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Molecular mechanism of alveolar macrophage polarization and cell communication with alveolar epithelial cell

Dissertation zum Erwerb des Doktorgrades der Naturwissenschaften

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I dedicate this work to my family.

Content

1. Zusammenfassung

Alveolarmakrophagen (AM) besiedeln die Oberfläche des Alveolarepithels und übernehmen so die Abwehrfunktion der unteren Atemwege. Zudem spielen AM eine wichtige Rolle bei der Pathogenese vieler entzündlichen Lungenerkrankungen wie z.B. Asthma und COPD. Eine notwendige Voraussetzung für diese besonders spezialisierten geweberesidenten Makrophagen ist ihre Plastizität, die es ihnen erlaubt sich entsprechend der jeweiligen Umweltgegebenheiten anzupassen und ihre Aktivierungsform (auch Polarisierung genannt) zu verändern. So können auch AM wie für andere Makrophagen bekannt, klassisch aktiviert werden (M1 Form) und einen pro-inflammatorischen Status einnehmen, oder alternativ (M2 Form) in einen anti-inflammatorischen Phänotyp polarisiert werden.

Das Immunoproteasom (IP) ist eine besondere Form des Proteasom. Es besitzt drei Interferon gamma (IFNγ) induzierbare katalytisch aktive Untereinheiten, nämlich die Low Molecular Mass Protein 2 (LMP2) und 7 (LMP7), sowie die Multicatalytic Endopeptidase Complex-Like 1 (MECL-1) Untereinheit, welche die katalytischen Untereinheiten 1, 2, und 5 des konstitutiven Proteasomes entsprechend ersetzen können. Abgesehen von seiner wichtigen Rolle bei der Antigenpräsentation, werden neuerdings für das IP neben Funktionen in der erworbenen Immunität auch Wirkungswege in der angeborenen Immunität beschrieben. Am besten untersucht wurde unlängst jedoch seine Funktion in der T-Zell Biologie, insbesondere bei T-Zell Survival und Expansion sowie bei der Differenzierung von Th17 Zellen.

In Anbetracht der erwähnten zentralen Bedeutung der Zellplastizität von Alveolarmakrophagen für die Abwehrfunktion und Homöostase der Lunge, und in Verbindung mit der Rolle des Immunoproteasom für zellbiologische und immunologische Pathways, wird im Kapitel 1 (Capter 1) die Untersuchung der Funktion des IP bei der AM Polarisation beschrieben. Wir zeigen hier, dass primäre aus der Mauslunge isolierte AM, ex vivo durch Stimulation mit LPS/IFN in die M1, bzw. durch IL-4 in M2-Phänotypen polarisiert werden können. Beide AM Aktivierungsformen weisen dabei eine verstärkte Expression und Aktivität der IP Untereinheiten LMP2 und LMP7 auf. Die Verwendung von aus Knockout-Mäusen isolierten AMs zeigte weiter, dass die Induktion der M1-Marker in LMP2 und -7 defizienten (LMP2-/-; LMP7-/-) nicht verändert wurde. Die M2-Marker dagegen waren in LMP2-/- und LMP7-/- AM eindeutig erhöht. Demen sprechend konnten wir weiter darstellen, dass unter M2-Bedingungen, die für den IL-4 signalweg bekannte Phosphorylierung der Mediatoren AKT und STAT6 verstärkt auftrat. Darüber hinaus zeigte sich auch der für die M2-Polarisierung wichtige Transkriptionsfaktor IRF-4 sowohl nach IL-4 als auch IL-13 Stimulation in seiner Expression in LMP7-/- Zellen stark erhöht. Schließlich konnten wir zeigen, dass die Protein aber nicht die mRNA Expression des für die IL-4 und -13 Signaltransduktion gemeinsamen Rezeptors IL-4R in LMP2-/- und -7-/- Zellen verstärkt war. Die daraus abgeleitete Hypothese, dass ein Mangel an IP Aktivität in LMP2-/- und -7-/- AM zu einer verstärktem IL-4R Expression führt und damit zu einer gesteigerten Sensibilität für die IL-4/13 Stimulation, welche wiederum eine erhöhte M2-Marker Expression bewirkt, konnte durch die Verwendung des neuen LMP7-spezifischen Inhibitors ONX0914 bestätigt werden.

Zusammenfassend zeigen unsere in Kapitel 1 dargelegten Untersuchungen, dass eine LMP2 oder -7 Hemmung die M2-Polarisierung von Alveolarmakrophagen durch Modulation der IL-4 Rezeptor Expression verstärkt. Da diese Wirkung für die LPS/IFN Stimulation bedeutungslos ist wird die M1-Polarisierung durch die LMP-Aktivität nicht beeinflusst. Die gezielte pharmakologische Hemmung von Immunoproteasom-Untereinheiten stellt somit eine neue therapeutische Möglichkeit dar die Immunität der Lunge gezielt zu beeinflussen.

Das 2. Kapitel (Chapter 2) befasst sich mit Zell-Zell Kommunikation von Alveolarzellen, nämlich der Alveolarmakrophagen mit den Typ 2 Pneumozyten (Typ 2 Alveolarepithelzellen; AEC-II). Ziel dieses Projektes war es zelluläre Faktoren zu identifizieren die von AMs in Abhängigkeit ihrer Aktivierung (Polarisation) freigesetzt werden und damit die AEC-II Funktion beeinflussen. Es wurden zu diesem Zweck Kontakt Co-Kulturen (Transwell Co-Kultur) der SV40 immortalisierten, murinen AM-Zelllinie MH-S mit der aus Maus Lungenadenom abgeleiteten AEC-II Zelllinie LA-4 durchgeführt. Zudem wurden AEC-II Kulturbedingungen mit AM-konditioniertem Medium benutzt um explizit die durch lösliche Faktoren meditierte Interaktion zu betrachten. Es zeigte sich, dass in Kontakt Co-Kulturen die M1 polarisierten MH-S Zellen die Expression von einigen immunrelevanten AEC-II Genen, insbesondere Il6, Tgfb1, Lcn2, Csf2, Ccl2 und Cx3cl1 in den LA-4 Zellen stark induzieren. Die Kultivierung von LA4 Zellen in von M1 polarisierten MH-S Zellen konditioniertem Medium, beeinflusste dagegen nur die Expression von Il6, Lcn2, Ccl2 und Cx3cl1 jedoch nicht die von Tgfb1 und Csf2. Die Verwendung von mit unbehandelten (M0) bzw. M2 polarisierten MH-S Zellen konditioniertem Medium wiederum stimulierte die LA-4 Zellen die Expression von

Cxc3cl1 zu verstärken. Die prototypischen proinflammatorischen Zytokine TNF- und IL-1 werden hauptsächlich von aktivierten Makrophagen sezerniert, und sind dafür bekannt während der akuten Entzündungsreaktion das Alveolarepithel inflammatorisch zu stimulieren. In unserem in vitro Modell zeigte sich, dass LA4 Zellen die Expression von Ccl2, Cx3cl1 und Csf2 stark durch TNF-, wohingegen die Expression von Lcn2 hauptsächlich durch IL-1 Gabe reguliert wurde.

Diese Ergebnisse deuten darauf hin, dass im Alveolarepithel die Stimulation der Tgfb1 und Csf2 Expression evtl. den Kontakt mit M1 polarisierten AM benötigt, wo hingegen Il6, Ccl2 und Lcn2 Expression auch durch von M1 AM freigesetzten, löslichen Faktoren stimuliert wird. Für die Induktion von Csf2 und Ccl2 ist von AM sezerniertes TNF- ein guter Kandidat und für Lcn2 entsprechend IL-1. Interessanter Weise war die epitheliale Cx3cl1 Expression unabhängig von IL-1 und wurde sowohl für pro- als auch anti-inflammatorische Versuchsbedingungen beobachtet. Letzteres passt gut zu der angenommen Funktion des membrangebundenen CX3CL1 Zytokins als AM Chemoattractant, welches die Chemotaxis der AMs zu Orten der Pathogen-Phagozytose (M1 Kondition) bzw. zur Efferozytose apoptotischer Zellen (M2 Kondition) steuern soll.

2. Summary

Alveolar macrophages (AM) play an important role during inflammatory lung diseases and provide the first line of defense of the lower airways. A major requirement for resident macrophages is their plasticity, which allows them to adapt according to the respective environment by modulating their state of activity, called polarization towards pro-inflammatory (classically activated, M1) or anti-inflammatory (alternatively activated, M2) phenotypes. The immunoproteasome is a specialized form of the proteasome which contains the three IFNγ-inducible catalytically active subunits: low molecular mass protein 2 (LMP2), multicatalytic endopeptidase complex-like 1 (MECL-1), and LMP7. They can replace their constitutive catalytic counterparts β1, β2, and β5, respectively. Apart from its major role in antigen presentation, immunoproteasomes have emerging functions in multiple innate and adaptive immune responses such as T cell survival and expansion, and Th17 differentiation. In view of the described key relevance of the plasticity of AMs for pulmonary host defense and homeostasis, combined with the impact of the immunoproteasome on cell-biological and immunological pathways, the study in chapter 1 was aimed to investigate the role of IP function in AM polarization. Here, we demonstrate that AMs can be polarized into M1 or M2 phenotypes after LPS/IFN-γ or IL-4 treatment, respectively. Both M1 and M2 AMs showed increased expression and activity of the IP subunits LMP2 and LMP7. The immunoproteasome kinetic study revealed increased expression and activity of LMP2, MECL-1, and LMP7 during both M1 and M2 polarization of AMs. While the induction of M1 markers was not affected in LMP2 and -7-deficient AMs, the expression of M2 markers was clearly increased in LMP2 and -7-/- cells. Accordingly, we found that also phosphorylation of

AKT and STAT6, which are the most important signaling pathways involved in M2 activation, were enhanced in LMP2 and -7-/- cells. In addition, our data showed that IRF-4 expression, a crucial transcription factor driving M2 polarization, was upregulated in IL-4/IL-13 treated AMs, and also further induced in LMP 7-/- cells. In further experiments, we figured out that the increased M2 markers and signaling is due to increased expression of IL-4Rα in LMP2 and -7-deficient AMs and confirmed our finding from knockout cells by using the LMP7 specific inhibitor ONX0914. Taken together, the study in chapter 1 demonstrated that LMP2 or LMP7 ablation enhances M2 polarization of AMs by modulating the IL-4 receptor expression, while it is dispensable for M1 polarization. These results suggest that inhibition of individual immunoproteasome subunits might present a new avenue to modulate innate immunity in the lungs.

The study of chapter 2 focused on the communication between polarized AM and epithelial cell. This study was aimed to identify the cellular factors derived from AMs which affect alveolar epithelial cells by using a trans-well co-culture and conditioned medium model. Here, we found that M1 polarized AMs enhanced the expression of several immune-related factors such as Il6, Tgfb1, Lcn2, Csf2, Ccl2 and Cx3cl1 in LA4 cells, whereas conditioned medium from M1 AMs induced expression of Il6, Lcn2, Ccl2 and Cx3cl1 in LA4 cells but not of Tgfb1 and Csf2. However, conditioned medium from M0 and M2 also markedly induced gene expression of Cx3cl1 in LA4 cells. The master pro-inflammatory cytokines TNF-α and IL-1β that are mainly secreted from macrophages have been well described to act on alveolar epithelial cells during acute inflammation. We found that mRNA expression of Ccl2, Cx3cl1 and Csf2 was strongly induced by TNF-α, whereas Lcn2 was strongly upregulated by IL-1β. These results indicated that

the induction of Tgfb1 and Csf2 expression in alveolar epithelial cells requires their contact with M1 polarized AM, whereas IL-6, CCL2 and LCN2 appeared already in alveolar epithelial cells and were further triggered by M1 AM released soluble factors. AM secreted TNF-α seems to represent a plausible candidate inducing the expression of Csf2 and Ccl2, whereas the induction of Lcn2 is dependent on IL-1β. Interestingly, the epithelial expression of Cx3cl1 was independent of IL-1β, but its induction was observed in both pro- and anti-inflammatory experimental conditions. The latter finding matches well with the assumed function of this membrane-bound cytokine to attract AMs for pathogen clearance by phagocytosis (M1 condition) as well as efferocytosis of apoptotic cells (M2 condition).

3. Chapter 1: Immunoproteasome composition impacts alveolar macrophage polarization

3.1. Introduction

3.1.1. Macrophage Overview: Development, Location, and Functions

Monocyte and macrophage development

Macrophages represent a group of immune cells which are widely distributed throughout the body and all tissues. Monocytes, a subset of circulating white blood cells in vertebrates, constitute 2% to 10% of all leukocytes in the human body (1), and can further differentiate into macrophage under certain conditions. Monocytes are continuously generated from bone marrow hematopoietic stem cells via macrophage and dendritic cell precursors and common monocyte progenitor (2). It has been known for a considerable amount of time that the growth factor Csf-1 and also as recently reported the cytokine IL-34 are important for the development of this lineage (2, 3). Accordingly mice deficient in the Csf-1 growth factor exhibit lower amounts of blood monocytes (4). It has further been demonstrated that there are two subsets of monocytes which exist in the human blood. They are named $CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{-}$ monocytes and have differential responsibilities in the stimulation process (5). In mice, $CSF1R^{+}$ monocytes are subdivided in distinct populations of $LY6C^{hi}CX3CR1^{mid}CCR2^{+}$ and $LY6C^{low}CX3CR1^{hi}CCR2^{-}$ monocytes, which are thought to be equal to their human counterparts (6).

