Activation of the Inflammatory Response by Fungal Components

LAURA TEIRILÄ

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LAURA TEIRILÄ

Activation of the Inflammatory Response by Fungal Components

FINNISH INSTITUTE OF OCCUPATIONAL HEALTH AND FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI
Activation of the Inflammatory Response by Fungal Components

Laura Teirilä

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Haartman Institute, Lecture Hall 2, on March 18th 2017, at 12 noon.

Helsinki 2017
White blood cells, the tiny multitaskers protecting our body, much already known, but there is still so much more to learn...
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Abstract

Fungi are associated with a wide range of diseases from superficial skin syndromes to invasive life-threatening conditions. Furthermore, exposure to non-infectious fungal components in the context of agricultural work or in water-damaged houses has been associated to illnesses in the respiratory tract. The inadequate knowledge of the immune mechanisms behind these illnesses has triggered an intense research effort attempting to understand how fungi can activate the defense mechanisms of immune system.

Vertabrates have a two-tiered immune system consisting of innate immunity and adaptive immunity. Their purpose is to protect the body from disease-causing microorganisms, physical stress or tissue damage. The innate immune system is the first to be activated; it facilitates the direct elimination of pathogens as well as initiating the inflammatory response. It also provides the necessary signals to trigger adaptive immunity if the pathogens evade or overwhelm innate immunity. Macrophages are leukocytes; these are cells which play a major role in triggering the innate immune response. They recognize pathogen specific structures (pathogen-associated molecular patterns, PAMPs) via their pathogen recognition receptors (PRRs), which trigger the rapid secretion of pro-inflammatory cytokines and chemokines and other pathogen eliminating actions.

This thesis focused on the inflammation triggered by the fungal components. The inflammatory response and related mechanisms were studied in vitro in the key defense cell of innate immunity, the macrophage, which were treated with a central cell wall component of fungi, (1,3)-\(\beta\)-glucan. In addition, we were also able to characterize the microbial component-related defense of lungs in a real-life situation, by studying the proteomic changes in bronchoalveolar lavage obtained from patients with illnesses associated with exposure of inhaled fungal and other microbial particles.

In these studies, we utilized the methods, where broad spectrum of parameters can be followed such as quantitative proteomics (iTRAQ, 2D-DIGE) and transcriptomics (microarray) together with traditional biomolecular techniques such as western blotting or RT-PCR.

One major outcome of this thesis project was the finding that \(\beta\)-glucan evokes a strong pro-inflammatory response via the IL-1 family cytokines in human macrophages. These cytokines are crucial mediators of inflammation, thus their secretion is highly regulated. Our study produced the first evidence that on its own \(\beta\)-glucan could cause the secretion of functional IL-1\(\beta\) by activating both dectin-1/Syk–signaling pathway and NLRP3 inflammasome. Beta-glucan-induced production of reactive oxygen species (ROS), cathepsin release and potassium efflux were required for activation of the NLRP3 inflammasome. Furthermore, we revealed that the secretion of IL-36\(\gamma\) in \(\beta\)-glucan stimulated macrophages was not
dependent on activation of NLRP3 inflammasome. These results indicate that cytokines of the IL-1 family play a role in inflammatory response induced by fungi, even in situations when the activation of the inflammasome is impaired.

Most of the members in IL-1 family lack the signal peptide and thus are not released via the classical protein secretion pathway. In addition to IL-1 cytokines, dectin-1 activation evoked an efficient unconventional secretion of other mediators of inflammation, which are secreted via vesicles, such as damage-associated molecules or integrins. Both IL-1β and vesicle-mediated protein secretions were suppressed by inhibition of inflammasome activity or by preventing the process of autophagy. Although the activation of protein secretion or inflammasome was prominent, the latter structure being known to facilitate pyroptosis, no significant cell death was observed after β-glucan stimulation. This indicates either the constitutive presence or the activation of cell viability sustaining factors. One of these factors could be the well-known myeloid cell growth factor, granulocyte-macrophage colony-stimulating factor, GM-CSF. This growth factor also seems to be one of the factors boosting the inflammatory response triggered by β-glucan, thus GM-CSF-generated macrophages displayed a more efficient secretion of IL-1 and other unconventionally secreted proteins than M-CSF-generated macrophages after β-glucan stimulation.

The results of this thesis highlight the potential of a major fungal cell wall component, β-glucan for initiating inflammation in human macrophages. Thus, the possibility of using β-glucan as a potential adjuvant in vaccines or treatments should be explored.

In an attempt to obtain direct information about conditions caused by the exposure to fungal and other microbial particles, we characterized the proteomic changes present in the bronchoalveolar lavage obtained from patients suffering illnesses associated with exposure to inhaled fungal and other microbial particles. The proteomic profiles of acute type of hypersensitivity pneumonitis (HP) were different from the profile of damp building-related illness (DBRI), indicating that these conditions are not closely associated. However, the increase in the levels of two well-known markers of inflammation (α-1-antitrypsin, galectin-3) was observed in both HP and DBRI, evidence for the activation of inflammatory mechanisms in both of these conditions.

This thesis provides novel knowledge concerning the inflammatory response and related mechanisms triggered by fungal components. These results may help to clarify the mechanisms behind the inflammatory symptoms experienced by individuals with fungal infections or exposure to fungal particles and will provide future tools for the treatment of fungal-related disorders.
Tiivistelmä


Tässä väitöskirjassa on keskitytty tutkimaan sienikomponentin aiheuttamaa tulehdusvastetta. Tulehdusvastetta ja sen mekanismeja on karakterisoitunut luontaisen immuniteetin keskeisimmässä puolustussolussa, makrofagissa sen altistuttua sienen seinäkomponentille, (1,3)-β-glukaanille. Lisäksi olemme tutkineet hengistyshiihin kohdistuvaa mikrobioloinneg perinteisiä menetelmiä kuten western blotting- ja RT-PCR -analyysejä.

Beta-glukaanin aktivoima voimakas IL-1 perheen tulehdusyyliini vasteen tämän vaihtosirjan yksi keskeisimmistä tuloksista. IL-1 sytokiinit ovat tärkeitä tulehdusta edistäviä sytokiinejä, minkä takia niiden tuotanto solussa on tiukasti

3
säädeltyä. Tutkimuksemme oli ensimmäinen, joka raportoi β-glukaanin yksistään pystyvän aktivoimaan signaaliereitit, jotka tarvitaan biologisesti aktiivisen IL-1β tulehdussytökiihin erityyteen ihmisen makrofagissa. Dektiini-1 –reseptorin tunnistaessa β-glukaanin aktivoitu signaaliereitti, joka johti IL-1β geeniulventaan ja proteiinin esimuodon syntymiseen solussa, sekä NLRP3 inflammasomin eli tulehdusvyväsens aktivaation, jonka seurauksena inflammasomin entsyymistä kasvaa 1 μmokkasi IL-1β sytykiihin sen biologisesti aktiiviseen muotoon. Beta-glukaanin induusomat solureaktiot, kuten reaktiivisten happyhydideiden tuotanto, katepsimentsymien vapautuminen solulimassa ja kaliumin ulosvirtaus solussa, todettiin tarvittavan NLRP3 inflammasomin aktiivioon makrofagissa. Beta-glukaanin aktivoima IL-36γ -erityys taal oli riippumaton NLRP3 inflammasomin aktivatiosta. Tuloksemme viittaa siihen, että IL-1 perheen sytykiiineillä on keskeinen rooli sienen aiheuttamassa tulehdusreaktiossa, ja tämä IL-1 erityysaste voi olla riippuvainen tai riippumaton NLRP3 inflammasomin aktiivioista.


Väitöskirjani tulokset korostavat β-glukaanin olevan sienen keskeinen immuunipuolustusta aktivoiva rakennesa, joka saa aikaa erittäin voimakkaan tulehdusreaktion ihmisen makrofagissa. Näiden tulosten valossa β-glukaanin käyttömahdollisuutta adjuvanttina rokotteissa tai hoidoissa pitäisi pitää enemmän.

Saadaksemme informaatiota home- ja muu mikrobialtistumisen aiheuttamisesta, tautiloista karakterisoimme keuhkohuhtelunesteessä tapahtuneita proteiinimuutoksia mikrobikomponenttialtistumiseen liittyvien sairausten kuten allergisen alveoliitin (hypersensitiivinen pneumoniitti) ja kosteusvauriorakennuksiin liittyvän sairastelun (damp building-related illness)
yhteydessä. Näiden kahden sairauden proteiiniprofiili erosi huomattavasti toisistaan viitaten siihen, että kyseessä on erilaiset tautiprosessit. Kummassakin tautitilassa havaittiin kuitenkin kahden tunnetun tulehdusproteiinimarkkerin (α-1-antitrypsiini, galektiini-3) nousu, mikä viittaa tulehdusmekanismien käynnistymiseen molemmissa sairauksissa.

Tämä väitöskirjatyö on tuottanut uutta tietoa liittyen sienen vaikutuksesta immuunipuolustukseemme ja karakterisoinut sienen rakennekomponenttien käynnistämää tulehdusvastetta. Nämä tulokset auttavat meitä ymmärtämään elimistössämme käynnistyviä immuunipuolustuksen mekanismeja, jotka johtavat tulehdusellisten oireiden ilmaantumiseen sieni-infektioiden ja sienikomponenttien aiheuttamien sairauksien yhteydessä. Tämä edesauttaa myös hoitojen ja diagnostiikan kehittymistä.
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Vantaa, February 2017

*Laura Teirilä*
List of original publications

This thesis is based on the following publications:


*, ** Equal contribution

The publications are referred to in the text by their Roman numerals.

Publication I is included in the thesis of Päivi Kankkunen (Activation of the inflammasome by (1,3)-β-glucans and trichothecene mycotoxins in human macrophages, Helsinki, 2014), and publication II is included in the thesis of Anna-Maria Lahesmaa-Korpinen (Computational approaches in high-throughput proteomics data analysis, Helsinki, 2012).

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIM2</td>
<td>absent in melanoma 2</td>
</tr>
<tr>
<td>AME</td>
<td>symptoms related to agricultural exposure of NIMPs</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis associated speck-like protein containing CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow–derived dendritic cells</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase-recruitment domain</td>
</tr>
<tr>
<td>CASP</td>
<td>caspase</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>DBRI</td>
<td>damp building–related illness</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DECTIN-1</td>
<td>dendritic cell-associated C-type lectin 1</td>
</tr>
<tr>
<td>2D-DIGE</td>
<td>two-dimensional difference gel electrophoresis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GBY</td>
<td>glucan from baker’s yeast</td>
</tr>
<tr>
<td>HP</td>
<td>hypersensitivity pneumonitis</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>innate lymphoid cells</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>isobaric tags for relative and absolute quantitation</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MSU</td>
<td>monosodium urate</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NIMP</td>
<td>non-infectious microbial particle</td>
</tr>
<tr>
<td>NLRP</td>
<td>protein complex containing NACHT, LRR and PYD domains</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide-binding domain leucine rich repeats-containing receptors</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>ODTDs</td>
<td>organic dust toxic syndrome</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen-recognition receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>pyrin domain</td>
</tr>
<tr>
<td>RAF1</td>
<td>v-raf-leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SARC</td>
<td>sarcoidosis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Src</td>
<td>proto-oncogene tyrosine protein kinase src</td>
</tr>
<tr>
<td>Syk</td>
<td>spleen tyrosine kinase</td>
</tr>
<tr>
<td>Th cells</td>
<td>T-helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Microscopic organisms, microbes, such as bacteria and fungal yeasts and molds are an intrinsic part of our everyday life. They are found in soil, water, and air and they compose the normal flora of our body. According to the latest knowledge, they seem to have a fundamental role in maintaining our health. This ubiquitousness of microbes means that we are exposed continuously to a low level of microbes and their components - this is usually harmless. On some occasions, for example due to some alteration in the normal flora or a breach in the integumentary barrier, microbes manage to cause an infection. Furthermore, in certain occupations and surroundings, an individual’s exposure to microbes can be significant, either in terms of quantity or quality, and these situations have been shown to associate with adverse health effects.

The innate immune system is the first line of defense against pathogens, facilitating their direct elimination and regulating the initiation of inflammation. It also provides the necessary signals to trigger the adaptive immunity. The crucial cellular players in innate immunity are leukocytes such as macrophages and dendritic cells, which recognize conserved structures of pathogens (pathogen-associated molecular patterns, PAMPs) via their pathogen recognition receptors (PRRs). This ligand recognition by receptor may stimulate the rapid activation of defense responses; production of cytokines, chemokines and reactive oxygen species (ROS), phagocytosis as well as antigen presentation to the cells of the adaptive immune system. The response of innate immune system was originally thought to be rather invariable and straightforward. According to recent knowledge, the mechanisms leading to innate immune response are much more complex and multifactorial than traditionally believed. Furthermore, innate immunity was recently shown to have its own immunologic memory, which was previously thought to be only a feature of adaptive immunity.

A decreased immune response due to advanced age, immunosuppressive medication, or congenital defects in immune mechanisms is known to increase the susceptibility to fungal infections. Immune-related mechanisms have been suggested to play a role in evoking the symptoms experienced by individuals exposed to non-infective fungal components. However, these mechanisms have remained poorly characterized.

This thesis focused on the characterization of the immune reaction triggered by fungal-associated molecular pattern, \( \beta \)-glucan. We studied the \( \beta \)-glucan-activated innate immune response and related mechanisms in the key innate immune defence cell, the macrophage. Furthermore, the immune response of the lungs was characterized by examining the proteomic changes in bronchoalveolar lavage collected from patients with illnessess related to exposure of fungal and other microbial components.
2. REVIEW OF THE LITERATURE

2.1. Innate immunity: the first line of defense

We encounter microorganisms constantly in our daily life, but they cause disease only occasionally. Vertebrates have developed a two-tiered immune system, consisting of innate immunity and adaptive immunity to protect the body from disease-causing microorganisms, pathogens (Figure 1).

**INNATE IMMUNITY**
- recognition of PAMPs, DAMPs by PRRs
- trained immunity

1. LINE
   - PHYSICAL
     • skin
   - MICROBIOLOGICAL
     • normal flora
   - CHEMICAL
     • stomach acids, antimicrobial peptides

2. LINE
   - COMPLEMENT CASCADE
   - CELLS OF INNATE IMMUNITY
     • macrophages
     • dendritic cells
     • granulocytes
     • mast cells
     • innate lymphoid cells

**ADAPTIVE IMMUNITY**
- specific recognition of antigen
- immunological memory

1. B-CELL IMMUNITY
   - HUMORAL IMMUNITY
     • plasma-, memory -B-cells

2. T-CELL IMMUNITY
   - CELL-MEDIATED IMMUNITY
     • cytotoxic-, helper-, suppressor -T-cells

Figure 1. The innate and adaptive immunity. The responses of innate immunity are rapid and non-specific. Innate immunity functions as the first line of defence against infection consisting physical barriers and bloodbourne factors resisting the invasion and spreading of the pathogen. The cellular defence is mainly dependent on PRRs, which recognize PAMPs present on a variety of microorganisms or danger-associated molecular patterns (DAMPs) released during cellular damage or stress. Innate immunity is recently proposed
to have its own immunological memory termed “trained immunity”. The adaptive immune response is slower to develop. The full development of adaptive immunity response requires the engagement of receptors (T-cell and immunoglobulin receptors from T- and B-cells, respectively) with their specific antigens, the expansion and differentiation of the responder cells, and the development of a memory for the specific antigen response.

In addition to pathogens, internal signal molecules emitted by our body during the stress or tissue damage (danger-associated molecular patterns, DAMPs) activate protective responses in our immune system. Innate immunity is the first system to become activated. Usually this response is sufficient on its own to eradicate the pathogen, however occasionally a pathogen will evade or overwhelm innate immunity and the adaptive immunity system has to be triggered by innate immunity. One important feature of both of the immune systems is that they can distinguish between self and non-self molecules or when the self molecule is located in places it normally should not exist, but these systems differ in the ways they perform this function.

The innate immune system comprises two types of defenses against invading pathogens: constantly present physical, microbiological and chemical barriers of the skin and mucosal epithelia in the airways, gut and urogenital tract and then the molecular and cellular responses, which are induced if a pathogen breaches these first barriers (Murphy, 2012). Thus, the response of innate immunity can be immediate or when induced, responses occur within a few hours with a duration of several days. The response of innate immunity is non-specific in its nature, similar kinds of defense mechanisms are activated against broad classes of pathogens (viruses, bacteria, fungi and parasites).

Epithelial cells, which are joined together by tight junctions, and the secretion of mucus and its outward flow driven by ciliated cells, provide the mechanical barrier against pathogens. The presence of the normal microbiota prevents colonization of pathogens e.g. by limiting the availability of nutrients and attachment sites on the epithelia. Previous studies have highlighted the crucial influence of the intestinal microbiota to the host’s immune system and its potential effect in the development of several diseases in addition to its important role in sustaining homeostasis (Cerf-Bensussan and Gaboriau-Routhiau, 2010). Chemical defense mounted by the epithelia refers to the secretion of antimicrobial peptides such as defensins, cathelicidins and histatins. These agents are released by epithelia, innate immune cells or salivary glands. Most of these peptides need proteolytic steps to achieve their active stage when they can disrupt the cell membrane of the microbe. (De Smet and Contreras, 2005). In addition, the acidic pH of the stomach and digestive enzymes of the upper gastrointestinal tract, lysozyme in tears and saliva represent chemical barriers to infection.

Microorganisms that breach these defenses are met by molecules of the complement system, which have a major role in the humoral defense of innate
immunity. Complement is a system of plasma proteins, which are constantly present in blood and other body fluids in their inactive form. Complement proteins can be activated via three different pathways: directly by pathogens or indirectly by pathogen-bound antibody (classical pathway), via spontaneous hydrolysis of C3 and its binding to bacterial surface sites (alternative pathway) and via mannose-binding lectins and ficolins binding to carbohydrate structures on the bacterial cell wall (lectin pathway) (Gasque, 2004). An encounter with a pathogen triggers the complement cascade, where the consecutive complement molecules starting from C3 are activated by cleavage, leading directly to lysis of the pathogen by disrupting its cell membrane or opsonization of the pathogen with C3b and C5a components, which signal the innate immune cells to first engulf and then eradicate the pathogen.

There are three types of phagocytosing cells in the innate immune system: monocytes and macrophages, granulocytes and dendritic cells; these will be discussed in more detail in the following chapter. The innate immune cells identify microbes by a limited number of germline coded receptors called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). These receptors recognize regular molecular patterns present on many microorganisms which do not occur on the body’s own cells. Some of these receptors induce the secretion of effector molecules such as cytokines and chemokines in the cells of innate immunity, which convey the signal to other immune cells and/or affect the function of the original cell. These cytokines and chemokines released by activated innate immune cells, especially macrophages, initiate the process known as inflammation. As a part of the inflammation defense, related proteins and cells are recruited from the blood into infected tissue to destroy the pathogen, this process is an important means to combat the infection. Inflammation also increases the flow of lymph from infected area to nearby lymphoid tissue carrying pathogens and antigen-presenting cells such as dendritic cells, where they activate lymphocytes and initiate the adaptive immune response. This highlights the crucial role of the innate immune system, in addition to providing early defense against infections, it also triggers and drives the adaptive immunity to respond effectively to infection (Medzhitov and Janeway, 2002).

Once adaptive immunity has been triggered, the next step in inflammation is recruiting the antibody molecules and effector-T cells to the site of infection. This response may take days to develop because the few B- and T-lymphocytes specific for that pathogen must first undergo clonal expansion before they differentiate into the effector cells that migrate to the site of infection. This specific recognition of antigens via antigen receptors on the B- and T-lymphocytes is generated through somatic gene rearrangements and hypermutation, allowing adaptive immunity to specifically recognize many types of microorganisms. In this way, adaptive immunity possesses more specific defense mechanisms to overcome invading pathogens that have evaded or overwhelmed innate immunity. The antibodies produced and the activated lymphocytes can persist after the original infection has
been eliminated, thus they help to prevent immediate reinfection as well as ensuring long-lasting immunity should a second infection occur many years later. Thus, the response against the same pathogen is usually faster and more intense. This immunological memory of adaptive immunity can last for a lifetime (Bonilla and Oettgen, 2010, Murphy, 2012). Previously, the innate immune system was not considered to possess an immunologic memory and its actions were generally thought to be identical with every encounter with a pathogen. This assumption has now been challenged with recent results suggesting that certain infections or vaccines are able to induce reprogramming of the innate immune system, leading to protection against reinfection. This shows a new type of immunological memory in a process proposed to be called trained immunity (Quintin et al., 2014, Netea et al., 2016).

Over time, it has became clear that components of the innate immune response have a major role in sustaining the normal homeostasis of the body but when functioning in an improper manner, they participate in the pathogenesis of many of the illnesses classified as autoinflammatory diseases. These autoinflammatory diseases (e.g. gout) differ from autoimmune diseases (e.g. lupus) usually in a way that the classical hallmarks of autoimmunity, namely high-titer autoantibodies and antigen specific-T-cells, the components of adaptive immunity, are absent. (Masters et al., 2009).

2.1.1. Cells of innate immunity

Innate immune cells comprise a set of tissue resident, circulating or recruited leukocytes, which mediate the process of inflammation when sensing a pathogen or tissue damage through their germline encoded receptors in order to eliminate the infection. These defense cells of innate immunity consist of macrophages, monocytes, granulocytes (neutrophils, basophils, eosinophils), mast cells, dendritic cells, and innate lymphoid cells (ILC). At the onset of infection, usually tissue-resident cells such as macrophages, dendritic cells and mast cells are the first cells to encounter the pathogen. They respond to the pathogen by initiating the process of inflammation and produce potent inflammatory mediators: cytokines and chemotactic proteins to recruit the circulating neutrophils and monocytes into the affected tissue. Through the increased release of antimicrobial proteins, more efficient phagocytosis, and possibly the activation of adaptive immune responses, the activities of innate immune cells are directed to eliminate the infection and restore the tissue homeostasis.

Leukocytes of the immune system develop from pluripotent hematopoietic stem cells in bone marrow, passing through different developing steps and progenitor stages. There are two main progenitor types for leukocytes in the immune response: lymphoid and myeloid cells (Kondo, 2010). Recently, it has also been postulated that there is a lineage of myeloid cells with an independent origin from hematopoietic stem cells (Schulz et al., 2012). The common myeloid
progenitor is a precursor for most of the innate immune cells such as monocytes, macrophages, granulocytes and dendritic cells. It is possible that a minor subset of dendritic cells arises also from the common lymphoid progenitor (Manz et al., 2001). Most of the leukocytes in adaptive immunity, T-and B-lymphocytes, develop from the other branch, from the common lymphoid progenitor. There is also a subset of cells, ILCs, that are functionally grouped into the innate immunity, but known initially to develop in the fetal liver and in the later stages from common lymphoid progenitors (Spits et al., 2013, Sonnenberg and Artis, 2015).

In contrast with adaptive immune cells, innate immune leukocytes have a reduced proliferative capacity, and during infection, they are mainly replenished by controlling the proliferation and differentiation of hematopoietic stem and leukocyte progenitor cells, which are then mobilized and transported in the bloodstream into the affected tissues (Takizawa et al., 2012). It is also known that a distinct process exists in which tissue-resident macrophages undergo in situ (local) proliferation in order to increase the local population density during the immune response of T-helper 2-type cells (Jenkins et al., 2011).

Despite the limited capacity of proliferation, mature innate defense cells display remarkable plasticity towards diverse cellular functions by changing their phenotype or function when they are exposed to different microenvironmental factors during the inflammation or steady-state conditions. This represents one crucial mechanism for modulating the immune response that immune cells are capable of adapting to the prevalent situation by modifying their function (Galli et al., 2011).

Macrophages are one of the key defense cells in innate immunity, they are long-lived and resident cells in almost all tissues, thus being the first cells to encounter the invading pathogen. Their role in innate immunity will be discussed in more detail in the following chapter. Monocytes circulate in blood and when migrating to the tissues, they differentiate into macrophages, thus replenishing the macrophage population in the infected tissue. The phagocytic uptake of pathogens is a crucial step in innate immune defense. Both of these cell types are able to recognize, engulf and kill the invading pathogens. This initial contact with the pathogen activates multiple antimicrobial mechanisms in these phagocytes, leading to secretion of signaling molecules, which trigger inflammation and recruit other type of immune cells such as granulocytes to the site of infection. Neutrophils are short-lived but numerically they are the most common cells from the group of granulocytes circulating in blood; they are quickly recruited to the infected site. They are capable of phagocytosing pathogens and once the pathogen has been engulfed, it is moved into intracellular vesicles where it is destroyed by degradative enzymes. (Kumar and Sharma, 2010). The action of inflammatory cells causes inflammatory tissue damage, in particular neutrophils are considered to be tissue-destructive cells since they release many degradative components in their environment during their activation. Thus phagocytes have also a crucial role
in restoring tissue homeostasis by engulfing and clearing the cellular debris and apoptotic cells. This task mainly falls on the shoulders of macrophages.

Basophils and eosinophils are other, less abundant groups of granulocytes circulating in the blood. Their cytotoxic products form a host-defence that is mainly involved in protection against multicellular parasites, such as helminths. They are also known to participate in allergic responses. Mast cells have also large granules in their cytoplasm that are released when they are activated. The blood-borne progenitor for mast cell is not well defined. In contrast to granulocytes, which circulate in the blood, mast cells are located in mucosal and connective tissues. They comprise one part of the defense against parasites, but they are also known to be activated during bacterial infection and having a crucial role in orchestrating allergic responses. The components released by mast cells and basophils seem to more directly act on the mucosal epithelia, smooth muscles and vasculature by limiting the spread of parasites and promoting their expulsion from the host rather than harming the parasite itself (Voehringer, 2013).

ILCs are important effector cells in the innate immune system; they belong to the lymphoid lineage, but lack expression of specific antigen-receptors. They act in the early stages of infection and tissue damage by secreting cytokines. In addition, they function in lymphoid organogenesis and tissue remodeling (Spits et al., 2013). One subset of ILCs is called the natural killer (NK) -cells, which are cytotoxic and specialized ILCs for combating intracellular pathogens, mainly viruses. NK -cells have two main functions: inducing the apoptosis of infected cells and producing cytokines, especially large quantities of interferon-γ (IFN-γ) (Lanier, 2005).

Like macrophages and neutrophils, dendritic cells (DC) are phagocytic cells that are able to degrade the pathogens they take up as well as producing the cytokines involved in host defense. Nonetheless, their main task is not the clearance of microorganisms. Instead, DCs initiate the adaptive response acting as professional antigen-presenting cells (APCs) (Mellman and Steinman, 2001). Furthermore, macrophages can act as an antigen-presenting cell. Both cell types possess the ability to engulf pathogens, process them to antigens, which they can present through their major histocompatibility complex (MHC) class I or II proteins, with the class type depending on the pathogen, to the cells of adaptive immunity. Activated APCs express also other cell-surface associated molecules and secrete cytokines which help to trigger the activation of adaptive immune system.

The function of innate immune cells forms the cornerstone for an efficiently working innate immune system. In addition, these cells can activate adaptive immunity, thus achieving more efficient elimination of pathogens by linking together the responses of innate and adaptive immune systems, especially when innate immunity has not been successful in eradicating the pathogen on its own.
Macrophages are key players in innate immunity. These cells were first identified by Elie Metchnikoff late in the 19th century on account of their phagocytic nature (macrophage meaning “the big eater” in Greek) (Cavaillon, 2011). Metchnikoff proposed that macrophages would be able of discriminating self from non-self and thus they could be capable of recognizing invading pathogens i.e. the crucial insight that subsequently led to concept of innate immunity. He was awarded the Nobel Prize in 1908 with Paul Erlich due to their work and pivotal conclusions in cellular and humoral immunity contributing to the fight against pathogens.

Macrophages have an essential role in both inflammatory and anti-inflammatory responses of innate immunity evoked by invading pathogen or damaged tissue. In addition, they are involved in the repair of damaged tissue, thus maintaining tissue homeostasis. They also affect tissue development during morphogenesis.

