Werner *et al*., 2009). A requirement for IL-17 in clearance of *A. fumigatus* from lungs was shown, as neutralization of IL-17 early in postinfection period increased dramatically fungal lung burden (Werner *et al*., 2009). In corroboration, higher lung fungal burden in $TLR9^{-/-}$ mice coincided with lower levels of IL- 17 in the lung (Ramaprakash *et al*., 2009), implying that IL-17 is involved in protective responses to *A. fumigatus*. In lung tissue,

innate immunity cells might be responsible for the early lung IL-17. Data from $p47^{phox}$ deficient mice imply that γ/δ T cells might be a source of IL-17 produced 2 days following infection (Romani *et al*., 2008). Enrichment in IL-17 content in lungs of infected rats early in postinfection period might rely on neutrophils as well, shown recently to be a significant source of early produced (day 1 p.i.) IL-17 in pulmonary aspergillosis in mice (Werner *et al*., 2011). Generation of IL-17 producing cells in draining lymph nodes of infected rats started at this time point, judging by gene expression data. However, spontaneous cytokine release by dLN cells was not detected, for what post transcriptional/translational processing (as well as environmental influences) might be responsible. Differential pattern of change of IL-17 mRNA levels (unchanged compared to sham infected at day 3, followed by a tendency to be lower than in sham infected in AO contrary to a tendency of higher mRNA levels in DA rats at day 7 p.i. than AO rats) might have accounted for lower spontaneous production, as well as lower responsiveness to stimulation in the former compared to the latter strain. Although generation of cytokine producing cells started early in lymph nodes, significant lung cell IL-17 production was observed later following infection. Such a pattern of IL-17 release by lung cells suggests either late migration of IL-17 producing cell from lymph nodes or suppression of cytokine production in migrating cells early in infection. Given the pattern of cytokine production by lung cells (IFN-γ earlier than IL-17) and that inhibition of generation of IL-17 producing cells by IFN-γ is reported (Harrington *et al*., 2005; Zhou *et al*., 2009) suppression of IL-17 seems possible. Late production of IL-17 by lung cells point out the need for IL-17 later in infection, when a decrease in the amounts of IFN-γ (compared to early production) was observed. In this regard, the importance of IL-17 production in settings of relative IFN-γ deficiency was shown in murine pulmonary aspergillosis (Zelante *et al*., 2007). Late production of IL-17 by lung cells concomitantly with presence of cytokine producing cells in draining lymph nodes imply T cells as a source of this cytokine in lungs. In corroboration, anti-CD3 stimulated conventional (α/β T-cell receptor expressing) T cells isolated from lungs of infected $p47^{phox}$ deficient mice at day 6 p.i. (when measured) were shown as the main source of this cytokine in *A. fumigatus* pulmonary infection (Romani *et al*., 2008). Although generation of IL-17 producing cells in lung-draining lymph nodes is already shown in mice (Zelante *et al*., 2007) it is measured at one time point (day 7 p.i.) solely.

Our data showed that they are present during the entire period post infection in DA rats or from early infection stages in AO rats (i.e. from day 3 p.i.). Draining lymph node $CD4^+$ cells are main producers of IL-17, judging by similar level of production by total and $CD4^+$ dLN cells. Highly increased production of IL-17 by BAL cells at day 14 p.i. when no further rise (compared to antecedent time points) in dLN cell cytokine release and gene expression was observed, suggests additional differentiation of IL-17 producing cells in the lung microenvironment. Along with that, lack of strain differences in the levels of IL-17 produced by lung cells as opposed to lower production by dLN cells of AO rats suggest positive regulation of this cytokine in the lung environment in AO rats.

The relevance of IL-17 for antifungal activities of innate immune effectors is however not clear, as decreased capacity of neutrophils (exposed to IL-17 *in vitro*) to kill *A. fumigatus* conidia was demonstrated, despite increased production of effectors (myeloperoxidase and gelatinase) (Zelante *et al*., 2007).