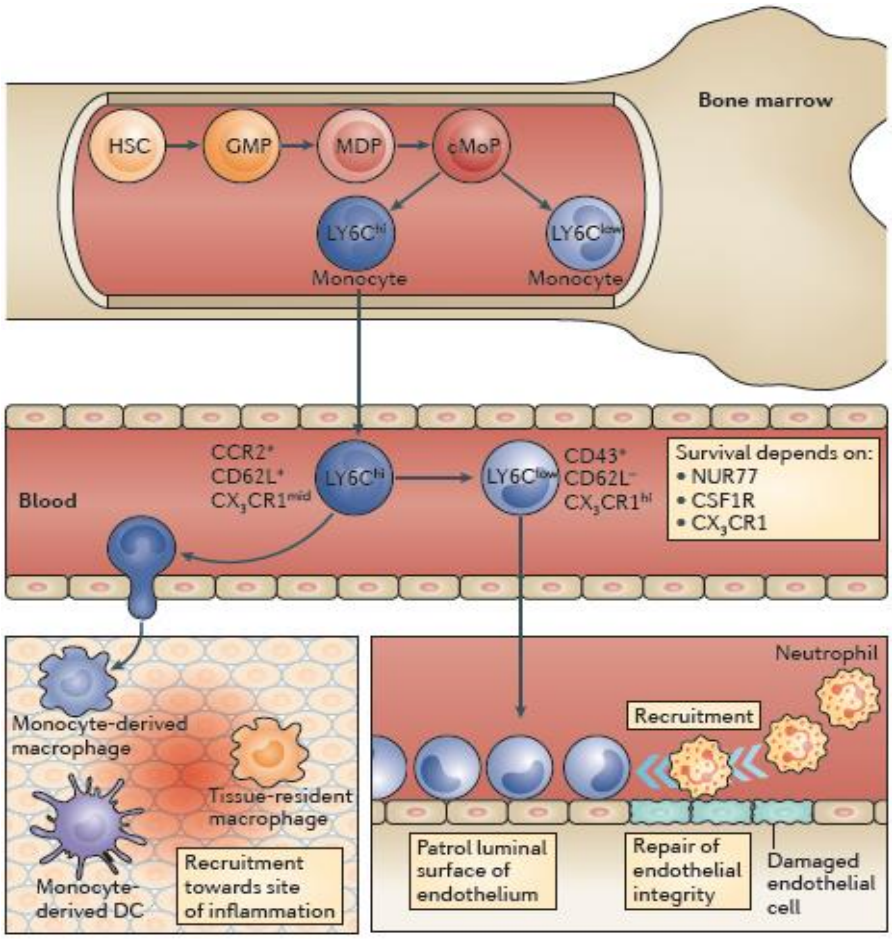

Fig 3.1: Scheme of monocyte and macrophage development. In the bone marrow, monocytes are continuously generated from hematopoietic stem cells (HSCs) via macrophage and dendritic cell (DC) precursor (MDP) and common monocyte progenitor (cMoP) intermediates. In the steady state, there are two functionally distinct monocytes called LY6C^{hi} and LY6C^{low} subsets circulating in blood vessel to form a developmental continuum (BOX 1). Macrophage-like LY6C^{low} cells patrol the endothelial surface and coordinate its repair by recruiting neutrophils. LY6C^{hi} monocytes are rapidly recruited to sites of inflammation and sites of tissue remodeling, where they extravagate and can give rise to monocyte-derived DCs and monocyte-derived macrophages. Copied from (6).

3.1.2. The overview of tissue macrophage location and origin

Tissue-resident macrophage populations are found in the majority of tissues in the body including microglia in the brain, alveolar macrophages in the lung, and Langerhans cells in the skin and Kupffer cells in the liver (7). They are unique phenotypes according to

their distinct micro-niches that are extremely heterogeneous, which makes them exhibit tissue specific functions and adapt to the tissue environment in which they reside (7). Tissue-resident macrophages were firstly recognized as phagocytic cells for invading pathogens. They are considered to be the frontline of tissue defense (8). Intensive studies revealed that tissue macrophages play an essential role in immune response and inflammation, such as the clearance of microbes and necrotic and apoptotic cells, initiation of the innate immune response to infection and the resolution of inflammation (9). Studies on the role of the specific function of tissue macrophage in each inflammatory related disease according to its site will help us to understand their pathogenic contribution to disease. For instance, Kupffer cells (KC), also known as Browicz-Kupffer cells, reside within the lumen of the liver sinusoids (10), which enable them be easily exposed to toxic components arising in the blood such as bacterial endotoxins, ethanol and toxic substances from the gut (11, 12). Therefore, Kupffer cells have been reported to be involved in many liver diseases including acute liver injury, alcohol-related liver disease and liver infections (13). Microglia, another type of resident macrophage reside in the brain and spinal cord, and thus is believed to play the crucial role in brain infectious disease, Alzheimer's disease, Parkinson's disease, multiple sclerosis and several psychiatric disorders (14, 15).

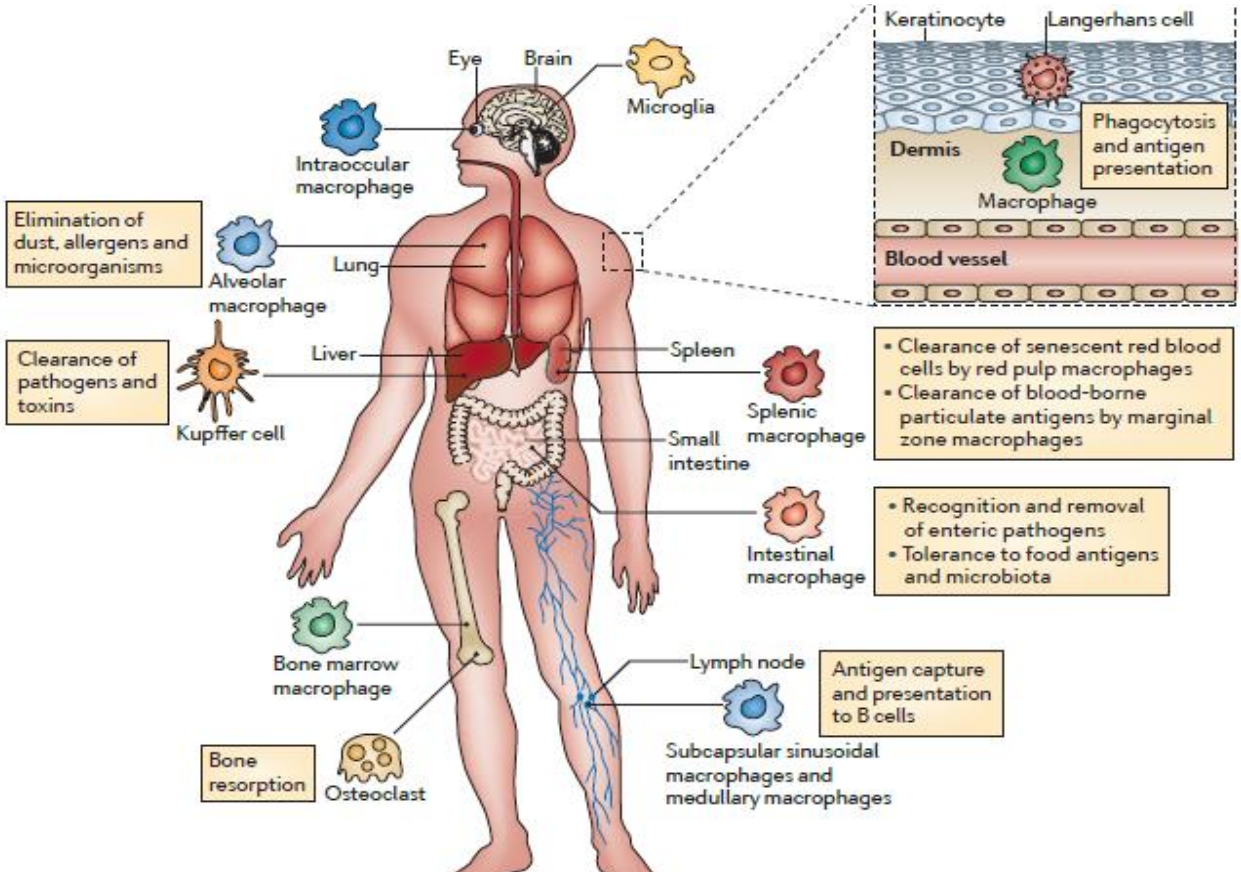

Fig 3.2: Scheme of localization of tissue macrophage. Copied from (16)

It has been well documented that bone marrow hematopoietic stem cells (HSCs) give rise to circulating monocytes, which can differentiate in tissues into macrophages. However, a recent study showed that the mouse embryo yolk sac is a sufficient source of specific tissue macrophage subtypes in the liver, skin and central nervous system (CNS) in the absence of HSCs (17).

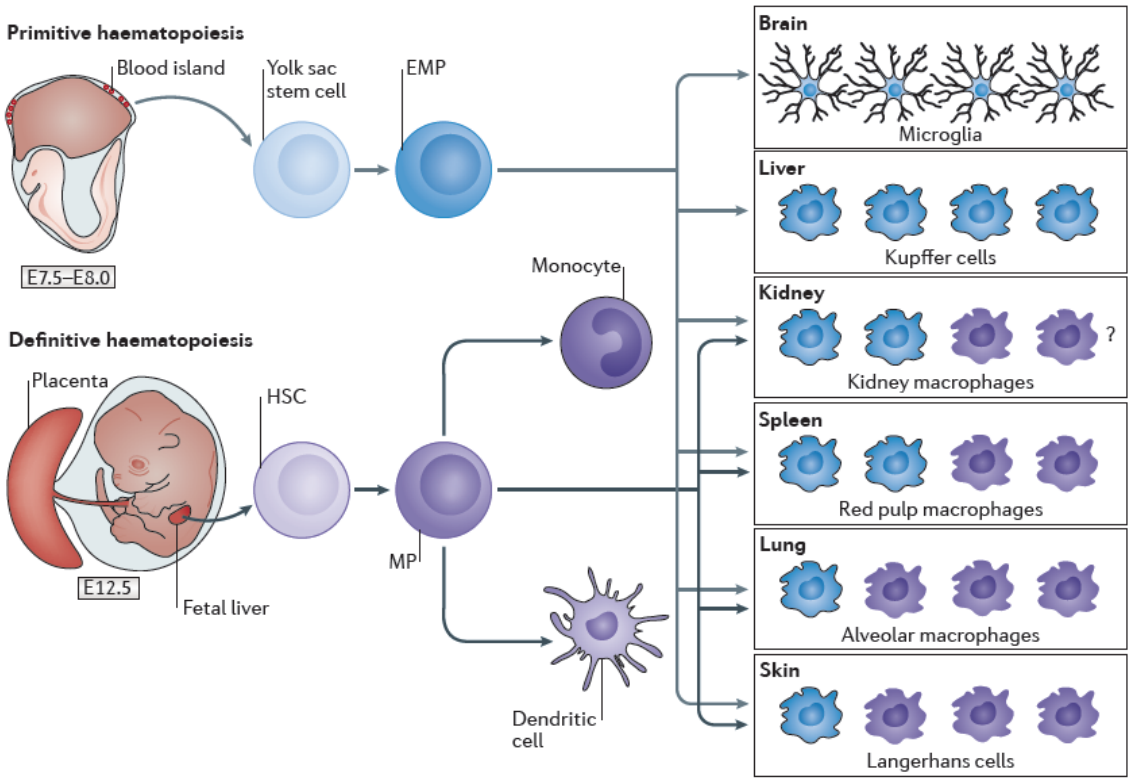

Fig 3.3: Scheme of origin of tissue macrophages. At embryonic day 7.5 (E7.5)–E8.0, a process, called primitive hematopoiesis, is a transient early wave of myeloid cell development. At this period, in blood islands of the yolk sac, cells with stem cell potentials develop. Their progeny erythromyeloid progenitors (EMPs) further differentiate and populate several tissues, including the brain, where they become tissue macrophages that potentially have longevity and a high capacity for self-renewal. Taken from (18).

Alveolar macrophages (AM)

Pulmonary macrophages are considered to exist within at least two anatomically distinct compartments. The alveolar macrophage (or dust cell) predominantly set in the airspace of alveoli where they are in close contact with the respiratory epithelium. They act as the lung's first defense line against inhaled pathogens and environmental pollutants (19). The plasticity of alveolar macrophages is required to adapt to a unique airway microenvironment (17), which finally causes alveolar macrophage to be an unusual phenotype in many respects compared with other lung resident macrophages (17). The

interstitial macrophage, on the other hand, resides in the lung parenchyma (20). Although these cells share many common features of alveolar macrophage, in mice, alveolar macrophages are easily distinguished from interstitial macrophages by their unusual phenotype (Table1) (17).

Surface marker	Interstitial macrophage	Alveolar macrophage
CD11b	Intermediate expression	Not expressed
CD11c	Not expressed	High expression
CD14	Intermediate expression	Low expression
CD200R	Intermediate expression	High expression
DEC205	Expression unknown	Intermediate expression
F4/80	Low expression	Low expression
Mannose receptor	Intermediate expression	High expression
MHC class II	Intermediate expression	Low expression
SIGLEC-F	Not expressed	High expression

Table 1: The specific phenotype of mouse macrophages from different sites. Adapted from (17).

In addition, it has been found that alveolar macrophages have many properties of dendritic cells (DC) (21). For example, they have a better antigen-presenting capability than peritoneal lavage-derived macrophages (PLM) (21). The dendritic cell marker CD11c has also been found to be more highly expressed in the alveolar macrophage compared to other macrophages (22).

It has been controversial for many years whether AMs are derived from their blood precursor monocytes. Newly published studies have indicated that AMs develop from fetal monocytes rather than arise from circulating blood monocytes and adopt a stable

phenotype shortly after birth in response to instructive cytokines, and then self-maintain throughout life (18, 23, 24). However, the signals and molecular mechanisms that drive AMs and when such signals are provided are not yet fully understood. A recent study has shown that the CSF2 induced expression of the nuclear receptor PPAR-γ is essential for the differentiation of AMs from fetal monocytes. (24, 25).