One key feature of macrophages is their ability to engulf solid particles and then to destroy and eliminate them in their internal vesicles called phagosomes. This process is called phagocytosis. Phagosomes fuse with other intracellular vacuoles, such as lysosomes. Inside these phagolysosomes, particles are degraded in very acidic environments with the help of ROS and nitric oxide (NO), as well as antimicrobial enzymes such as cathepsins (Flannagan et al., 2012). During homeostasis, macrophages serve as a common “janitorial” cell of the body, phagocytosing and removing the cellular debris and dead, apoptotic cells. This kind of clearance function does not activate the inflammatory mechanisms of macrophages, especially if other inflammatory stimuli are missing from the environment (Mosser and Edwards, 2008). Encounters with the pathogen or substances leaking from damaged tissue are needed to activate the macrophages. This may initiate the other crucial function performed by the macrophages, secretion of inflammatory mediators. These cytokines promote inflammation by recruiting and activating other defense cells, which may lead to the manifestation of typical inflammatory symptoms: swelling and redness in the infected site or even to systemic symptoms like fever. An encounter with pathogen may also trigger the activation of adaptive immunity. Macrophages are able to present antigens from phagocytosed pathogens on their surface MHC-molecules to the cells of adaptive immunity and thus they act as APCs. Nonetheless, other defense cells of innate immunity, i.e. dendritic cells, are more potent at triggering activation of T-cells and adaptive immunity.

In adult mammals, tissue macrophages are found in virtually all tissues, where they display great phenotypical and functional diversity. On the one hand, tissue macrophages exhibit tissue specific morphological and functional phenotypes, thus macrophages have been given different names according to the tissue in which they reside, such as osteoclasts (bone), alveolar macrophages (lung), microglia (brains), Kupffer cells (liver) and Langerhans cells (skin) etc. On the other hand,
tissue macrophages share some common functions in all tissues such as maintaining the tissue homeostasis (clearance of damaged and defective cells) and have an important role in initiating, developing and resolving the inflammatory response during the infection (Wynn et al., 2013, Ginhoux et al., 2016).

Recent studies have led to the development of the hypothesis of dual origin for tissue macrophages (Schulz et al., 2012, Gomez Perdiguero et al., 2015). According to work emerging from a murine model, it seems that during embryogenesis, tissue macrophages are derived from the yolk sac and fetal liver progenitors. Populations of tissue-resident macrophages (such as F4/80 bright resident macrophages) in many organs, such as liver, skin and brain, seem to have a prenatal origin and persist throughout life. The first identified molecule as controlling the migration of fetal macrophage progenitors to their final destination was just recently revealed. The endothelium-specific molecule, plasmalemma vesicle-associated protein (PLVAP), regulated the migration of fetal monocyte-derived macrophages to tissues in mice (Rantakari et al., 2016). After birth and in adulthood, the hematopoiesis passes from the yolk sac and fetal liver to the bone marrow. Monocyte-derived macrophages originate from the pluripotent hematopoietic stem cells in bone marrow. These macrophage precursors, monocytes, constantly replenish tissue macrophages in various organs, such as intestine (Bain et al., 2014). The numbers of tissue macrophages can increase during inflammation (Italiani and Boraschi, 2014). This is mainly due to a replenishment of the tissue macrophage population by circulating monocytes which migrate to the infected tissue and develop into macrophages. However, there is a consensus that some tissue resident macrophages are also able to proliferate and although it is not clear how this occurs, it has been suggested to take place through self-renewal or through the proliferation of local progenitors (Gentek et al., 2014).

Another way to classify macrophages, in addition to their origin, is through their functional status. In their basal state during the homeostasis, resident tissue macrophages show great diversity in their functional capabilities, morphologies, and transcriptional profiles i.e they have adapted to their environment (Gautier et al., 2012, Wynn et al., 2013). The term polarization describes the capacity of macrophages to modify their function in a plastic manner in response to changes in their microenvironment. In general, macrophages can change their activation states in response to microbial components, damage-associated components, growth factors, other cytokines and potentially any other entity that they are capable of recognizing with their receptors. This explains how macrophages are able to adapt to different conditions and respond with appropriate functions to distinct situations. This functional diversity is a key feature for macrophages. In principle, macrophages can modify their function from a wound-healing and tissue repairing type (commonly called M2 or alternatively activated macrophages) to a killing and microbicidal active type, which causes tissue damage (commonly called M1 or classical activated macrophages) (Sica and Mantovani, 2012). An encounter with a
pathogen or with environmental irritants, or tissue injury are all factors that may trigger the phenotypic change in local tissue macrophages to adopt an ‘inflammatory phenotype’, M1 type. Classical activation of the macrophages is induced by exposure to IFN-γ and/or recognition of Toll-like receptor ligands, leading to the activation of the NF-kB and STAT1 signalling pathways. This, in turn, enhances the response of antimicrobial defense by increasing the production of reactive oxygen and nitrogen species, and proinflammatory cytokines, such as TNFα, IL-1 and IL-6 (Wynn et al., 2013).

In contrast, some epithelium-derived alarmins and IL-4 and IL-13 cytokines produced by Th2 cells, may trigger so called ‘alternative activation’ in macrophages. These cells have been especially associated with wound healing and tissue repair, as well as the formation of fibrosis. They participate in the remodeling of the extracellular matrix by scavenging collagen and extracellular matrix components. In addition, alternatively-activated macrophages express immunoregulatory functions by producing a variety of immunomodulatory proteins such as arginase-1 and IL-10 that regulate the magnitude and duration of immune responses. Therefore, in contrast to classically-activated macrophages that activate immune defense and promote inflammation, alternatively-activated macrophages are typically involved in the re-establishment of homeostasis and suppression of inflammatory responses (Wynn et al., 2013).

This M1/M2 classification is a very simplistic way to describe the functional plasticity of macrophages. This binary classification is unable to represent the complex in vivo environment in which numerous cytokines and other factors interact simultaneously and thus it is not capable of defining the polarization steps or the final differentiated state of macrophages. It is also noted that macrophages can change their function when responding to the external challenges (such as a pathogen) on a second occasion: they can become less reactive (tolerance) or then display enhanced inflammatory response (trained immunity) (Netea et al., 2016). The molecular mechanisms to explain these diverse properties have not yet been elucidated. Epigenetic reprogramming has been suggested to be one of the main reasons for these changes (Netea et al., 2016). Another interesting mechanism possibly involved in innate memory involves changes in metabolic processes (Italiani and Boraschi, 2015).

The presence and function of macrophages are central to many diseases. They are important for tissue morphogenesis and thus the lack of macrophages or their dysfunction may lead to developmental abnormalities (Pollard, 2009). Macrophages, as professional phagocytes, have a crucial role in clearing the dead cells during homeostasis, and since they are non-responsive to self-antigens that may arise during the clearance process, they suppress the possibility of inflammation and autoimmunity (Savill et al., 2002). Since macrophages are also essential promoters of inflammatory response, they have been suggested to play a role in several inflammatory diseases such as atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis and fibrosis (Murray and Wynn,
Their contribution in these diseases is usually related to their pro-inflammatory potential, especially to their ability to secrete powerful inflammatory mediators, which promote inflammation causing collateral tissue damage. However, it is important to remember, that in addition to promoting inflammation, macrophages are involved in the recovery phase of many diseases. In fibrosis, macrophages participate in ECM remodelling and dampen the immune response that contributes to tissue injury.

2.1.2. Sensing of foreign or danger

Innate immunity is an ancient part of the host defense system against infection. The same molecular mechanisms are exploited by many organisms, from plants to animals, whereas adaptive immune system, as a relative newcomer, arises as late in the evolutionary scale as in the phylum of vertebrates. The recognition in the innate immune system relies on a limited number of germline-encoded receptors, PRRs. These PRRs recognize structures, PAMPs that are conserved features of microbial pathogens such as viral nucleic acids or bacterial and fungal cell wall components, consisting of repeated sequences of carbohydrates, lipids and proteins - patterns not found in the host. PAMPs usually represent molecules that are essential for the viability of the microbes, thus it is very improbable that pathogens could avoid detection by simply altering their PAMPs. The recognition of these PAMPs allows the innate immune system to distinguish infectious non-self from non-infectious self. This innate immunity recognition theory of PRRs and PAMPs was first introduced by Charles Janeway Jr. in 1989 (Janeway, 1989).

However, this theory could not explain why non-pathogenic microbes did not cause activation of the innate immunity or why the immune system is activated after tissue transplantation or in autoimmune disease when there is no infective pathogen present.

Polly Matzinger presented “The danger hypothesis” in 1994 (Matzinger, 1994). She suggested that in addition to PAMPs, the innate immunity system relies on the recognition of signal molecules released during conditions of cell distress or injury. These endogenous signal molecules are called danger – or damage-associated molecular patterns (DAMPs).

DAMPs are host molecules, which are normally found inside the cells, but they can passively leak out of the damaged tissue into the extracellular space if there is tissue injury or cellular distress (Matzinger, 1994, Matzinger, 2002, Kono and Rock, 2008). In addition to their release from necrotic cells and injured tissue, several DAMP molecules are also secreted from activated inflammatory cells in response to microbial components or cytokines via nonclassical protein secretion pathway (Bianchi, 2007).

DAMPs can be recognized by the same pattern-recognition receptors as PAMPs such as Toll-like receptors (TLRs) or by specialized receptors such as RAGE (Bierhaus et al., 2005). PAMP or DAMP recognition may stimulate cell
differentiation, cell death, or the secretion of inflammatory or anti-inflammatory cytokines, depending on the cell expressing the receptor and the other signals, which the cell gathers from its environment. As an example of the different effects of DAMPs, ATP (adenosine triphosphate) is known to stimulate inflammation, while adenosine can behave in an opposite way, acting as an inhibitor of inflammation (Sitkovsky and Ohta, 2005, Ferrari et al., 2006).

2.1.2.1. Pattern recognition receptors

The initial sensing of pathogens or tissue injury is mediated by innate germline-encoded PRRs, expressed not only by professional immune cells such as macrophages and DCs, but also by other cell types such as epithelial cells, endothelial cells and fibroblasts (Takeuchi and Akira, 2010).

Several classes of PRR families have been identified. These include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain, leucine-rich repeat–containing receptors (NLRs) and RIG-I-like receptors (RLRs). In addition, a family of enzymes, such as oligoadenylate synthase (OAS) proteins and cyclic GMP-AMP synthase (cGAS), which function as intracellular sensors of nucleic acids are included into the PRRs (Takeuchi and Akira, 2010, Wu and Chen, 2014).

PRRs can be characterized by their structure and specificity. They can also be classified into two groups according to their cellular localization: plasma membrane-bound receptors or cytosolic receptors. In the cytosol, these receptors can be found in membrane-bound compartments such as endosomes or lysosomes. The detection of extracellular pathogens is mediated mainly by PRRs expressed on the plasma membranes including CLRs and TLRs, which are specialized in the recognition of common components of fungal and bacterial cell walls. CLRs comprise a group of membrane-bound receptors characterized by the presence of carbohydrate-binding domain, which detects carbohydrate structures on fungi, bacteria and viruses (Takeuchi and Akira, 2010). They play a crucial role in antifungal immunity, thus these receptors and their function will be described in detail in chapter 2.2.2.

TLRs are among of the best characterized PRR families; in humans there are 10 identified and in mice 12 TLRs have been found (Takeuchi and Akira, 2010). The membrane-bound TLRs, including TLR1, TLR2, TLR4, TLR5 and TLR6, are specialized in the recognition of common components of bacteria, viruses, fungi and parasites (Takeuchi and Akira, 2010, Becker et al., 2015).

The innate recognition of viruses is mediated by a group of endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) detecting viral nucleic acids. Another group of PRRs, which were mentioned earlier, RIG-I-like receptors, are intracellular receptors, which are specialized in detecting viral nucleic acids (Pichlmair and Reis e Sousa, 2007).
Membrane-bound TLR4 is the main receptor for lipopolysaccharides (LPS) (Poltorak et al., 1998). They are major components of the outer membrane of gram-negative bacteria, which makes them one of the prime bacterial PAMPs recognized by the immune system. LPS is an endotoxin; during a bacterial infection, LPS may be responsible for the dangerously reduced blood pressure that occurs in conjunction with septic shock (Beutler and Rietschel, 2003). In addition to TLR4, LPS stimulation occurs through a series of interactions with several co-receptors and proteins including CD14, MD-2 and the LPS binding protein (Miyake, 2007). TLRs are composed of a leucine-rich repeats (LRRs), which are involved in ligand binding such as PAMP, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor, the (TIR) domain, that interacts with TIR domain-containing adaptor molecules such as myeloid differentiation-primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-b (TRIF), TIRAP/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM)(O’Neill and Bowie, 2007). TLR signaling is usually divided into two distinct and main pathways, which depend on the usage of the following adaptor molecules, MyD88 and TRIF. Most TLRs, with the exception of TLR3, seem to be dependent on the expression of MyD88 for their functions (Takeuchi and Akira, 2010). The LPS activated signaling cascade through TLR4 induces the activation of transcription factor NF-κB, which subsequently translocates from the cytoplasm into the nucleus and triggers the transcription of specific genes involved in the production of proinflammatory cytokines and other immune response related genes. One feature of MyD88-independent signaling is the induction of Type I interferons and interferon-inducible genes (Lu et al., 2008).

The cytoplasmic receptors of NLR family are emerging as important regulators of immunity as they are involved in detecting and mediating the immune response to a broad variety of pathogens and damage- and stress-associated substances. At present, a total of 22 NLR genes have been identified in humans (Bauernfeind et al., 2011). Members of the NLR family share a similar pattern of domain organization. They are composed of a central nucleotide-binding domain (NBD), and different kinds of N-terminal regions; in most NLRs, these harbor protein-binding motifs, such as CARD in NLRCs, pyrin (PYD) domain in NLRPs or a baculovirus inhibitor of apoptosis protein repeat (BIR) in NLRBs. In addition, there are NLRs with an acidic activation domain (NLRAs) and a family with no strong homology to the N-terminal domain of any other NLR subfamily member (NLRXs) (Ting et al., 2008). At the other end of the receptor, C-terminal leucine-rich repeats (LRR) motifs are believed to be involved in sensing microbial and danger-associated molecules and modulating the NLR activity. The precise mechanisms to explain how LRRs sense their ligands are still largely unknown. Two intensively studied members of NLRCs, NOD1 and NOD2, initiate NF-kB signaling, leading to the transcription of proinflammatory cytokines and the activation of antimicrobial responses. These receptors recognize the distinct fragments of bacterial peptidoglycans (PGN), gamma-D-glutamyl-meso-
diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively (Caruso et al., 2014).

Several NLRPs harboring a pyrin domain or NLRBs harboring a BIR domain in their N-terminus are not involved in the transcriptional activation of pro-inflammatory cytokines. They are components of the cytosolic macromolecular protein complexes termed ‘inflammasomes’ that regulate caspase-1 (cysteinyld aspartic protease-1) activation and activation of the inflammatory responses (Martinon et al., 2002, Man and Kanneganti, 2015).

Innate immune system is able to recognize multiple PAMPs and DAMPs, expressed by a pathogen or released due to ongoing infection or a stress situation, through the PRRs which exhibit different specificities and cellular locations. This enables effective detection and successful combatting of the microbial challenge with as few side effects as possible to the host.

2.1.2.2. The inflammasomes

Inflammasomes are crucial receptors and sensors of innate immune system for recognizing invading pathogens, host-derived danger signals and environmental irritants. They are involved in the production of biologically active proinflammatory cytokines of IL-1 family, thus they promote the generation of an inflammatory response (Man and Kanneganti, 2015). Inflammasomes are cytosolic multimeric complexes that comprise three main structures: a sensor protein belonging to the NLR (nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing) family, or non-NLR PRRs, such as AIM2 (absent in melanoma 2)-like receptor or interferon, gamma inducible protein 16 (IFI16), an adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and an inactive cysteine-protease enzyme, procaspase-1 (Latz et al., 2013).

Several sensor proteins can trigger the formation of inflammasomes and the amount of different sensor molecules behaving in such a manner may still grow in the future as more research is conducted. Inflammasomes are designated according to their sensor molecules. Most of the inflammasomes that have been described to date contain a NOD-like receptor (NLR) sensor molecule, with the structure of NOD-, LRR- and pyrin domain or NOD-, LRR- and CARD-domain. These include NLRP1, NLRP3, NLRP6, NLRP7 and NLRP12 inflammasomes or the NLRC4 inflammasome (also called IPAF), respectively (Latz et al., 2013). Two other non-NLR containing inflammasomes have been described, absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16) both belong to PYHIN (pyrin and HIN domain-containing protein) family (Hornung and Latz, 2010). AIM2 possesses a pyrin domain to recruit ASC and a HIN domain for DNA-binding, whereas IFI16 has two DNA-binding HIN domains instead of one.

In the NLR family of the inflammasomes, the NLRP3 (previously called “NALP3”, “PYPAF1”, or “cryopyrin”) has been the most intensively studied inflammasome, which plays a crucial role in innate immunity by sensing a wide
range of multiple pathogen (bacterial, fungal, viral)-, environmental- and host-derived factors (Table 1. on page 19). NLRP3 has also been implicated in the pathogenesis of several inflammatory diseases such as atherosclerosis and gout (Martinon et al., 2006, Duewell et al., 2010).

NLRP1 was the first described member of NLR family to be part of the inflammasome complex and caspase-1 activation (Martinon et al., 2002). The trigger for oligomerization and activation for NLRP1 has been suggested to be the bacterial cell wall component, muramyl dipeptide, along with the simultaneous detection of host ribonucleoside triphosphates (Faustin et al., 2007). In contrast, one of the murine NLRP1 isoforms, NLRP1b, is activated by the Bacillus anthracis lethal toxin (Boyden and Dietrich, 2006). The structure that triggers NLRP6 has remained unknown, but the function of NLRP6 is associated in maintaining the intestinal health and homeostasis (Elinav et al., 2011).

NLRP6 deficiency results in impaired mucin granule exocytosis from goblet cells (Wlodarska et al., 2014), defective mucus secretion is known to increase susceptibility to persistent microbial infections. The NLRP7-containing inflammasome is found in humans, is activated by bacterial lipopeptides and it is not expressed in mice (Khare et al., 2012) and furthermore, the specific factors triggering NLRP12 have not been characterized. It has been shown to play a role in Yersinia pestis infection via activating production of IL-18, which is a crucial cytokine for clearance of the Yersinia infection (Vladimer et al., 2012).

NLRC4 is critical in anti-bacterial defenses; it is activated by components of bacterial type III secretion system and flagellin. Unlike other inflammasomes, NLRC4 activation requires additional host molecules, NAIPs, which has been postulated to function as an actual receptor for the inflammasome, which then triggers NLRC4 (Zhao and Shao, 2015).

Even though the NLRs are categorized as PRRs, in most cases, the mechanisms of direct interaction between NLRs and their activating stimuli have not been clarified and in some cases, NLRs seem to function rather as adaptors of inflammasome. Unlike NLRs, AIM2 and IFIT16 directly bind to their ligand. AIM2 is typically activated by viral or bacterial double-stranded (ds) DNA in the cytosol originating from viruses such as mouse cytomegalovirus and vaccinia virus or cytosolic bacteria such as Francisella tularensis and Listeria monocytogenes (Hornung et al., 2009, Fernandes-Alnemri et al., 2010, Rathinam et al., 2010). The IFIT16 inflammasome is located in the nucleus; it is activated by DNA from Kaposi sarcoma-associated herpes virus (Kerur et al., 2011).

Upon activation, using the NLRP3 inflammasome as an example, oligomerization of NLRP3 leads to recruitment of the adaptor protein, ASC, through interaction between the pyrin structure (PYD) of ASC and the N-terminal pyrin (PYD) of the NLRP3. This oligomerization requires ATP to bind to the NBD structure of the NLRP3 (Duncan et al., 2007). ASC in turn brings monomers of procaspase-1 into close proximity with its CARD domain, forming a multimeric complex termed the inflammasome and initiating the self-cleavage of procaspase-1.
into its active form. Active caspase-1 proteolytically cleaves two essential pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, into their biologically active forms (Latz et al., 2013). Recently, it has been shown that the recruitment of ASC and the formation of the ASC oligomers, so-called ASC specks, is one the most important phenomena in inflammasome assembly and activation of caspase-1. The formation of ASC specks and inflammasome activity are regulated via ASC phosphorylation (Hara et al., 2013). These ASC specks act also in cell-to-cell communication. Inflammasome activation has been shown to accumulate ASC oligomers in the extracellular space, where they continue to process extracellular pro-IL-1β. Phagocytosis of these extracellular ASC specks by macrophages induces lysosomal damage and IL-1β production in the recipient cells, indicating that ASC specks can function as danger signals (Franklin et al., 2014).

In addition to the production of active IL-1 family cytokines, inflammasome dependent caspase-1 activity can occasionally trigger the form of cell death known as pyroptosis, and thus affect the survival of myeloid cells during inflammation (Bergsbaken et al., 2009). Pyroptosis shares characteristics with both apoptosis, such as DNA fragmentation and necrosis, such as cellular swelling and plasma membrane rupture and the release of proinflammatory intracellular content. Pyroptosis occurs independently of proapoptotic caspases (caspase-3, caspase-6, caspase-8) (Bergsbaken et al., 2009) In other respects, the regulation of pyroptosis is not well defined. However, increased inflammasome activity appears to increase the extent of pyroptosis.

The proinflammatory cytokine IL-1β can also be processed by non-canonical pathways involving other caspase types. In mice, it has been demonstrated that NLRP3-dependent caspase-1 activation and IL-1β processing can also be triggered by an indirect, non-canonical pathway downstream of caspase-11. Activation of caspase-11 (caspase-4 and caspase-5, human orthologues) is triggered by cytosolic LPS and it seems to occur independently of all known inflammasomes. It is still unclear how caspase-11 mediates the caspase-1 activation downstream of the NLRP3 inflammasome (Lamkanfi and Dixit, 2014). In addition, the non-canonical caspase-8 inflammasome composed of mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), adaptor protein ASC and caspase-8 mediates IL-1β maturation after sensing fungal components via the dectin-1 receptor on human dendritic cells (Gringhuis et al., 2012).
Table 1. Multiple signals lead to NLRP3 inflammasome assembly and caspase-1 activation

<table>
<thead>
<tr>
<th>NLRP3 activator</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAMPs</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Microbial cell wall components</strong></td>
<td></td>
</tr>
<tr>
<td>Bacterial muramyl dipeptide (MDP)</td>
<td>(Martinon et al., 2004)</td>
</tr>
<tr>
<td><strong>Microbial nucleotides</strong></td>
<td></td>
</tr>
<tr>
<td>Bacterial and viral DNA, RNA</td>
<td>(Kanneganti et al., 2006, Muruve et al., 2008)</td>
</tr>
<tr>
<td>(Kailasan Vanaja et al., 2014)</td>
<td></td>
</tr>
<tr>
<td><strong>Microbial pore-forming toxins</strong></td>
<td></td>
</tr>
<tr>
<td>Hemolysins (from <em>Staphylococcus aureus</em>)</td>
<td>(Munoz-Planillo et al., 2009)</td>
</tr>
<tr>
<td>Nigericin (from <em>Streptomyces hygroscopicus</em>)</td>
<td>(Mariathasan et al., 2006)</td>
</tr>
<tr>
<td>Streptolysin (from <em>Streptococcus pyogenes</em>)</td>
<td>(Harder et al., 2009)</td>
</tr>
<tr>
<td><strong>DAMPs</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ion channel activator</strong></td>
<td></td>
</tr>
<tr>
<td>ATP (ligand for P2RX7)</td>
<td>(Mariathasan et al., 2006)</td>
</tr>
<tr>
<td><strong>Disease associated crystalline material, particles, protein aggregates</strong></td>
<td></td>
</tr>
<tr>
<td>Amyloid-β (Alzheimer’s disease)</td>
<td>(Halle et al., 2008)</td>
</tr>
<tr>
<td>Calcium pyrophosphate dihydrate (CPPD)</td>
<td>(Martinon et al., 2006)</td>
</tr>
<tr>
<td>(pseudogout)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol crystals (atherosclerosis)</td>
<td>(Duewell et al., 2010, Rajamäki et al., 2010)</td>
</tr>
<tr>
<td>Monosodium urate, MSU (gout)</td>
<td>(Martinon et al., 2006)</td>
</tr>
<tr>
<td><strong>Mediators of acute inflammation</strong></td>
<td></td>
</tr>
<tr>
<td>Serum amyloid A (SAA, acute-phase protein)</td>
<td>(Niemi et al., 2011)</td>
</tr>
<tr>
<td>C3a (complement protein)</td>
<td>(Asgari et al., 2013)</td>
</tr>
<tr>
<td><strong>ENVIRONMENTAL IRRITANTS</strong></td>
<td></td>
</tr>
<tr>
<td>Alum (vaccine adjuvant)</td>
<td>(Hornung et al., 2008, Li et al., 2008)</td>
</tr>
<tr>
<td>Inorganic compounds: asbestos, silica</td>
<td>(Dostert et al., 2008, Hornung et al., 2008)</td>
</tr>
<tr>
<td>Long needle-like carbon nanotubes</td>
<td>(Palomäki et al., 2011)</td>
</tr>
<tr>
<td>Urban air pollutants: dust and emission particles</td>
<td>(Hirota et al., 2012)</td>
</tr>
</tbody>
</table>

The majority of the data derived from (Bauernfeind et al., 2011)
2.1.2.3. Regulation of NLRP3 inflammasome activation and secretion of IL-1β

NLRP3 inflammasome is activated by a wide range of different substances. The exact mechanisms through which these ligands activate the canonical NLRP3 are largely unknown. Even although the NLRP3 is categorized as a PRR, it is believed that rather than having a direct interaction between NLRP3 and its activating ligand, NLRP3 activators induce cellular events that lead to assembly of NLRP3 inflammasome and caspase-1 activation. Currently, there are some generally agreed-upon events known to activate NLRP3 inflammasome i.e. potassium efflux ($K^+$) out of the cell (Munoz-Planillo et al., 2013), generation of mitochondrial ROS (Zhou et al., 2011), the release of mitochondrial DNA or cardiolipin (Shimada et al., 2012, Iyer et al., 2013), the secretion of cathepsins into the cytosol as a result of phagolysosomal membrane destabilization (Hornung et al., 2008) and the translocation of NLRP3 to the mitochondria (Zhou et al., 2011, Subramanian et al., 2013).

However, not all of these mechanisms are accepted by all investigators in this field, such as involvement of cathepsin B or ROS in NLRP3 activation (Dostert et al., 2009, Munoz-Planillo et al., 2013). Thus the precise activation mechanism of NLRP3 is still unclear.

Recently, several regulators have been suggested to be involved in NLRP3 inflammasome activation; these include double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5) and NIMA (never in mitosis gene a)-related kinase 7 (Nek7). PKR, originally studied as an intracellular receptor for viral dsRNA, was shown to have a broader role and interact physically with inflammasome components and to mediate the activation of inflammasomes (NLRP1, NLRP3, NLRC4, AIM2) in its response to a wide array of stimuli such as double-stranded RNA, ATP, monosodium urate (MSU), aluminium or anthrax lethal toxin (Lu et al., 2012). GBP5 has been reported to promote NLRP3 inflammasome activation in response to soluble substances (ATP, nigericin) and bacteria, but not to particulate matter (Shenoy et al., 2012). Nek7 is a part of the NIMA-related kinase family, which is involved in the regulation of mitotic progression and the response to DNA damage. Nek7 was shown to be required for NLRP3 inflammasome activation in a response to ATP, nigericin, MSU crystals and alum (He et al., 2016, Shi et al., 2016). The catalytic domain of Nek7 interacts with the LRR domain of NLRP3 inflammasome, although the kinase activity was not required for activation of the Nek7-mediated inflammasome (He et al., 2016, Shi et al., 2016).

The most prominent outcome of NLRP3 inflammasome signaling, along with the induction of pyroptosis, is considered to be the secretion of biologically active proinflammatory cytokines, IL-1β and IL-18 (Lamkanfi and Dixit, 2012). These cytokines exert systemic effects and they promote a wide range of inflammatory responses (Lamkanfi and Dixit, 2012). Therefore, these cytokines are largely
beneficial to the host during an infection. Thus, activation of the inflammasome, which leads to secretion of these cytokines, must be kept silent during the normal homeostasis and even during an infection, it must be finely controlled to avoid excessive tissue damage.