Similar lung content of IL-4 during the entire postinfection period and unchanged production of this cytokine by lung cells of infected animals (as well as BAL cells) suggests minor relevance of this cytokine for immune response to *A. fumigatus.* Increased IFN-γ production, a major cytokine inhibitor of development of IL-4 producing T cells in various microbial infections (Maggi *et al*., 1992) might be responsible for lower IL-4 production. Increased IL-4 production (numerically) by lung cells of infected AO rats at day 7 p.i. as opposed to unchanged levels in draining lymph nodes at this time point suggest upregulation of production of this cytokine in lungs as well. Increased levels of IL-4 might have contributed to positive regulation of this cytokine, as differentiation of primed to effector T cells is dependent on IL-4 (Schulze *et al*., 1998). Although the negative impact of IL-4 in aspergillosis was recognized earlier, these are the first data to show inhibition of production of this cytokine that starts from the beginning of infection and is present until it is eradicated. Lack of change of IL-4 production by dLN cells of infected animals of either strain (except at day 3 p.i. in DA rats) suggests minor relevance of this cytokine for immune response to *A. fumigatus* in rats. A decrease in mRNA (at day 3 p.i.) and of stimulated production of this cytokine suggest an inhibition of development of IL-4 producing cells in draining lymph nodes. Decrease in production of this cytokine in dLN in response to stimulation

with conidia, suggests an inhibition of development of IL-4 producing cells in draining lymph nodes. Decrease in IL-4 production might be beneficial for the host, as this cytokine was shown to enhance susceptibility to invasive pulmonary aspergillosis (Cenci *et al*., 1999). A decrease in IL-4 production following stimulation at unchanged levels of mRNA at day 7 p.i. in both strains might rely on post transcriptional/translational events. IFN-γ, a major cytokine inhibitor of development of IL-4 producing T cells in various microbial infections (Maggi *et al*., 1992) might be responsible for lower levels of dLN cell IL-4 production in both strains. A decrease in IL-4 production might be beneficial for the host, as this cytokine was shown to enhance susceptibility to invasive pulmonary aspergillosis (Cenci *et al*., 1999).

Although unchanged IL-10 lung tissue content was observed in infected compared to sham-infected animals of both strains, progressive increase of IL-10 level produced by lung cells was noted in infected animals from day 3 to day 14 p.i.. High levels of lung cell-derived IL-10 that exceeded the levels of both proinflammatory cytokines in both strains might reflect a need for balanced levels of (potentially) antagonistic cytokines to ensure efficient (but restrained) response. In the light of such considerations, it could be assumed that higher levels of IL-10 production by lung cells of AO rats might reflect the need for stringent control of proinflammatory cytokine production in these rats. Increase of IL-10 production in pulmonary aspergillosis shown in our study is in contrast to mouse data that showed low (Cenci *et al*., 1998) or no production (Balloy *et al*., 2005) of this cytokine. The difference in time point analyzed (day 1 p.i. or shorter period of analysis, i.e. four days post infection) in these studies compared to 14-day period in our study might be responsible. Increase in IL-10 production by draining lymph node cells suggests that lymphocytes are source of this cytokine with $CD4^+$ cells as major contributors to dLN cell IL-10 production. Lower production of IL-10 by cells of this subset at day 3 p.i. suggest other cell sources in draining lymph nodes. In this regard, production of IL-10 by $CD8^+$ cells was reported under certain settings (Richards *et al*., 2000).

Higher (compared to DA rats) levels of IL-10 concomitantly with lower levels of IFN-γ were noted in draining lymph nodes of AO rats that are less susceptible to induction and expression of local damaging inflammatory reaction to contact allergen in experimental contact hypersensitivity (Popov *et al*., 2011). However, in the light of data

that showed other activities of IL-10 which might be relevant for antifungal response in lungs including increase of phagocytosis (Roilides *et al*., 1997) and the role in cell migration (Rivera *et al*., 2009) upregulation of IL-10 production by lung cells of AO rats might reflect an effort of AO rats to enhance local antifungal defense. IL-10 can exert other activities, beside inhibition of proinflammatory cytokines, which might be relevant for antifungal response in lungs. In this regard, proinflammatory activity of IL-10 concerning promotion of some local effector mechanisms in inflammation was proposed (Mocellin *et al.,* 2003) and regulation of the balance between proinflammatory and antiinflamatory activities of IL-10 by some cytokines was demonstrated *in vitro* (Sharif *et al*., 2004). Although IL-10 inhibited reactive oxygen species production and damage of *A. fumigatus* hyphae by human monocytes *in vitro*, no interference with intracellular killing was noted and stimulation of phagocytosis was observed (Roilides *et al*., 1997). Recent data suggested the role for IL-10 in migration of *A. fumigatus* specific T cells to airways of infected mice (Rivera *et al*., 2009), what illustrates further diverse effects of IL-10 in aspergillosis.