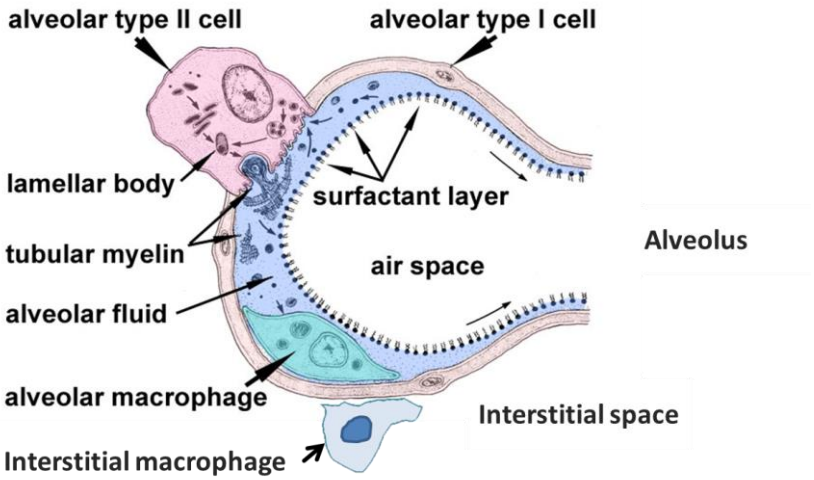

Fig 3.4: localization of alveolar macrophage in the alveolus. Alveolar macrophages are localized on the top of alveolar type I cell which is surrounded by the alveolar fluid. The main content of alveolar fluid is surfactant which are secreted by the alveolar type II cells. Adapted from (26).

3.1.3. Macrophage functions

The phagocyte is a type of leukocyte that protects the body by engulfing and ingesting harmful foreign particles, bacteria, and dead or dying cells (27, 28). Professional phagocytes include many types of leukocytes (such as neutrophils, monocytes, macrophages, mast cells, and dendritic cells). Macrophages are one type of phagocytes, which are responsible for recognition, engulfing and killing of pathogens and apoptotic cells (29). Another essential role of macrophages is alerting the immune system to the

presence of invaders and immune disorders. In addition, macrophages are able to repair the tissue injuries (30).

3.1.3.1. Phagocytosis

Monocytes and macrophages are recruited to the site of injury, inflammation and infection. When macrophages come into contact with a pathogen or apoptotic cell, macrophages use a mechanism called phagocytosis to engulf the pathogens into cells. Phagocytosis is a specific form of endocytosis involving the vesicular internalization of solids such as bacteria and environmental particles. Phagocytosis is derived from the Greek words

"phagein" meaning “to eat” and is the word used to describe the engulfing and destruction of pathogens. Phagocytosis was first discovered by Élie Metchnikoff in 1882 (31, 32). Upon engulfment, a vesicle called a phagosome is formed around the microbe by the cell membrane which then fuses with a lysosome specialized vesicle that contains digestive enzymes to destroy the pathogens (33, 34). Some macrophages act as scavengers, removing dead or necrotic cells while others provide host immunity by engulfing microbes (35, 36). Most macrophages can live for several months and can kill hundreds of different bacteria before they die. In this process, macrophages provide a non-specific or innate immunity. The precise process of phagocytosis depends upon the particle being internalized, its size and whether it controls its own fate (37). In broad terms, the uptake process usually requires receptors which include Fc-receptors, CD44, MARCO and CD36 in order to collect around the particle (38-41), to archive signaling to promote membrane extension and polymerization of the underlying actin cytoskeleton,

and subsequent maturation of the internalized vacuole (the phagosome) to fuse with lysosomes and initiate particle degradation (42).

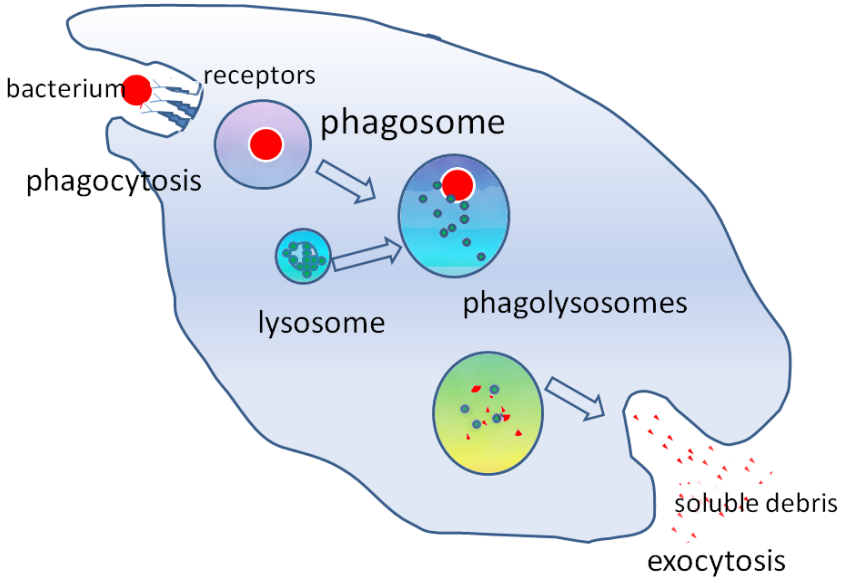

Fig 3.5: A bacterium phagocytosed by a macrophage. Binding of phagocyte surface receptors causes the internalization of bacterium into phagosome. The phagosome ingested with bacterium is then fused with the lysosome, forming a phagolysosome and leading to degradation of bacterium. Taken from (http://en.wikipedia.org/wiki/Phagocyte)

3.1.3.2. Bridging Innate and Adaptive Immunity

The immune system is typically divided into two categories: innate and adaptive although these distinctions are not mutually exclusive (43). Innate immunity refers to the nonspecific defense mechanisms that occur immediately or within hours of the appearance of pathogens. The macrophage, being the first line of defense against many common pathogens, is thought to be the crucial player of the innate immune system (44). In order to recognize pathogens, pattern recognition receptors expressed on macrophages are able to distinguish between self and nonself, which then leads to the activation of an immune signaling pathway and production of immune mediators such as

the cytokines and antimicrobial peptides (45). On the one hand, cytokines can amplify the immune response locally by binding to their receptors, on the other hand they can recruit lymphocytes which are a sign of involvement of the adaptive immunity (46). This is how macrophages alert the immune system to microbial invasion. In addition, macrophages can process and present foreign antigens as well as dendritic cells to a corresponding T cell. The antigen being displayed is attached to an MHC class II molecule, which acts as a signal to activate T cells (47). Moreover, T cells also stimulate B cells to generate specific antibodies to each antigen. This "signature" antigen is also remembered by T cells and B cells, which allow them to target the antigen again in future (48). Therefore, the macrophage is one of the crucial white blood cells that are able to bridge innate and adaptive immunity.

3.1.3.3. Macrophage activation and polarization

The macrophage is a particularly dynamic cell during anti-pathogens immune responses, inflammation, resolution and tissue wound healing (49, 50). Under such conditions, macrophages of different origin, being monocytes or tissue macrophages can acquire distinct functional phenotypes depending on their surrounding microenvironment. Two well-studied polarized subsets have been established according to their functions and distinct gene expression profiles, which are the classically activated macrophages (M1 macrophages) and alternatively activated macrophage (M2 macrophages)(51). M1/M2 paradigms are analogized with Th1 (T helper 1)/Th2 (T helper 2) type immunes responses. It is worth noting that inducible expression of iNOS

and arginine Arginase1 were well described as markers for respective characterization of M1 and M2 macrophages (51-54).

The M1 polarized macrophage, whose prototypical activating stimuli are IFNγ and LPS, and alternatively activated macrophages (or M2) are further subdivided into in M2a (stimulation of IL-4 or IL-13), M2b (immune complexes in combination with IL-1beta or LPS) and M2c (IL-10, TGF-β or glucocorticoids). M1 macrophages show strong anti-microbe properties with high production of pro-inflammatory cytokines (TNF-a, IL-1b, and IL-12) and antimicrobial effectors (nitric oxide and defensins), which are mainly presented during acute infection and inflammation (50). M2 polarized macrophages are believed to play a crucial role in tissue repair and resolution of inflammation due to their high phagocytic clearance of apoptotic neutrophils and the secretion of anti-inflammatory cytokines (e.g. IL-10)(51). M2a macrophages induced by IL-4 or IL-13 trigger a Th2 type like immune response, and are involved in anti-infectious responses to parasites e.g. against Helminth Parasites (55). In addition, new evidence indicates that M2 macrophages have a pro-fibrotic role in fibrosis (56). M2b macrophages are considered immunity regulation and are induced by LPS, IL-1 and immune complexes. Besides IL-10 they also produce IL-1, IL-6 and TNF-a. M2c macrophages are induced by IL-10/TGF-β and also exhibit anti-inflammatory functions (57). It has also been shown that the M2c but not M2a macrophages induce regulatory T cells (Tregs) from CD4+CD25- T cells in vitro and are more effective than M2a macrophages in protecting against tissue injury (57). Moreover, thorough studies have evidenced that polarized macrophages control immune responses and inflammation by a chemokine repertoire that recruits other immune cells; for instance, M1 macrophages express the chemokines CXCL1,

CXCL2, CXCL3, CXCL5, CXCL9, and CXCL10 and CCL2, CCL3, CCL4,CCL5, CCL11, and M2 macrophages increase expression of CCL2, CCL17, CCL22, and CCL24 (58, 59)

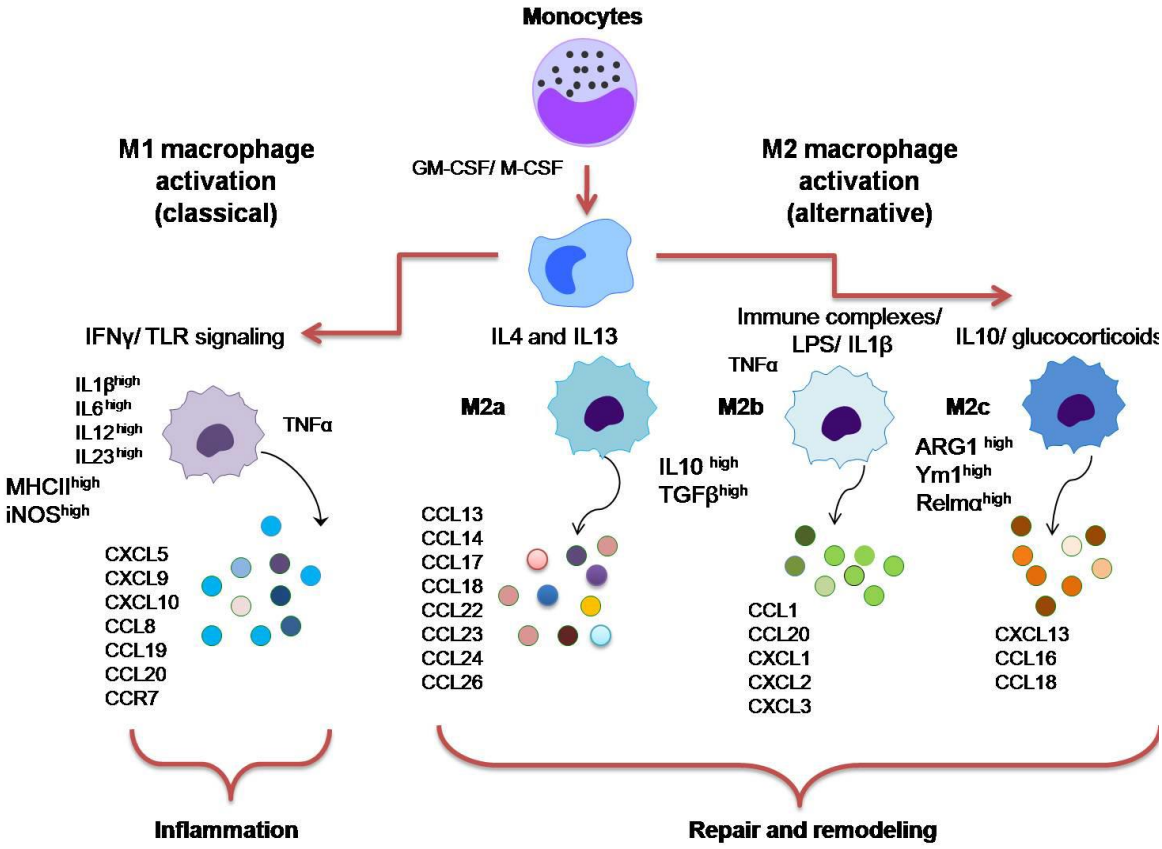

Fig 3.6: Scheme of M1 (classical) and M2 (alternative) macrophage polarization. Several cytokines and chemokines are involved in the classical and alternative activation of macrophages. Monocytes get differentiated into macrophages which in turn polarize to M1 type on exposure to LPS or IFNγ. Various signals define the different forms of alternative activation of macrophages. IL-4 or IL-13 induces M2a subtype; IL1β or LPS or immune complexes induces M2b macrophages; and IL10 or glucocorticoids results in M2c macrophages. Taken from (http://cdn.intechopen.com/pdfs-wm/46529.pdf).

3.1.4. Transcriptional networks of macrophage polarization

A variety of studies have been carried out to understand the signaling pathways, transcription factors, and epigenetic regulation during macrophage polarization. By using the technique of mouse genetic deletion of genes in macrophages, a number of pathways were identified to be involved in molecular mechanisms of macrophage polarization. In the following sections, the key transcription factors are discussed for their roles in a polarized macrophage phenotype.