It has been postulated that the full activation of NLRP3 inflammasome in macrophages involves two-steps (Bauernfeind et al., 2011) (Figure 2). The first is a priming step; the prototypical example is the binding of bacterial lipopolysaccharide (LPS) to TLR4 that leads through NF-κB signaling to upregulation of pro-IL-1β and onwards to an increase in the cellular expression of NLRP3 components (Bauernfeind et al., 2009). The production of pro-IL-18 does not require this first step as it is expressed constitutively in many leukocytes (Puren et al., 1999). Post-translational modifications are known to be crucial in almost every step of inflammasome activity, including ubiquitination, deubiquitination, phosphorylation, and degradation. Even priming can induce deubiquitination of NLRP3, which is essential for its assembly and activation (Juliana et al., 2012, Lopez-Castejon et al., 2013). Inflammasome activation requires also a post-translational modification of the adaptor protein ASC, this needs to become linearly ubiquitinated and phosphorylated before inflammasome assembly can occur (Hara et al., 2013, Rodgers et al., 2014).

Once primed, the activation of NLRP3 requires a second, distinct stimulus such as the presence of a microbial toxin or a DAMP before the second step is taken, where the components are assembled into the inflammasome structure followed by the secretion of IL-1β and IL-18.

The requirement of two signals for NLRP3 inflammasome activation suggests that macrophages do not activate their production of proinflammatory cytokine IL-1β when receiving only the second stimulus. Requirement of the crucial priming signal via other PRRs, that indicates either the presence of infection or the presence of other activated defence cells (via activation of PRRs by microbial products or danger signals, or receptors recognizing pro-inflammatory cytokines), along with the second signal may have the purpose of preventing accidental NLRP3 activation. Uncontrolled activation of NLRP3 can have devastating consequences for the host such as triggering of an autoinflammatory disease (Bauernfeind et al., 2011).
Figure 2. Two distinct signals (priming and activation) are needed for activation of NLRP3 inflammasome and production of biologically active IL-1β.

2.1.2.4. NLRP3-associated diseases

Our knowledge of how inflammasomes contribute to the pathology of many autoinflammatory diseases has significantly increased in recent years.
Inflammasomes mediate the effective and rapid innate immune response against the infectious or non-infectious agents, leading to the elimination of the threat. Inflammasomes have also been suggested to play a role in the development of adaptive immunity (Dostert et al., 2013). Therefore, any uncontrolled function of the inflammasome, such as continuous production of proinflammatory cytokines may be harmful since it can lead to severe outcomes for the host. In this chapter, the focus will be placed on the NLRP3 inflammasome and diseases associated with its dysfunction.

Several inherited autoinflammatory disorders are caused by gain of function mutations in NLRP3, thus highlighting its importance in the inflammatory process (Masters et al., 2009). There are several well known examples e.g. the cryopyrin-associated periodic fever syndromes (CAPS, also called cryopyrinopathies), which include Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), and neonatal onset multi-system inflammatory disease (NOMID) (Hoffman et al., 2001, Feldmann et al., 2002). The cryopyrin (or CIAS1) was the name formerly used for NLRP3, which was given to the gene and its protein product because of its role in cold-induced fevers (Hoffman et al., 2001, Feldmann et al., 2002, Ting et al., 2008). These cryopyrinopathies differ in the level of symptom severity, but their general symptoms are urticarial skin rashes, prolonged episodes of fever, arthritis, and other types of localized inflammation (Hoffman et al., 2001, Feldmann et al., 2002, Masters et al., 2009). It has been postulated that the CAPS-associated mutations may cause conformational changes and trigger the spontaneous assembly of NLRP3, which lead to persistent activation of caspase-1 and the disproportionate production of IL-1β (Dowds et al., 2004).

There is evidence accumulating from the majority of knockout mouse studies with some supporting human data, implicating NLRP3 in the pathogenesis of several complex diseases. It is notable that many of these diseases such as metabolic disorders (atherosclerosis, gout, type 2 diabetes) and neurogenerative diseases (Alzheimer’s disease) have a high impact on public health. One common feature of these diseases is aberrant accumulation of certain metabolites, such as cholesterol crystals in atherosclerosis, MSU crystals in gout or islet amyloid polypeptides (IAPP) in type 2 diabetes, or the presence of misfolded protein aggregates such as amyloid-β peptide plaques in Alzheimer’s disease (Martinon et al., 2006, Halle et al., 2008, Duewell et al., 2010, Masters et al., 2010, Rajamäki et al., 2010, Heneka et al., 2013). These components have been proved to function as endogenous DAMPS and activators of the NLRP3 inflammasome. Thus, they activate IL-1β signaling, leading to inflammation and cell death, the general phenomena encountered in these diseases (Chen et al., 2006, Halle et al., 2008, Duewell et al., 2010, Masters et al., 2010).

The NLRP3 is activated also by a variety of exogenous agents, ranging from microbes (e.g. viruses, bacteria, fungi) and their components to certain inorganic particles found in the environment (e.g. minerals). The data from patients with
lung diseases and animal knockout studies suggests that a number of inhaled inorganic particles can cause NLRP3 inflammasome activation in the lung; these particles include asbestos, silica and bleomycin, this last compound is exploited in an experimental model of idiopathic pulmonary fibrosis. The production of IL-1β and IL-18 cytokines via activated NLRP3 inflammasome recruits additional immune cells into the lung and boosts the inflammatory response, potentially leading to initiation or progression of the pulmonary disease (De Nardo et al., 2014).

IL-1β plays a major role in NLRP3 activated inflammatory response. Thus IL-1 antagonism has been found to be successful in the treatment of many IL-1 associated diseases. Inhibition of IL-1 signaling with the recombinant IL-1 receptor antagonist, anakinra, or with the soluble decoy IL-1 receptor, rilonacept, or with the neutralizing IL-1 antibody, canakinumab, has proven extremely beneficial in CAPS patients and metabolic disorders such as type 2 diabetes and gout (Dinarello and van der Meer, 2013). There has also been intense research into the molecules inhibiting NLRP3 directly, as this may be one way to avoid the side effects which may occur when IL-1 signaling is totally blocked. One of these side effects is excessive immunosuppression, which may lead to an increased risk of infection, because the IL-1 response triggered by other antimicrobial inflammasomes, such as NLRP1 and NLRC4, is also inhibited. Recently, MCC90, a highly potent specific inhibitor of the activation of canonical and non-canonical NLRP3 has been characterized (Coll et al., 2015). Treatment with MCC90 decreased neonatal lethality in a mouse model of CAPS and inhibited NLRP3 in ex vivo samples collected from patients with Muckle-Wells syndrome (Coll et al., 2015). Future trials with these direct NLRP3 inhibitors will determine whether they represent a new, more efficient, treatment for NLRP3 associated diseases.

### 2.1.2.5. Proinflammatory cytokines of IL-1 family

Interleukin-1 family cytokines are central mediators of innate immunity and inducers of local and systemic inflammation. The IL-1 family also includes members which suppress inflammation (Dinarello, 2013). Their production is part of the defense response of host against invading pathogens and other noxious agents. These cytokines play key roles in the function and differentiation of cells from both innate and adaptive immunity system (Garlanda et al., 2013). Because of their wide-scale impact, activation and signaling of IL-1 family cytokines are tightly regulated at many levels: gene transcription, protein processing, receptor binding, balancing between the expression of receptor agonists and antagonists, and post-receptor signaling by naturally occurring inhibitors (Palomo et al., 2015).

The IL-1 family consists of seven ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38) and one cytokine with anti-inflammatory features (IL-37) (Garlanda et al., 2013). They share a common C-terminal three-dimensional protein structure
and most of them are synthesized as precursor proteins, with non-existent or very little biological activity and thus to achieve their full biological potential, they require proteolytic processing, which is one crucial step in the control of the activation of IL-1 cytokines (Sims and Smith, 2010, Dinarello, 2013, Garlanda et al., 2013). All the IL-1 family members, with the exception of the IL-1Ra, lack an N-terminal hydrophobic signal sequence and thus are not secreted via the conventional endoplasmic reticulum-golgi pathway (Sims and Smith, 2010, Garlanda et al., 2013). Unconventional secretion mechanisms of IL-1 cytokines are still mainly remained uncharacterized. This chapter will focus on the function of proinflammatory agonists of IL-1 family (Table 2).

Table 2. Proinflammatory cytokines of IL-1 family and their main functions

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Alternative name</th>
<th>Receptor</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>IL-1F1</td>
<td>IL-1R1, IL-1R2</td>
<td>DAMP, Th17 responses, inflammation</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1F2</td>
<td>IL-1R1, IL-1R2</td>
<td>Antimicrobial resistance, Th17 responses, inflammation</td>
</tr>
<tr>
<td>IL-18</td>
<td>IL-1F4</td>
<td>IL-18Rα</td>
<td>Antiviral resistance, Th1 responses, inflammation</td>
</tr>
<tr>
<td>IL-33</td>
<td>IL-1F11</td>
<td>ST2</td>
<td>DAMP, Th2 responses, inflammation</td>
</tr>
<tr>
<td>IL-36α</td>
<td>IL-1F6</td>
<td>IL-1Rrp2 (IL-36R)</td>
<td>Tissue-restricted inflammations (skin, lung)</td>
</tr>
<tr>
<td>IL-36β</td>
<td>IL-1F7</td>
<td>IL-1Rrp2 (IL-36R)</td>
<td>Tissue-restricted inflammations (skin, lung)</td>
</tr>
<tr>
<td>IL-36γ</td>
<td>IL-1F8</td>
<td>IL-1Rrp2 (IL-36R)</td>
<td>Tissue-restricted inflammations (skin, lung)</td>
</tr>
</tbody>
</table>

The majority of the data derived from (Garlanda et al., 2013)

IL-1β is a highly inflammatory cytokine and it has been the most extensively studied member of the IL-1 family. IL-1 exerts systemic effects, it can affect virtually nearly every organ or cell. It is involved in a wide range of biological activities including development of fever, vasodilation, angiogenesis, hematopoiesis, leukocyte recruitment, function and survival and it also have a role in the adaptive immune system by affecting lymphocyte activation and antibody synthesis (Dinarello, 2009, Garlanda et al., 2013). It has been reported that IL-1β induces the development of Th17 cells (Sims and Smith, 2010, Garlanda et al., 2013), the subclass of activated CD4+ helper T-cells in adaptive immunity which are associated with the immune response against extracellular bacteria and fungi (Luckheeram et al., 2012). IL-1β has been implicated in a wide range of inflammatory diseases such as type 2 diabetes and gout (Dinarello and van der Meer, 2013). IL-1β is primarily a product of innate immune defense cells such as monocytes, macrophages and dendritic cells, released mostly in response to inflammatory stimuli such as PAMPs or proinflammatory cytokines e.g. TNF or IL-1 itself (Dinarello, 2009). IL-1β and IL-18 are produced as biologically inert pro-peptides that need to be cleaved in their N-terminal region before they can bind to their receptors and induce cellular responses. As demonstrated in a
previous chapter, the preform of IL-1β, as is also the case with IL-18, must be cleaved by caspase-1 enzyme, which in turn needs the assembly and activation of intracellular protein complex named the inflammasome for its activation (Schroder and Tschopp, 2010). In addition, pro-IL-1β or IL-18 can also be cleaved independently of caspase-1 activation by neutrophil serine proteases, such as proteinase 3 and elastase and proteases released by invading microorganisms or mast cell-derived chymase and NK cell-derived granzyme B (Netea et al., 2015).

In contrast to IL-1β, IL-18 precursor is constitutively expressed in most cells, it is not pyrogenic (a fever inducer) and it does not induce the production of acute-phase proteins or proinflammatory cytokines such as TNF or IL-6 (Novick et al., 2013). IL-18 has been associated with the Th1 polarized cell-response, which contributes to the immune response by inducing the production of IFN-γ and activation of natural killer cells (NK cells) (Garlanda et al., 2013). The Th1 type response is usually encountered in viral infections, but may also be seen in the presence of intracellular pathogen (Luckheeram et al., 2012). Clearly elevated levels of serum IL-18 are detected in patients with infections caused by viruses such as Epstein-Barr virus, cytomegalovirus and human immunodeficiency virus (ten Oever et al., 2012, van de Veerdonk et al., 2012). However, depending on the surrounding cytokine milieu, IL-18 can display various inflammatory activities, e.g. in the absence of the IL-12 or IL-15 cytokines, it can promote Th2 responses (Nakanishi et al., 2001). A role for IL-18 has been postulated in several autoimmune diseases, such as Crohn’s disease and psoriasis and other diseases such as myocardial dysfunction, metabolic syndromes, macrophage activation syndrome, and acute kidney injury (Novick et al., 2013). In addition to IL-18’s proinflammatory role of, it has displayed protective features in some models of diseases and has been suggested to act also as an important regulator of homeostasis (Novick et al., 2013). This proposal is supported by the finding that IL-18 deficiency in mice results in development of the metabolic syndrome with obesity and lipid abnormalities (Netea et al., 2006) as well as the fact that administration of exogenous IL-18 promoted mucosal healing in caspase-1 knockout mice subjected to dextran sulfate sodium (DSS) induced colitis (Dupaul-Chicoine et al., 2010).

IL-1α and IL-33 are the IL-1 family members, which have a dual function, acting as proinflammatory cytokines or as transcription factors. Both of these cytokines are consistently found in cell nuclei and their precursor forms contain nuclear localization sequences in their N-terminal region that can bind to DNA (Werman et al., 2004, Carriere et al., 2007). Intracellular precursor proteins of IL-1α and IL-33 can be released in situations of cell damage or cell death and stimulate early inflammatory responses by acting as DAMPs. These full length precursors of IL-1α and pro-IL-33 can bind to their respective receptors and induce cell signaling, although their activities are significantly enhanced after protease cleavage. IL-1α is processed by calpain, a membrane-associated, calcium-dependent cysteine protease (Kobayashi et al., 1990). IL-33 is cleaved to its more
potent mature forms by calpain and the neutrophil serine proteases, cathepsin G and elastase, whereas caspase-1 cleavage has resulted in a loss of IL-33 activity (Garlanda et al., 2013, Netea et al., 2015).

IL-1α binds to the same receptor (IL-1R) with IL-1β, either as an unprocessed precursor or as a processed protein. In practical terms, IL-1α shares similar kinds of biological properties as IL-1β. On the other hand, the unique features of IL-1α include its role in initiating so-called sterile inflammation by inducing the expression of inflammatory cytokines and chemokines and recruiting neutrophils when released by dying cells, whereas secretion of IL-1β needs the initial activation of the inflammasome and it is more involved with the recruitment and retention of macrophages. (Rider et al., 2011). In contrast to IL-1β, IL-1α is constitutively expressed as precursor protein in epithelial cells, endothelial cells and keratinocytes (Garlanda et al., 2013). IL-1α is rarely secreted from cells, but its membrane-anchored form is fully biologically active and can induce gene expression in neighboring cells (Kaplanski et al., 1994). The different effects of IL-1α and IL-1β seem to occur more due to the different cell sources of these cytokines as well as their distinctive cellular locations and release mechanisms, rather than being attributable to differences in downstream signaling following the IL-1R engagement (Garlanda et al., 2013). IL-1α has been shown to play a role in atherosclerosis and also in some experimental models of brain injury (Dinarello, 2009, Luheshi et al., 2011).

IL-33 is the most recently identified member of IL-1 family and it is expressed constitutively or in an inducible manner in several stromal, parenchymal and hematopoietic cell types. The dominant biological activity of IL-33 on immune cells is to polarize the immune response towards allergic response and the Th2 direction i.e. inducing the production of Th2 cytokines (IL-4, IL-5, IL-13) and triggering the M2-polarization of macrophages. IL-33 acts directly on mast cells and eosinophils may be due to abundant expression of ST2 in these cells. It promotes the maturation, survival and cytokine production of mast cells, as well as elevates the survival and expansion of eosinophils (Schmitz et al., 2005, Oboki et al., 2010, Sims and Smith, 2010, Garlanda et al., 2013). IL-33 plays a role as a mediator in the Th2 type inflammatory conditions such as allergic lung inflammation, whereas in helminth infections, it seems to have a protective role (Garlanda et al., 2013). In addition to its Th2 polarizing effect, it seems to possess anti-inflammatory features by reducing the gene transcription of those proinflammatory cytokines which are activated by transcription factor NF-κB (Ali et al., 2011).

The IL-36 is a subgroup of the IL-1 family, but its function has remained poorly characterized. It includes three agonists: IL-36α, IL-36β, and IL-36γ. The expression of IL-36 cytokines is mainly observed in keratinocytes, bronchial epithelium, monocytes/macrophages and lymphocytes (Gresnigt and van de Veerdonk, 2013). IL-36 cytokines are predominantly expressed in skin and there are some reports that these cytokines are involved in the pathogenesis of skin
diseases such as psoriasis (Johnston et al., 2011, Marrakchi et al., 2011).
Furthermore, a role in the pathology of pulmonary diseases has been suggested. In mice, the gene encoding IL-36γ is located in the locus of allergen induced bronchial hyperresponsiveness-1 \((\text{Abhr1})\). Furthermore, the expression of IL-36γ in the lung was increased in an animal model where the mice are asthma susceptible compared to asthma resistant mice, suggesting that IL-36γ may be involved in allergen-induced inflammation in the lung (Ramadas et al., 2006). In addition, the intratracheal administration of IL-36α or IL-36γ to mice induced a rapid influx of neutrophils into the BALF, one of the hallmarks of inflammation in lungs (Gresnigt and van de Veerdonk, 2013). Bronchial epithelial cells express IL-36γ, when stimulated with proinflammatory cytokines such as IL-1β, TNF, IL-17, or microbial substances, whereas IL-36γ induces chemokine IL-8 and the Th17 chemokine CCL20 in human lung fibroblasts (Vos et al., 2005, Chustz et al., 2011).

The IL-36 cytokines, secreted by keratinocytes or other epithelial cells, have a significant effect on dendritic cells, inducing the production of many cytokines and chemokines such IL-1β, TNF, IL-6, IL-12, IL-23, CXCL1 and GM-CSF in the dendritic cells. These mediators are associated with Th1 and Th17 responses, thus in this way, IL-36 signaling can promote not only the innate responses but also the responses mediated via adaptive immunity by affecting the T-cell polarization (Vigne et al., 2011, Mutamba et al., 2012).

IL-36 cytokines do not contain caspase-1 cleavage sites, however, similarly to IL-1β and IL-18, in a recent report it was shown that N-terminal truncation, although artificial in this case, increased dramatically the proinflammatory activity of IL-36 (Towne et al., 2011). However, currently it is not understood how endogenous IL-36 undergoes maturation into its bioactive form and which enzyme or enzymes are involved in this processing, and even whether this occurs in humans under pathologic conditions. The IL-36 signaling in many respects resembles IL-1α and IL-1β signaling, but IL-36 cytokines seem to be expressed in a more tissue-restricted manner than either IL-1α or IL-1β. Therefore, they cannot be considered to be simple surrogates for IL-1α or IL-1β. It has been proposed that they have a role in IL-1α/β independent inflammatory responses and have evolved to regulate tissue specific immune responses (Gresnigt and van de Veerdonk, 2013).

2.1.3. Protein secretion as a response to immune system activation

The secretion of immune mediators such as cytokines, chemokines, DAMPs and other substances affecting the immune system by both immune and non-immune cells plays a crucial role in determining the course of the inflammatory response. Some cytokines are released in order to maintain homeostasis in tissues or when the balance must be restored after combatting an environmental challenge
such as the presence of a dangerous pathogen. In contrast, proinflammatory cytokines are released in the response to a pathogen or other harmful signals in order to promote the inflammatory response and achieve the eradication of this harmful stimulus. The production of proinflammatory cytokines in cells is regulated at many levels by cell signaling, as well as at the levels of mRNA and protein synthesis. Thereafter, an essential role in the activation and release of the cytokines is carried out by intracellular trafficking machinery, which performs the necessary modifications in proteins and arranges their transport to their final destinations. This trafficking machinery and the related secretion pathways of the cytokines are complex and highly regulated in many cells, involving specialized organelles, membrane structures and multiple participating molecules (Stow and Murray, 2013).

2.1.3.1. Conventional and unconventional protein secretion

The vast majority of eukaryotic proteins are secreted through the conventional or the so-called classic protein secretion pathway which refers to protein secretion via the endoplasmic reticulum (ER)-to-Golgi membrane pathway. These conventionally secreted proteins contain an N-terminal or an internal signal-peptide that directs them into the lumen of the ER. This step is followed by the vesicular transport of secretory proteins in COPII-coated vesicles to the Golgi membranes and thence to the cell surface where the cargo vesicle is fused with the plasma membrane and its contents are released into the extracellular space (Dancourt and Barlowe, 2010). One form of conventional secretion is constitutive exocytosis, which is the predominant mechanism for cytokine release from macrophages and DCs, which do not have the same kind of cytosolic secretory granules as eosinophils and cytokines are transferred direct from Golgi to plasma membrane via recycling endosomes and secreted into the extracellular milieu. Several proinflammatory cytokines such as TNF, IL-6, IL-12 and a group of chemokines are secreted via constitutive exocytosis only after being synthesized in response to microbial or inflammatory stimuli (Lacy and Stow, 2011).

Proinflammatory cytokines such as IL-1 family members or several DAMPs such as calgranulins (S100 proteins) or galectins lack the protein signal sequence required for ER entry and are thus released from cells via the unconventional protein secretion pathways (Bianchi, 2007, Garlanda et al., 2013). Currently, unconventional secretion is distinguished into nonvesicular and vesicular pathways, and these are classified further into four subtypes (I-IV) based on their detailed secretion mechanisms (Rabouille et al., 2012). The non-vesicular pathways involve direct protein translocation across the plasma membrane by lipid-induced oligomerization and membrane insertion (type I) or proteins are transported through the plasma membrane by ABC-transporters (type II). Type III unconventional secretion is based on protein trafficking in vesicles and occurs at least in four different ways. The protein cargo can be translocated from the cytosol
to the plasma membrane inside the secretory lysosomes or autophagosomes. The fusion of these lysosomes or autophagosomes with the plasma membrane leads to the release of their protein content into the extracellular space. The cytoplasmic proteins might also be packed into the endosomal vesicles; this leads to the formation of multivesicular bodies. When these multivesicular bodies fuse with the plasma membrane, endosomal vesicles are released into the extracellular space as exosomes. Lastly, direct microvesicle shedding from the plasma membrane can also capture intracellular proteins in their lumen and lead to the release of these proteins. Type IV pathway and the second type of unconventional vesicular secretion is a variation within the classic secretory pathway, where the proteins bypass the Golgi complex after trafficking in ER and reside in the plasma membrane. Generally, type I-III pathways mediate the secretion of cytoplasmic proteins, in contrast, the type IV pathway is used for integral membrane proteins, which are not released outside of the cell (Nickel and Rabouille, 2009, Rabouille et al., 2012).

2.1.3.2. Secretion of IL-1 via unconventional protein secretion pathway

Proinflammatory cytokines of the IL-1 family are known to be secreted via unconventional secretion pathways with the secretion of IL-1β being studied most extensively (Garlanda et al., 2013). Caspase-1 is one of the so-called inflammatory caspases; it contains a CARD-domain in its N-terminus, and plays a role in inflammation since it is a part of the inflammasome complex and thus it can regulate the activation of the proinflammatory cytokines pro-IL-1β and pro-IL-18 (Martinon and Tschopp, 2007). The function of caspase-1 differs from apoptotic caspases, such as caspase-3, in that it does not participate in apoptotic cell death. However, the pyroptotic type of cell death (pyroptosis) is considered to be mainly dependent on caspase-1 (Bergsbaken et al., 2009). Caspase-1 has been proposed to participate in the regulation of unconventional protein secretion (Keller et al., 2008). Secretion of IL-1β and IL-18 is known to be impaired in caspase-1 deficient macrophages. Surprisingly, also the release of IL-1α, which is not a substrate for caspase-1, seems to be dependent on caspase-1, although not on its protease activity (Kuida et al., 1995, Li et al., 1995, Gross et al., 2012). It has been suggested that depending on the type of NLR activator (phagocytozed particle or soluble NLRP3 agonist such as ATP or nigericin), the release of IL-1α is correspondingly either caspase-1 independent or dependent, but in both cases IL-1α processing depends on calpain protease activity (Gross et al., 2012, Yazdi and Drexler, 2013).

The activation of the inflammasome is associated with the secretion of its central components (caspase-1, ASC, NLRP3), as well as the secretion of caspase-1 substrates IL-1β and IL-18, and also several other proteins, which lack signal peptides, into the extracellular milieu (Qu et al., 2007, Keller et al., 2008, Gross et
al., 2012). Phagocytosis of secreted inflammasome components such as ASC specks by macrophages has been shown to induce an inflammatory response in these cells (Franklin et al., 2014), indicating that activation of unconventional secretion might be one of the immune mechanisms capable of promoting and spreading the inflammatory response.

The release of IL-1β was one of the earliest discovered examples of unconventional protein secretion (Rubartelli et al., 1990) but its secretion mechanisms are still being intensely debated. The release of soluble IL-1β into the extracellular milieu has been proposed to occur via ABC transporters (Hamon et al., 1997, Ikeda et al., 2007) or through the uptake of secretory lysosomes, which fuse with the plasma membrane (Andrei et al., 2004, Carta et al., 2006). Nonetheless, there are other studies that point to the secretion of IL-1β packaged into the vesicles such as microvesicles (MacKenzie et al., 2001) or exosomes (Qu et al., 2007). According to these results, it is very likely that there are multiple factors (stimulus, cell type, or culturing conditions) which may play a role in dictating the secretory mechanisms used for IL-1β release (Stow and Murray, 2013).

Recently, it has been demonstrated that a process called autophagy participates in the regulation of unconventional secretion of IL-1β and other proteins (Dupont et al., 2011, Ponpuak et al., 2015). The research of autophagy has been the centre of attention in 2016 due the award of the Nobel Prize in physiology or medicine to biologist Yoshinori Ohsumi for his work in 1990s, which revealed the mechanisms underlying autophagy in a yeast model. Autophagy, which is mainly recognized via the cytoplasmic autodigestive process during starvation and removal of protein aggregates or dysfunctional organelles, also affects protein secretion by a process termed secretory autophagy (Ponpuak et al., 2015). Secretory autophagy is known to facilitate the unconventional secretion of the cytosolic and leaderless proteins such as IL-1 family members IL-1β and IL-18, and DAMPs such as HMGB1 (Dupont et al., 2011, Piccioli and Rubartelli, 2013). In addition, the autophagic machinery plays a role in excreting more complex cytoplasmic cargo and particulate substrates, and it has also been shown to influence conventional secretory pathways (Ponpuak et al., 2015). Thus, autophagy and autophagic factors are closely connected at many levels with secretion and the polarized sorting of proteins inside cells. It has recently been reported that the secretory autophagy-related ATG factors such as Atg5, which govern the biogenesis of autophagic membranes, are required for the unconventional secretion of IL-1β (Dupont et al., 2011), confirming that the secretion of several unconventional secretory proteins such as IL-1β involves autophagosome-like vesicular intermediates. In addition, it has been demonstrated that in cells where the autophagy process was induced by starvation, the secretion of IL-1β was enhanced when these cells were stimulated by known inflammasome activators (nigericin, alum, silica or amyloid-β fibrils) (Dupont et al., 2011). However, there are also reports which indicate that autophagy suppresses inflammasome activation by maintaining mitochondrial...
homeostasis by preventing the release of ROS (Zhou et al., 2011) or mitochondrial DNA (Nakahira et al., 2011). In addition, the negative regulation of inflammasome activation and IL-1β release is also proposed to occur due to the autophagic degradation of inflammasome components and IL-1β (Harris et al., 2011, Shi et al., 2012). These results indicate that autophagy has a dual role in the regulation of inflammatory response. It affects the secretion of IL-1β and other unconventional secreted proteins, furthermore, it can reduce the inflammatory response by targeting the components and substrates of the inflammasome to be broken down and degraded.

2.1.3.3. Extracellular vesicles –as conveyors of the immune system

Extracellular vesicles can be formed from the plasma membrane by direct shedding; they are then called microvesicles or they can originate from an intracellular compartment, the multivesicular bodies (MVB) which are released by exocytosis. These latter vesicles, once released into the extracellular environment, are called exosomes. It is thought that extracellular vesicles serve as signal conveyors in intercellular communication, both locally and systemically, as they can transfer their contents into the new cells. Extracellular vesicles may contain membrane and intracellular components that include proteins, lipids and nucleotides, but their composition can differ with respect to site of vesicle biogenesis (Robbins and Morelli, 2014). There are examples of releasing molecules such as a Fas ligand which when present in extracellular vesicles, experience reduced degradation by surface proteases, augmenting their local concentration which may ultimately improve their biological activity by favouring their aggregation (Zuccato et al., 2007).