Increase in number of macrophage that have capacity to ingest conidia is in line with data from pulmonary aspergillosis in mice that showed increased phagocytic capacity of alveolar macrophages early (day 1) in post infection period (Philippe *et al*., 2003). Phagocytosis of conidia and subsequent production of reactive oxygen species (ROS) are the main effector activities of alveolar macrophages in murine aspergillosis (Philippe *et al*., 2003). In line with this, our data showed increased oxidative activity of lung cells (judjing by increase in number of NBT^+ cells, and intracellular MPO activity) in both rat strains. Differences in the numbers of activated (NBT^+) lung cells in AO and DA rats in settings of comparable levels of IFN-γ, cytokine necessary for phagocyte activities that are required for killing of ingested *A. fumigatus* conidia and hyphal damage (Roilides *et al,* 1993; Shao *et al,* 2005) suggest higher sensitivity of lung cells from DA rats to this proinflammatory cytokine. However, our measurements of overall (in lung homogenates) cytokine content showed higher amounts of IFN-γ in DA as compared to AO rats at respective time points, what might have contributed to increased numbers of activated macrophages (and neutrophils) in lung cell population of DA rats. Lower MPO activity in lung leukocytes of infected AO as compared to DA rats is in line with data that showed lower expression of MPO in peripheral blood granulocytes of

AO rats in settings of systemic inflammation (Belij *et al*, 2012). Although neutrophils are traditional sources of MPO (Hampton *et al*., 1998), macrophages can also increase their MPO content when activated (Rodrigues *et al*., 2002). Transient and lower degree of activation of lung cells from AO rats (as compared to DA) might be responsible for lower efficiency of fungal removal in this strain.

6. CONCLUSIONS

6. Conclusions

1. Rats of both strains successfully control the infection with *A. fumigatus* in lungs judjing by fungal removal from lungs, but trhe process was more efficient in DA rats.

2. Elimination of fungus from lungs occurs in environment enriched in proinflammatory cytokines (IFN-γ and IL-17) and unchanged Th2/anti-inflammatory cytokines (IL-4 and IL-10) in both rat strains. However, differences in the levels of IFN-γ (lower in AO rats) as well as in the dynamics of proinflammatory cytokine appearance (longer in DA rats), that was transient in AO rats, as well as longer presence of IL-17detectability attendance were observed.

3. In both rat strains infection resulted in inflammation in lungs, but the response was generally more pronounced in DA rats judging by:

- greater numbers of neutrophils that accumulated in the lungs
- oxidative activity of leukocytes recovered from lungs by enzyme digestion (i.e. NBT reduction and MPO activity)
- higher numbers of $CD4^+$ cells in population of BAL cells

4. No strain-related differences were observed concerning proinflammatory (IFN-γ and IL-17) cytokine production by total lung leukocytes. Transient (day 7 p.i.) increase in IL-4 production was observed in AO compared to DA rats. Increases in IL-10 production was observed in both strainn, but the levels produced were higher in AO rats.

5. BAL leukocytes from both strains respond to infection by IFN-γ and IL-17 production, but higher levels of these cytokines were observed in AO rats as compared to DA rats late in infectio. There was no IL-4 response in either of strains. Transient IL-10 production (similar in levels in both AO and DA rats) was noted.

6. Similar cellularity as well as proliferative activity of draining lymph node (dLN) cells was observed. In both rat strains dLN cells differentiate toward IFN-γ and IL-17-

producing cells. However, differences in the intensity and/or dynamics of cytokine responses were observed:

- Both spontaneous as well as conidia-stimulated IFN-γ production where higher in infected AO compared to DA rats. Increased levels of IFN-γ mRNA in AO (unchanged in DA rats) were observed, that are followed by a decrease (compared to uninfected AO rats) later in infection.
- Similar levels of spontaneous IL-17 production, but lower responsiveness of dLN cells to stimulation with fungus was observed. Production and IL-17 gene expression start earlier in DA rats.
- Transient decrease in spontaneous IL-4 production in both rat strains was noted. IL-4 response to stimulation with conidia was suppressed as well, but only early in AO rats and during the whole post infection period in DA rats. There was a decrease in IL-4 mRNA in both ratb strains.
- Similar levels of spontaneous as well as stimulated IL-10 production were observed, but there was higher (at the start and later in infection) and longer (during the entire post infection period) responsiveness to stimulation in DA compared to AO rats.