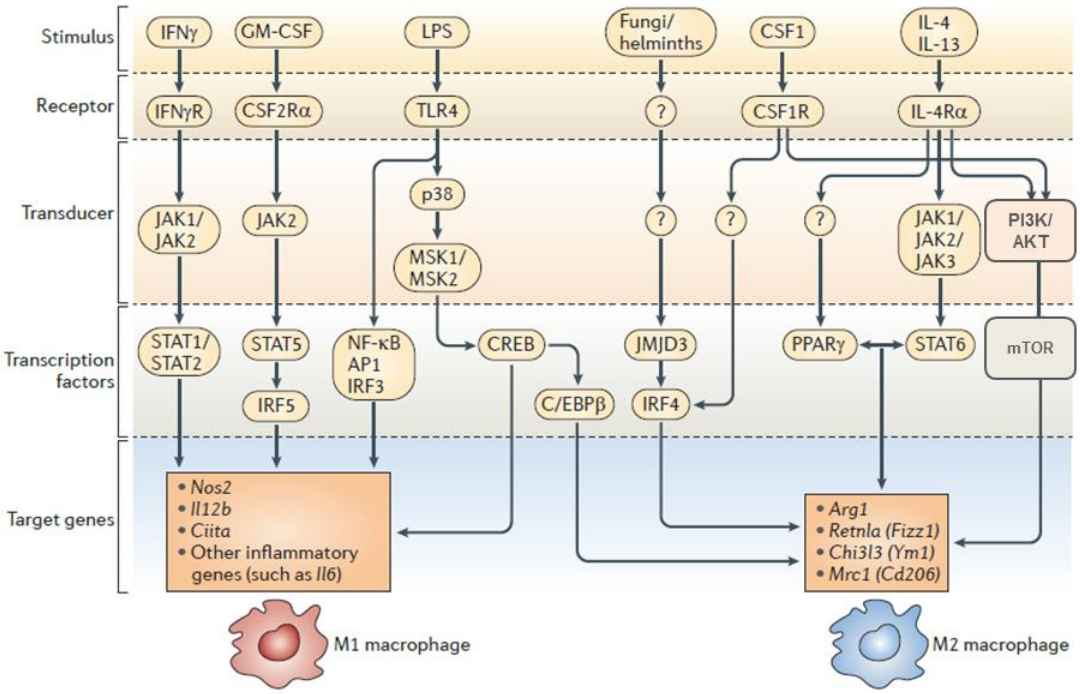

Fig 3.7: Signaling pathways in M1 and M2 macrophage polarization. IFNγ, LPS and CSF2 or Fungi/helminths, CSF1 and IL-4/IL-13 respectively induce the M1 or M2 polarization. The main genes that are characteristic of either the M1 or the M2 polarized state are also shown. The main marker genes used for M1 characterization are Nos2, Il12b, Ciita and Il6, and Arg1, Chi3l3, Retnla and Mrc1 are used as M2 marker. The main transcription factors involved in M1 polarization are STAT1/STAT2, STAT5, IRF5, NF-κB, AP1 and IRF3, IRF4, and PPARγ, C/EBPβ, STAT6 and mTOR for M2. Taken from (60).

3.1.4.1. The NF-κB /STAT1 signaling axis

Polarized (M1) macrophages show increased anti-microbial activity by enhanced expression of NOS2, increased MHC class II expression, and increased secretion of IL-12 which promotes the Th1 immune response (61). IFNγ-mediated Janus kinase–signal transducer and activator of transcription (JAK–STAT) are supposed to regulate transcription of those genes (62), which are characterized by IFNγ receptor triggered JAK-mediated tyrosine phosphorylation and subsequent dimerization of STAT1 which binds to IFN gamma-activated sequences in the promoters of the M1 markers gene (60). LPS is often a co-stimulus of IFNγ for M1 polarization as it binds to the Toll like receptor TLR4 and leads to activation of nuclear factor kappaB (NF-κB) which induces expression of pro-inflammatory cytokines such as Tnf, Il1b, Il6 and Il12. The NF-κB p65 and p50 heterodimer complexes with the inhibitory protein IκBα in the cytosol but is released after the phosphorylation of IκBα by IκB kinase (IKK) and translocated into the nucleus where it binds to the NFκB response element (TRE) (63). In addition, LPS induces the production of IFNβ which in turn binds to the IFNα/β receptor and triggers the formation of STAT1–STAT2 heterodimer to induce the M1 signature gene expression, such as Nos2, Tnf and Il12b. Therefore, it is clear that both NF-κB and STAT1 activity is crucial for M1 macrophage polarization (64). It has been shown that STAT1-deficient mice have severe malfunctions in immunity, which causes them to be hypersensitive to bacterial and viral pathogens infection (65).

3.1.4.2. The JAK–STAT6-SOCSs signaling pathway

Cytokines IL-4 and IL-13 have been well established to induce M2a polarization of macrophages. They are supposed to bind to the interleukin 4 receptor alpha (IL-4Rα) and interleukin13 receptor alpha 1 (IL-13Rα1) and lead to phosphorylation of JAK1 and JAK3 to further trigger the phosphorylation of STAT6. Following homo-dimerization, STAT6 translocate into the cell nucleus where it recruits the IRF4 and initiates the transcription of M2 markers genes, including arginase 1 (Arg1), macrophage mannose receptor 1 (Mrc1; also known as Cd206), resistin-like-α (Retnla; also known as Fizz1) and chitinase 3-like 3 (Chi3l3; also known as Ym1). Additionally, STAT6 also induces expression of the transcription factor PPAR-γ, which acts in synergy with STAT6 to promote the expression of M2-specific genes and macrophage polarization (66, 67). It has been investigated that mice harboring the specific knockout of the Il4ra and STAT6 are not able to polarize to M2 macrophages which leads to a disorder in TH2 cell-mediated inflammation (66). Suppressors of cytokine signaling (SOCS) are important regulators of LPS and cytokine responses. They are the endogenous inhibitors of STAT proteins, which inhibit the JAK-STAT pathway by negative feedback of cytokine signaling. Corresponding studies have reported that SOCS1, -2, and -3 are induced in response to cytokine stimulation, and the corresponding SOCS proteins inhibit cytokine-induced signaling pathways (68). SOCS family members modulate signaling by several molecular mechanisms, which include inactivation of the Janus kinases (JAKs), blocking the binding of the signal transducers of transcription (STATs) to receptors, and ubiquitination of signaling proteins and their subsequent targeting to the proteasome (68). Recent studies have suggested that SOCS2, and SOCS3 differentially contribute to

macrophage M1 and M2 polarization (69). It has been demonstrated that there is a bias toward M1-macrophage polarization in SOCS2-deficient mice, whereas SOCS3-deficient macrophages express surface markers associated with M2-macrophage polarization (69, 70).

3.1.4.3. IFN Regulatory Factors (IRF-3, IRF-4 and IRF-5)

Interferon regulatory factors are proteins which regulate transcription of interferons. In mammals, the IRF gene family consists of nine members: IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, IRF-8, and IRF-9 (71). IRFs are also involved in many immune processes, including anti-bacterial and virus immunity, Th1-cell responses, dendritic cell development, and inflammation (72). IRFs are also found to play a crucial role in the regulation of macrophage polarization. It has been suggested that IRF-3 is associated with inflammatory microenvironments and contributes to the polarization toward a M1 macrophage phenotype. Two adaptors, MyD88 and TRIF, mediate the signaling downstream of TLR4 (73, 74). The signaling through the TRIF adaptor pathway activates IRF-3 which leads to the secretion of type I interferons, such as IFN-α and IFN-β (73, 75). Then, these type I interferons induce the activation of the transcription factor STAT1 and the transcription of M1 marker genes such as CXCL9 and CXCL10 by binding to the type I interferon receptor (IFNAR) (73, 76). Another recently described interferon regulatory factor in the regulation of M1 polarization is IRF-5. Previous studies have shown that IRF-5 is needed for the optimal expression of IL-12 and pro-inflammatory cytokines in mice (77). CSF2-polarized M1 macrophages exhibited up-regulated expression of IRF-5. M1 markers genes expression were increased by the

overexpression of IRF-5 and inhibited slightly by IRF-5 interfering RNA (siRNA). The capability of IRF-5 in regulating these M1 gene expressions is due to the direct recruitment of IRF-5 to gene promoters such as Il12b, but it represses transcription of Il10, probably also by binding to an ISRE in the gene promoter which has to be further investigated (78).

IRF-4 is described as a lymphocyte-specific transcription factor of the IRF family, and is a negative regulator of Toll-like-receptor (TLR) signaling which is central to the activation of the innate and adaptive immune systems (79). However, recent studies have shown that IRF-4 was able to specifically regulate M2 macrophage polarization in response to IL-4 and parasites or the fungal cell-wall component chitin. The regulation of macrophage polarization by IRF-4 involves histone demethylase JMJD3 which could remove an inhibitory histone modification called H3K27me3. Cells devoid of JMJD3 are not able to polarize into the M2 phenotype while not having a role in regulation of M1 macrophages (80).

3.1.4.4. PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway which is important in apoptosis and hence cancer (81). Moreover, it has been recognized that this pathway also has broad roles in innate and adaptive immune cells, including neutrophils, monocytes, macrophages and dendritic cells as well as B and T lymphocytes (82). It has been shown that the PI3K/mTOR pathway is activated by a broad array of different stimuli via specific receptors, including the BCR, TCR, cytokine receptors (eg, interleukin 2), insulin receptor, insulin-like growth factor I receptor, but also TLRs (82). LPS and IL-4

used to induce the M1 and M2 macrophages respectively have both been shown to induce phosphorylation of AKT and PI3K which further leads to activation of mTOR. Hence, in recent years, scientists have become increasingly interested in examining its role in regulation of macrophage polarization. AKT (also known as PKB) is a family of three serine/threonine protein kinases (AKT1, AKT2, and AKT3) that regulate many cellular functions. A study has unexpectedly shown that AKT2−/− macrophages are hypo-responsive to LPS stimulation, exhibiting the opposite phenotype to AKT1−/− macrophages (83). Moreover, AKT2−/− macrophages show an M2 phenotype attributed to reduced expression of miR-155 which targets C/EBPβ that is a key regulator of M2 polarization (83). mTOR was first named as the mammalian target of rapamycin, that integrates both intracellular and extracellular signals, and serves as a central regulator of cell metabolism, growth, proliferation, survival and the immune response (84). Newly published studies have indicated an existence of an mTORC1-AKT regulatory loop in the IL-4 signaling pathway in which the receptor engagement of the IRS/PI3K/AKT pathway leads to mTORC1 activation that in turn attenuates AKT signaling. Genetic loss of either TSC1 or TSC2 leads to constitutive mTORC1 activation. It has been demonstrated that TSC1−/− macrophages have a marked defect in M2 polarization in response to IL-4, while the inflammatory response to LPS is enhanced (85).

3.1.5. The role of alveolar macrophage polarization in chronic lung diseases

The lung is a major site of continuous immune reactions as it encounters various foreign particles and antigens entering the respiratory system. Alveolar macrophages are among the most abundant immune cells in the respiratory tract, and they are a unique

type of mononuclear phagocytes that populate the surface of the lung in steady state. They form the first line of defense against pathogens invading the alveolar space. Although alveolar macrophages exhibit unique properties compared with other resident macrophages, they could also polarize into distinct phenotype of M1 and M2 macrophage in vitro (86). When exposed to a specific microenvironment, macrophages acquire either M1- or M2-polarized phenotypes associated with inflammation and tissue remodeling, respectively. With the dramatic changes of the micro-environment during chronic inflammatory lung diseases, the alveolar macrophage accordingly polarizes into the characteristic M1 or M2 phenotype. A number of studies have shown that alveolar macrophage polarization has a crucial role in the pathogenesis of chronic lung inflammatory diseases including chronic obstructive pulmonary disease (COPD), asthma and idiopathic pulmonary fibrosis due to their contribution in the initiation, regulation and termination of inflammation.

3.1.5.1. Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterized by progressive lung function decline and an abnormal inflammatory response in the airways, and is mainly caused by cigarette smoke. The accumulation of immune cells including macrophages, neutrophils, CD8+ -lymphocytes and B-cells has been proven to be associated with the severity of COPD (87). Alveolar macrophages play a critical role in the pathophysiology of COPD and are a major target for an anti-inflammatory therapy in future. Alveolar macrophages from COPD patients have an increased baseline and stimulated secretion of inflammatory proteins, including certain cytokines, chemokines, reactive oxygen

species and elastolytic enzymes, which together could contribute to all of the pathophysiological features of COPD (88). With the intensive studies about macrophage polarization in both in vivo and in vitro, hence, the role of the distinct macrophage polarized phenotypes in COPD gained the attention of immunologists and pulmonologists. Based on studies with COPD patients, initially M1 polarization was expected to play a crucial role in COPD (89). It is well known that thousands of compounds presented in cigarette smoke, including the LPS as a natural contaminant of tobacco smoke can polarize macrophages into M1 in vitro, characterized by high expression of iNOS (90). Previous studies have already proven that iNOS is induced in the lungs of COPD patients (91). Moreover, many studies have shown that COPD patients showed higher concentrations of the pro-inflammatory M1 cytokines, IL-1β, IL-6, and TNF-α (92) which are partially released from alveolar macrophages. MMP9, a protease of the matrix metalloproteinase (MMP) family, is suggested to be involved in the breakdown of the extracellular matrix in COPD (93). M1 macrophages have also been found to secrete MMP9, which presumably facilitate macrophage migration during inflammation (94). All of these studies have indicated the essential role of polarized M1 macrophages in COPD pathogenesis. However, a study by Lisette Kunz showed contradictory results. They showed that the percentage of macrophages with M2-type characteristics is significantly higher in the BAL from ex-smokers than in current smokers with COPD but this increased anti-inflammatory phenotype is not necessarily accompanied by a decrease in inflammatory parameters (89). Therefore, this study indicates the important role of M2 macrophage polarization in COPD. Alternatively activated M2 was induced by the Th2 –biased cytokines IL-4 and IL-13. It have been

demonstrated that IL-13 were induced in COPD patients and was thought to contribute to inflammation, emphysema, and mucus metaplasia (95). This also raises a possibility that IL-13 overexpression induces M2 polarization in COPD. MMP12, another type of matrix metalloproteinase, is well known for its role in COPD and emphysema progress (96). Previous studies showed that MMP12 could be induced in IL-4-stimulated M2 macrophages (97). In summary, some evidence indicates the role of M2 activation in COPD, which may contribute to the development of COPD. So far, no studies have been conducted to exactly characterize the phenotype and role of M1 and M2 in COPD.