Exosomes are the best characterized group of secreted membrane vesicles and thus their function is discussed in more detail in this chapter. Exosome vesicles (50–100 nm) are secreted from viable cells, either constitutively or in an induced manner, but they are not released from either lysed or apoptotic cells. Many cell types such as hematopoietic cells, intestinal epithelial cells, neuronal cells and tumor cells have been described to release exosomes in vitro. Exosomes are found in vivo in plasma and several other biological fluids such as urine, saliva, breast milk, semen, bronchoalveolar lavage fluid and sputum (Thery et al., 2009, Record et al., 2011). Macrophages infected with intracellular pathogens have been shown to secrete exosomes (Bhatnagar et al., 2007) and in general, exosome secretion is thought to be a response to environmental challenges (pathogen encounter, cell stress), and could be considered to be one of the mechanisms used by cells and tissues to adapt to these changes (Thery et al., 2009). The contents of lipid and protein in exosomes differ from the contents of microvesicles or apoptotic blebs. Exosomes have a high content of proteins and may include many different kinds of proteins (Exocarta, a web-based compendium of exosomal
proteins, RNAs and lipids hosts currently over 40,000 proteins) (Keerthikumar et al., 2015). The proteins that are frequently seen enriched in exosomes are those related to vesicle genesis or their trafficking (TSG101, ALIX, annexins), signal transduction (kinases and G-proteins), cytoskeleton organization (actin and tubulin), antigen presentation (MHC class I and II molecules, heat shock proteins) and vesicle targeting either towards the recipient cells or to the extracellular matrix (adhesion molecules such as integrins and tetraspanins) (Robbins and Morelli, 2014).

Initially, exosomes were thought to be simply a mechanism for removing unneeded proteins from the cells, especially for cells that have poor capacities to degrade proteins or are located towards a drainage system such as the kidney tubule or gut (Record et al., 2011). Subsequently, it was revealed that exosomes have important roles in the regulation of the immune system by promoting or suppressing the immune response and thus they possibly are to able to drive inflammatory, autoimmune and infectious disease pathology. It is also known that exosomes carry both antigenic material and peptide-MHC complexes which can activate the recipient cells such as antigen-presenting cells (APCs) or T-cells. Exosomes released from Mycobacterium -infected macrophages contained pathogen-derived antigens and could stimulate the proinflammatory response in vitro and in vivo (Bhatnagar et al., 2007). Tumor-derived exosomes carrying tumor-proteins and antigens are emerging mediators of tumorigenesis and can activate directly macrophages, NK -cells and also T-cells via dendritic cell presentation (Bobrie et al., 2011, Peinado et al., 2012). On the other hand, they may also carry ligands such as Fas L or TGFβ which inhibit immune cell proliferation or activation (Bobrie et al., 2011). The exosomes that are released by APCs contain antigens with surface MHC class I and class II molecules and therefore potentially can directly stimulate preactivated CD8+ and CD4+ T cells, respectively. In contrast, if they are to activate naïve T cells which require high levels of T-cell receptor crosslinking and co-stimulation, the content of exosomes must be captured and presented by DCs (Bobrie et al., 2011). Secreted vesicles may also possess immunosuppressive properties and induce a kind of tolerance. As mentioned before, tumor-derived microvesicles or exosomes have been shown to suppress the function of immune cells, for example by inducing T cell apoptosis via FasL (Andreola et al., 2002) or galectin-9 (Klibi et al., 2009). In addition, in immune cells, activated T-cells can secrete FasL containing vesicles and induce an activation-induced cell death (AICD) of bystander T-cells (Monleon et al., 2001). Vesicles purified from body fluids with immunosuppressive features have also been described, for example exosomes in human breast milk have been demonstrated to reduce T cell activation in vitro (Admyre et al., 2007). Exosomes, secreted by virally infected cells can also suppress the host immune response and
in that way, favour the spread of an infection. Exosomes released by HIV-infected cells were able to trigger apoptosis in uninfected bystander T cells (Lenassi et al., 2010).

In summary, exosomes carry numerous signals between immune cells or non-immune cells, resulting in multiple functions affecting our immune response, but which of these functions are really important in vivo and which of them could be utilized in the clinic, still remain to be elucidated.

2.2. The innate immune response to fungal particles

Fungi are present everywhere, thus we are continually inhaling a low level of airborne fungal particles from our environment. Fungi are mainly found in soil and water, but they can live on materials with even a low level of moisture. Fungi belong to our body’s normal flora (microbiome), which have an essential role in sustaining our health by preventing the overgrowth and colonization of pathogenic microbes. These common environmental or commensal fungi such as *Aspergillus fumigatus*, *Cryptococcus neoformans* or *Candida albicans* can cause fungal diseases, mycoses, when their recognition by our immune system is defective and cannot resist the fungal burden or when the inflammatory response becomes overactivated while attacking the fungi and causes widespread damage to the host (Romani, 2011, Iliev et al., 2012). Fungi are associated with a wide spectrum of diseases, such as respiratory allergy and skin diseases.

In immunocompromised patients, fungi may cause inflammatory diseases and severe life-threatening infections (Romani, 2011). As the population of immunocompromised individuals is growing significantly (increased prevalence of individuals receiving immunosuppressive treatments or suffering from autoimmune diseases), so too has the incidence of fungal diseases and the need for effective diagnosis tools and treatments (Pappas, 2010).

In addition to the mycoses, which are characterized by tissue invasion by fungi and their spread and growth in the host, also exposure to the environmental fungal particles, which are not infective, has been associated with the development of several diseases such as hypersensitivity pneumonitis and damp building–related illness. These diseases will be discussed in chapter 2.3.

2.2.1. Fungal cell wall components as activators of innate immunity

Fungi are eukaryotic organisms that are traditionally classified, based on their morphologic features, into single-celled and multicellular filamentous forms such as yeasts and moulds, respectively. The fungal cell wall components represent an
important group of fungal PAMPs which are recognized by the PRRs on immune cells (Romani, 2011, Becker et al., 2015). For example, about 90% of the cell wall of a well-known fungal pathogen, *C. albicans* is made up of carbohydrate structures. The three major polysaccharide components of fungal cell wall, which are known to be recognized by the immune system and are found in all medically important fungi, are the inner cell wall structures: β-glucans (polymers of glucose) and chitin (a polymer of N-acetylglucosamine), and components of the outer cell wall: mannans (chains of mannose molecules attached to fungal proteins via N-or O-linkage) (Gow and Hube, 2012).

The composition of the fungal cell wall varies and depends on different factors such as the fungal morphotype, the current growth stage of the fungus, as well as environmental factors such as temperature, oxygen levels, availability of nutrients or the presence of anti-fungal drugs (Verwer et al., 2012, Shepardson et al., 2013, Beauvais et al., 2014). After budding, β-glucans are surface-exposed in the bud scar, but during the hyphae stage of *C. albicans*, the β-glucans are masked and cannot elicit a strong inflammatory response via their specific PRR, dectin-1 (Gantner et al., 2005). Other inner layer components of fungal cell wall also become exposed on the surface during the budding process. In addition, conidia, which usually are immunologically rather inert, expose the polysaccharides of the inner cell wall when the outermost rodlet-layer of conidia is degraded, for example during germination (Aimaniannda et al., 2009). Chitin is the other major polysaccharide in the inner cell wall and studies with purified or fungal chitin have shown that it is associated with the recruitment of eosinophils and it can also promote M2 macrophage activation (O’Dea et al., 2014, Van Dyken et al., 2014). In contrast to β-glucan, a specific and distinct recognition receptor for chitin has yet not been fully characterized. The outer layer of the cell wall of *C. albicans* contains proteins, which are highly glycosylated with mannose-containing polysaccharides. These mannans are recognized by several Toll-like receptors and C-type lectin receptors (Netea et al., 2008). Both mannans and mannoproteins of *C. albicans* exert important immunostimulatory activities by stimulating the proinflammatory cytokine production and inducing dendritic cell maturation (Garner et al., 1994, Pietrella et al., 2006), as well as activating T-cell immunity (Mencacci et al., 1994, Gomez et al., 1996).

### 2.2.1.1. The immunomodulatory nature of 1,3-β-glucan

Beta-glucans are carbohydrate structures (polysaccharides) found naturally in fungi, plants and some bacteria. Glucan has a 1,3-β-glucan backbone with varying numbers of 1,6-β branches; it is the major glucan structure in the fungal cell wall (Figure 3) (Tsoni and Brown, 2008). Fungal 1,3-β-glucans have long been recognized as stimulants of innate immunity and thus they are essential fungal pathogen-associated molecular patterns (Brown and Gordon, 2005). Large particulate (insoluble form) β-glucans are known to activate the innate immune
response via dectin-1 receptor after the formation of a phagocytic synapse (Goodridge et al., 2011) and in this way, they induce both phagocytosis and the production of cytokines and reactive oxygen species (ROS) by leukocytes. In contrast, the soluble and small β-glucans seem to inhibit these responses, when they become attached to the dectin-1 receptor (Brown and Gordon, 2001, Brown and Gordon, 2003, Goodridge et al., 2011). In addition to the effects to the innate immunity, particulate β-glucans also influence adaptive immunity by promoting dendritic cell maturation and antigen presentation, which trigger the activation of T-cells (LeibundGut-Landmann et al., 2007, Leibundgut-Landmann et al., 2008).

Figure 3. The basic structures of linear (1,3) (A) and branched (1,6) (B) β-glucans

2.2.2. Recognition of fungal particles by innate immunity

The first innate defence mechanisms recognizing the entry of fungi and initiating the clearance of the pathogen are constitutively existing molecules: defensins, collectins, surfactant and the proteins of the complement system. Furthermore, conserved cell wall structures and nucleic acids of the fungi trigger activation of phagocytes via PRRs (Table 3). The direct killing and clearance of the fungi by phagocytosis is one of the main defense mechanisms in antifungal immunity and it is mediated by macrophages, neutrophils and monocytes. In
addition, phagocytes produce oxidative (respiratory burst) or nonoxidative substances (antimicrobial peptides) to kill the extracellular and intracellular fungal pathogens (Brown, 2011). In most cases, in order to achieve the full protection against the fungal pathogens, the activation of adaptive immunity is needed. This is mainly initiated and directed by dendritic cells, which after uptake of the fungi, become mature and promote the differentiation of naïve T-cells into different subtypes of T-helper (Th) cells. Th17-cell activation has been shown to play an important role in the defense of fungal infections, induced mainly through downstream signaling of C-type lectin receptors (CLRs) in phagocytes (Vautier et al., 2010). Defects or abnormalities in the Th17 response have been related to fungal re-infections, chronic mycosis and autoimmune diseases (Harrington et al., 2005, Puel et al., 2010, Vautier et al., 2010, Borghi et al., 2014). Several cytokines are involved in promoting the Th17 differentiation including IL-1β, IL-6, IL-21, IL-23 and TGF-β. In turn, activated Th17-cells produce IL-17 cytokines, which increase the antifungal activity of neutrophils and secretion of antimicrobial peptides from epithelial cells (Vautier et al., 2012, Borghi et al., 2014). Th1 cells also play a key role in the antifungal defense, they are induced by IL-12 cytokine and produce IFN-γ and TNF, both of which activate phagocytes (Vautier et al., 2012, Borghi et al., 2014). Th2 cell activation, induced in response to IL-4 and IL-13, is associated with the suppression of protective Th1 cell responses and the promotion of the alternative activation of macrophages that favour fungal infections and allow the development of fungi-associated allergic responses (Muller et al., 2007, Borghi et al., 2014).

Table 3. The main groups of pattern-recognition receptors (PRRs) involved in the recognition of fungal molecules and the response of innate immunity during a fungal infection

<table>
<thead>
<tr>
<th>PRRs</th>
<th>Receptor localization</th>
<th>Selected fungal ligands</th>
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<tbody>
<tr>
<td>C-type lectin receptors, CLRs</td>
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<tr>
<td>DC-SIGN</td>
<td>Plasma membrane</td>
<td>N-linked mannans</td>
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<td>Dectin-1</td>
<td>Plasma membrane</td>
<td>β-glucan</td>
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<td>Dectin-2</td>
<td>Plasma membrane</td>
<td>α-mannan</td>
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<td>Dectin-3</td>
<td>Plasma membrane</td>
<td>α-mannan</td>
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<td>Mannose receptor</td>
<td>Plasma membrane</td>
<td>N-linked mannans, α-glucan, chitin</td>
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<td>Galectins</td>
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<td>Galectin-3</td>
<td>Extracellular</td>
<td>β-mannosides</td>
</tr>
<tr>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptors, NLRs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NLRP3</td>
<td>Cytosol</td>
<td>β-glucan</td>
</tr>
<tr>
<td>Scavenger receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td>Plasma membrane</td>
<td>β-glucan</td>
</tr>
</tbody>
</table>
Multiple pattern-recognition receptors of innate immunity are involved in the recognition of fungal PAMPs during a fungal infection or exposure to fungal particles. Activation of these PRRs induces pathogen eliminating responses in the cells including phagocytosis, respiratory burst, and cytokine release. The best characterized PRRs involved in fungal recognition are Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs).

Three different TLRs have been claimed to be the main TLRs involved in the recognition of most fungal pathogens: TLR2, which forms a heterodimer with TLR1 or TLR6, TLR4 and TLR9 (van de Veerdonk et al., 2008). However, there are contradictory reports concerning the individual TLR receptors and their role in the antifungal response against different fungal pathogens (Calich et al., 2008). It has been shown that mice lacking the signaling and adaptor protein MyD88, which is shared by most TLRs, are susceptible to *C. albicans* and *A. fumigatus* infections and MyD88 signaling is essential in DCs for promoting the Th1 response aimed at eliminating fungi (Bellocchio et al., 2004). TLR polymorphisms have also linked to increased sensitivity to fungal infections in humans (Brown, 2011). Despite these associations, no increased susceptibility to fungal diseases has been found in patients with defects in MyD88 (von Bernuth et al., 2008, Picard et al., 2010), indicating that TLRs are not the main PRRs mediating the antifungal response in humans.

CLRs are considered to be the central PRRs for fungal recognition (Hardison and Brown, 2012, Plato et al., 2015). CLRs are transmembrane receptors, which recognize a wide range of ligands and have at least one C-type lectin-like domain (CTLD). CLRs are expressed mainly on myeloid cells such as macrophages and DCs. Several CLRs such as dectin-1, dectin-2, mannose receptor, DC-SIGN (DC-specific ICAM3-grabbing non-integrin) and Mincle can induce intracellular signaling after they have recognized the presence of fungi (Hardison and Brown, 2012). Dectin-1 was the first CLR to be characterized (Figure 4). It is the major...
CLR for fungal β-glucan and its signaling has been widely studied (Brown and Gordon, 2001, Drummond and Brown, 2011). Clustering of dectin-1 is activated by aggregated or particulate β-glucan, which leads to a phagocytic synapse and activation of signaling molecules downstream of the receptor.

![Dectin-1 Structure](image)

Figure 4. Structure of dectin-1. The major receptor for β-glucan is dectin-1, which activates intracellular signaling pathways via a cytoplasmic ITAM-like motif. This signaling leads to a number of innate immune responses involving the recruitment of Syk and Src kinases, the activation of transcription factor NF-kB through CARD9, as well signaling which can activate pathways of mitogen-activated protein kinases (MAPKs) and nuclear factor of activated T-cells (NFAT). In addition, dectin-1 can signal independently from Syk through Raf-1 kinase (Goodridge et al., 2011, Kerrigan and Brown, 2011).

Dectin-3 recognizes α-mannans; when it forms a heterodimeric receptor with dectin-2, this complex has even higher sensitivity for fungi, making it possible to mount a more potent anti-fungal response (Zhu et al., 2013). Fungal binding to these receptors leads most often to phagocytosis of the fungi and the induction of antifungal effector mechanisms such as the production of cytokines. In addition, these receptors promote the development of an adaptive immune response, particularly towards Th1 and Th17 responses (Hardison and Brown, 2012).
The primary signal transduction molecule for CLRs is Syk (spleen tyrosine kinase). Some of the receptors (Dectin-2, Mincle) couple to Syk via the Fc receptor common \(\gamma\)-chain. Syk signaling through PKC\(\delta\)–CARD9–Bcl-10–MALT1 leads to activation of transcription factor NF-\(\kappa\)B, which facilitates the gene transcription and production of inflammatory mediators. Syk signaling may also lead to activation of the transcription factor NFAT, which regulates the transcription factors of the early growth response genes, as well as those of the inflammatory mediators (Goodridge et al., 2007). There is also a Syk-independent signaling route through Raf-1 (v-raf-leukemia viral oncogene homolog 1), which also modulates the function of NF-\(\kappa\)B (Hardison and Brown, 2012). The importance of CLRs and their downstream signaling components in fungal immunity has been highlighted in studies with knockout mice, in which the deficiency of CLR signaling led to defective immunity towards fungal pathogens (Saijo et al., 2007, Saijo et al., 2010, Strasser et al., 2012). In humans, a deficiency in dectin-1 and CARD9 was shown to lead to susceptibility to certain fungal infections, especially mucocutaneous candidiasis (Ferwerda et al., 2009, Glocker et al., 2009).

The exact mechanisms through which fungal ligands activate and interact with NLRs, especially NLRP3, are still mainly unknown. Mutations in the genes of the NLRP3 inflammasome have been linked to elevated susceptibility to vaginal candidiasis (Lev-Sagie et al., 2009). The activation of the NLRP3 inflammasome is a crucial step for the secretion of biologically effective IL-1\(\beta\). Furthermore, polymorphism in IL-1\(\beta\) gene has been linked with an increased risk for fungal disease, e.g. invasive pulmonary aspergillosis (Sainz et al., 2008).

The recognition of fungi is orchestrated via multiple PRRs, and many of these receptors can collaborate with each other to achieve the optimal antifungal response (Hardison and Brown, 2012). One of the well-described interactions during the fungal infection is dectin-1 collaborating with MyD88-coupled TLRs leading to a synergistic increase in the secretion of TNF, IL-10 and IL-23, while the release other cytokines such as IL-12, is repressed (Dennehy et al., 2009).

### 2.3. Health effects related to exposure of inhaled non-infective microbial particles

Microbes are ubiquitous, countless numbers of bacteria and fungi are found in soil and water, and even closer, within our own bodies, where they belong to our commensal microbiota. Thus, we are continually exposed to a low level of microbial activity and their metabolites, a condition that is usually harmless. In certain occupations and surroundings, the exposure to microbial metabolites can be significantly elevated or the microbes may be different from normal which may lead to adverse health effects.
In this chapter, the focus will be placed on the health effects and diseases associated to inhalation of non-infectious microbial particles. Agricultural workers are routinely exposed to large numbers of environmental factors such as organic dust containing microbial cell wall components (bacterial endotoxin and peptidoglycan, fungal 1,3-β-glucan), which could lead to adverse respiratory health effects that predominately are non-IgE mediated (Poole and Romberger, 2012).

Most of the non-infective microbial particles which are inhaled become entrapped in the upper respiratory tract. They do not reach further than the bronchi because they are washed away by the mucosa layer with the help of cilia cells. The smaller particles (such as fungal hyphal fragments, microbial structure particles) less than 10 μm in diameter are tiny enough to reach the terminal bronchioles and alveoli (Geiser et al., 2000, Heyder, 2004). The airway macrophages recognize dust particles as foreign due to the presence of the PAMP-structures in the particles and they start to eradicate them. Depending on the other signals from the milieu, they can remove the dust particles as part of the normal maintenance work and sustain the homeostasis or they can respond by releasing cytokines that promote inflammation, activate epithelial cells and elicit neutrophil recruitment (Poole and Romberger, 2012).

It has been speculated that the inflammatory response elicited by the initial exposure to organic dusts becomes partially, but not completely, suppressed, leading to the development of the so-called chronic inflammatory adaptation response (Poole and Romberger, 2012). This may explain at least to some extent, the symptoms associated with diseases with exposure to organic dusts.

The inflammatory potential of non-infectious microbial particles is based on their ability to stimulate the innate immunity through PAMP-structures. Pattern recognition receptor signaling pathways involving TLRs (TLR4 and TLR2), dectin-1 and nucleotide oligomerization are partially responsible for mediating the inflammatory consequences attributable to organic dust environments (Charavaryamath et al., 2008, Poole et al., 2011). Genetic polymorphisms in pattern recognition receptors may be partially responsible for accounting for the variability in disease manifestations among exposed individuals (Poole and Romberger, 2012).

From the pathogenic point of view, these illnesses have features on one hand resembling autoimmune diseases (exaggerated immune reaction to harmless substance) and on the other hand, reminiscent of infectious diseases (PAMP recognition and inflammatory response) (Wolff, 2011). Although the microbial components play the major role in eliciting the inflammatory response, on their own they do not completely explain the airway inflammatory consequences observed after exposure to organic dust. Thus, further research will be necessary to elucidate the offending agents (endogenic, exogenic) and the participating signaling pathways.
Three illnesses associated with the inhalation of non-infectious microbial particles will be discussed in more detail. *Organic toxic syndrome* serves as an example of the acute reaction following a high level exposure to an organic dust. In *hypersensitivity pneumonitis*, the exposure levels are lower and a long-term exposure is usually required before the onset or progression of the disease. *Damp building-related illness* represents an illness where the individual is exposed, usually over the long-term, to mold and bacterial metabolites and other substances related to the conditions of damp buildings. There is now a consensus that there is an association between building dampness and the health effects (WHO, 2009), however, no specific causal agent or pathogenetic mechanisms of adverse health effects in damp buildings have been identified conclusively (WHO, 2009).

### 2.3.1. Organic dust toxic syndrome

Organic dust toxic syndrome (ODTS) is an acute febrile respiratory illness encountered after exposure to high levels of inhaled organic dusts or aerosols, principally bacterial endotoxins or mold (Hendrick et al., 2002, Seifert et al., 2003). Typically, the exposure occurs in an agricultural context, although other occupations involving the handling of dusty organic material poses some risk. The syndrome usually begins within hours of the exposure to high concentrations of organic dust with the appearance of influenza-like symptoms such as fever and myalgias, usually accompanied by nausea, headache, chest tightness and cough. The exposure concentrations of fungal spores have been shown to be notably higher in ODTS than those associated with hypersensitivity pneumonitis (Malmberg et al., 1993).

The symptoms of ODST are self-limiting, they rarely persist for more than a few days and there are no long-term consequences even with recurring episodes (Hendrick et al., 2002). Re-exposure to the antigen will often decrease the symptoms. ODTS does not depend on immunological sensitization, so it can occur during or immediately after the first exposure (Hendrick et al., 2002). Serum IgG antibodies to the suspected etiological agent are not detected (Seifert et al., 2003). The exact mechanisms of toxicity are not known, but microbial PAMPs: bacterial endotoxins, fungal spores, or mycotoxins are believed to play a role in triggering this disease.

The incidence of ODTS is probably declining because of modern working techniques in farming (mechanized work, workers protected with dust masks and respirators).
2.3.2. Hypersensitivity pneumonitis

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is an inflammatory disease of the lung alveoli resulting from repeated exposure to a wide range of airborne substances; thermophilic bacteria, animal proteins, fungi and even some inorganic materials such as chemicals. Fungal exposure has been implicated in HP in a variety of occupations such as farming, where haymaking may expose workers to different species of molds (e.g. *Absidia corymbifera* and *Wallemia sebi*) but it may also be responsible for a disease acquired in domestic environments such as summer-type HP in Japan (Selman et al., 2010). Reports from IOM and WHO have concluded that there is clinical evidence to associate the onset of hypersensitivity pneumonitis with mold and dampness-associated microbiological agents (IOM, 2004, WHO, 2009). Only a small proportion of the exposed individuals (1-15%) ever develop HP (Patel et al., 2001, McSharry et al., 2002). The two-hit hypothesis has been postulated to explain this phenomenon: preexisting genetic susceptibility or promoting environmental factors (the first hit) associated with repetitive antigen exposure (the second hit) lead to the development of HP (Selman et al., 2012).

Clinically, HP is typically categorized as displaying acute, subacute or chronic forms. HP is a complex syndrome, and its clinical forms overlap frequently with each other and also with other lung diseases, which make its diagnosis challenging (Jacobs et al., 2005, Girard et al., 2009, Selman et al., 2012). In general, acute HP is characterized by the appearance of respiratory infection-like symptoms such as chills and body aches occurring a few hours after antigen exposure. The symptoms gradually decline within some hours or days but often recur with re-exposure to the antigen. Furthermore, the acute and also the subacute form of HP can be associated with wheezing and bronchial hyperresponsiveness, however, the chest radiograph is usually normal. Subacute HP may result from repeated low-level exposure to inhaled antigens and its features may overlap with acute and chronic stages. It is a progressive disease, with coughing and shortness of breath becoming persistent; its classical histopathological features comprise chronic bronchiolocentric inflammation and poorly formed non-necrotising granulomas. Chronic HP occurs with long-term, low level of antigen exposure, and may occur without having preceding acute/subacute episodes of HP. The clinical presentation includes progressive dyspnea, cough, fatigue and weight loss and often fibrosis develops in its chronic stage (Hendrick et al., 2002, Malo et al., 2013).

The immunopathogenesis of HP has remained poorly understood. The patients have often IgG against antigens present in the microbes to which they have been exposed, however, these antibodies can also be detected in exposed individuals without HP (Hendrick et al., 2002). Immune complexes with this IgG are thought to be a central part of the pathogenesis in the acute phase of HP (Selman et al., 2012). However, there are some reports which dispute this hypothesis by claiming...
that there are too little clinical data to support it or that antibody complexes alone are not sufficient to evoke HP (Patel et al., 2001, Woda, 2008).

Lymphocytosis, often accompanied by an increase in macrophages, is seen in HP patients (Woda, 2008). Following the early influx of CD4+ cells, the proportion of CD8+ cells increases (Patel et al., 2001). The current view suggests that HP is caused by a cytotoxic delayed hypersensitivity involving CD8+ cytotoxic lymphocytes (Patel et al., 2001, Woda, 2008). Some experimental studies have shown that mice whose immune response has been skewed toward a Th1 response over a Th2 response, are more likely to develop HP (Butler et al., 2002, Irifune et al., 2003). There are recent studies confirming that IL-17-and IL-22-secreting Th17 cells are also involved in the immunopathogenesis of HP (Joshi et al., 2009, Simonian et al., 2009a). The Th17-polarized immune response seems to be associated with disease severity such as the development of lung fibrosis (Simonian et al., 2009a, Simonian et al., 2009b).

2.3.3. Damp building-related illness

The concept of “sick building syndrome” (SBS) was first described in the 1980s to explain the health problems related to indoor air quality in office buildings (WHO, 1983). One major, but not the only cause, for SBS is the presence of dampness in the buildings. Damp building-related illness (DBRI) represents illness associated with water damage in dwellings or workplaces. Hypersensitivity pneumonitis is included in these illnesses if it is caused by damp buildings, although this happens rather rarely (WHO, 2009). The typical DBRI is associated with a prolonged exposure to damp buildings, usually only a portion of the exposed population develops symptoms (Straus et al., 2003, Akpinar-Elci et al., 2008). There are currently no well-defined diagnostic criteria for the great majority of DBRIs. The symptoms tend to be quite diverse but often include fever, myalgias, headaches and respiratory symptoms like cough. It was recently demonstrated that exposure to water damage in buildings can be associated with an increase in the numbers of lymphocytes in BAL, although there was no change in the CD4/CD8 ratio, in contrast to the increase of CD8 cells usually seen in HP (Wolff et al., 2009).

There is abundant evidence that building-related exposure to mold and dampness results in an increased incidence of respiratory diseases (WHO, 2009). Continuation of the exposure to damp building will usually worsen the symptoms and result in persistent respiratory symptoms, especially of asthma. A number of studies have observed an increase in non-IgE (non-atopic) mediated asthma associated with mold and dampness (Cox-Ganser et al., 2009, Karvala et al., 2010). The symptoms usually diminish when the subject is away from the building and conditions associated with damp buildings are avoided. However, some patients develop increased sensitivity to many environmental factors with symptoms that persist even though the primary source of the exposure has been
eliminated from their environment (Redlich et al., 1997, Edvardsson et al., 2008, Karvala et al., 2013, Karvala et al., 2014).