7. $CD4^+$ cells are the main producers of IFN-γ and IL-17 in infected individuals of both strains, but the levels of IL-17 were lower in AO compared to DA rats. This T cell subset produce IL-10 in both strains as well, but only transiently in AO rats.

8. Differences in the pattern of cytokine responses by lung cells and draining lymph nodes suggest changes in the pattern of cytokine responses in lung microenvironment of AO rats:

- down-regulation of IFN-γ (higher production by dLN cells of AO vs DA rats, but similar by lung leukocytes)
- up-regulation of IL-17 (lower production by dLN cells of AO vs DA rats, but similar by lung leukocytes)

Data presented in this study showed for the first time that resistance to *A. fumigatus* in immunocompetent rats of two different strains was associated with immune responses that differed in the intensity as well as in the time course. The knowledge of genetically-based immune-relevant differences in mechanisms of defense against *A. fumigatus* in rats might be helpful in the future use of rat models of pulmonary aspergillosis in developing and testing immunotherapeutic interventions of fungal diseases.

7. REFERENCES

7. References

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Biography

Amal Atia Mhfoud El Muzghi was born in Tarhouna, Libya, on December 16th, 1972. She started her Bachelor studies in 1991 at the Faculty of Science, Al Fateh University, Tripoli, where she graduated in 1994.

From 1995 to 2005, Amal Atia El Muzghi worked as a teacher at the secondary school of Tarhouna, where she taught the following courses: Natural history, Anatomy and physiology, Histology, Microbiology, General biology.

From 2005 to 2008, Amal Atia El Muzghi worked as Inspector in the Ministry of Education specializing in life science curricula at the secondary level.

In 2003 she started Master studies at the Department of Science Life (Animal Physiology), Faculty of Arts & Sciences of Tarhouna, University of El Margueb, Libya and graduated in 2007.

From 2007 to 2008 she worked at the laboratory of the Department of Physiology at the Faculty of Arts & Sciences of Tarhouna, University of El Margueb, Libya to study Effect of Gibberellic acid on reproduction and sex hormones in rats.

In the period from March 2006 to June 2006 Amal Atia attended training course Design and analysis of experiments at Faculty of Arts & Sciences of Tarhouna, University of El Margueb.

Прилог 1.

Изјава о ауторству

Потписана Amal Atia Mhfoud El-Muzghi

број уписа Б3064/2010

Изјављујем

да је докторска дисертација под насловом

Имунски механизми резистенције имунокомпетентних јединки Dark Agouti и Albino Oxford пацова на пулмонарну инфекцију изазвану гљивом *Aspergillus fumigatus* Fresen. (Immune mechanisms in resistance of immunocompetent Dark Agouti and Albino Oxford rats to pulmonary infection with fungi *Aspergillus fumigatus* Fresen.)

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 02.06.2014.

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Amal Atia Mhfoud El-Muzghi

Број уписа Б3064/2010

Студијски програм Биологија; Имунобиологија

Наслов рада Имунски механизми резистенције имунокомпетентних јединки Dark Agouti и Albino Oxford пацова на пулмонарну инфекцију изазвану гљивом *Aspergillus fumigatus* Fresen. (Immune mechanisms in resistance of immunocompetent Dark Agouti and Albino Oxford rats to pulmonary infection with fungi *Aspergillus fumigatus* Fresen.)

Ментор др Ивана Мирков и др Милена Катарановски

Потписани Amal Atia Mhfoud El-Muzghi

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, 02.06.2014.

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Имунски механизми резистенције имунокомпетентних јединки Dark Agouti и Albino Oxford пацова на пулмонарну инфекцију изазвану гљивом *Aspergillus fumigatus* Fresen. (Immune mechanisms in resistance of immunocompetent Dark Agouti and Albino Oxford rats to pulmonary infection with fungi *Aspergillus fumigatus* Fresen.)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

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