3.1.5.2. Asthma

Asthma is a complex lung disease, which is characterized by airway inflammation and airway hyperresponsiveness (AHR). It has been well documented that alveolar macrophages play a crucial role in the development and progression of asthma (98). Asthma is a chronic inflammatory disease with increased influx of inflammatory cells in the lungs along with a prominent Th2 cytokine signature (99). Among inflammatory cells, macrophages are the most abundant leukocytes found in the airspaces, which suggest that they have an important role in fighting against pathogens and airway remodeling and eosinophilic inflammation in asthma (100).

Firstly, the pro-asthmatic role of M1 macrophages has been investigated in clinical and then in experimental asthma. On the one hand, it has been shown that asthmatic macrophage is insufficient to fight against with microbe infections in the respiratory tract (101), which indicate the dysfunction of M1 activation, on the other hand, asthmatic macrophage are able to release M1 related pro-inflammatory mediators such cytokines

and nitric oxide (102, 103). For instance, recent investigations revealed that the generation of ATP and uric acid upon airway exposure to allergens leads to the release of the IL-1β from alveolar macrophages through activation of an inflammasome complex which can cleave pro-IL-1β to mature IL-1β together with caspase-1. IL-1β production could further lead to the enhanced Th17 cell differentiation which contributes to the control of allergic asthma (104). Moreover, nitric oxide, as one of main products of M1 polarized macrophage, is believed to amplify lung injury during asthma due to DNA damage, inflammation, and increase mucus production in a murine model of allergen(105, 106). Furthermore, it has been well documented that LPS is involved in the initiation of asthma, and both the level of LPS and IFN-γ used for M1 induction in vitro increased significantly in severe asthmatic patients (107, 108). Thus, alveolar macrophage polarization toward the M1 subset can promote the development of asthmatic disease.

In the context of the Th2 like immune response in asthma, Th2 cytokines IL-4 and IL-13 were found to be abundantly expressed in asthmatic lungs (109), and therefore it is not surprising that alveolar macrophage from asthma patients also expressed M2 markers. Such as elevated levels of chitinase family members have been found in the serum and lungs of patients with asthma (110). In addition, it has been shown that asthmatic macrophages exhibit higher levels of M2 markers, including mannose receptor and transglutaminase 2 (111, 112). In other studies, it has been found that sequence variations in the MRC1 gene correlated with asthma severity (113). M2 cells that secreted FIZZ1 (a resistin-like molecule-α) were found to be overexpressed in asthma. Jun Fei Wang has found that FIZZ1 plays a role in the early stages of airway remodeling

in asthma by increasing the expression of α smooth muscle actin (α-SMA) and type I collagen through the activation of the PI3K/AKT signaling pathway in asthma (114). Moreover, increased expression of Th2 chemokines CCL17 and CCL22 in asthma have been reported (115). Previous work has demonstrated that CCL17 and CCL22 are responsible for the recruitment of $CCR4^{+}$T lymphocytes into asthmatic tissue which are a major source of TH2 cytokines IL-4 and IL-13 (116). Newly published research has indicated that there is an overexpression of CCL17 in alveolar macrophages of asthmatic patients, which correlated significantly with sputum eosinophilia (117). Therefore, this research again supports the important role of M2 phenotype in asthma pathogenesis.

In summary, complex cytokine networks are involved in the pathophysiological progress of asthma because of the multifactorial nature of asthma, which also give rise to the reason of involvement of both M1 and M2 macrophages in asthma. Therefore, future work should help us to understand how the balance between M1 and M2 macrophages contribute to this complicated chronic lung inflammatory disease.

3.1.5.3. Pulmonary fibrosis

Pulmonary fibrosis is a lung disease that is resistant to treatment and carries a high mortality rate. It is characterized by the progressive and irreversible destruction of the lung architecture caused by scar formation that ultimately leads to lung malfunction, disruption of gas exchange, and death from respiratory failure (118). Idiopathic pulmonary fibrosis (IPF), a particularly severe form of pulmonary fibrosis with unknown cause, primarily occurs in older adults, and is associated with the histo-pathologic

pattern of usual interstitial pneumonia (UIP) (119). It has been suggested that alveolar macrophages are integrated into all stages of the fibrotic process, which may be due to its key role in fibroblast recruitment, proliferation, and activation (120). Additionally they are involved in the recruitment of inflammatory cells to sites of tissue injury by releasing chemokines and degrading ECM components by secreted specific matrix metalloproteinases (121). Furthermore, it is well known that pro-fibrotic mediators, including TGF-β1 and PDGF that induce the proliferation and activation of collagen-secreting myofibroblasts (119), are released by alveolar macrophages. During pulmonary fibrosis the plasticity of alveolar macrophages is needed to allow them to be able to polarize in each distinct phenotype in response to the dynamic micro-environment changes in airs pace. So far, there are no substantial studies about the role of M1 macrophages in pulmonary fibrosis. But according to previous research, it is well feasible that M1 alveolar macrophages contribute to fibrosis particularly in the initial phases of the disease. In the earliest stages of tissue damage, epithelial cells or endothelial cells may release inflammatory mediators that can promote the M1 macrophage polarization. Once polarized toward M1, macrophages produce TNF-α, IL-1β, and oxygen radicals. Many studies have indicated that these inflammatory cytokines and oxygen radicals are associated with development of fibrosis with their ability to amplify the inflammatory response and cause further tissue damage (94).

Due to the importance of the Th2 inflammatory responses in the development of pulmonary fibrosis, there are many studies reporting on the role of M2 macrophage polarization in the fibrotic phase of lung fibrosis. It has been demonstrated that IL-13 and IL-13R are highly expressed in IPF patients correlating with disease severity (122).

Thus, it is not surprising that M2 macrophages were found to be increased in BALF of IPF patients (94, 123, 124). M2 macrophages secrete a number of inflammatory and pro-fibrotic mediators, among them Th2 chemokines such as CCL17, CCL18 and CCL22 that have been reported to be associated with fibrosis development by recruiting CCR4+ T cells (123). It has been demonstrated that serum CCL18 concentrations have a predictive value in IPF and may be a useful tool in the clinical management of patients with IPF (125). In a bleomycin induced mouse fibrosis model, one study showed that CCL17 is elevated in bleomycin treated mice compared with the control group, and that application of a CCL17 neutralizing antibody attenuated fibrosis and pulmonary inflammatory cell numbers (126). Other M2 markers have also been shown to be increased in IPF such as galectin-3. It has been reported that galectin-3 contributes to the transforming growth factor-β1-driven lung fibrosis and that TD139, an inhibitor of galectin-3, attenuated the late-stage progression of bleomycin caused lung fibrosis by inhibiting TGF-β–induced β-catenin activation in vitro and in vivo (127). However, there are also some contradictory findings suggesting that M2 macrophages could be anti-fibrotic by suppression and resolution of fibrosis and uptake of ECM components. One study has shown that mice lacking arginase-1 in M2 macrophages have signs of unresolved inflammation and fibrosis (128). Uptake of these components is mediated by different mannose receptors which are known as M2 markers, and mannose receptor has shown to attenuate fibrosis in different models (129).

To summarize, both M1 and M2 alveolar macrophages are important cells in the pathogenesis of fibrotic lung diseases. M1 macrophages are thought to be more important in the initial inflammatory phase while M2 macrophages contribute mainly to

the fibrotic phase. Therefore, understanding how these two phenotypes contribute to different phase of pulmonary fibrosis is very important in understanding the development of this disease.

3.1.6. Protein degradation by the proteasome

In 2004, the Nobel Prize in Chemistry was awarded to the scientists for their discovery of the ubiquitin-proteasome mediated protein degradation. The ubiquitin-proteasome system (UPS) is the primary means by which cellular proteins are degraded and is a highly regulated system for the elimination of misfolded or damaged proteins as well as proteins whose activity is acutely regulated by signaling pathways (130). Therefore, this system has been reported to play a central role in almost all the cellular processes including cell proliferation, transcriptional regulation, apoptosis, immunity, and development (131). The core structure of this system is the 26S proteasome, a dynamic multi-subunit proteolytic complex within the cell, which functions as the key enzyme for non-lysosomal protein degradation (132).

Ubiquitin (Ub) is a small protein (76-residue) that is evolutionarily highly conserved in all eukaryotes (130). The initial signal for the degradation of the targeted protein is the selective binding of ubiquitin to the target proteins. The conjugation of Ub to a target protein is a three step process that begins with a high energy thioester linkage with an Ub-activating enzyme, also called an E1. Afterwards, this E1 “activated” Ub is then delivered to the active-site cysteine of an Ub-conjugating enzyme (E2). In step three, the addition of ubiquitin to the protein substrate is catalyzed by one of many Ub-protein ligase (E3) s - a diverse group of proteins (133). The high specificity and selectivity of

the UPS system lies in the diversity of E3s different ubiquitin-protein ligase that can recognize a specific substrate (133).

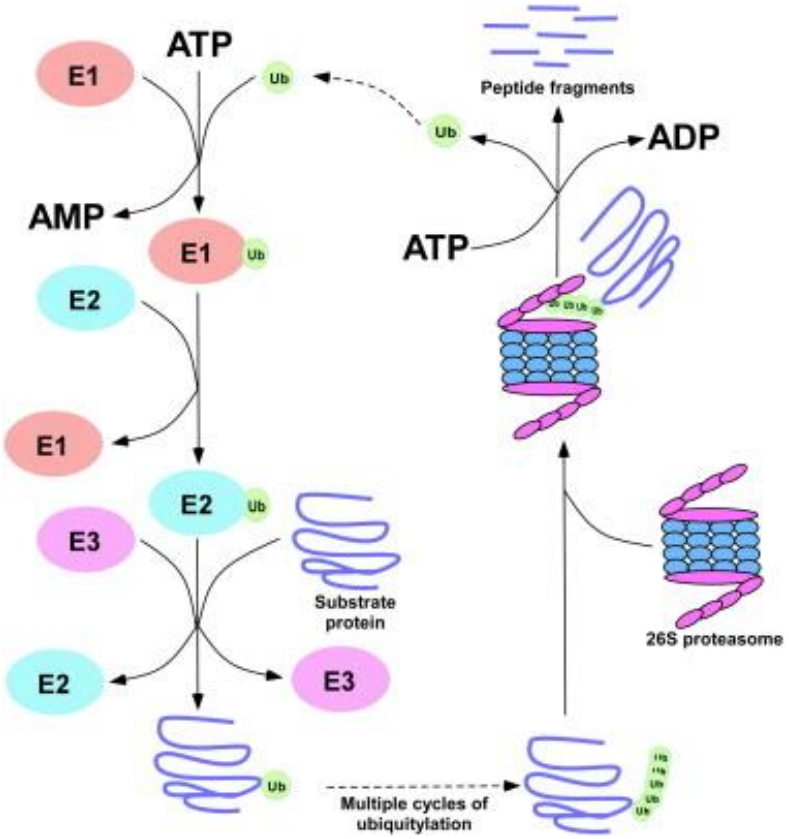

Fig 3.8: The schematic diagram showing ubiquitylation of substrate protein and its subsequent degradation by the 26S proteasome complex. An ubiquitin activating enzyme (E1) first forms a thioester bond with ubiquitin and then binds to an ubiquitin conjugating enzyme (E2). Subsequently, in the presence of an ubiquitin ligase enzyme (E3), the carboxy-terminus of ubiquitin forms an isopeptide bond with a K residue on target protein. The 26S proteasome recognizes, unfolds and degrades the polyubiquitylated-target protein into small peptides. Ub, ubiquitin. Taken from (134).

3.1.6.1. Structure of the proteasome

The proteasome is a self-compartmentalized protease. It carries out proteolytic activities deep within its interior, which means that it requires the appropriate features to gain access to the central proteolytic chamber. Once the delivery of the target protein to the

proteasome after complicated ubiquitin modification mediated by the chaperones and shuttling factors has taken place, most of proteasome actions are regulated by the regulatory subunit which feed substrates to the inner protease sites (135).

The 26S proteasome complex is a non-lysosomal proteolytic machine that consists of a 20S core particle (CP) and a 19S regulatory particle (RP), the latter of which can be further subdivided into lid and base sub-complexes. The 20S CP confers the proteolytic activities of the proteasome, whereas the 19S RP shows an ATP-dependence and specificity for ubiquitin protein conjugates (132). The 20S CP resembles a cylinder composed of four rings (two α and two β rings). Both of each α and β rings are composed of seven different α components (α1–α7) or β components (β1–β7) to form a β ring. In the β ring, three of the seven β-components were proven to be catalytically active, and are named by their substrate specificities: chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1) (134). The chymotrypsin-like activity cleaves proteins leaving hydrophobic residues, while the trypsin and caspase-like activities cleave, leaving basic and acidic residues, respectively (134). With the help of the 19S RP, the target proteins are delivered into the catalytic chamber of the 20S CP. It has been proven that the 19S is the proteasome regulatory particle (RP) responsible for recognition and processing of ubiquitinated substrates. Established as a highly dynamic proteasome activator, the RP has a large number of both permanent and transient components with specialized functional roles that are critical for proteasome function (136).

The 26S proteasome, also named constitutive proteasome, is found in most cells. In contrast to the constitutive form of proteasome, there is an inducible proteasome called

immunoproteasome (IP) which is tissue-specific and abundant in immune-related cells. The IP differs from its common counterpart. In the context of immune response, the IP is induced by the stimulation of cytokines such as IFN-γ and TNF-α, and then the β1, β2 and β5 components of the constitutive proteasome are replaced by low molecular mass protein 2 (β1i/LMP2), multicatalytic endopeptidase complex-like-1 (β2i/MECL-1), and β5i/LMP7. In addition, the IP also has an 11S regulatory structure or PA28 instead of the 19S RP of the 26S proteasome. Such replacement allows the IP to generate improved antigenic peptides for major histocompatibility complex (MHC) class I-mediated immune responses (137, 138).