There is a consensus that there is an association between dampness in buildings with health effects (Bornehag et al., 2004, Fisk et al., 2007, WHO, 2009). An increased moisture level may promote the growth of microbes such as fungi and bacteria on indoor materials and on structures within the building. Depending on the microbes and their activity, they are capable of emitting spores, fragments, microbial volatile organic compounds (mVOCs) and mycotoxins into the indoor air, which may be responsible for the health effects seen in individuals exposed to dampness in buildings (WHO, 2009). There are studies which suggest that the exposure to one structural component in the fungal cell wall, i.e. 1,3-D-glucan, may be responsible for the airway inflammation and symptoms, but the results are mixed, and specific symptoms and potential underlying mechanisms associated with exposure have still not been identified (Douwes, 2005). Moisture may also trigger the degradation of building materials and contribute to the release of non-microbial chemicals into the indoor air (IOM, 2004). However, there is no specific quantity or quality of microbial particles or other substances related to condition of damp building which can be stated to designate an exposure state capable of causing significant health effects. In other words, no specific causal agent for the adverse health effects associated with damp buildings has yet been identified conclusively (WHO, 2009, Mendell et al., 2011).

Mechanisms underlying the health effects associated with dampness and mold are largely unknown and several factors are thought to be involved (WHO, 2009). Inflammatory, non-allergic responses have been considered to play a role in the development of DBRI (Wolff, 2011). This explanation is biologically plausible as inflammation–related diseases such as asthma are associated with exposure to damp buildings (WHO, 2009). However, also the development of an allergic response against indoor microbial agents has been suggested to be one of the disease causing mechanisms (WHO, 2009).
3. AIMS OF THE STUDY

Inhaled fungal and other microbial components can activate the immune response in the lung and cause symptoms in the airways. The aim of this study was to characterize the inflammatory mechanisms and proteomic changes associated with the innate immune response triggered by the central cell wall component of fungi, β-glucan, in human macrophages. In order to obtain direct information of the illnesses associated with the exposure of inhaled fungal particles, the proteomic changes in the bronchoalveolar lavage obtained from patients with these illnesses were analysed.

The specific aims of the study were:

1. to characterize the β-glucan-triggered pro-inflammatory response by analysing the production and secretion of IL-1 family cytokines in human primary macrophages (I, IV)

2. to obtain a global view of the innate immune response elicited by β-glucans; the protein secretion pattern and the associated intracellular signaling pathways were characterized in human primary macrophages after β-glucan stimulation (II).

3. to reveal the proteomic changes associated with the immune defense in the lungs after exposure to inhaled fungal particles we characterized the proteomic changes occurring in bronchoalveolar lavage fluid obtained from patients with illnesses associated with exposure to inhaled fungal and other microbial particles (III)
4. SUMMARY OF THE MATERIAL AND METHODS

The material and methods used in the study are described here briefly. The more detailed descriptions of the protocols can be found in the original publications, which are referred in the text with the Roman numerals (I-IV).

4.1. Sample material

4.1.1. Human macrophages (I, II, IV)

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service). PBMCs were isolated from leukocyte-rich buffy coats with the low-speed density gradient centrifugation as previously described (Pirhonen et al., 1999). PBMCs were then plated and non-adherent cells were washed away and adherent monocytes were allowed to differentiate into macrophages six days in macrophage serum-free medium (Macrophage-SFM, Life Technologies) supplemented with GM-CSF (10 ng/ml, Biosource International) and antibiotics. After this six days of cell culturing, macrophages were used in the experiments (I, II, IV). Culturing M-CSF-generated macrophages, GM-CSF was replaced with 50 ng/ml rh M-CSF (ImmunoTools) (IV).

4.1.2. Mouse bone marrow-derived dendritic cells (II)

Mouse bone marrow–derived dendritic cells (BMDCs) were derived from bone marrow cells isolated from the femurs of wildtype C57BL/6 (NOVA-SCB AB) and dectin-1 knockout mice (Saijo et al., 2007). Briefly, bone marrow was flushed out from mice femur, the red blood cells were lysed from the bone marrow cell suspension and the remaining bone marrow cells were washed before culturing in RPMI 1640 media (BioWest) supplemented with 30 ng/ml GM-CSF (Biosource International), 10% FBS, 1% L-glutamine, 1% HEPES, 0.05 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids and antibiotics (Gibco). In general, after nine days of culturing the BMDCs were used in experiments. The animal study was approved by the Health Services of State Provincial Office of Southern Finland (II).
4.1.3. Mouse bronchoalveolar lavage fluid cells (IV)

Female mice, C57BL/6 and dectin-1 knockout mice (Saijo et al., 2007) were used at 8 to 10 weeks of age. C57BL/6 or dectin-1 -/- mice were intranasally exposed to curdlan (100 µg in 50 µl of PBS) 6 hrs before sacrifice and sample collection. The controls in all studies received PBS only. The lungs were lavaged with sterile PBS (800 µl for 13 s) via a tracheal tube. The bronchoalveolar lavage (BAL) sample was cytocentrifuged on a slide, and the cells were stained using May Grünwald-Giemsa (MGG) and counted under light microscopy (Leica DM 4000B). The remaining BAL cell suspension was centrifuged, supernatant was removed and stored at -70°C for cytokine analysis, whereas the cells were quick-frozen and kept at – 70°C until RNA isolation. The animal study was approved by the National Animal Experiment Board and the Regional State Administrative Agency for Southern Finland (IV).

4.1.4. Patient samples: bronchoalveolar lavage fluid and plasma (III)

Bronchoalveolar lavage fluid samples were collected from patients having symptoms compatible with damp building-related illness (DBRI) or symptoms related to agricultural exposure (AME) to non-infectious microbial particles (NIMPs). The criterion for agricultural NIMP exposure was the handling of an organic moldy material. The samples were collected from patients being examined in the Finnish Institute of Occupational Health (FIOH). BAL fluids from patients with hypersensitivity pneumonitis (HP) served as the reference material for an established lung disease associated with NIMPs, and sarcoidosis (SARC) served as the reference material for a lung disease with no direct association with NIMPs. The reference BAL samples originated from an earlier study from the Meltola Hospital (Wolff et al., 2003). The controls comprised samples from healthy individuals from the personnel of the Meltola Hospital and FIOH. All tested plasma samples (available from control, hypersensitivity pneumonitis and sarcoidosis groups) were from the Meltola Hospital. Preparation of BAL samples was performed as described in (Taskinen et al., 1994). Patient studies were approved by The Coordinating Ethics Committee at the Hospital District of Helsinki and Uusimaa (III).
4.2. Cell experiments in vitro

The main cell model used in vitro studies was a human GM-CSF-differentiated monocyte-derived macrophage (I, II, IV). Other utilized cells models were M-CSF-differentiated monocyte-derived human macrophages (IV), and mouse bone marrow-derived dendritic cells (II).

4.2.1. Stimulants (I,II,IV)

The main stimulant in the experiments has been (1,3)-β-glucan, the polysaccharide found ubiquitously in the fungal cell wall. Four different (1,3)-β-glucan products were used: curdlan from *Alcaligenes faecalis* (I,II,IV), glucan from baker’s yeast (*Saccharomyces cerevisiae*) (I,II), paramylon from *Euglena gracilis* (I) and zymosan from *Saccharomyces cerevisiae* (I). In general, in the secretome analyses, the cells were stimulated for 18 hours and the cells subjected to transcriptomic analyses for 6 hours. Stimulation times are described in more detail in the original publications (I,II,IV). All β-glucans were purchased from Sigma-Aldrich.

LPS is an endotoxin, which is produced by a variety of gram-negative bacteria. In this study, LPS (*Escherichia coli* O111:B4, Sigma-Aldrich) was used as a control of well-known microbial and inflammatory stimulus unrelated to the fungi (I,II). The stimulation times for LPS were the same as for β-glucans, which have been described above.

Macrophages were stimulated with influenza A-virus (A/Udorn/72(H3N2) or transfected with polyI:C (a cytosolic viral dsRNA analog from Sigma-Aldrich) 18 hours to obtain positive controls for cell death assays (II). Influenza A-virus was kindly provided by Professor Ilkka Julkunen (National Institute for Health and Welfare (THL), Helsinki, Finland). Influenza A-virus was cultured in embryonated hen eggs and stored at −70°C. The hemagglutination titer was 256, as measured by standard methods. In infection experiments, virus dose of 2.56 hemagglutination U/ml was used. PolyI:C was used at 10 μg/ml and transfection was performed according to the manufacturer’s instructions (Sigma-Aldrich).

Table 4. The main stimulants used in the experiments

<table>
<thead>
<tr>
<th>Main stimulants</th>
<th>Concentration</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan from baker’s yeast</td>
<td>10, 100 μg/ml</td>
<td>I, II</td>
</tr>
<tr>
<td>Curdlan</td>
<td>10, 30, 100 μg/ml</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Paramylon</td>
<td>10, 100 μg/ml</td>
<td>I</td>
</tr>
<tr>
<td>Zymosan</td>
<td>10, 100 μg/ml</td>
<td>I</td>
</tr>
<tr>
<td>LPS</td>
<td>100 ng/ml, 1 μg/ml</td>
<td>I, II</td>
</tr>
</tbody>
</table>
4.2.2. Inhibitors (I,II,IV)

The inhibitors used in the in vitro experiments are described in table 5. Generally, the inhibitors were added for the cells one or half an hour before the stimulus except for Brefeldin A, where the inhibitor was given one hour after stimulus. More detailed information about the inhibition times and concentrations is described in the original publications.

Table 5. The inhibitors used in the cell studies

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>(Description)</th>
<th>Concentration</th>
<th>Manufacturer</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-METHYLADENINE, 3-MA</td>
<td>(Inhibition of autophagosome formation)</td>
<td>10 mM</td>
<td>Sigma-Aldrich</td>
<td>II</td>
</tr>
<tr>
<td>BREFELDIN A</td>
<td>(Inhibition of ER to Golgi trafficking)</td>
<td>100 ng/ml</td>
<td>Sigma-Aldrich</td>
<td>II</td>
</tr>
<tr>
<td>BUTYLATED HYDROXYANISOLE, BHA</td>
<td>(ROS inhibitor)</td>
<td>100 μM</td>
<td>Sigma-Aldrich</td>
<td>I</td>
</tr>
<tr>
<td>CA-074-Me</td>
<td>(Cathepsin B inhibitor)</td>
<td>10 μg/ml or 25 μM</td>
<td>Calbiochem/Millipore</td>
<td>I, IV</td>
</tr>
<tr>
<td>CASPASE-1 INHIBITOR VI, Z-YVAD-FMK</td>
<td>(Caspase-1 inhibitor)</td>
<td>50 μM</td>
<td>Santa Cruz Biotechnology</td>
<td>I</td>
</tr>
<tr>
<td>CASPASE-1 INHIBITOR VI, Z-YVAD-FMK</td>
<td>(Caspase-1 inhibitor)</td>
<td>25 μM</td>
<td>Calbiochem/Millipore</td>
<td>II, IV</td>
</tr>
<tr>
<td>CYTOCHALASIN D</td>
<td>(Inhibitor of phagocytosis)</td>
<td>1 μg/ml</td>
<td>Sigma-Aldrich</td>
<td>I</td>
</tr>
<tr>
<td>DECTIN-1 Ab</td>
<td>(Blocking the Dectin-1-receptor signaling)</td>
<td>9 μg/ml</td>
<td>R&amp;D Systems</td>
<td>I</td>
</tr>
<tr>
<td>MCC950</td>
<td>(Inhibitor of NLRP3 inflammasome)</td>
<td>1 μM</td>
<td>Avistron</td>
<td>IV</td>
</tr>
<tr>
<td>POTASSIUM CHLORIDE, KCl</td>
<td>(Used for blocking of potassium efflux)</td>
<td>added 1 mM</td>
<td>Sigma-Aldrich</td>
<td>I</td>
</tr>
<tr>
<td>PP2</td>
<td>(Src tyrosine kinase inhibitor)</td>
<td>5 or 10 μM</td>
<td>Sigma-Aldrich</td>
<td>I, II</td>
</tr>
<tr>
<td>SRC I INHIBITOR</td>
<td>(Src tyrosine kinase inhibitor)</td>
<td>20 μM</td>
<td>Sigma-Aldrich</td>
<td>II</td>
</tr>
<tr>
<td>SYK II INHIBITOR</td>
<td>(Syk tyrosine kinase inhibitor)</td>
<td>1, 5, 10 μM</td>
<td>Calbiochem/Millipore</td>
<td>I, II, IV</td>
</tr>
</tbody>
</table>

4.2.3. Small-interfering RNAs (I,II)

Macrophages were transfected twice with non-targeting control small-interfering RNA (SiRNA) and with two different Beclin-1 (Hs_BECN1_1, Hs_BECN-1_3) or NLRP3 (Hs_CIAS1_6, Hs_CIAS1_9) siRNAs before the β-glucan stimulation. HiPerfect Transfection Reagent was used in the SiRNA experiments according to the manufacturer’s instructions. All the reagents for siRNA experiments were purchased from Sigma-Aldrich. More details of the SiRNA procedure is described in the original publications (I, II).
4.3. Proteomic methods

4.3.1. Isobaric tag for relative and absolute quantification (iTRAQ) -labeling and mass spectrometry (II)

4plex iTRAQ (AB Sciex) labelling was used for relative quantitation of secreted proteins in human macrophages after β-glucan stimulations (curdlan and baker’s yeast) or after lipopolysaccharide stimulation (LPS), the latter served as a control inflammatory stimulus unrelated to fungi. Before stimulation, the cells were washed and the culture media was changed to serum-free RPMI 1640-medium. After stimulation, the same amount of cell culture media was collected from every study group and concentrated as described in 4.3.3. Next, the proteins from the concentrated media fraction were precipitated with 2-D Clean-Up Kit (GE Healthcare), followed by protein alkylation, trypsin digestion, and iTRAQ labeling of the resulting peptides according to the manufacturer’s instructions (Applied Biosystems). After labeling, the samples were pooled and the peptides were prefractionated with strong cation exchange chromatography (SCX) using an Ettan HPLC system (Amersham Biosciences) connected to a Poly-SULFOETHYL A - column. Each SCX-fraction containing the labeled peptides was analyzed twice with nano-LC-ESI-MS/MS (nano-liquid chromatography-electrospray ionization-tandem mass spectrometry) using Ultimate 3000 nano-liquid chromatograph (Dionex) and QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems) with nano-ESI ionization as described previously (Lietzen et al., 2011). MS data were acquired automatically using Analyst QS 2.0 software (II).

Data analysis for iTRAQ

ProteinPilot 2.0.1 interface (AB Sciex) with Paragon search algorithm (Shilov et al., 2007) was used for protein identification and relative quantitation. Database searching was done against UniProt human database (version 2008-01-28 with 20330 human sequences) and ‘decoy’ database (the reverse amino acid sequence for false discovery rate estimation). The search criteria were: cysteine alkylation with MMTS (methyl methanethiol sulfonate), trypsin digestion, biological modifications allowed, thorough search, and detected protein threshold of 95% confidence (Unused ProtScore > 1.3). The MS/MS results from two biological replicates were combined to obtain one list of differentially secreted proteins from β-glucan (curdlan and GBY) and LPS stimulated macrophages. For the proteins identified (p-values under 0.05) in both replicate experiments, the relative quantitation was averaged if the fold change values between the replicates were under 2.0 or if both values were over 4.0. In addition, the following criteria were used for averaging the fold change values between the replicates: the fold change...
value in one replicate was under 4.0, the fold change difference was under 3.0 and both quantifications had p-values under 0.05. The protein quantitation result from an individual experiment was included into the list if the quantitation had a p-value less than 0.05.

The identified proteins were classified based on their Gene Ontology annotations using GeneTrail (http://genetrail.bioinf.uni-sb.de/) (Backes et al., 2007) and GoMiner (http://discover.nci.nih.gov/gominer/index.jsp). SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to determine the proteins secreted via classical pathways, and ExoCarta version 3.2 database (Mathivanan and Simpson, 2009) (http://www.exocarta.org/) was used to determine the proteins released via exosomal vesicles.

4.3.2. 2D-DIGE (two-dimensional difference gel electrophoresis) and DeCyder analysis

Approximately 10 ml of each BAL sample was concentrated to 100 μl by ultrafiltration (Amicon Ultra-15 tubes with 5000 MWCO, Millipore). The sample was then depleted of albumin and immunoglobulin G with ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma Aldrich) to facilitate higher sample loading, and to improve detection of low-abundance proteins. BALF proteins were precipitated with 2-D Clean-Up Kit (GE Healthcare) and dissolved in DIGE labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris-HCl, pH 8.8). Then, 10 μl from each sample was taken for the internal standard and 40 μg of BALF proteins were labelled using 200 pmol Cy3 or Cy5 dyes (CyDye DIGE Fluor minimal dyes; GE Healthcare) according to the Ettan 2-D DIGE instructions. The Cy2 dye was used as an internal standard. An internal standard was used to align spots during the analysis and to decrease technical variation. CyDye labelled protein samples were separated by isoelectric focusing (IEF) using IPG strips (13 cm, pH 3–10 NL, GE Healthcare). IEF was performed using Ettan IPGphor II (GE Healthcare) and the second dimension separation of proteins according to their size with polyacrylamide gel electrophoresis (SDS-PAGE). Gels were scanned and the fluorescence of CyDyes was measured using an Ettan DIGE Imager (GE Healthcare). After scanning, the gels were silver stained for spot excision. The images of scanned gels were analyzed for differences in protein expression by means of internal Cy2 labelled standard using DeCyder 2D 7.0 software (GE Healthcare). Gel spots with at least a 1.5-fold spot volume ratio change and a Student’s t-test p-value below 0.05 were picked for identification.

**Protein identification and bioinformatics**

Significantly up- or down-regulated protein spots were in-gel digested and the resulting peptides were extracted as previously described (Shevchenko et al., 2006,
Rostila et al., 2012). The dried extracts were dissolved in 2% formic acid. Each peptide mixture was analyzed with an automated nanoflow capillary LC–MS/MS using CapLC system (Waters) coupled to an electrospray ionization quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters). The obtained mass fragment spectra were analyzed with in-house Mascot v.2.1 (Matrix Science Ltd.) and searched against human or mammalian entries in the NCBI nr database. One missed cleavage was allowed, and the searches were performed with fixed carbamidomethylation of cysteines, and variable oxidation of methionine, histidine, and tryptophan residues. A minimum number of two matched peptides or a Mascot score higher than 70, was considered significant. Multivariate analysis of protein expression was performed with DeCyder Extended Data Analysis software (Version 7.0, GE Healthcare). The Gene Ontology classification of the identified proteins based on the DeCyder 2D 7.0 software data. Protein enrichment analysis was performed with a functional annotation tool: DAVID Bioinformatics Resources 6.7. (http://david.abcc.ncifcrf.gov/) (III).

4.3.3. Enzyme-linked immunosorbent assay (ELISA) and Luminex (I-IV)

The levels of secreted human or mouse cytokines were determined after cell stimulation from the cell supernatants with ELISA analysis according to the manufacturer’s instructions: hIL-1β (Diaclone) (I,II), mIL-1β (eBioscience) (II) and hIL-18 (Bender MedSystems) (I). The total IgG was measured from human BAL samples with ELISA assay kit from eBioscience (III). In addition, Luminex Bio-Plex Pro immunoassay from Bio-Rad Laboratories was used for measuring the secretion of human cytokines and chemokines from the cell supernatants: IL-1α (IV), IL-1β (IV), IL-18 (IV), CCL2 (II), CCL5 (II) and TNF (II). The Luminex assay was performed according to the manufacturer’s instructions using Bio-Plex 200 system hardware and version 4.1.1 of Bio-Plex 200 software.

4.3.4. Immunoblotting (I-IV)

Samples from macrophage experiments (I,II,IV)

To analyze the secreted proteins, macrophages were stimulated in RPMI-growth medium (BioWest) supplemented with L-glutamine, antibiotics and 10 mM HEPES. After stimulation, the cell culture media were collected and concentrated with centrifugal units with 10 kDa nominal molecular weight cut-off (purchased from Millipore) to obtain the enriched fraction of secreted proteins. This enriched protein fraction was directly used for immunoblotting.

To analyze the intracellular proteins, the macrophage cells after stimulation were lysed, and homogenized with ultrasound sonicator. The total protein concentration of the lysate was determined with Bio-Rad Dc Protein Assay (Bio-
Rad Laboratories) and 10 μg or 20 μg of protein was used for immunoblotting (I,II,IV).

To confirm equal loading and transfer of the proteins, membranes were stripped and stained with ready-to use SYPRO Ruby Protein Blot Stain (Sigma-Aldrich). The major protein band detected and basally expressed was utilized as a loading control. Showing the equal expression of GAPDH protein served also as a loading control (GAPDH Ab from Santa Cruz Biotechnology).

Patient samples: BAL and plasma (III)

To validate proteins identified from 2-DIGE and DeCyder analysis the relative protein expression of semenogelin, histones 2B and 4, α-1-antitrypsin and galectin-3 in BAL and in plasma were evaluated by Western blot analysis. Volumes of 15 μl of BALF samples or 1.5 μl of plasma were used for immunoblotting. A pool of all of the BALF or plasma samples was used as an internal standard on each gel to provide a reference against which to normalize the results.
**Table 6. The antibodies used for western blot-analysis**

<table>
<thead>
<tr>
<th>Abs against</th>
<th>Manufacturer</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-antitrypsin</td>
<td>AbFrontier</td>
<td>III</td>
</tr>
<tr>
<td>Alix</td>
<td>Santa Cruz Biotechnology</td>
<td>II</td>
</tr>
<tr>
<td>α/β-tubulin</td>
<td>Cell Signaling Technology</td>
<td>II, IV</td>
</tr>
<tr>
<td>Annexin I</td>
<td>Santa Cruz Biotechnology</td>
<td>II, IV</td>
</tr>
<tr>
<td>ASC</td>
<td>Chemicon /Millipore</td>
<td>I, IV</td>
</tr>
<tr>
<td>Atg7</td>
<td>Cell Signaling Technology</td>
<td>II</td>
</tr>
<tr>
<td>β2-integrin</td>
<td>Kindly provided by Prof. Carl Gahmberg</td>
<td>II</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>Sigma-Aldrich</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Cell Signaling Technology</td>
<td>I, II</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Calbiochem /Millipore</td>
<td>II, IV</td>
</tr>
<tr>
<td>Cathepsin D</td>
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<td>IV</td>
</tr>
<tr>
<td>Histone 2B (H2B)</td>
<td>Abcam</td>
<td>III</td>
</tr>
<tr>
<td>Histone 4 (H4)</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Kindly provided by Prof. Ilkka Jukunen (Pirhonen et al, 1999)</td>
<td>I, II</td>
</tr>
<tr>
<td>IL-18</td>
<td>Kindly provided by Prof. Ilkka Jukunen (Pirhonen et al, 1999)</td>
<td>I, II, IV</td>
</tr>
<tr>
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<td>R&amp;D Systems</td>
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<td>ITGAX</td>
<td>Novus Biologicals</td>
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<td>III</td>
</tr>
<tr>
<td>Tsg 101</td>
<td>Santa Cruz Biotechnology</td>
<td>II</td>
</tr>
</tbody>
</table>

**4.4. Other methods**

**4.4.1. Cell death assays (II)**

The level of cell death in cultured and stimulated macrophages was monitored with three different assays (II). The leakage of LDH, which is the marker of cell necrosis was measured with Cytotoxicity Detection Kit Plus (LDH) from Roche Diagnostics. To detect the level of cell apoptosis, the cells were treated according to the APOPercentage Apoptosis Assay (Biocolor Life Science Assays) and
photographed with an Olympus DP70 Digital microscope camera, connected to an Olympus IX71 light microscope, and using software in DP Controller (version 2.2.1.227) and DP Manager (2.2.1.195). The viability of the cells was determined by measuring the intracellular ATP using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

4.4.2. Electron microscopy of extracellular vesicles (II)

The extracellular vesicles were isolated from the supernatants of stimulated and control cells. The vesicles were then fixed with 1% paraformaldehyde, transferred into the Pioloform-carbon–coated copper grids, and allowed to absorb for 20 min. The grids were subsequently washed and contrasted with uranyl acetate to visualize proteins and viewed with a Jeol 1200 EX II transmission electron microscope (II).

4.4.3. Vesicle enrichment (II)

Macrophages were stimulated in RPMI 1640-media (BioWest) supplemented with L-glutamine, antibiotics and 10 mM HEPES, after which the cell culture media were collected. Enrichment of the extracellular vesicles was performed using centrifugal filter units (100 kDa cut-off, from Merck Millipore), and the flow-through was further concentrated with 10-kDa centrifugal filter units (Merck Millipore). The fractions of enriched vesicles were directly used for Western blotting or alternatively the vesicles were further purified by diluting the fraction with PBS and ultracentrifuging the diluted fraction twice at 100,000 x g for 1 h. The purified vesicles were resuspended in PBS and used for electron microscopy or Western blotting (II).

4.4.4. Gene expression microarray (II)

The microarray used in our study was an Agilent Whole Human Genome 4 X 44K 1-Color Array (Agilent Technologies). The microarray experiments were performed at Biomedicum Genomics (Helsinki, Finland). Fold change values of the genes were calculated for each stimulation versus the control, after which the values from three biological replicates were combined using the median value. Gene Ontology enrichment was calculated with Fisher exact test using a genome-wide reference set for human genes as the reference. SPIA was utilized for pathway enrichment analysis (Tarca et al., 2009) (21). All data analyses were performed with the freely available Anduril framework (Ovaska et al., 2010).
4.4.5. Quantitative real-time RT-PCR assay (I,II,IV)

The total cellular RNA, extracted with RNeasy Plus Mini Kit from Qiagen, was reverse transcribed into cDNA by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The gene expression analysis was performed by amplifying cDNA in TaqMan Fast universal PCR master mix (Applied Biosystems) or PerfeCta qPCR FastMix (Quanta Biosciences) with Predeveloped TaqMan assay primers and probes. The quantitative Real Time-PCR analysis was performed with ABI Prism 7500 Fast Sequence Detector using the 7500 Fast System SDS Software v1.4 (Applied Biosystems). The amplification values of endogenous 18S rRNA were determined from the analyzed samples to control the equal amount of added cDNA. The gene expression of the target cytokine genes were presented in relative units, which were calculated by a comparative C<sub>T</sub> method (Schmittgen and Livak, 2008).
5. RESULTS

5.1. (1,3)-β-glucans activate the NLRP3 inflammasome in human macrophages (I)

The major cell wall structure present in fungi is (1,3)-β-glucan (Tsoni and Brown, 2008), which is considered to be one of the main pathogen-associated molecular patterns affecting our immune system. The NLRP3 inflammasome is a cytosolic protein complex; its activation is associated with the recognition of microbial and other compounds released upon infection or cell stress (Bauernfeind et al., 2011). The activation of the NLRP3 inflammasome triggers the secretion of biologically active pro-inflammatory cytokines IL-1β and IL-18 through the function of proteolytic enzyme caspase-1 (Martinon et al., 2002, Bauernfeind et al., 2011). In the following experiments, we have studied the pathways and mechanisms which are involved in activation of NLRP3 inflammasome by (1,3)-β-glucans in one of the most important cell types involved in innate immunity, the macrophage.

5.1.1. (1,3)-β-glucans activate the NLRP3 inflammasome via dectin-1/Syk pathway (I)

Large particulate (insoluble form) β-glucans are known to be activators of innate immunity. First in this study, we compared the pro-inflammatory cytokine response of IL-1β in human macrophages towards β-glucan and to another microbial cell wall structure, LPS. Both curdlan (large particulate linear (1,3)-β-glucan) and LPS efficiently activated the transcription of the IL-1β gene (I, Fig. 1A). In contrast, only curdlan was able to induce the secretion of biologically active IL-1β in human macrophages (I, Fig. 1BC). A similar type of effect was also seen in human primary dendritic cells (I, Supplemental Fig. 1). In addition, other large particulate β-glucans (paramylon, glucan from baker’s yeast and zymosan) elicited IL-1β secretion in human macrophages, albeit at a lower level than curdlan (I, Fig. 1D). Thus curdlan was mainly used in the following experiments as a representative (1,3)-β-glucan.