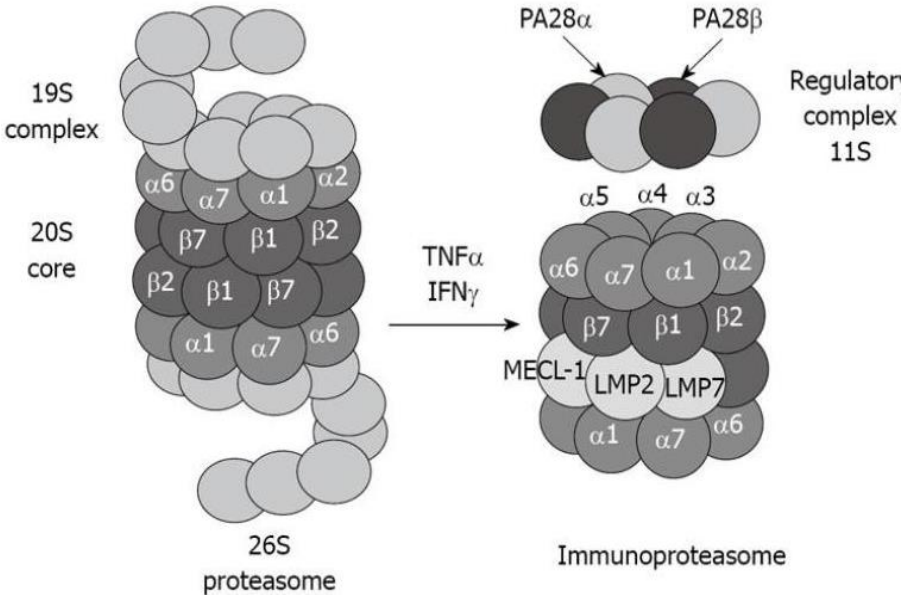

Fig 3.9: The structure of the constitutive proteasome and immunproteasome. Tumor necrosis factor (TNF-α) and interferon (IFNγ) induce formation of immunoproteasome subunits LMP7, LMP2 and MECL-1 subunits which replace the constitutive catalytic subunits β5, β1 and β2, respectively. Taken from (139).

3.1.6.2. Function of constitutive proteasome and Immunoproteasome

3.1.6.2.1. Proteasome function in MHC class I antigen processing

The ubiquitin–proteasome system is central in protein quality control and degradation in the mammal cells, which allow them to be involved in many of cellular processes including the cell signaling transduction, cell division, cell death, differentiation and migration(140, 141). However, the functions of proteasome in immune cell are still largely unclear. It has been well described that the proteasome plays a crucial role in MHC I antigen processing. Antigen recognition by cytotoxic T lymphocytes (CTLs) occurs through the interaction of their T cells receptors (TCRs) with peptide–MHC class I complexes. Both, intercellular and extracellular proteins are sources of antigenic peptides which are generated though the proteasome degradation (142). The proteasome is the protease that determines the carboxy-terminal anchor residues of MHC class I binding peptides and produces peptides of 8–9 amino acids that can bind directly to the peptide binding cleft of MHC class I molecules. In addition, amino terminally extended precursor peptides are also produced by proteasome, and are then processed further by aminopeptidases in the cytoplasm (143). It is generally assumed that the immunoproteasome improves quality and quantity of generated class-I ligands (144). It has been demonstrated that immunoproteasomes intensively increase the abundance and diversity of class-I ligands (145). Due to the recent study of the crystal structures of the constitutive proteasome and immunoproteasome, it was able to provide us an explanation for enhanced antigen processing by immunoproteasomes. It has been found that the β1i substrate binding channel is lined with hydrophobic amino acids, which finally leads to the enhancement of degradation of peptides into small nonpolar

residues (146). The β5i have the peptide bond hydrolysis ability which is favored by an increased hydrophilicity of the active site and additional hydrogen bonds shaping the oxyanion hole (146).

3.1.6.2.2. Modulation of immune signaling pathways

In recent years, it became apparent that both constitutive proteasome and immunoproteasomes not only function to process MHC-I ligands, but also possess additional immunological functions. It has been reported that LMP2/β1i-deficient bone marrow-derived dendritic cells infected with an influenza virus produced less IFN-α, IL-1β, IL-6 and TNF-α as compared to wild-type counterparts, indicating the crucial role of immunoproteasome in innate immune responses. The reduced ability to produce cytokines in LMP2/β1i-deficient cells has been associated with compromised NF-kB signaling (147). It has been shown that the constitutive ubiquitin-proteasome system is involved in NF-κB pathway activation through at least three steps: degradation of the NF-κB inhibitor IκB, processing of NF-κB precursors and activation of the IκB kinase (IKK) through a degradation-independent mechanism (148). However, contradictory findings have been reported on the role of the immunoproteasome for the degradation of IκBα and the activation of the canonical NF-κB pathway. Evidence in both knockout mice samples and humans with immunoproteasome mutations implicate a contradictory role of the immunoproteasome in modulating NF-κB signaling. One study reported that, immunoproteasome-deficient mice showed a defect in proteolytic processing of NF-κB precursors (p100/p105) and decreased degradation of IκBα (149, 150). Contrary to this research, another group using chemical genetic approaches showed that the catalytic

activity of the immunoproteasome subunits β1i and β5i is not required for canonical NF-kB activation (151). This difference may be due to the different cell line models, which were used in their laboratories. Therefore, more solid studies need to be performed to find out how immunoproteasomes may modulate the NF-κB pathway.

3.1.6.2.3. The role of immunoproteasomes in immune cell

T cell differentiation

CD4 T cells play the critical roles in regulating adaptive immunity to a variety of infectious diseases. They are also involved in autoimmunity and chronic inflammatory diseases including arthritis, inflammatory bowel disease (IBD), asthma, and IPF. Naive CD4+T cells can differentiate into different cells lineages such as Th1, Th2, and Th17 and regulatory T cells depending on the cytokines in the microenvironment (152). Several studies have demonstrated that immunoproteasomes shape the T cell repertoire and are responsible for the survival and expansion of T cells after virus infection (153, 154). Apart from that, it has been reported that immunoproteasome subunit LMP7 deficiency and inhibition suppresses Th1 and Th17 but enhances regulatory T cell differentiation. This study may be able to explain the therapeutic effect of LMP7 inhibitor ONX 0914 in experimental diabetes, arthritis, and colitis mice models (155).

Regulation of macrophage activation

It has been previously demonstrated that the proteasome serves as a central regulator of inflammation and monocyte and macrophage function (156). One of the monocyte and macrophage functions is the response to inflammatory stimuli such as LPS and

releases a large amount of the proinflammatory cytokines TNF-α, IL-1β and IL-6. It has been demonstrated that inhibition of proteasome activity by the proteasome inhibitor MG132 modulates proinflammatory cytokines production and expression of their receptors in the macrophage cell line U937 cells which involved the inhibition of NF-κB and AP-1 activation (157). It has also been that reported pretreatment of RAW 264.7 macrophage-like cells with the proteasome inhibitor lactacystin resulted in a dose dependent inhibition of LPS-induced TNF-α. Further studies proved that lactacystin blocked the LPS-induced ERK phosphorylation but failed to inhibit IRAK-1 kinase activity (158). However, so far, there are only few studies about the role of immunoprotesome in macrophage function. One study by Julia Reis suggested that constitutive proteasome subunits are replaced by immunoproteasome subunits after LPS treatment of RAW264.7 cells. Macrophages derived from mice with LMPs knockout exhibited dysregulated cytokine production in response to LPS in vitro (159). Specifically, NO production and IL-1β and IL-6 secretion from LMP deficient macrophages were markedly reduced compared to the Wt counterpart, whereas TNFα levels were unexpectedly unchanged in LMP-/- macrophages. Further studies indicated that the LPS-induced MyD88 pathway was normal, while the TRIF/TRAM and IRF-3 pathways were defective in LMP-/- macrophages (160). These studies reveal a novel active function of the immunoproteasome subunits, which suggest the complexes of immunoproteasome in the regulation of immune cells.

Though some studies were carried out to evaluate the function of proteasome in macrophages, the role of proteasome, particularly of the immunoprotesome, in alveolar macrophage biology is largely unknown.

3.2. Project aims

Accumulating evidence indicates a crucial role of immunoprotesome for macrophage activation. In view of the contribution of impaired polarization of alveolar macrophages in acute and chronic lung diseases, the aim of this study was to characterize the function of the immunoprotesome on the regulation of alveolar macrophage polarization.

The following objectives were pursued:

- Using different sources of alveolar macrophages, primary and cell lines to establish an LPS/IFNγ or IL-4 induced M1 or M2 polarization model in vitro.
- Investigating whether the proteasome and immunoproteasome subunit expressions were induced at both protein and mRNA levels during M1 or M2 polarization.
- Monitoring the kinetics of the expression and activity of the immunoproteasome expression and activity during macrophage polarization.
- Determining whether the immunoproteasome subunits LMP2 and -7 are functionally relevant for macrophage polarization.
- Identifying polarization associated changes of intracellular signaling related to the LMP2 and -7 deficiencies using appropriate knock out mice.
- Investigating whether an immunoproteasome subunit LMP7 specific inhibitor has a similar effect as a LMP7 knock out.

3.3. Chapter 1 – Results

3.3.1. Characterization of the polarization pattern of different alveolar macrophage types

A wide variety of sources of macrophages has been used for macrophage polarization studies (161). Although there are several reports about the polarization of alveolar macrophages under different diseases conditions, here we are describing the polarization of alveolar macrophages into respective M1 and M2 phenotypes at the in vitro level for the first time. To gain insight whether the polarization depends on the alveolar macrophage (AMs) background, we first characterized the plasticity of primary AMs isolated from BALB/c and C57BL/6 mice, as well as the SV40 immortalized BALB/c-derived AM cell line MH-S. To trigger polarization, cells were treated either with LPS and IFNγ to induce M1-like phenotypes or with IL-4 for induction of M2 polarization.

To determine if polarized alveolar macrophages show different cell morphology, bright field microscopy was used to examine the cell morphology after 24 h of polarization. Cell morphology of AMs was clearly altered depending on the stimulus as exemplarily shown for C57BL/6 AMs (Figure 3.10): In comparison to untreated and non-polarized cells (M0), the shape of M1-polarized macrophages was globular, while M2-polarized macrophages exhibited a flattened and adherent morphology.

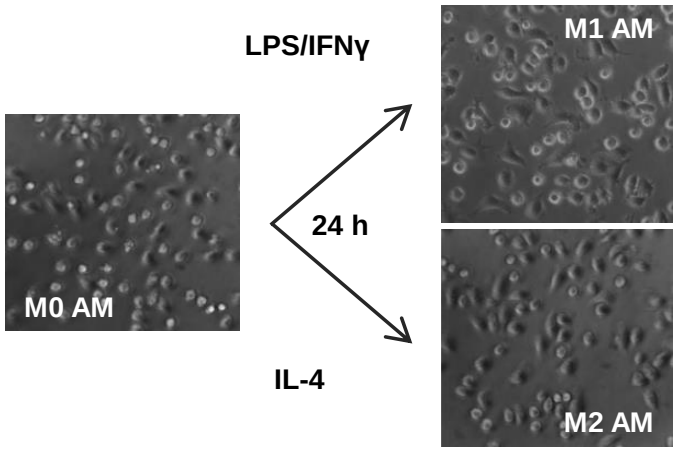

Figure 3.10 Representative morphology of 24 h polarized M1 and M2 alveolar macrophage. Primary alveolar macrophages from C57BL/6 mice were polarized into M1 or M2 for 24 h by stimulation with LPS/IFNγ or IL-4, respectively. Non-polarized cells (M0) served as controls. Cell morphology was examined by bright field microscopy with 20-fold magnification. Results are representative for at three independent experiments.

To better understand the global gene expression patterns in polarized AMs, we used Illumina microarrays to identify up-regulated genes in M1 and M2 cells. We found that 162 genes were induced in M1 condition (see heat map in Figure 3.11).

Figure 3.11 Heat map analysis of global gene expression in M1 polarized alveolar macrophages. Visualization of gene expression changes in 24 h polarized primary AMs from C57BL/6 mice as a heat map. Up-regulated genes in M1 are shown in red. The genes with $P<0.05$ were displayed. Results are from three individual C57BL/6 wt mice.

As we expected, a number of M1 markers as described in the literature were upregulated in our microarray analysis, among them Tnf, Il1b, and Il12b. However, Nos2, as one of most frequently used M1 marker, was not shown to be induced in M1 cells, which might be due to a technical reason. We also selected these markers to characterize the polarization profile of the different AMs. Comprehensive gene expression analysis by qPCR for activation of specific genes confirmed markedly increased expression of these M1 marker genes in M1 polarized cells compared to untreated (M0) and M2 polarized cells (Fig. 3.12).

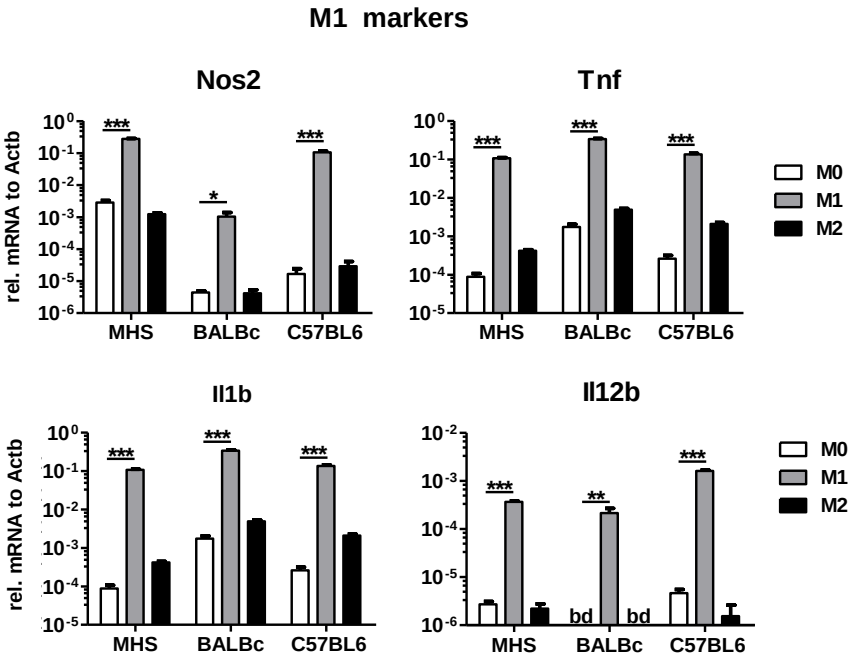

Figure 3.12 Alveolar macrophages show characteristics of M1 polarization by marker genes profiling. M1 marker gene expression analysis of MH-S cells or primary alveolar macrophages from BALB/c or C57BL/6 mice polarized for 24 h: Nos2, Tnf, Il1b, Il12b, relative to Actb (β-actin) expression. Results are representative for three independent experiments, bd, below detection.

To investigate the genes associated with M2 AMs polarization, microarray analysis identified 35 genes to be induced at M2 condition, which are displayed in the heat map below (Figure 3.13). Among these genes, Arg1, Retnla and Ccl17 have been well described as M2 markers genes. They were selected for the characterization of M2 polarization of AMs from different sources.

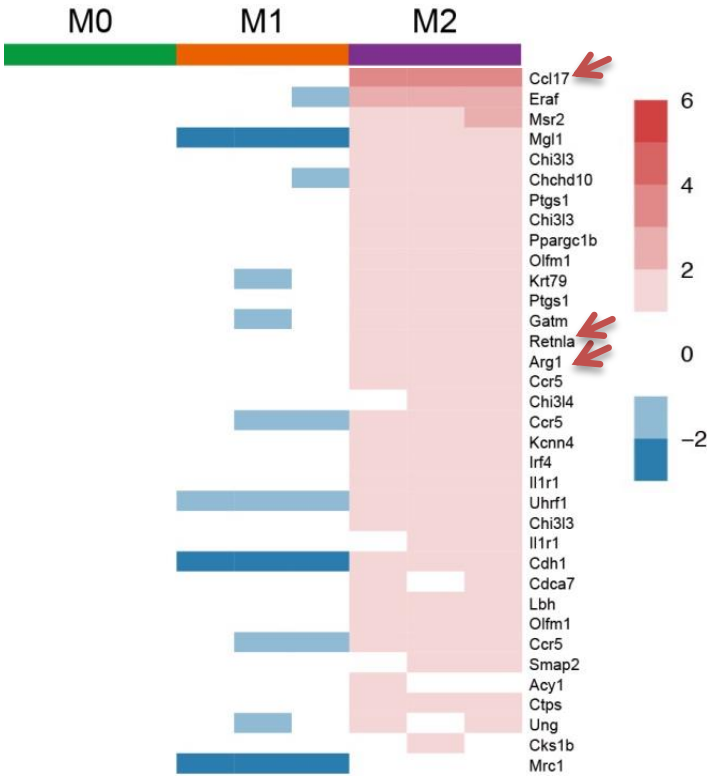

Figure 3.13 Heat map analysis of global genes expression in M2 polarized alveolar macrophages. Visualization of gene expression for 24 h polarized primary AMs from C57BL/6 mice as a heat map. Up-regulated genes in M2 are shown as pink. The genes with $P<0.05$ were displayed. Results are from three individual C57BL/6 wt mice.

IL-4 treatment induced uniform expression of M2 marker genes in primary AMs while marker gene expression was less consistent in IL-4 treated MH-S cells: Expression of Arg1 and Mrc1were stimulated in a M2 specific manner in all three types of AM, while

Ccl17 and Retnla (Fizz-1) were only found elevated in primary AMs, but not in MH-S cells (Fig. 3.14).

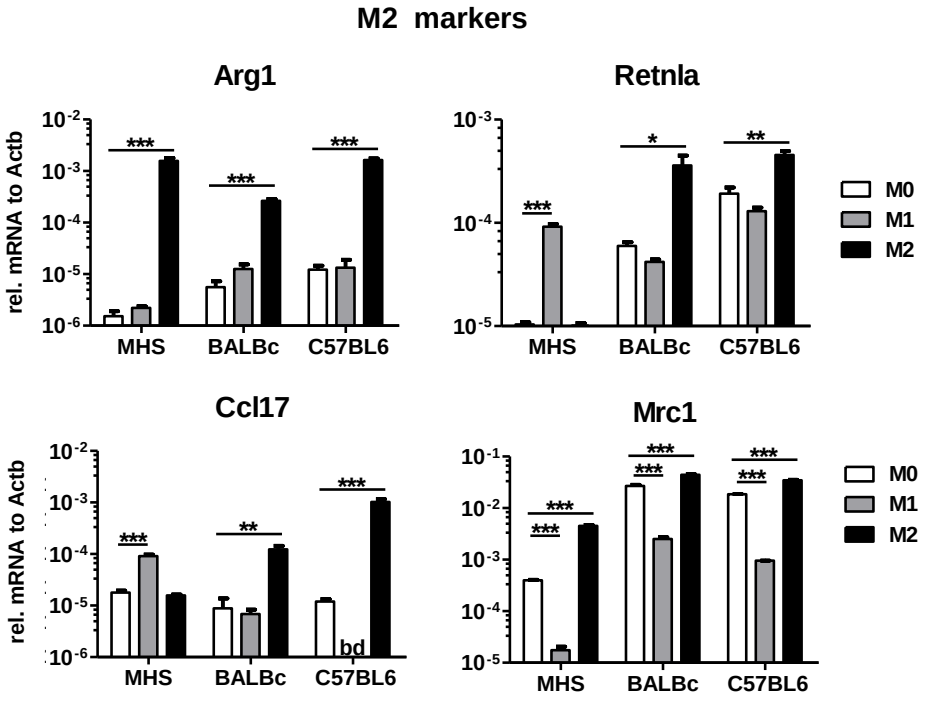

Figure 3.14 Alveolar macrophages show characteristics of M2 polarization by maker genes profiling. M2 marker gene expression analysis of MH-S cells or primary alveolar macrophages from BALB/c or C57BL/6 mice polarized for 24 h: Arg1 (Arginase1), Ccl17, Retnla (Fizz-1), Mrc1 (mannose receptor 1), relative to Actb expression. Results are representative for at three independent experiments bd, below detection.

To further confirm the alveolar macrophage polarization at the protein level, we selected the most well-known marker gene iNOS for M1 and Arginase1 for M2 to perform western blot analysis. Polarization of alveolar macrophages was confirmed at the protein level for primary AMs after 24 h of cytokine stimulation with elevated iNOS (NOS2) expressions in M1 and Arg1 in M2 polarized cells, respectively (Fig. 3.15).

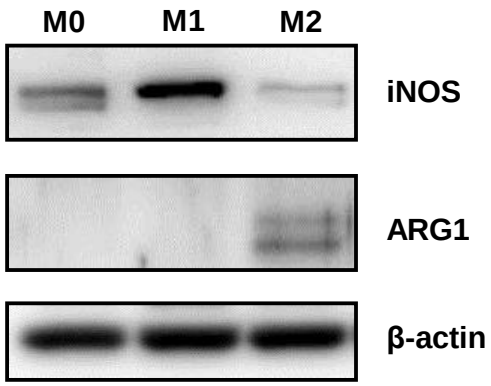

Figure 3.15 M1 (iNOS) and M2 (Arginase1) protein analysis shows characteristic M1/M2 alveolar macrophage polarization. M1 (iNOS) and M2 (Arginase1) markers were evaluated on protein level in primary alveolar macrophages from C57BL/6 mice polarized for 24 h. Results are representative for three independent experiments.

3.3.2. Proteasome and Immunoproteasome expression and activity during alveolar macrophage polarization.

To investigate regulation of proteasome related genes in polarized AMs, we screened the expression pattern of 60 genes as shown in the heat map (Figure 3.16). We found that a number of constitutive proteasome and immunoproteasome subunits were induced in M1 polarized macrophages but generally not in M2-polaized AMs.

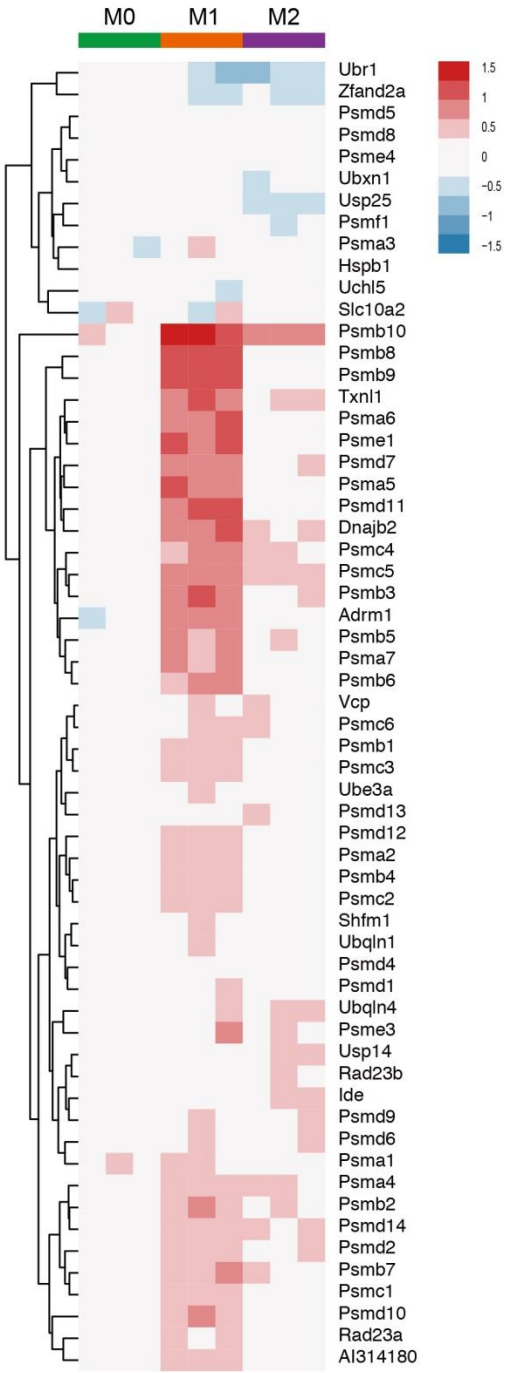

Figure 3.16 Heat map analysis of proteasome related genes expression in polarized alveolar macrophages. Visualization of gene expression for 24 h polarized primary AMs from C57BL/6 mice as a heat map. Up-regulated genes in M1 and M2 are shown as red. The genes with $P<0.05$ were displayed. Results are from three individual C57BL/6 wt mice.

To validate the regulation of the expression of constitutive proteasome subunits and proteasome regulators during AM polarization, gene expression of the PSMA3, PSMD11, PSME1/2/3 and PSMB5/6/7 subunits was profiled by qPCR. Interestingly, the mRNA levels of all the proteasome subunits were uniformly induced after 24 h at M1 Fig. 3.17).

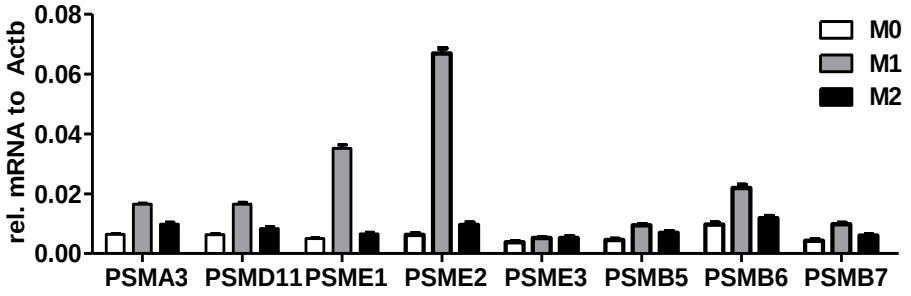

Figure 3.17: mRNA expression of proteasome subunits is induced in both M1 and M2 polarized alveolar macrophages. Proteasome subunit gene expression in MH-S cells or primary alveolar macrophages from BALB/c or C57BL/6 mice polarized for 24 h: PSMA3, PSMD11, PSME1/2/3 and PSMB5/6/7 expression displayed relative to Actb. Results are representative for three independent experiments. Note, that we used a linear scale to depict the minor changes in gene expression levels.

It has been well described that IFNγ can induce expression of immunoproteasome subunits in a variety of cell types through the STAT1 pathway (162), which causes the replacement of constitutive proteasome by immunoproteasomes. As we used IFNγ plus LPS to induce the M1 macrophage phenotype, we firstly speculated that the expression of the immunoproteasome subunits LMP2, MECL-1, and LMP7 is induced in M1 macrophages. According to our expectation, our microarray data revealed that immunoproteasome subunits LMP2, MECL-1, and LMP7 were induced in M1 AMs. The gene expression of these immunoproteasome subunits was further analyzed by qPCR in both M1 and M2. While mRNA levels of all three immunoproteasome subunits (Psmb8,

9, 10 for LMP7, LMP2, and MECL-1, respectively) were uniformly induced after 24 h, under conditions of M1 polarization, no reproducible changes were observed for M2 polarization conditions (Fig. 3.18).

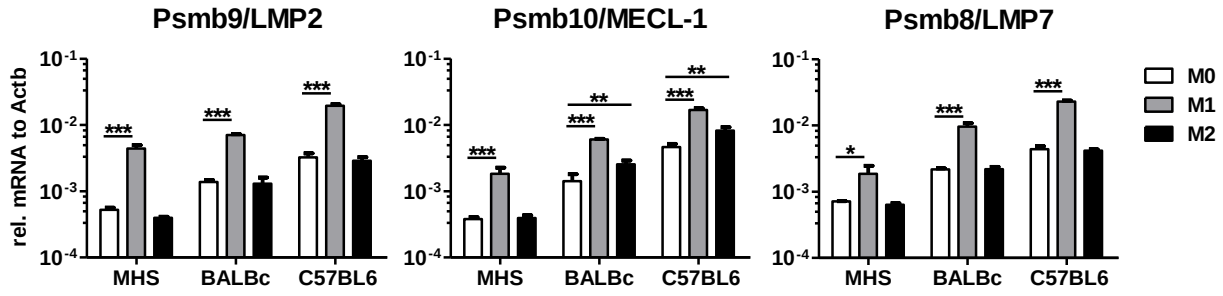

Figure 3.18: mRNA expression of immunoproteasome subunits LMP2 and LMP7 is induced in M1 but not in M2 polarized alveolar macrophages. Immunoproteasome subunit gene expression in MH-S cells or primary AMs from BALB/c or C57BL/6 mice polarized for 24 h: Psmb9 (LMP2), Psmb10 (MECL-1), and Psmb8 (LMP7) expression displayed relative to Actb. Results are representative for three independent experiments.