Activation of caspase-1 is known to process the pro-form of IL-1β into its mature form and to promote its secretion (Martinon et al., 2002, Keller et al., 2008). Caspase-1 activation was observed after curdlan stimulation, but not with LPS, in human macrophages (I, Fig. 6A). In addition, the inhibition of caspase-1 activation with a specific inhibitor, Z-YVAD-FMK totally blocked the curdlan-induced IL-1β secretion, even though it did not affect the curdlan-induced IL-1β gene transcription (I, Fig. 6BC). These results demonstrate that β-glucan is able on
its own to induce IL-1β transcription and trigger the secretion of IL-1β via activation of the inflammasome associated caspase-1.

Dectin-1 is mainly expressed on the membrane of antigen-presenting cells and is the main pattern-recognition receptor for β-glucans (Brown and Gordon, 2001). It contains a short cytoplasmic tail with ITAM-like sequences, which utilize a spleen tyrosine kinase (Syk) as the signal transduction molecule (Goodridge et al., 2011, Kerrigan and Brown, 2011). The blocking anti-dectin-1 antibody and the specific inhibitor for Syk both diminished the curdlan-induced IL-1β transcription and secretion in human macrophages (I, Fig 2ABCD). The other IL-1 cytokine, IL-18, is constitutively expressed and needs only activation of caspase-1 to be processed into its active form which is secreted into the extracellular space. Both anti-dectin-1 and the Syk inhibitor totally blocked IL-18 secretion induced by curdlan (I, Fig. 5AB). This indicates that, in addition to inducing the IL-1β transcription, the dectin-1/Syk signaling pathway is essential for the activation of the inflammasome and caspase-1 in response to β-glucan stimulation.

It has been reported that the activation of cytoplasmic sensor, the NLRP3 inflammasome complex by MSU and silica is dependent on the phagocytosis of these crystals (Hornung et al., 2008). Blockade of the macrophages’ phagocytotic properties with cytochalasin D before the curdlan treatment did not exert any effect on curdlan-induced IL-1β mRNA levels (I, Fig. 3A), but it abolished totally the secretion of IL-1β triggered by curdlan (I, Fig. 3B). This indicates that in addition to the recognition of β-glucan with membrane-bound dectin-1, the formation of the phagocytic synapse (Goodridge et al., 2011) is also essential for the activation of the inflammasome and secretion of IL-1β.

To confirm the activation of NLRP3 inflammasome after the β-glucan stimulus, the NLRP3 gene was silenced with specific NLRP3 siRNAs in human macrophages. NLRP3 gene-silencing decreased the levels of secreted IL-1β (I, Fig. 4B), but had only a marginal effect on IL-1β mRNA levels after β-glucan treatment (I, Fig. 4C). Furthermore, NF-κB –dependent upregulation of NLRP3 has been shown to be essential for the inflammasome activation (Bauernfeind et al., 2009). Curdlan treatment led to the induction of NLRP3 mRNA in human macrophages, which was also observed with LPS (I, Fig. 4C). Activating the NLRP3 gene transcription with LPS-priming and blockade of the dectin-1 pathway with a Syk-inhibitor before curdlan-treatment of the same cells abolished the IL-18 secretion in human macrophages (I, Supplemental Fig. 2.) emphasizing that β-glucan-induced Syk activation is also needed for NLRP3 inflammasome activation and IL-18 secretion for reasons other than simply inducing the NLRP3 gene transcription. To summarize, our results indicate that β-glucan activates NLRP3 inflammasome via the dectin-1 pathway to trigger the IL-1β and IL-18 response.
5.1.2. (1,3)-β-glucan- induced NLRP3 inflammasome activation is dependent on ROS formation, K+ efflux and cathepsin activity (I)

The formation of mitochondrial reactive oxygen species (ROS) induced by crystalloid substances (MSU, silica, aluminum) as well as the release of cathepsin B into the cytosol after phagocytosis of these crystals have been suggested to be triggering events in the activation of the NLRP3 inflammasome (Hornung et al., 2008, Zhou et al., 2011). To study the roles of ROS formation and release of cathepsin B in β-glucan-induced NLRP3 activation, a known ROS inhibitor, BHA, or a specific cathepsin B inhibitor, CA-074-Me was added to the human macrophages prior to the β-glucan stimulus. Both of these inhibitors had little effect on the curdlan-triggered IL-1β mRNA expression (I, Fig. 6D and Fig. 7A), but in contrast, they both completely abolished the curdlan-induced secretion of IL-1β (Fig. 6D and Fig. 7B). Potassium efflux (K⁺) and a low intracellular potassium concentration have also been associated with NLRP3 inflammasome activation in a response to particulate substances ((Munoz-Planillo et al., 2013). The effect of potassium efflux in curdlan-induced NLRP3 inflammasome activation was studied in the presence or absence of additional potassium chloride in the media. The enhanced potassium chloride did not affect the curdlan-induced IL-1β mRNA levels (I, Fig. 7C), but it totally blocked the curdlan-induced secretion of IL-1β (I, Fig. 7D). These data indicate that ROS formation, K⁺ efflux and cathepsin activity are essential events in the β-glucan- triggered activation of the NLRP3 inflammasome.

5.2. (1,3)-β-glucans activate unconventional protein secretion in human macrophages (II, IV)

Many cytokines and chemokines mediating the immune response are secreted through the conventional protein secretion pathway. These conventionally secreted proteins contain an N-terminal signal-peptide that directs their secretion to occur via the endoplasmic reticulum (ER)-to-Golgi membrane pathway. Some proteins (IL-1, DAMPs) lack a protein signal sequence required for ER entry and are thus secreted from macrophages by unconventional protein secretion pathways. Vesicle-mediated protein transport and release into the extracellular space are one of the central mechanisms in unconventional protein secretion.
5.2.1. Both (1,3)-β-glucans and LPS activate significant changes in gene transcription (II)

To analyze the global transcriptional response for β-glucans, human macrophages were stimulated either with curdlan or glucan from baker’s yeast (GBY). LPS served as a control for a well-known inflammatory microbial particle unrelated to fungi. The gene expression analysis was performed with microarray technology. The number of identified genes with >2-fold increase or a decrease were as follows: 767 for curdlan, 1447 for GBY and 1683 for LPS (II, Supplemental Table II). The overall transcription profile of curdlan and GBY resembled each other, and this profile of β-glucan differed from LPS, indicating the different downstream signaling triggered by β-glucans and LPS in human macrophages (II, Fig. 1B). According to the KEGG pathway analysis, both stimulants induced genes related to chemokine signaling, cytokine-cytokine receptor interactions and MAPK signaling pathways. In contrast, genes related to cytosolic DNA-sensing pathways, Jak-STAT signaling pathway and NOD-like receptor signaling pathways were overrepresented after LPS and underrepresented after β-glucan stimulation (II, Fig. 1B).

5.2.2. (1,3)-β-glucans, but not LPS, activate robust protein release via vesicle-mediated unconventional secretion pathways (II, IV)

To characterize the global secretome response for β-glucans, curdlan or GBY, or LPS, which served as a control here, human macrophages were stimulated for 18 hrs. iTRAQ labeling combined with LC-MS/MS analysis was utilized for quantitation and identification of the secreted proteins from the cell medium. The protein secretion was robust after β-glucan stimulation compared to the LPS stimulus. In addition, the secretomes of curdlan and GBY resembled each other (Figure 5).
Figure 5. The secretomes of curdlan- and GBY-induced macrophages resembled each other. There were 1597 reliably quantified distinct proteins analyzed with the iTRAQ method from two independent biological experiments. The results shown here are combinations of those two replicates.

A. The average ratios of total secreted protein compared between stimulated and untreated cells

<table>
<thead>
<tr>
<th>THE AVERAGE RATIO OF PROTEIN DETECTED</th>
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<tbody>
<tr>
<td>LPS: CTR</td>
</tr>
<tr>
<td>CURDLAN: CTR</td>
</tr>
<tr>
<td>GBY: CTR</td>
</tr>
<tr>
<td>1.1</td>
</tr>
<tr>
<td>2.7</td>
</tr>
<tr>
<td>3.7</td>
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B. Secretome of β-glucan. The Venn diagram illustrates the identified proteins with relative quantity >2.0 from curdlan-and GBY-induced macrophage secretomes.

Most of these β-glucan induced secreted proteins were classified as intracellular proteins according to Gene Ontology analysis (II, Fig. 1A). To confirm that these proteins were actively secreted in response to the β-glucan stimuli and not a result of cell death, evidence of apoptosis and necrosis was studied and excluded based on the results. We could not detect any morphological signs of cell death. On the contrary, the cells appeared to be very intact and viable after β-glucan stimulation (II, Fig. 2). The characterization of the secretion mechanisms using the SignalP software revealed that 26% and 20% of the curdlan- and GBY-induced proteins, respectively, had a signal peptide sequence needed for the conventional type of protein secretion (II, Fig. 3A, Supplemental Table IIIA). Cytokines and chemokines, most of which are secreted through conventional signal-peptide mediated pathways, were increased in the β-glucan secretome, as also at a more moderate level in the LPS secretome (II, Fig. 4AB). Treating the cells with Brefeldin A, which inhibits the transport of signal-peptide including proteins from
ER to Golgi, completely prevented β-glucan-induced conventional secretion of cytokines and chemokines, but not IL-1β, which is secreted via unconventional pathways (II, Fig. 4C). It is known that proteins lacking the protein signal sequence are released by unconventional protein secretion pathways. One form of unconventional secretion is secreting the proteins into the extracellular milieu through membrane vesicles called exosomes and microvesicles (Rabouille et al., 2012). Based on the exosomal protein database Exocarta, 49% of secreted proteins after curdlan and GBY stimulation have been reported to be released via exosomes (II, Fig. 3A, Supplemental Table IIIIB). When these were classified according to the biological processes, most of the β-glucan induced proteins were designated to those processes where the proteins are known to be released by exosomes: protein metabolic processes, vesicle-mediated trafficking, signaling, cytoskeleton organization and cell adhesion and migration (II, fig. 3B). To confirm the vesicle-mediated nature of the β-glucan-induced protein secretion, the enriched fraction containing the possible extracellular vesicles was prepared from the cell media after β-glucan stimulus. Analysis of this fraction with electron microscopy showed exosomal-type vesicles in the sample (II, Fig. 3C). Furthermore, the western blot analysis of typical exosomal marker proteins and visualization of the total protein amount with silver staining from this vesicle-fraction confirmed that β-glucan stimulation increased robustly the secretion of exosomal proteins (II, Fig. 3DE). Vesicle-mediated protein secretion (annexin I, ITGAX, α/β-tubulin) was more extensive in β-glucan-induced GM-CSF-macrophages than in M-CSF-macrophages (IV, Fig. 4).

5.2.3. NLRP3 inflammasome activation via dectin-1/Syk pathway is crucial for β-glucan-activated unconventional protein secretion (II, IV)

Activation of the NLRP3 inflammasome is associated with the secretion of its central components and secretion of caspase-1 substrates IL-1β and IL-18 (Qu et al., 2007, Keller et al., 2008). Cytokines of the IL-1 family are thought to be secreted via unconventional protein secretion pathways (Garlanda et al., 2013). The secretion of mature IL-1β and IL-18, and inflammasome components and regulators were detected during the β-glucan stimulation (II, Fig. 5A). The secretions of IL-1β, IL-18 and caspase-1 were more abundant in GM-CSF-macrophages than in M-CSF-macrophages, indicating that these former macrophages display a more powerful activation of inflammasome after β-glucan stimulus (IV, Fig. 3AC). Interestingly, the preform of caspase-1 (p45) was expressed at higher levels in GM-CSF-macrophages than in M-CSF-macrophages (IV, Fig.5). One other remarkable observation was that β-glucan induced the expression of GBP5, the putative regulator of NLRP3 inflammasome activation, in GM-CSF-macrophages (IV, Fig. 5). These two results may explain the more
efficent activation of the inflammasome in β-glucan stimulated GM-CSF-macrophages.

To study in more detail the secretion mechanisms of inflammasome-components and -regulators after β-glucan treatment, the enriched extracellular vesicle fraction was prepared from the cell media and used for secretion analysis. Mature forms of IL-1β and IL-18 and all the isoforms of ASC were detected only in the flow-through fraction, indicating that these molecules had not been secreted inside vesicles after β-glucan stimulus (II, Fig. 5B). In contrast, although the preforms of cathepsins were also detected in the flow-through fraction, their mature forms were seen only in the fraction containing vesicles (II, Fig. 5B). To characterize the effect of β-glucan-induced NLRP3 activation to the unconventional protein secretion, a specific inhibitor for caspase-1 (Z-YVAD-FMK) was used. The caspase-1 inhibitor abolished the β-glucan-induced secretion of IL-1β and exosomal marker proteins, but it did not have any effect on cathepsin D (II, Fig. 5C). These results indicate that caspase-1 regulates the secretion of IL-1β and several vesicle-mediated proteins, but not the secretion of molecules, which are activated upstream of caspase-1.

According to our earlier results, β-glucan activates the NLRP3 inflammasome via signaling through the dectin-1 pathway in human macrophages. We wanted to confirm the role of dectin-1 in the secretion of inflammasome related components and vesicle-mediated proteins. The secretions of mature forms of IL-1β and cathepsins were abolished in β-glucan stimulated bone marrow derived dendritic cells from dectin-1 -/- mice (II, Fig. 6AB). However, the β-glucan-induced increase in the levels of IL-1β mRNA was not totally inhibited in cells from dectin-1 -/- mice (II, Fig. 6C). BMDCs were used because mouse bone-marrow-derived macrophages are not responsive to β-glucans (Goodridge et al., 2009a).

Src and Syk are kinases, which are linked downstream of dectin-1 signaling (Goodridge et al., 2011). Syk inhibition clearly decreased the secretion of the mature forms of IL-1β, cathepsins and ASC p10 in macrophages (II, Fig. 6D). In contrast, Src inhibitors had no effect on the secretion of these proteins (II, 6D and Supplemental Fig. 1A). Exosomal protein secretion of annexin I and tubulin were dependent on Syk, whereas the Src inhibitor, on its own, did not have any effect on these proteins (II, Fig. 6F).

5.2.4. (1,3)-β-glucan –induced unconventional protein secretion is dependent on autophagy (II)

Autophagy is a highly conserved process in cells, since it is mainly involved for degradation and removal of dysfunctionsing organelles and aggregates. It has been
previously shown that autophagy can participate in the regulation of protein secretion (Ponpuak et al., 2015).

Our secretome data revealed that many autophagy–associated proteins were secreted after the β-glucan stimulus in human macrophages (II, Fig. 7A, Supplemental Table IIIC). We also observed that β-glucan increased the conversion of LC3-I to LC3-II, which is found on autophagic membranes and is indicative of the activation of the autophagy process (II, Fig. 7B). To study the role of autophagy on β-glucan induced protein secretion, the process of autophagy was blocked with 3-MA, which inhibits the activity of class III phosphatidylinositol-3-OH-kinase (PI(3)K) and prevents the formation of autophagosomes. The inhibition of autophagy suppressed the β-glucan-induced total protein secretion and the secretion of proteins related to exosomes, inflammasome and autophagy (II, Fig. 7DE). The effect of autophagy on the β-glucan secretome was also studied by silencing the function of the beclin-1 gene with the SiRNA approach. Beclin-1 is a key component of the class III PI(3)K-complex. In line with the 3-MA results, the decreased beclin-1 expression led to an inhibition of total protein secretion and decreased the secretion of exosomal and inflammasome- and autophagy-related proteins during the β-glucan stimulation (II, Fig. 7FG). In contrast, beclin-1 silencing did not affect the β-glucan-triggered conventional secretion of chemokines (II, Fig. 7H).

To conclude, activation of autophagy is required for β-glucan-induced unconventional protein secretion, but not for conventional protein secretion.

5.3. (1,3)-β-glucans activate production of IL-1 family cytokines in human macrophages (I, IV)

Cytokines of interleukin-1 family have a key role in innate immunity as inducers of local and systemic inflammation. Most of the pro-inflammatory cytokines of IL-1 family lack the signal peptide and are thus not released via the classical protein secretion pathway (Garlanda et al., 2013). The activation of NLRP3 inflammasome mediated caspase-1 is known to drive the secretion of IL-1β and IL-18, but the impact of inflammasome activation in the secretion of other IL-1 members has remained mainly uncharacterized.

5.3.1. GM-CSF -macrophages are more potent producers of IL-1 family cytokines compared to M-CSF -macrophages (IV)
The granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are essential cytokines regulating the developing process, survival and function of macrophages (Hamilton, 2008, Louis et al., 2015). In this study, we compared the response of pro-inflammatory IL-1 cytokines in both GM-CSF -and M-CSF-differentiated human monocyte-derived macrophages after β-glucan stimulation. The characterization was performed by analyzing the transcription and secretion of IL-1 family members. After 6 h of β-glucan stimulation, the induction in the mRNA levels of IL-1α, IL-1β and IL-36γ was seen in both macrophage types (IV, Fig. 1). The induction observed in mRNA levels of IL-36α and IL-36β after β-glucan treatment was not significant (IV, Fig. 1). The mRNA levels of IL-33 were only increased in β-glucan-stimulated GM-CSF- macrophages (IV, Fig. 1), in contrast, there were no differences in the mRNA levels of IL-18 between the controls and β-glucan exposure groups or different macrophage types (IV, Fig. 1).

The secreted concentrations of IL-1α, IL-1β and IL-18 were higher in GM-CSF -macrophages than in M-CSF- macrophages after 18 hours’ β-glucan stimulation (IV, Fig. 3AC). Beta-glucan-induced secretion and processing of IL-36γ was studied with immunoblotting in parallel to the assays of IL-1β and IL-18. Thus far, IL-36 cytokines have not being reported to contain caspase-1 cleavage sites similarly to IL-1β and IL-18. The biologically active form of IL-1β (p17) was detected only in β-glucan-stimulated GM-CSF-macrophages (IV, Fig. 3C). In addition, the secretions of both the pro-form (p24) and the mature protein of IL-18 (p18) were more abundant in the supernatant collected from GM-CSF -macrophages after β-glucan treatment (IV, Fig. 3C). The amount of pro-IL1β was at the same level in the lysates collected from the both stimulated macrophage types (IV, Fig. 3B). There was little difference in proIL-18 expression in untreated cells or in β-glucan- stimulated GM-CSF -and M-CSF –macrophages (IV, Fig. 3B).

IL-36γ was detected only in the supernatant of β-glucan stimulated GM-CSF-macrophages. The amount of secreted IL-36γ cytokine correlated with the intracellular produced cytokine in β-glucan-treated GM-CSF –macrophages (IV, Figure 3B and 3C). The molecular weight of the detected IL-36γ protein, both inside the cell and in the supernatant, was approximately 19 kDa. These results indicate that β-glucan-induced IL-1 cytokine secretion is more efficient in GM-CSF-macrophages than in M-CSF-macrophages, even though the β-glucan induced the gene expression of IL-1-family cytokines in both GM-CSF- and M-CSF-macrophages.

5.3.2. (1,3)-β-glucan-induced IL-1α and IL-36γ transcription is dependent on cathepsin B activity (IV)

The activation of dectin-1 and NLRP3 inflammasome and their effect to the β-glucan-induced gene transcription of IL-1 family cytokines were studied. Human
GM-CSF-macrophages were used in this experiment since according to our earlier results, these cells display a more prominent IL-1 response when exposed to β-glucan. To examine the role of dectin-1 signaling in the β-glucan induced IL-1 response, a specific Syk inhibitor was used. This inhibitor blocked totally the β-glucan induced gene transcription of IL-1α, IL-1β and IL-36γ (IV, Fig. 6A). In addition to the human macrophages, the mRNA levels of IL-1 cytokines were studied in the bronchoalveolar lavage cells collected from intranasally β-glucan exposed C57BL/6 wild type and dectin-1 knockout mice to elucidate the role of dectin-1 in the response of IL-1 family cytokines in mouse lung. Treatment with β-glucan significantly increased the transcription levels of IL-1α, IL-1β and IL-36γ in BAL cells obtained from wild type mice (IV, Fig. 2). In contrast, a deficiency of dectin-1 impaired the transcription of β-glucan-induced IL-1 cytokines. These results indicate that the dectin-1 pathway is needed for β-glucan-activated gene expression of IL-1 family cytokines in human macrophages and in vivo activated mouse BAL cells.

To elucidate the role of caspase-1 and cathepsin B in β-glucan-triggered gene expression of IL-1 cytokines, the specific inhibitors Z-YVAD-FMK and CA-074-Me were used, respectively. The caspase-1 inhibition had no inhibitory effect in β-glucan induced IL-1α, IL-1β and IL-36γ genes (IV, Fig. 6A). In contrast, CA-074-Me clearly blocked the β-glucan-induced transcription of IL-1α and IL-36γ genes, but exerted no effect on IL-1β (IV, Fig. 6A). These results suggest that activation of cathepsin B, in addition to its recognized role in the secretion of IL-1β, has an effect on the gene expression of IL-1α and IL-36γ.

5.3.3. Secretion of IL-36γ is not dependent on NLRP3 inflammasome (IV)

The inhibition of NLRP3 activation by cathepsin B and caspase-1 inhibitors led to a decreased secretion of IL-1β in human macrophages after β-glucan stimulus (I, Fig.6, IV, Fig. 6B). We wanted to study the effect of the same inhibitors on the secretion of other IL-1 family cytokines, IL-1α and IL-36γ. The cathepsin B-inhibitor blocked the secretion of IL-1α, in contrast, the caspase-1 inhibitor had only a partial effect on the secretion of IL-1α after β-glucan treatment (IV, Fig.6B). Cathepsin B -and caspase-1 inhibitors inhibited totally the β-glucan-induced secretion of mature IL-1β (IV, Fig. 6C). In contrast, the caspase-1 inhibitor only partially decreased the secretion IL-36γ (IV, Fig. 6C). To confirm the role of NLRP3 inflammasome in the secretion of IL-1 family cytokines, we used a specific inhibitor for the NLRP3 inflammasome, MCC950 (Coll et al., 2015). Secretion of β-glucan-induced IL-1α and IL-1β was prevented by NLRP3 inhibition (IV, Fig. 6DE). In contrast, NLRP3 inhibition did not block the secretion of IL-36γ (IV, Fig. 6E). Taken together, these results show that β-glucan-
induced secretion of IL-1α, IL-1β and IL-36γ cytokines is differentially regulated by cathepsin B, caspase-1 and NLRP3 inflammasome in human macrophages.

5.4. **Proteomic changes of the alveolar lining fluid are different between the illnesses related to exposure to non-infective microbial particles (III)**

Individuals living in certain surroundings or in certain occupations have an increased risk of being exposed to microbial dust including non-infective particles from fungi and bacteria. These microbial exposures are associated with diseases which manifest airway symptoms such as hypersensitivity pneumonitis and damp building-related illness (WHO, 2009, Selman et al., 2010), but rather little is known about the proteomic changes of the alveolar lining fluid related to these diseases.

5.4.1. **Protein expression pattern of bronchoalveolar lavage is different between the damp-building related illness and hypersensitivity pneumonitis-like conditions**

In order to identify possible diagnostic markers for illnesses related to exposure to non-infectious microbial particles (NIMPs), we collected the BAL samples from individuals who had been exposed to NIMPs in the context of DBRI or in the context of agricultural environment and manifesting hypersensitivity pneumonitis-like symptoms (AME). Samples from patients diagnosed with acute type of HP were used as a reference for inflammatory lung disease related to NIMP exposure. Samples from healthy individuals served as controls (CTR) and samples from sarcoidosis patients (SARC) served as a reference for inflammatory lung disease with no direct association with NIMP exposure. More detailed information about the patients is provided in Tables 1 and 2 (III). The quantitative proteomic analysis was performed for BAL samples utilizing the 2D-DIGE and DeCyder analysis. Subsequently, the identification of differentially expressed protein spots was performed with LC/MS-MS. A total of 63 protein spots and 34 different proteins were identified as being differentially expressed between the control versus one or all the other study groups, or between the DBRI and AME (III, Fig. 1 and Table S1). According to the Gene Ontology classification analysis, most of the identified proteins were grouped as extracellular proteins, had some function in antigen or protein binding and were part of certain biological processes such as the immune response, the function of platelets, iron homeostasis, transmembrane transport and response to reactive oxygen compounds (III, Fig. 2AB). David enrichment analysis
showed that extracellular secreted proteins, especially plasma proteins, were an abundant group in the analyzed BAL (III, Table S2).

The hierarchical clustering of the 63 identified proteins from BAL (III, Fig. 2C) revealed the differences in protein expression patterns between the study groups. The abundant expression of most of the identified proteins in both AME and HP located them into the same main cluster. The other groups, CTR, DBRI and SARC formed their own main cluster, indicating that these groups have a different protein expression pattern than hypersensitivity pneumonitis and hypersensitivity pneumonitis-like condition. In this latter main cluster, CTR and DBRI formed their own subgroup, indicating a similar type of overall protein expression between these two. A similar kind of clustering of the groups was also seen in the principal components analysis performed for spot maps based on the variance in their protein expression (III, Fig. 2D). In addition, the lymphocyte percentages of BAL samples chosen for DIGE analysis were compared and according to this result, DBRI differed from the control samples, but not from the other study groups (III, Figure S2).

According to the DeCyder analysis, none of the identified 34 proteins (III, Table S1) were upregulated specifically in only one of the study groups. Apolipoprotein A1 was the only identified protein upregulated in NIMP-related diseases (AME, DBRI, HP) compared to control, but there was no difference compared to sarcoidosis.

5.4.2. Semenogelin and histone 4 proteins are more abundant in bronchoalveolar lavage of hypersensitivity pneumonitis-like conditions than in damp-building related illness

We could not identify any protein which was a specific marker for NIMP-exposure associated diseases, i.e. none of the identified proteins were upregulated only in NIMP-related disease group (AME, DBRI, HP) compared to control and sarcoidosis. Thus the proteins for immunoblotting-based validation and quantitation were chosen according to their reasonable robustness in disease groups compared to healthy controls. The proteins chosen were: alpha-1-antitrypsin (A1AT), galectin-3 (GAL3), histone H4 (H4) and semenogelin I (SEM) (III, Fig. 3AB). The protease inhibitor, A1AT and the unconventionally secreted carbohydrate-binding lectin, GAL3 are proteins, which levels are known to increase during inflammation. According to immunoblot quantitation results, expressions of A1AT and GAL3 were elevated in all disease groups compared to control group (III, Fig. 3C). There were no differences in the expression of A1AT between the AME and DBRI, but their A1AT expression differed from HP. With respect to GAL3, there were no significant changes between the disease groups (AME, DBRI, HP, SARC) when they were compared with each other. Semenogelin, a protein involved in the formation of sperm coagulum (Lilja, 1985),
was more abundant in AME, HP and SARC groups than in controls (III, Fig.3C). Semenogelin expression remained at the control level in the DBRI group, which differed from its expression in hypersensitivity pneumonitis (p=0.0001 for DBRI vs. HP) and hypersensitivity pneumonitis-like condition (p=0.0009 for DBRI vs. AME). Histones can elicit an inflammatory response functioning as endogenous danger signals (Chen et al., 2014). The immunoblot validation was performed for histone 4, from a protein band of approximately size of 50 kDa. This size is aberrant for histone, thus this particular protein band was confirmed by mass spectrometric analysis from the immunoblot membrane. The increased amount of histone 4 was found in BAL of AME, HP and SARC (III, Fig. 3C). The levels were different between DBRI and AME/HP (p=0.01 for DBRI vs. AME, and p=0.0031 for DBRI vs. HP).

All the samples used in this study were obtained from patients with a non-smoking background. When six BAL-samples collected from healthy controls with a smoking background were included in the immunoblot validation and the results were compared to non-smoking controls, only GAL3 levels were increased in the group of smokers (p=0.0482).

Furthermore, the immunoglobulin response was determined from the study groups with the ELISA assay. The concentrations of total IgG were increased in BAL collected from AME, HP and SARC groups (III, Fig. S4). In addition to BAL, plasma samples were available from control, HP and SARC groups. It was possible to quantify the expression levels of galectin-3 and histone 2B (H2B) by immunoblot from the samples without any interference by the abundant plasma proteins. In plasma, the detected size of H2B protein band was predicted to be 17 kDa. There were no differences between the groups in the expression of galectin-3 (III, Fig. 4A). Levels of H2B were elevated in HP and SARC groups compared to control (III, Fig. 4B). The difference in abundance of H2B between HP and SARC was not statistically significant (data not shown).
6. DISCUSSION

The invasion and proliferation of pathogenic microbes can lead to tissue damage and disease. These microbes are recognized by our immune system via their conservative and unique microbial structures (pathogen-associated molecular patterns, PAMPs). The microbial structural components can trigger different kinds of responses in our immune system when recognized by pattern recognition receptors (PRRs). Macrophages are the central defense cells of innate immunity, and usually the first cells to confront the pathogen or pathogenic particles. The recognition of pathogenic substances or danger signals released from damaged tissue may lead to activation of signal cascades downstream in the macrophage’s PRRs as well as the production of pathogen killing compounds (ROS, NO) and the secretion of chemokines and cytokines. These latter agents promote the inflammatory response by activating and recruiting the immune cells into the site of microbial encounter and tissue damage. Furthermore, antigen presentation to the lymphocytes by activated macrophages may lead to the initiation of adaptive immune response. In contrast to the role of macrophages as activators of inflammation, macrophages also sustain the body homeostasis by taking care of tissue repair, dampening the inflammatory reaction by secreting anti-inflammatory factors and having a certain threshold for environmental signals that will initiate the inflammatory cascades.

The exposure to inhaled non-infective microbial particles in certain occupations and environments is associated with diseases manifesting airway symptoms. These particles contain the same pathogen-associated molecular patterns as an intact pathogen, and thus may trigger the immune response. The innate immunity system is the first line of defense for pathogens; it is involved in the initiation of inflammatory response and for the most part, it is sufficient on its own to eradicate the threat. Thus it is important to characterize the mechanisms by which these microbial structures affect our innate immune system and furthermore to determine whether these activated mechanisms could explain the pathogenesis behind the diseases associated with the exposure of microbial particles or infection with viable pathogen.

6.1. NLRP3 inflammasome is activated by β-glucan via dectin-1 signaling pathway in human macrophages

NLRP3 inflammasome is a cytosolic protein complex, which is an important sensor of the innate immune system for recognizing the invading pathogens, host-
derived danger signals or other harmful substances. The induction of the NLRP3 inflammasome triggers activation of caspase-1 enzyme, which catalyses the pro-inflammatory cytokines of IL-1β and IL-18 into their biologically active forms (Martinon et al., 2002, Bauernfeind et al., 2011).

A deficiency of NLRP3 or other inflammasome components (ASC, caspase-1) is associated with increased susceptibility to fungal diseases, highlighting their importance in antifungal defense. Mice lacking a functional NLRP3 inflammasome or IL-1 receptor were more susceptible to infections of most common fungal pathogen, *Candida albicans* (Gross et al., 2009, Hise et al., 2009, Joly et al., 2009). In contrast to NLRP3, deficiency for other Nlrps (Nlrp1, Nlrc4, Nlrp6, Nlrp12) did not change the IL-1β response of the cells to *C. albicans* (Gross et al., 2009, Joly et al., 2009). The activation of NLRP3 inflammasome was also crucial for the antifungal immune response against *Aspergillus fumigatus* in a human monocyte cell line (Said-Sadier et al., 2010).

The major carbohydrate structure of the fungal cell wall and the crucial immunostimulatory PAMP of fungi is (1,3)-β-glucan (Tsoni and Brown, 2008). Concurrently with our studies, the activation of NLRP3 inflammasome by fungal β-glucan was shown in mouse thioglycolate-elicited peritoneal macrophages and bone marrow-derived dendritic cells (Kumar et al., 2009). In our study, we characterized the potential of (1,3)-β-glucan to activate pathways and mechanisms, which are involved in the activation of NLRP3 inflammasome and secretion of IL-1β in human primary macrophages.

It has been postulated that the secretion of IL-1β in macrophages requires two different signals (Bauernfeind et al., 2011). The first signal, providing the so-called priming step, is mediated by the membrane-bound PRR and leads to upregulation of *IL-1β* and *NLRP3* gene expression via transcription factor NF-κB (Bauernfeind et al., 2009, Dinarello, 2009). In *in vitro* experiments, the priming of the macrophages is typically induced by LPS, which binds to TLR4 and triggers the NF-κB signaling. After priming, the second and usually distinct signal, for example mediated by microbial toxin or DAMP, is required for activation of NLRP3, which leads to the assembly of the inflammasome complex and the secretion of mature IL-1β.

Our results revealed that both β-glucan and other microbial PAMP, bacterial LPS induced the gene expression of IL-1β in human macrophages. In contrast, only β-glucan activated the secretion of IL-1β, which was abolished when the activation of caspase-1 was inhibited or the gene for NLRP3 was silenced. Moreover, we observed that β-glucan triggered IL-1β secretion in human dendritic cells, the main APCs, which may have an influence in activation and polarization of adaptive immunity during fungal infection or exposure to β-glucans. These results show that in contrast to LPS, β-glucan is capable of providing both of these signals for NLRP3 inflammasome activation and secretion of IL-1β. This indicates that a major component of the fungal cell wall on its own can trigger the inflammatory response by inducing the secretion of IL-1β in human macrophages.
The membrane-bound C-type lectin receptor, dectin-1 is the main PRR for β-glucans; it is known to facilitate phagocytosis and the oxidative burst, and activate expression of pro-inflammatory cytokines via the NF-κB signaling (Brown and Gordon, 2001, Drummond and Brown, 2011). The importance of dectin-1 receptor and its downstream signaling components in fungal immunity has been demonstrated in studies with human and mice, where the deficiency of dectin-1 has led to increased susceptibility to fungal infections, especially a higher prevalence of mucocutaneous candidiasis in humans (Saijo et al., 2007, Ferwerda et al., 2009, Glockler et al., 2009, Strasser et al., 2012). Our results revealed that the dectin-1 receptor was required for β-glucan-induced increase of IL-1β mRNA and secretion of IL-1β in human macrophages. In β-glucan-stimulated mouse bone marrow-derived dendritic cells, dectin-1 deficiency blocked the secretion of IL-1β, but did not totally prevent the induction of IL-1β mRNA, indicating that also other receptors may be involved in the β-glucan-induced IL-1β transcription in mice. These additional receptors may include scavenger receptors such as CD36 and SCARF1 (Means et al., 2009) or complement receptor CR3 (Goodridge et al., 2009b), which have been reported to recognize β-glucan and mediate the antifungal response. Other CLRs, such as dectin-2, are known to activate NLRP3 inflammasome in response to helminth antigens (Ritter et al., 2010), and when forming a heterodimer with dectin-3, it facilitates an effective antifungal response via the same downstream signaling components (Syk tyrosine kinase, NF-κB transcription factor) as dectin-1. However thus far, these receptors have been linked with the recognition of fungal α-mannans, not β-glucans (Zhu et al., 2013).

The spleen tyrosine kinase (Syk) is the main molecule mediating the signal transduction after dectin-1 activation (Kerrigan and Brown, 2010). Recent studies have shown that Syk controls both the synthesis of pro-IL-1β and inflammasome activation during C. albicans or A. fumigatus stimulation (Gross et al., 2009, Said-Sadier et al., 2010). Our results revealed that the activation of Syk signaling was needed for β-glucan-induced increase of IL-1β mRNA and secretion of IL-1β in human macrophages. Moreover, Syk inhibition abolished β-glucan-induced secretion of IL-18. The pro-form of IL-18 is known to be expressed constitutively in macrophages. It has been shown that NF-κB dependent upregulation of NLRP3 expression is needed for inflammasome activation (Bauernfeind et al., 2009). Priming human macrophages with LPS, to ensure Syk-independent upregulation of NLRP3 mRNA, did not rescue the defect in secretion of IL-18 in Syk-inhibited and β-glucan stimulated cells. This suggests that Syk signaling has also other roles in the activation of the inflammasome. This has also been observed in other studies, where NLRP3 ligands such as malarial hemozoin or carbon nanotubes have failed to activate the NLRP3 inflammasome, when Syk is inhibited (Shio et al., 2009, Palomäki et al., 2011), which points to a more general role for Syk in activating the inflammasome. According to recent studies, Syk is involved in the phosphorylation of the inflammasome component, ASC, during the activation of NLRP3, with signaling being triggered by several NLRP3 ligands such as ATP,
nigericin, MSU and alum. Phosphorylation of ASC is believed to control the formation of ASC specks and subsequently to the activation of inflammasome-mediated caspase-1 (Hara et al., 2013, Lin et al., 2015).

In addition to Syk, the activation of Src kinase in upstream of Syk has been linked to dectin-1 signaling and activation of NLRP3 (Shio et al., 2009, Kerrigan and Brown, 2010). In our macrophage experiments, the src inhibitors exerted no effect on the β-glucan induced gene transcription or protein secretion of IL-1β. This suggests that Syk can act also independently of Src kinases. To summarize, the dectin-1/ Syk pathway not only controls the production of pro-IL-1β cytokine, but is also crucial for activating the NLRP3 inflammasome and IL-1β secretion as a response for β-glucans in human macrophages.

Previous studies have revealed that crystalloid structures such as MSU and silica are phagocytized into the cell cytosol prior to activation of NLRP3 inflammasome (Hornung et al., 2008). Dectin-1 has been shown to be the primary receptor for initiating the phagocytosis of fungi exposing the cell wall β-glucans (Goodridge et al., 2012). Blockade of the internalization of β-glucan in human macrophages prevented the secretion of IL-1β, but not the transcription of IL-1β mRNA. This indicates that the formation of the phagocytic synapse is involved in dectin-1 signaling. Dectin-1 has been demonstrated also to induce the release of IL-1β independently of pathogen internalization via a non-canonical caspase-8 inflammasome in human dendritic cells (Gringhuis et al., 2012). Our results, where blockade of antigen internalization totally prevented the secretion of IL-1β, imply that there are differences between macrophages and dendritic cells in activating the canonical and noncanonical inflammasome pathways.

The exact molecular mechanisms, which trigger the activation of NLRP3 are mainly unknown. Ligands activating the NLRP3 inflammasome have been shown to induce cellular events which lead to NLRP3 assembly and subsequently to caspase-1 activation.

The main phenomena linked to the activation of canonical NLRP3 include changes in cell ion balance (K⁺ efflux) (Munoz-Planillo et al., 2013), redox state (release of oxygen radicals, ROS) (Zhou et al., 2011) and lysosomal destabilization and cathepsin activity (Hornung et al., 2008).

Phagocytosis of particulate matter has been shown to cause the disruption of the lysosomal membrane and the leakage of lysosomal cathepsins into the cytosol which led to the activation of the NLRP3 inflammasome (Hornung et al., 2008). Cathepsins are protease enzymes, which are involved in the proteolytic degradation of lysosomal compartments (Turk et al., 2012). In addition to silica crystals and aluminum salts, several other particulate substances such as amyloid-β (Halle et al., 2008), cholesterol crystals (Duewell et al., 2010, Rajamäki et al., 2010), long needle-like carbon nanotubes (Palomäki et al., 2011) and monosodium urate crystals (Välimäki et al., 2013) have been shown to activate NLRP3 depending on cathepsin-B activity. In line with this, by inhibiting the event of phagocytosis, or inhibiting the activity of cathepsin B with CA-074-Me, it was
possible to inhibit completely the β-glucan-induced release of IL-1β in our experiments with human macrophages. Some other studies using cathepsin B-deficient cells or pharmacologic inhibitors have reported different results, with a role of cathepsin B in activation of NLRP3 (Halle et al., 2008, Hornung et al., 2008, Dostert et al., 2009, Gross et al., 2009, Joly et al., 2009, Riteau et al., 2012). The reasons for these discrepancies might be differences in cell models being used (Gross et al., 2009, Joly et al., 2009) and possible off-target effects (targeting also other cellular proteases) by the inhibitor being used (CA-074-Me) (Newman et al., 2009). It is also possible that cathepsin B ablation in knockdown models is compensated by alterations in the functions of other cellular cathepsins. To summarize, our results confirmed that lysosomal destabilization is needed for β-glucan induced NLRP3 activation. However, in the future, it would be beneficial to clarify the role of cathepsin B in β-glucan induced NLRP3 activation, and exclude the possible effect of other proteases with additional methods.

The production of ROS is a conserved reaction of macrophages and it is associated with phagocytosis of the pathogen. In addition, ROS are also involved in sensing stress and cellular damage, thus suggesting that the release of ROS may be a potential signal for activating the inflammasome. Many known NLRP3 activators such as microbial toxins and crystalloid substances have been shown to trigger the formation of ROS (Dostert et al., 2008, Martinon et al., 2009, Schroder and Tschopp, 2010, Zhou et al., 2011, Latz et al., 2013). There are previous studies that ROS, especially those released from mitochondria, are involved in the activation of the NLRP3 (Dostert et al., 2008, Zhou et al., 2011, Heid et al., 2013). In our experiments, inhibition of ROS abrogated the β-glucan induced IL-1β release, pointing to a role for ROS in β-glucan induced inflammasome activation.

However, several studies have questioned the role for ROS or mitochondrial perturbation in the activation of NLRP3 (Munoz-Planillo et al., 2013, Allam et al., 2014). Instead, there are studies implying that these NLRP3 triggering substances, which have been described to activate the inflammasome via lysosomal disruption or ROS, could share a common signal for activating the NLRP3: potassium efflux (Petrilli et al., 2007, Munoz-Planillo et al., 2013). This suggests that compromised membrane integrity could represent the common feature leading to activation of NLRP3 since this has been observed with almost all stimuli examined thus far. In addition in our studies, β-glucans induced NLRP3 activation via the K⁺ efflux dependent mechanism. Recently, it has been postulated that potassium efflux could act as the principal trigger for NLRP3 activation in both canonical and noncanonical pathways (Rivers-Auty and Brough, 2015). Suppression of potassium efflux blocked the noncanonical activation pathway of NLRP3, by diminishing the release of IL-1β triggered by transfected LPS (Rivers-Auty and Brough, 2015). The exact mechanism to explain why this change in ion balance affects the NLRP3 assembly and activation via canonical and noncanonical pathways still remains to be determined.
Pyroptosis, the caspase-1 or mouse caspase-11 (caspase-4/5 in humans) activity dependent form of cell death is triggered by many canonical and noncanonical inflammasome activating substances (Lamkanfi and Dixit, 2014). The activation of caspase-1 is needed for the production of active inflammatory cytokines such as IL-1β, but occasionally it can lead to pyroptosis, characterized by rupture of the plasma-membrane and leakage of proinflammatory intracellular contents (Bergsbaken et al., 2009). Interestingly, in our studies, β-glucan induced a robust secretion of proinflammatory cytokines, as also intracellular proteins, but no signs of active cell death were observed with assays measuring the levels of cell death. One possible explanation may be the robust secretion of growth factors (GM-CSF, M-CSF) during the β-glucan stimulation (Article II, Figure 4). In addition to their role in the myeloid developing process, GM-CSF and M-CSF are essential cytokines regulating the survival and function of macrophages, both in steady-state and during inflammation (Hamilton, 2008, Louis et al., 2015).

This observation makes β-glucan an even more interesting study target compared to other non-infective microbial components since it on its own provides all of the signals, which are needed for activation of NLRP3 and secretion of proinflammatory cytokines. At the same time, it boosts the viability of the macrophages preventing them from undergoing pyroptosis and presumably, in that way, it strengthens the level of the immune reaction.

6.2. Unconventional protein secretion as an innate immune response to the β-glucans

Production and secretion of proteins in macrophages are regulated at many levels within the cell such as gene transcription, protein synthesis, and intracellular protein trafficking.

In our study, we analyzed the global transcriptional and secretional responses to β-glucans (curdlan or GBY) in human macrophages. LPS, a TLR4 ligand was used as a control, representing a well-known inflammatory microbial structure unrelated to fungi. We demonstrated that the activation of dectin-1 pathway induced significant changes in gene expression and robust protein secretion utilizing both conventional and unconventional protein secretion pathways. LPS induced also significant expression of genes, which was different from the type of expression activated by β-glucans.

The level of protein secretion after LPS stimulus was less robust compared to that seen with the β-glucans and the most extensively secreted proteins were mainly classical agents such as chemokines and cytokines, which are primarily regulated at the level of gene expression. This is line with the previous results of Meissner and co-workers where two third of the secreted proteins were reported to possess a signal peptide or transmembrane region after LPS stimulus in human
There was a notable group of proteins among the β-glucan-induced proteins; these are reported to be released through extracellular vesicles (EVs). This vesicle-mediated form of unconventional secretion is assumed to deliver signaling molecules more efficiently to adjacent cells than conventional secretion, where the proteins readily diffuse throughout the extracellular milieu (Record et al., 2011). One major group of inflammatory proteins identified in our β-glucan secretome data were the proteins that have been demonstrated or suggested to act as danger-associated molecular pattern molecules (DAMPs) (e.g., galectins, heat shock proteins, HMGB-proteins, S100-proteins) (Gallucci and Matzinger, 2001, Bianchi, 2007).

DAMPs are host molecules, which normally have a well-defined intracellular function, but they are released or become exposed following a tissue injury, cell death or a stress. Apart from their passive release during cell injury and death, many of the endogenous danger signal proteins are known to be secreted from activated inflammatory cells through the unconventional pathways (Bianchi, 2007). Caspase-1 activation related cell death, pyroptosis, is linked to leakage of intracellular proteins into the extracellular space (Bergsbaken et al., 2009). However, no signs of active cell death were observed during the stimulation evoked by β-glucan, therefore the identification of large amount of the DAMPs in the β-glucan secretome was considered to reflect the active secretory processes occurring in β-glucan-induced macrophages. All the DAMPs identified in the β-glucan secretome, except for HMGBs, can be found in the database of ExoCarta (Article II, Supplemental Table IIIB, link to ExoCarta). This confirms that these particular DAMPs are secreted in exosomes utilizing unconventional secretion mechanisms. Galectin-3 was one of the DAMPs, the secretion of which was robustly activated after β-glucan stimulus. Galectin-3 can facilitate chemoattraction and elicit the oxidative burst in leukocytes, and act also as a PRR, which recognizes the carbohydrate structures on the cell wall of Candida albicans (Sato et al., 2009). In addition, a recent report revealed that galectin-3 directly associates with dectin-1 and has an essential role in proinflammatory response induced by pathogenic C. albicans (Esteban et al., 2011). It is likely that the β-glucan-induced active secretion of DAMPs leads to enhanced antifungal defense e.g. DAMPs can act as chemottractants, they can also activate signaling of PRRs (dectin-1, TLRs, RAGE) or even boost the adaptive immunity response by binding to antigenic peptides and facilitate their transport to the antigen presenting cells (Srivastava, 2002, Bierhaus et al., 2005, Sato et al., 2009, Esteban et al., 2011).

In our study, the other major group of proteins for which β-glucan clearly induced secretion were proteins involved in leukocyte migration. These proteins included a significant amount of adhesion proteins such as integrins and proteins that regulate the reorganization of the actin cytoskeleton. This robust release of integrins and related cytoplasmic cargo proteins in extracellular vesicles upon β-glucan stimulation of macrophages has also been confirmed by Cypryk and co-
workers (Cypryk et al., 2014). These proteins are vital for the surveillance functions of leukocytes, especially for their ability to migrate from blood to tissue into site of infection. In addition, the same proteins are believed to mediate the interaction between the secreted vesicles and recipient cells, and thus to facilitate cell-cell communication (Thery et al., 2009). The interaction between the vesicle proteins and cell surface of the recipient cell may lead to the fusion of the membranes and to the release of vesicle cargo into the cytoplasm of the recipient cell, which then can affect the function of the recipient.

The main cell model used in our studies was the human GM-CSF-induced monocyte-derived macrophages. The phenotype of GM-CSF-macrophages has been reported to resemble one of the human alveolar macrophages (Akagawa et al., 2006), which normally take care of the cleansing of inhaled noxious particles in the alveolar space of the lung. However, there are some discrepancies encountered with the GM-CSF–macrophage model, implying that it better represents dendritic cells than macrophages. Nonetheless, based on the results obtained from bioinformatics analyses of macrophage and dendritic cell transcriptomes (Robbins et al., 2008, Crozat et al., 2010), it has been postulated that GM-CSF-generated cells are closer to macrophages than dendritic cells. M-CSF–generated macrophages have been widely used as a representative in vitro model for tissue macrophages (Martinez et al., 2006, Way et al., 2009). It was reported that mouse bone marrow-derived macrophages, cultured in the presence of M-CSF growth factor, were not responsive to β-glucans, because the dectin-1-CARD9 signal route failed to activate NF-κB (Goodridge et al., 2009a); these results favored the usage of the mouse GM-CSF-generated dendritic cells in β-glucan studies.

The present study compared the β-glucan-induced protein secretion between the human GM-CSF-differentiated macrophages and M-CSF-differentiated macrophages. The protein markers of exosomal secretion were more abundant in β-glucan induced GM-CSF macrophages, as were also cytokines of IL-1 family, which are also known to be secreted via unconventional pathways. These results are in line with the knowledge that M1-type macrophages, so-called classically activated macrophages, usually mount a strong inflammatory reponse against microbial ligands (Wynn et al., 2013). Mimics of these M1- macrophages can be derived from monocytes by culturing them with GM-CSF. In contrast, M2-type macrophages, so-called alternatively activated macrophages, are typically thought to participate in the re-establishment of homeostasis and are involved in the suppression of inflammatory responses (Wynn et al., 2013). The growth factor M-CSF is used to aid in their culturing in vitro. One reason for more efficient protein secretion in GM-CSF macrophages can be that GM-CSF enhances the expression and function of dectin-1 in GM-CSF-differentiated macrophages (Willment et al., 2003, Serezani et al., 2012). In addition, it is believed that the GM-CSF provides the additional signal for β-glucan by boosting its immunomodulatory activity and capabilities to initiate a robust cytokine and chemokine response (Min et al.,
According to our results, human monocyte-derived GM-CSF-macrophages represent an optimal cell model for \textit{in vitro} studies investigating the inflammatory response induced by β-glucans.

Activation of vesicle-mediated unconventional protein secretion has been reported to occur after exposure to several pathogens and disease-associated substances such as influenza A (Lietzen et al., 2011), herpes simplex virus 1 (Miettinen et al., 2012) and monosodium urate crystals (Välimäki et al., 2013). Our study revealed that also β-glucan activates robust secretion of proteins via vesicle-mediated unconventional pathways. This was confirmed in the study of Cypryk and co-workers, where they characterized in greater detail, the proteomics of vesicles released during the β-glucan stimulation in human macrophages (Cypryk et al. 2014) Our results (Article I), in conjunction with other reports (Kumar et al., 2009), have highlighted that β-glucans are potent activators of the NLRP3 inflammasome. MSU has also been reported to activate NLRP3 inflammasome and induce release of IL-1β (Martinon et al., 2006). LPS provides only the first priming signal for activation of NLRP3, and is not capable of delivering the second signal for activation of NLRP3 inflammasome in human macrophages (Bauernfeind et al., 2011). Thus, it is convenient to speculate that activation of the NLRP3 inflammasome is required for activation of vesicle-mediated unconventional protein secretion. Indeed, active caspase-1 has been shown to be a regulator of unconventional protein secretion (Keller et al., 2008). In our study, the inhibition of caspase-1 in β-glucan-induced macrophages abolished the release of unconventionally secreted proteins, IL-1β, and also inhibited that of exosome-transported tubulin and annexin I. Caspase-1 inhibition had no effect on conventionally secreted proteins (TNF, chemokines), indicating that activation of the inflammasome via the dectin-1 pathway triggers unconventional secretion, but is not essential for the release of classically secreted proteins. The secretion of the inflammasome’s central components is followed by inflammasome activation (Keller et al., 2008). This was in line with our results, where β-glucan induced secretion of caspase-1 and ASC, and cathepsins B and D, i.e. core components and regulators of NLRP3 inflammasome, respectively. Further analysis revealed that the mature forms of cathepsins had been secreted in exosomes after the β-glucan stimulus. Thus, exosomes may facilitate the transport of these active proteases to adjacent cells and result in NLRP3 inflammasome activation also in these recipients.

To confirm the role of the dectin-1 signaling pathway in β-glucan induced unconventional secretion, the participation of dectin-1 receptor, and Src and Syk kinases downstream of dectin-1 were investigated. The experiment with bone marrow-derived dendritic cells from wild and dectin-1 knockout mice revealed that secretions of mature forms of IL-1β and cathepsins were dectin-1 dependent. Src-family kinases have been shown to act upstream of Syk and this kinase signaling pathway has been linked to NLRP3 inflammasome activation (Shio et al., 2009, Kerrigan and Brown, 2010, Hara et al., 2013, Lin et al., 2015). Inhibition
of Syk kinase completely prevented the β-glucan-induced secretion of IL-1β, and via vesicles-secreted mature forms of cathepsins, tubulin and annexin. In contrast, the results obtained after Src inhibition revealed that Src kinases have only a minor role in the β-glucan-induced unconventional secretion. Thus indicating that Syk can act independently of Src and it has a more crucial role in signaling pathway, which triggers the unconventional secretion after dectin-1 activation.

To conclude, dectin-1/Syk kinase-signaling pathway and inflammasome activity are essential for β-glucan-induced unconventional protein secretion.

Recently, it was demonstrated that autophagy participates in the regulation of unconventional protein secretion (Ponpuak et al., 2015). Autophagy is an evolutionary conserved process involved for removal of long-lived proteins, insoluble protein aggregates or dysfunctional organelles. This kind of selective autophagic degradation is activated in response to these latter mentioned components or alternatively by the other cellular stress factors such as cytosol-invasive bacteria and viruses (Kuballa et al., 2012). During starvation, when the amount of nutrients is limited, autophagy is activated, leading to cytoplasmic autodigestion (Rubinsztein et al., 2012). Autophagy is believed to be involved in the defense of many bacterial infections, however, very little is known about its role in antifungal defense. The process through which autophagy affects protein secretion is called secretory autophagy (Ponpuak et al., 2015). This secretory autophagy is known to facilitate unconventional secretion of the leaderless proteins such as IL-1 family cytokines, DAMPs, or cytoskeletal proteins (Ponpuak et al., 2015).

Our data demonstrated that the β-glucan stimulus increased the amount of lipid-associated LC-II, which is located on autophagy membranes and is a well-known marker of an activated autophagic process. In addition, the activation of dectin-1 elicited a robust secretion of autophagy-associated proteins. These results indicate that the dectin-1/syk -signaling pathway activates autophagy. Furthermore, inhibition of autophagy after β-glucan treatment blocked IL-1β release and the other proteins known to be secreted via unconventional routes. However, inhibition of autophagy did not affect the β-glucan-induced conventional protein secretion in this study. In line with our results, it has recently been shown that unconventional secretion of IL-1β requires secretory autophagy-related ATG factors, which are involved for the biogenesis of autophagic membranes (Dupont et al., 2011). In addition, another recent study detected the enhanced secretion of IL-1β in cells, where the autophagic process was induced by starvation, and the cells were treated by known NLRP3 inflammasome activators (i.e. alum, amyloid-β fibrils, nigericin, or silica fibrils) (Dupont et al., 2011). However, there are also reports which indicate that autophagy negatively regulates secretion of IL-1β and suppresses the activation of the inflammasome. The autophagic process has been shown to prevent release of ROS or mitochondrial DNA by maintaining the mitochondrial homeostasis and in this way, it suppresses the organelle stress-mediated activation of inflammasome (Zhou et al., 2011, Saitoh and Akira, 2016).
In addition, the autophagic process negatively regulates the activation of the inflammasome and inhibits the IL-1β release by targeting the ubiquitylated inflammasome components and IL-1β for autophagic degradation (Harris et al., 2011, Shi et al., 2012, Saitoh and Akira, 2016). In addition, a deficiency in the autophagic process in myeloid cells has been shown to evoke an aberrant activation of the inflammasome and to promote the development of inflammatory diseases (Saitoh and Akira, 2016).

These recent studies emphasize the dual role for autophagy in the regulation of inflammation. First, it is required for secretion of proteins via the unconventional pathway, second, it can reduce the inflammatory response by targeting the components and substrates of the inflammasome so that they are broken down by degradation.

In conclusion, autophagy has a role in antifungal defense by affecting the unconventional protein secretion and secretion of inflammatory factors induced via dectin-1 signaling pathway.

6.3. The pro-inflammatory response of IL-1 cytokines induced by β-glucans

Members of the interleukin-1 family are the main pro-inflammatory cytokines in innate immunity but this family also has members with anti-inflammatory features (Garlanda et al., 2013). The most widely studied and best known IL-1 cytokine is the highly inflammatory IL-1β. It triggers inflammation in multiple ways by mediating the development of fever, vasodilation and recruitment and function of leukocytes. Our study, as well as others, has shown IL-1β to be one of the cytokines released during the exposure of fungal component (Article I)(Kumar et al., 2009), which plays a crucial role in the immune response against fungal infections (Vonk et al., 2006, Sainz et al., 2008, Gross et al., 2009, Hise et al., 2009, Lev-Sagie et al., 2009). IL-1β, with other IL-1 family members, IL-1α and IL-36, has been reported to participate in the induction of adaptive immunity and development of Th17 cells, which are involved in the host’s protective immunity against fungi (Gresnigt et al., 2013, Underhill and Pearlman, 2015).

Cytokines of the IL-1 family exert widescale effects and thus their production and signaling are tightly regulated at many levels (Palomo et al., 2015). In our study, we focussed on the proinflammatory agonists of IL-1 family (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and in their expression after a β-glucan stimulus.

The mRNA levels of IL-1α, IL-1β and IL-36γ were increased in both GM-CSF- and M-CSF-derived macrophages after β-glucan treatment. Accordingly, the induction of IL-36γ was also observed previously in human PBMCs after infection with fungi (Gresnigt et al., 2013). In contrast, β-glucan induced the transcription of
IL-33 only in GM-CSF- macrophages. Interestingly, this recently identified member of the IL-1 family is associated with immune cell polarization towards an allergic response and the Th2 direction as well as promoting the M2-polarization of macrophages (Schmitz et al., 2005, Oboki et al., 2010), whereas cytokines IL-1α/β and IL-36 are more linked to the Th1-and Th17 responses.

The importance of dectin-1 signaling in β-glucan induced transcription of IL-1 genes was revealed in an in vivo-model utilizing mice with wild type or dectin-1 knockout genotype. Curdlan exposure via the intranasal route induced transcription of IL-1α, IL-1β and IL-36γ cytokines in BAL cells sampled from these animals. A deficiency of dectin-1 decreased the levels of β-glucan-induced IL-1 cytokines, but did not block completely the transcription, suggesting that also receptors other than dectin-1 may be involved in the induction of IL-1 family cytokines after β-glucan exposure.

In addition, the role of dectin-1 signaling in β-glucan induced IL-1 expression was studied in human GM-CSF- macrophages by inhibiting the function of Syk kinase. This blocked totally the β-glucan activated transcription of IL-1α, IL-1β and IL-36γ. This indicates that the gene transcription of IL-1 family cytokines triggered by β-glucan is predominantly dependent on the dectin-1/Syk pathway.

Pro-inflammatory cytokines of IL-1 family lack an N-terminal signal sequence and thus are not secreted via the conventional route involving the endoplasmic reticulum-golgi pathway (Sims and Smith, 2010, Garlanda et al., 2013). According to our results, the secretion of IL-1 family cytokines was much higher in GM-CSF -macrophages compared to M-CSF -macrophages after β-glucan stimulus. The secretion of both IL-1β and IL-18 are known be controlled by activation of the inflammasome and caspase-1 (Martinon et al., 2002, Bauernfeind et al., 2011). Caspase-1 has also been associated with the regulation of unconventional protein secretion (Keller et al., 2008). In addition to IL-1β and IL-18, secretion of central components of the inflammasome has been reported to follow the activation of the inflammasome (Qu et al., 2007, Keller et al., 2008).

In our study, GM-CSF –macrophages were more efficient than M-CSF- macrophages at secreting IL-1β, IL-18 and the active form of caspase-1 in their response to the β-glucan stimulus. These results are evidence of more efficient activation of the inflammasome in GM-CSF-macrophages after β-glucan recognition. Interestingly, there were no clear differences after β-glucan treatment in the levels of intracellular NLRP3, ASC, or in the preform of IL-1β between the cell types. The preform of IL-18 was even more abundant in M-CSF-marophages. In contrast, the inactive form of caspase-1 (p45) was considerably more abundant in GM-CSF-macrophages. This may explain the more powerful outcome of GM-CSF-macrophages with respect to the secretion of active caspase-1 and IL-1β and IL-18.

Furthermore, β-glucan induced a clear expression of GBP5 protein in GM-CSF-macrophages, which may boost the activation of the NLRP3 inflammasome. GBP5 are guanylate-binding proteins, which belong to the family of interferon-
induced GTPases (Kim et al., 2012). They have recently been reported to be regulators of canonical and noncanonical inflammasome activation and to be an important component of host defense (Kim et al., 2012, Kim et al., 2016). GBP5 is known to facilitate the activation of NLRP3 inflammasome in a response to soluble material and live bacteria, but not to particulate matter (Shenoy et al., 2012). It has been proposed that GBP5 affects the inflammasome activation via a physical interaction. GBP5 has been demonstrated to bind to NLRP3 and promote the formation of the inflammasome complex through its own self-assembly (Shenoy et al., 2012). There is another theory suggesting that GBPs lyse either pathogen-containing vacuoles or bacteria directly to release ligands for recognition by central components of canonical or noncanonical inflammasome activation pathways (Kim et al., 2016). Although NLRP3 responses to particulate and sterile DAMPs such as alum and monosodium urate are largely unaffected by IFN-γ and seem to be regulated without GBP5 (Shenoy et al., 2012, Kim et al., 2016), there is a need to clarify the role of GBP5 in β-glucan induced NLRP3 inflammasome response since β-glucan represents a particulate-like PAMP.

GM-CSF is a well-known growth factor recently shown to possess pro-inflammatory features. It was reported to enhance the expression and function of dectin-1 (Willment et al., 2003, Min et al., 2012, Serezani et al., 2012). The basal level of dectin-1 was higher in GM-CSF-macrophages than in M-CSF-macrophages and this may partially explain the different responses between the macrophage types in our study. Recently, it was demonstrated that GM-CSF could elevate the expression of Rab39a (Khameneh et al., 2011), the GTPase, which is involved in caspase-1 dependent IL-1β secretion (Becker et al., 2009). Thus, GM-CSF was speculated to enhance the exocytosis and unconventional secretion of IL-1β (Khameneh et al., 2011). In addition, Khameneh and co-workers suggested that GM-CSF could enhance the synthesis of the preform of IL-1β and in that way, it could modulate the level of secretion by strengthening the LPS-induced NF-kB signaling. A similar kind of synergism has been revealed also between the dectin-1 and GM-CSF in mouse dendritic cells (Min et al., 2012). However, there were no dramatically differences between the two human macrophage types examined here with respect to either β-glucan-induced gene transcription or the production of preforms of IL-1β and IL-18.

Most of the IL-1 cytokines need to be cleaved in their N-terminal region before they can achieve their full biologically active form capable of inducing cellular responses via IL-1 receptors. The pro-peptides of IL-1β and IL-18 must be cleaved by caspase-1 enzyme, and its function is triggered by the activation and assembly of inflammasome (Martinon et al., 2002, Bauernfeind et al., 2011). Cytokines of IL-36 do not contain caspase-1 cleavage sites, however recently it was shown that artificial N-terminal truncation clearly increased the proinflammatory activity of these cytokines (Towne et al., 2011). It is still uncertain if the endogenous IL-36 protein undergoes maturation in humans during pathological conditions. Thus, we studied the secretion and processing of IL-36γ
protein, in parallel to those of IL-1β and IL-18, after β-glucan treatment in human GM-CSF-and M-CSF-macrophages. The secretion and intracellular production of IL-36γ were more efficient in β-glucan-induced GM-CSF-macrophages. The molecular weight was the same for the secreted and the intracellular forms of IL-36γ, indicating that β-glucan-induced IL-36γ is not cleaved before secretion in human macrophages. The production of a similar size of IL-36γ protein has been observed after *Mycobacterium tuberculosis* infection in human macrophages (Ahsan et al., 2016), where dectin-1 is known to be involved in the recognition of the mycobacterium (Yadav and Schorey, 2006). *M. tuberculosis* expresses α-glucan instead of β-glucan within their outer capsule, however, the exact ligand of *M. tuberculosis* for dectin-1 has not yet been identified.

The NLRP3 inflammasome is known to control the activation of caspase-1 and via the release of IL-1β (Bauernfeind et al., 2011). We determined whether inflammasome activation also plays a role in secretion of IL-1α or IL-36γ. Secretion of these cytokines was induced by β-glucan in GM-CSF-macrophages. The release of IL-1α has been associated with the activation of caspase-1, albeit independently of its catalytic activity (Gross et al., 2012). Even less is known about the impact of the NLRP3 inflammasome or caspase-1 in IL-36 secretion. The presence of particulate matter has been shown to activate the NLRP3 inflammasome and induce the secretion of IL-1β depending on cathepsin-B activity (Hornung et al., 2008, Rajamäki et al., 2010, Palomäki et al., 2011, Välimäki et al., 2013). Human macrophages preincubated with CA-074-Me secreted less IL-1α and IL-36γ after the β-glucan stimulus. We previously observed similar results with IL-1β. It has been reported that IL-1α secretion, activated by particulate matter, is blocked when cathepsin activity is impaired (Gross et al., 2012). To confirm that CA-074-Me does not exert any effect on gene transcription, we analyzed the β-glucan-induced mRNA levels of IL-1 cytokines in CA-074-Me treated macrophages. Interestingly, CA-074-Me did not have any impact on IL-1β transcription, whereas this inhibitor prevented the transcription of IL-1α and IL-36γ. Cathepsin B is a cysteine protease, which is involved in the degradation of proteins, by targeting them to the lysosomal system of the cell. According to our current knowledge, the involvement of cathepsins in the regulation of β-glucan-induced transcription of IL-1 genes is a novel observation. Cathepsins have been reported to play a role in NF-kB activation *in vivo* and *in vitro* studies (Schotte et al., 2001, Wang et al., 2013, Ni et al., 2015). Our results suggest that cathepsin B regulates the transcription of β-glucan-induced IL-1 cytokines in different ways. This may be due to its impact of NF-kB signaling or other signaling pathways, which are known to trigger IL-1 expression after dectin-1 activation.

Inflammasome NLRP3 and caspase-1 play a central role in the processing and secretion of IL-1β. Accordingly, GM-CSF-macrophages preincubated with the specific NLRP3 inhibitor, MCC950 (Coll et al., 2015) or a caspase-1 inhibitor (Z-YVAD-FMK) totally blocked the secretion of IL-1β after the β-glucan stimulus.
The caspase-1 inhibitor diminished partially and the NLRP3 inhibitor prevented completely the β-glucan-induced IL-1α response. In contrast to IL-1α and IL-1β, neither caspase-1 nor NLRP3 inhibition blocked the secretion of IL-36γ in response to β-glucans.

IL-1α is not a substrate for caspase-1, instead it is processed by calpain cysteine protease. However, the release of IL-1α has been associated with the activation of inflammasome, albeit independently of caspase-1’s catalytic properties (Gross et al., 2012). IL-1α secretion induced by ATP, nigericin toxin or viable C. albicans was shown to be dependent on the components of the NLRP3 inflammasome, whereas the secretion pathway of IL-1α induced by particulate matter was independent of the NLRP3 inflammasome (Gross et al., 2012). Our results with β-glucan suggest that the activation of IL-1α secretion is majorly inflammasome-dependent. IL-1α binds to the same receptors, IL-1RI and IL1RACP with IL-1β (Garlanda et al., 2013) and in practical terms, they share similar kinds of biological properties. In contrast to IL-1β, the cytokine IL-1α is constitutively expressed as a precursor protein in epithelial cells and is mainly released in situations of cell damage or cell death, and seldom via active secretion (Garlanda et al., 2013). The role of IL-1α in regulating the inflammatory response of lungs against fungal pathogens is less well understood than the role of IL-1β. IL-1α has been postulated to orchestrate the recruitment of leukocytes, especially neutrophils, in mice with pulmonary Aspergillus fumigatus infection (Caffrey et al., 2015). Our study revealed that IL-1α was secreted in human macrophages after β-glucan stimulus, implying that the activation of dectin-1 pathway can trigger the secretion of IL-1α during the pulmonary fungal infection.

The impaired secretion of β-glucan-induced IL-36γ observed after blockade of the activities of inflammasome and caspase-1 was consistent with a previous report; in that study, caspase-1 inhibition or its deficiency decreased the levels of produced IL-36γ after mycobacterium infection (Ahsan et al., 2016). The Ahsan and colleagues also suggested that endogenous IL-1β and IL-18 could further amplify production of IL-36γ (Ahsan et al., 2016). This elevating impact of inflammasome-regulated IL-1β and IL-18 on the synthesis of IL-36γ could thus explain the possible indirect effects of the inflammasome and caspase-1 on the secretion of IL-36γ. Cytokines of the IL-36 family are known to play a prominent role in inflammation and neutrophil recruitment in the lungs independently from IL-1α and IL-1β (Chustz et al., 2011, Ramadas et al., 2011). Furthermore, they have been associated with allergen-inflammation in the lung (Ramadas et al., 2006). The effect of IL-36 signaling in many ways is reminiscent of IL-1α and IL-1β signaling, but the expression of the IL-36 cytokines seems to more tissue-restricted. Therefore, cytokines of the IL-36 family cannot be considered to be simple substitutes for IL-1α or IL-1β. Most likely they have a role in regulation of tissue specific immune responses, which might be potentiated by endogenous IL-1β and IL-18. Our studies highlighted the induction of IL-36γ in human
macrophages in the response for fungal β-glucan. Further studies will be needed to reveal the role of IL-36γ in fungi-induced lung inflammation.

6.4. Proteomic profiles of alveolar lining fluid are different in the illnesses associated with exposure to non-infective microbial particles

To obtain information from real-life examples of microbial exposures, we investigated the proteomic changes associated with immune defense of the lung in NIMP–related diseases. We also attempted to identify possible diagnostic markers for diseases related to exposure of inhaled NIMPs.

The proteomic profile of BAL collected from individuals with exposure to NIMPs in the context of DBRI or AME was studied. Patients in this latter group manifested HP-like symptoms. BAL samples from patients with a diagnosis of acute type of HP were used as a reference. Samples from SARC patients served as a reference for inflammatory lung disease with no direct association with NIMP exposure. In addition, BAL from healthy individuals served as controls.

Our results from the proteomic analysis revealed a substantial difference in protein expression in bronchoalveolar lining fluid between DBRI and HP / HP-like conditions. The pattern of protein expression in DBRI resembled more the patterns of healthy controls and SARC than that observed in HP and AME. A part of the explanation could be that exposures of microbial dust are usually known to be less intense among individuals with DBRI than with HP. The symptoms commonly reported for HP are fever, cough and dyspnea (Hendrick et al., 2002). In contrast, the symptoms related to DBRI are very variable including asthmatic symptoms associated with proximal airways or irritation of mucous membrane in the nasal area in the upper airways and irritation of eyes and skin have also been reported (WHO, 2009). Generally, HP is an illness known to preferentially affect the alveolar space of the lung, while DBRI seems to have a greater impact in the proximal airways. It seems that these two diseases affect the alveolar space to different extents, which serves as one further explanation for the differences seen in the protein expression patterns between HP and DBRI. Thus our proteomic results suggest that the DBRI, at least with respect to its typical clinical presentation, is different from HP. The symptoms related to DBRI typically develop after prolonged exposure to the conditions present in water-damaged buildings. It is possible that the proteomic pattern from the initial phase of DBRI could display greater similarities with the acute type of HP, especially because it is known that conditions of damp building can sometimes cause clinical HP (Enriquez-Matas et al., 2007).

Four different proteins (alpha-1-antitrypsin, galectin-3, histone 4/2B, semenogelin I) were analyzed in more detail according to their well-known status
in inflammation or their novelty value in lung inflammation. All of these proteins were detected with reasonable robustness in the disease groups in contrast to the healthy controls, although none of them seemed to be a specific biomarker for NIMP-exposure related diseases.

The expression of alpha-1-antitrypsin and galectin-3, which are considered as markers of inflammation, was increased in BAL collected from all conditions, DBRI, HP and SARC. Alpha-1-antitrypsin (A1AT) is a traditional and well-known biomarker for lung inflammation. A1AT protects lungs against proteolytic destruction by inhibiting the activities of serine proteases such as leukocyte elastase (Greene et al., 2008). In addition, it has also been reported to have other anti-inflammatory and tissue-protective properties (Petrache et al., 2006, Pott et al., 2009). A1AT deficiency is known to associate to hyper-responsiveness of the lung in cases when there has been organic dust exposure (Sigsgaard et al., 2000). Different pulmonary exposures increase levels of A1AT (Gomzi et al., 1989) and elevated levels have been detected in BAL of patients with interstitial lung disease (Wattiez et al., 2000, Kriegova et al., 2006, Okamoto et al., 2012).

The other inflammatory protein detected, galectin-3, has been linked to inflammation and microbial infection in lungs. In addition, it has been reported to play a role in the development of lower airway hyperresponsiveness (Zuberi et al., 2004, Ge et al., 2010). Galectins are beta-galactoside binding lectins (Barondes et al., 1994), which are released passively from the cytosol of dying cells or actively secreted by immune cells during inflammation (Sato et al., 2009). This active secretion of galectin-3 is known to utilize the unconventional routes of protein secretion (Bianchi, 2007). Interestingly, in our previous study, we demonstrated that β-glucan could activate a robust unconventional protein secretion and galectin was one of the molecules, the secretion of which was induced by β-glucan in human primary macrophages (Article II). Galectin-3 is involved in many processes activating innate immune response (e.g. chemoattraction of leukocytes, activation of oxidative burst in neutrophils (Yamaoka et al., 1995, Sano et al., 2000). In addition to its function as a damage-associated molecular pattern (DAMP), it can also act as a PRR, which binds to the glycan structures of microbes (Sato et al., 2009). This increased expression of A1AT and galectin-3 suggests that an inflammatory response has been activated at some level in all of these conditions, both in interstitial lung diseases and in diseases associated with the exposure to NIMPs. This is in line with earlier published findings with HP, which detected an increase in macrophage numbers and the levels of cytokine IL-1β in BAL of HP patients (McSharry et al., 2002).

In our proteomic study, we investigated another DAMP capable of triggering inflammation, i.e. histone. Histones form the structural unit and component called the nucleosome with nucleotides (Kornberg and Lorch, 1999) and are located in the nuclei of eukaryotes. High levels of histones in blood have been detected in diseases associated with enhanced cell death (Holdenrieder and Stieber, 2009). Histones play a role in hyperinflammatory syndromes, and have been shown to
mediate death in a mouse model of sepsis (Xu et al., 2009, Huang et al., 2011). In lung diseases, elevated levels of histones have been observed in patients with chronic obstructive pulmonary disease (COPD) (Hacker et al., 2009). In our study, the expression of histone H4 was increased in the BAL samples in all disease groups other than DBRI.

In plasma, where samples were available from CTR, HP and SARC, the levels of histone H2 were elevated in two groups, HP and SARC. This may indicate that H2B could be viewed as a marker for the inflammation in the lungs. In the future, it would be interesting to analyze histones in blood also from DBRI patients and examine if this inflammatory protein could be detected with an elevated expression also in that group. Based on our results of a high level of histone expression in BAL and plasma samples obtained from HP and SARC patients, it can be speculated that histones may play at least some role in the pathogenesis of interstitial lung diseases. The molecular weight of the detected histones in BAL samples was aberrant. This might be due to alterations in normal post-translational modifications such as phosphorylation, acetylation, methylation and ubiquination. These are processes which regulate chromatin structure and gene expression. It needs to be clarified whether factors leading to development of interstitial lung disease can cause the appearance of aberrant size histones in BAL fluid and possibly altered activation of post-translational modification.

One of the novel findings in this proteomic study was the detection of high levels of semenogelin protein in BAL of HP, AME and SARC patients. Semenogelins I and II are the major proteins secreted from glandular epithelium and epididymis; they are involved in the formation of the gel-like structure of semen (Lilja, 1985). Fragments of semenogelin have been reported to possess also antimicrobial effects and they also display a high zinc ion binding capacity (Bourgeon et al., 2004, Jonsson et al., 2005, Edström et al., 2008). Thus far, it seems that our proteomic study is the first to have identified semenogelin in BAL fluid of two groups of patients i.e. those with SARC and those exposed to inhaled non-infectious microbial particles. If one considers the reported features of semenogelin, this protein may play a role in the regulation of mucus viscosity. For example, deficient mucus clearance in the airways and lungs has been associated with microbial infections and other respiratory diseases (Randell et al., 2006). Another putative role for semenogelin might be in the regulation of zinc homeostasis. The perturbation of zinc homeostasis has been linked with allergic inflammation (Truong-Tran et al., 2002). In addition, it has been reported that airway epithelial cells are highly susceptible to oxidant-induced apoptosis when labile zinc is depleted (Carter et al., 2002, Bao and Knoell, 2006).

Taken together, our results showed that the proteome of alveolar lining fluid in DBRI was different from HP/HP-like conditions thus suggesting they represent different disease entities. We did not find that the expression of any of the studied proteins could be considered as specific for a NIMP-associated illness. However, one interesting and novel finding was the increase in the levels of two well-known
markers of inflammation in the alveolar lining fluid of DBRI patients, indicating that there is an activation of inflammatory mechanisms also in this condition.
Fungi are ubiquitous in the environment and belong to our normal microbiological flora. Sometimes, fungi manage to cause an infection due to an alteration in the microbiota, breach in the integument barrier or since the individual is receiving immunosuppressive therapy. Exposure to the non-infective components of fungi or other microbes such as can occur in agricultural work or water-damaged buildings is also sometimes associated with illnesses.

This thesis focused on the characterization of the immune reaction triggered by fungal components at two different levels. First, the innate immune response and especially, inflammation related mechanisms activated by the fungal cell wall component, (1,3)-β-glucan, were studied in the key innate immune defence cell, the macrophage. Second, the immune response of lungs were characterized by studying the proteomic changes in bronchoalveolar lavage collected from patients with illnesses related to exposure of fungal and other microbial components.

One major result of the study was that β-glucan stimulates a strong response of cytokines from the IL-1 family in human macrophages. These cytokines are crucial inducers of local and systemic inflammation, thus their secretion is highly regulated. The secretion of IL-1β requires the activation of two signaling pathways, the first via a membrane-bound receptor and the second through an intracellular protein complex, the inflammasome. This is the first evidence indicating that on its own β-glucan could trigger both of these pathways in human macrophages, via activation of the dectin-1/ Syk –signaling pathway and the NLRP3 inflammasome. Beta-glucan-induced activation of NLRP3 inflammasome was stimulated by the production of ROS, cathepsin release and potassium efflux. Today, these findings from our group’s first β-glucan related study have been widely cited (Article I). In the latest study in this series, we demonstrated that β-glucan-induced the secretion of two other IL-1 family members, IL-1α and IL-36γ, and this process was totally blocked by inhibition of cathepsin B. However, the secretion of IL-36γ was not dependent on the NLRP3 inflammasome. In summary, our results suggest that IL-1 cytokines play a role in the inflammatory response induced by fungi, even when the activation of inflammasome is impaired.

Beta-glucan induced a robust secretion of many classically secreted proteins such as chemokines and cytokines, which contributes to the innate immune response during a microbial infection. Furthermore, an extensive secretion of proteins via the unconventional secretion pathway was activated by β-glucan in human macrophages. In addition to the IL-1 cytokines, dectin-1 activation led to the efficient secretion of vesicle-mediated proteins, which are known to mediate inflammation, such as danger-associated molecules or cell adhesion and migration.
proteins. It has been postulated that if molecules are secreted via vesicles, then they reach the target in adjacent cells more efficiently than classically secreted proteins, which have to diffuse throughout extracellular milieu. Despite the efficient activations of protein secretion and the inflammasome, the latter structure being known to facilitate pyroptosis, no extensive cell death was observed during the β-glucan treatment. This indicates either the presence or the activation of factors sustaining the viability of human macrophages. Further investigations will be needed to characterize these factors; they could well provide us with useful tools for influencing the direction of the response of the immune system.

Inhibition of inflammasome activity or the process of autophagy suppressed dectin-1-induced IL-1β and vesicle-mediated protein secretion. Autophagy is an evolutionary conserved lysosomal pathway involved in the degradation of cellular substances during starvation; under normal conditions, it may function as an intracellular disposal process. Selective autophagic degradation is emerging as a part of antimicrobial defense; this phenomenon is activated in response to cytosol-invasive bacteria and viruses or other cellular stress signals. Our results indicate that autophagy is activated during β-glucan stimulation and it acts through the unconventional secretion of proteins, evidence that the process of autophagy is one of the mechanisms regulating the immune response against fungi in human macrophages.

The function of macrophages is affected by many factors present in the environment, some potentiating, some counteracting their effects. Colony-stimulating factors are long-known for their ability to generate mature myeloid cells from precursor cells. Recently, they have been shown to be also essential cytokines, regulating the survival and function of macrophages, both under steady-state conditions and during inflammation. In our study, the GM-CSF-generated macrophages demonstrated a more efficient secretion of IL-1 and other unconventionally secreted proteins than M-CSF-generated macrophages in response to the stimulation with β-glucan. The more abundant expression of procaspase-1 and GBP5, which are the central component and the regulator of NLRP3 respectively, in β-glucan-stimulated GM-CSF-macrophages may explain the difference between these cell types. These results imply that GM-CSF is one of the cytokines boosting the inflammatory response of macrophages activated by β-glucan. There is already some preclinical data suggesting that manipulating the biology of colony-stimulating factors might be beneficial in inflammatory and autoimmune diseases (Wicks and Roberts, 2016). Further investigation will be needed to unravel the role of colony-stimulating factors in fungal-related diseases and if they will confer any therapeutic benefits.

The results (Figure 6.) highlight the importance of β-glucan as a crucial immunomodulatory structure of fungi and signaling pathways of dectin-1 and the NLRP3 inflammasome in the immune response triggered by fungi. Furthermore, these results favor the exploitation of β-glucan as a potential adjuvant in vaccines or treatments where the immune response needs to be enhanced.
Figure 6. The activation of the inflammatory response by fungal component, 1,3-β-glucan in human macrophages. The detection of β-glucan by dectin-1 tiggers significant transcriptional changes and activates both conventional and unconventional secretion of inflammation promoting factors. The unconventional protein secretion induced by β-glucan was affected by activation of NLRP3 inflammasome, active autophagy process and myeloid growth factors (GM-CSF, M-CSF).

Inflammatory symptoms have been described in individuals who have been exposed to fungal components when handling moldy hay in agricultural work or who have worked or lived in water-damaged houses where fungal components can be found in the building materials and the indoor air. We studied the proteomic changes present in bronchoalveolar lavage collected from patients exposed to fungi in these kinds of surroundings, and who were manifesting symptoms of acute type of HP or symptoms associated with DBRI. The proteomic profiles of HP and DBRI were different, the proteomic changes being more robust in HP than in DBRI when compared to healthy controls. We were able to differentiate between these two conditions both of which are associated with an exposure to microbial particles and at the same time, we observed increases in the levels of two markers of inflammation (α-1-antitrypsin, galectin-3) in both HP and DBRI. In addition, an increase in a novel protein, semenogelin, was detected in samples of HP and SARC. It could be argued that its main role might be to regulate mucus viscosity,
which is an important factor in the clearance of inhaled matter from the respiratory tract.

This thesis attempts to clarify the immunological mechanisms behind the symptoms experienced by the individuals, who have been exposed to fungal components. Its results provide novel knowledge about the inflammatory response triggered by the major cell wall structure of fungi, β-glucan, which is an immune-stimulating ligand; cells are exposed to this compound not only during fungal infections but also if exposed to noninfectious fungal components. When macrophages encounter β-glucan they activate different secretory pathways and secrete copious amounts of chemokines, DAMPs and multiple members of IL-1 family cytokines, highlighting the importance of fungal components in this regard. We were also able to study the inflammation induced by microbial components in a real life situation, however, much additional work will be required before we can bridge the gap between in vitro-studies and human illnesses.

Future research efforts could be directed at elucidating the mechanisms which regulate the initiation and the strength of inflammation such as the recently reported noncanonical activation of NLRP3 inflammasome resulting in pyroptosis, whose role in fungal infections still needs to be clarified. Another interesting question is the recent findings of memory in innate immunity, designated as trained immunity. This finding has the potential to be fundamental in understanding the illnesses associated with exposures to microbial components. Genetic and epigenetic factors are also likely to affect the propensity of an individual to suffer these illnesses and require further studies.
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Activation of the Inflammatory Response by Fungal Components

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