Western blot analysis for the standard 20S proteasome α1-7 and the two immunoproteasome subunits LMP2 and LMP7 revealed moderate basal expression of immunoproteasome subunits which was strongly upregulated in M1-polarized AMs compared to unstimulated controls. Remarkably, however, protein expression of both, LMP2 and in particular LMP7, were elevated in M2 cells (Fig. 3.19) indicating posttranscriptional regulation of immunoproteasome expression upon M2 polarization of AMs. The protein level of α1-7 was also induced in both M1 and M2 cells.

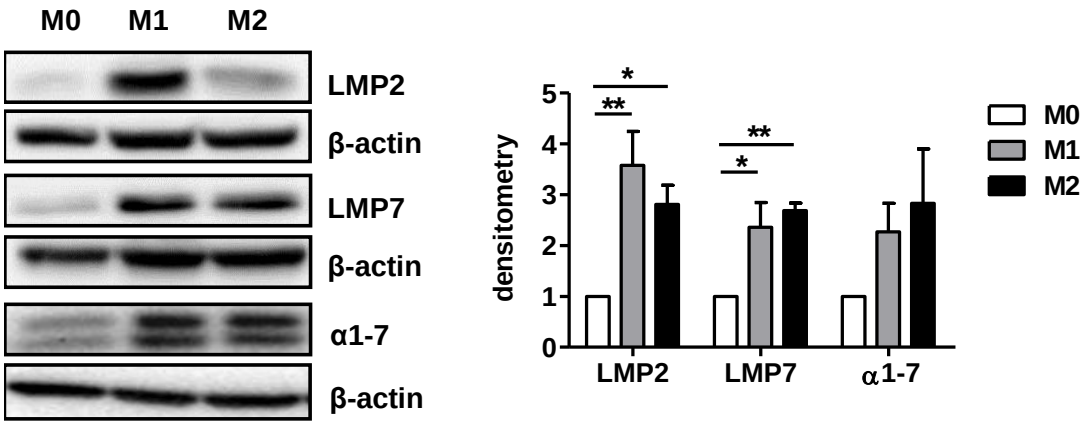

Figure 3.19: Protein expression of constitutive proteasome and immunoproteasome subunits LMP2 and LMP7 were induced in both M1 and M2 polarized alveolar macrophages. Immunoproteasome subunit LMP2 and LMP7 protein expression in primary alveolar macrophages from C57BL/6 mice polarized for 24 h. Densitometric analysis was done from three independent experiments.

Specific activities of the immunoproteasome subunits can be analyzed using specific activity based probes which have been developed recently (163, 164). To further corroborate the existence of catalytically active immunoproteasomes in polarized AMs we assessed the activity of immunoproteasome and standard proteasome subunits using a set of fluorescently labeled activity-based probes (ABPs) with distinct binding specificities to the different active sites after 24 h of cytokine stimulation (Fig. 3.20). These ABPs covalently bind to the active-site threonine of the catalytic subunits and can be used to label active proteasome complexes in native lysates. Labeled lysates are then separated by SDS-PAGE to attribute activities to single subunits. . Both, immuno- and constitutive proteasome (β1, β2 and β5) activities were significantly elevated in M1 polarized alveolar macrophages compared to the control M0 state but only β1 was considerably elevated also in M2-polarized AMs.

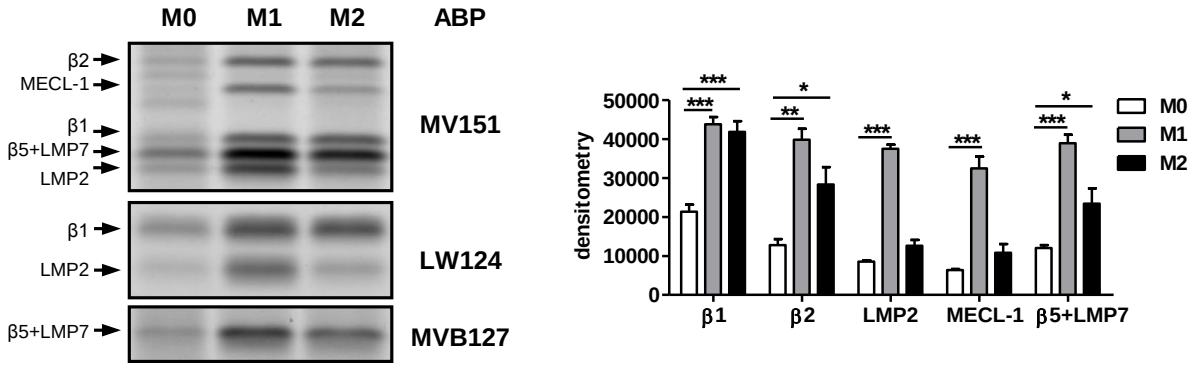

Figure 3.20: Activity of constitutive proteasome and immunoproteasome subunits LMP2 and LMP7 were induced in both M1 and M2 polarized alveolar macrophages. Proteasome activity, in polarized alveolar macrophages from C57BL/6 mice detected after 48 h stimulation by activity-based probes (ABP) MV151 (labeling all catalytically active β-subunits), LW124 (β1 and LMP2) or MVB127 (β5 and LMP7). Densitometric analysis displays the combined data from three experiments. Picture provided courtesy of Oliver Vosyka.

3.3.3. Time course of immunoproteasome subunit expression during alveolar macrophage polarization.

The majority of studies focused on only one single time point of macrophage polarization, which cannot provide full information about the dynamic changes of genes during the whole range of polarization. To study the kinetics of the increased expression of LMP2, MECL-1, and LMP7 during IFNγ-driven AM polarization, primary macrophages were treated with LPS or IFNγ or IL-4 for 6, 24, 48, and 72 h, respectively. Firstly, we investigated the expression of respective M1 marker Tnf or M2 maker Ccl17. At M1 polarizing conditions, gene expression analysis revealed maximal levels of the M1 marker Tnf after 6 h of LPS or IFNγ treatment which returned to baseline after 72 h (Fig. 3.21). At the same time, expression levels of LMP2, MECL-1, and LMP7 were increased after 6 h and remained elevated for 72 h. In M2 polarized AMs, expression of the M2 marker Ccl17 increased dramatically up to 72 h. For immunoproteasome subunits,

however, we only observed a moderate increase of MECL-1 but no change in gene expression levels of LMP2 and LMP7.

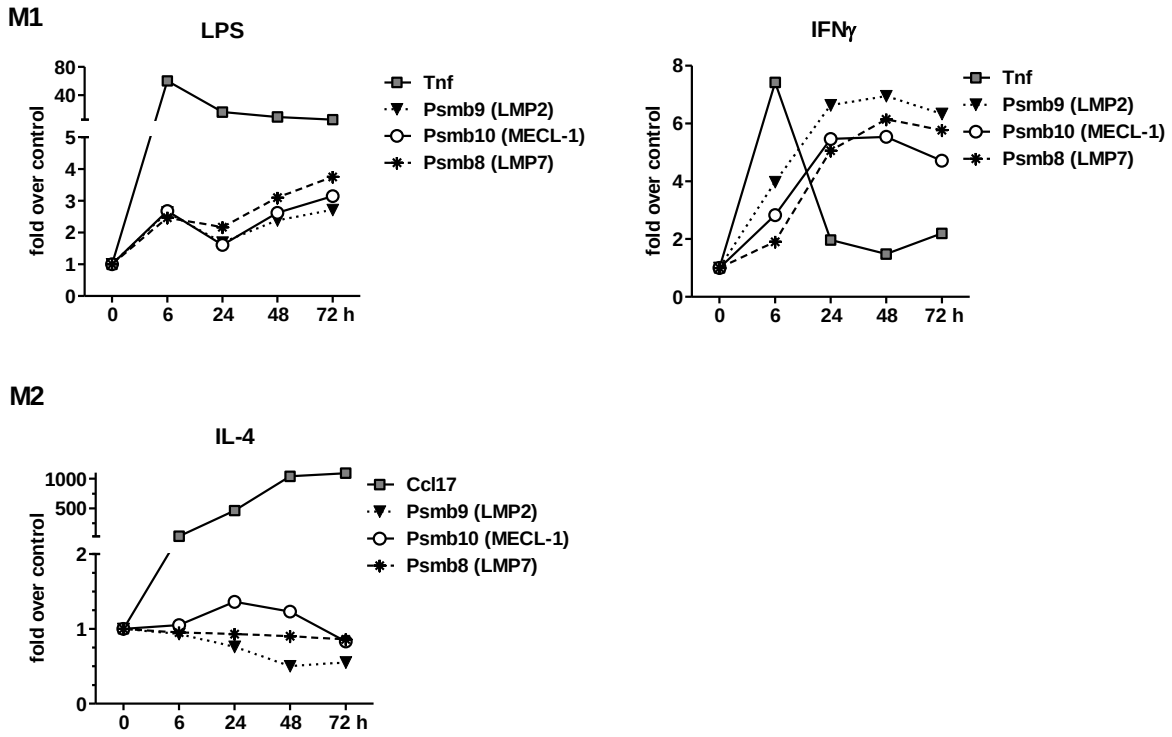

Figure 3.21: Gene expression analysis of immunoproteasome subunits during primary alveolar macrophage polarization. Alveolar macrophages from C57BL/6 mice were treated with IFNγ or IL-4 for 6, 24, 48 and 72 h and mRNA was analyzed to detect expression of Tnf, Ccl17, Psmb8 (encoding LMP7), Psmb9 (LMP2) and Psmb10 (MECL-1). Results are displayed as fold change over control relative to Actb expression and are representative for three independent experiments.

Following the mRNA profile, we investigated protein expression of constitutive proteasomes and immunoproteasomes in primary macrophages and MH-S. Cells were treated with LPS or IFNγ and IL-4 or IL-13 for 6, 24, 48, and 72 h, respectively. In contrast to the mRNA results, protein levels of constitutive proteasome and immunoproteasome subunits increased further from 24 to 72 h in both M1 and M2 polarizing conditions in primary AMs (Fig. 3.22 A) and in the MH-S cell line (Fig. 3.22 B).

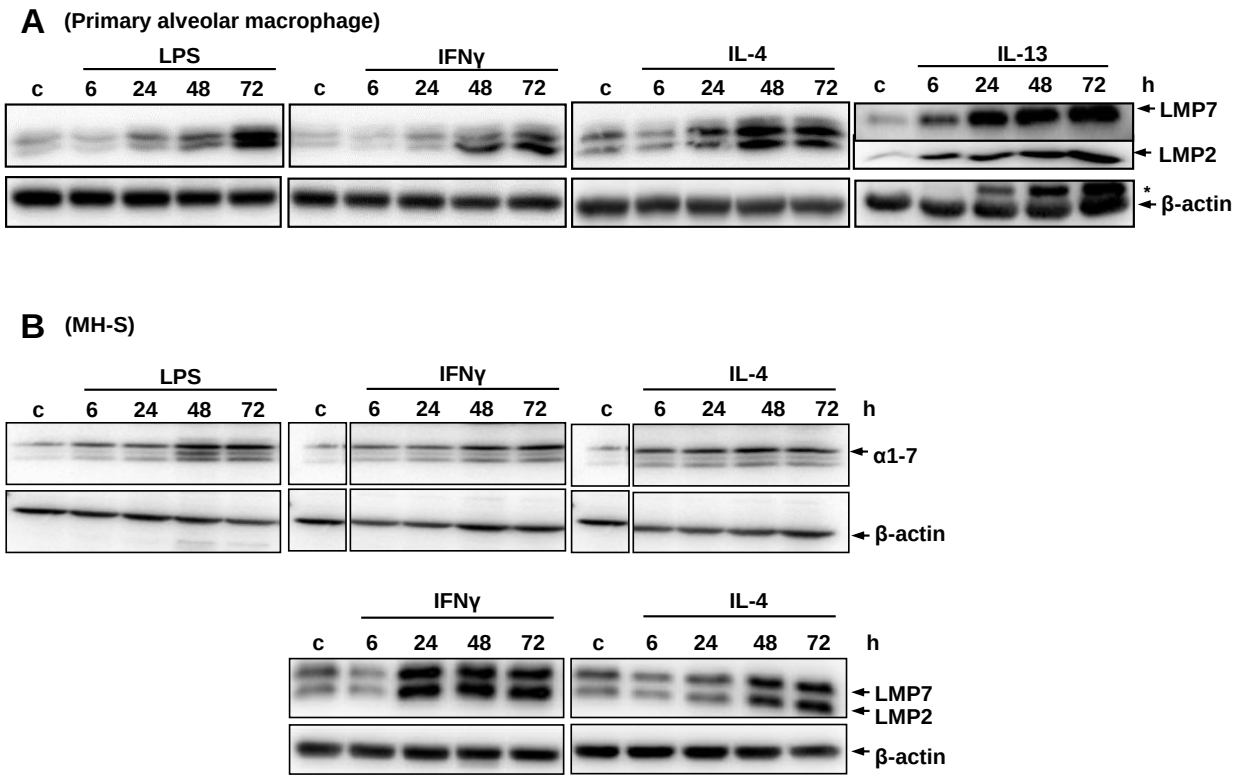

Figure 3.22: (A, B) Proteins level analysis of constitutive proteasome and immunoproteasome subunits during primary alveolar macrophage polarization. Alveolar macrophages from C57BL/6 mice and MH-S cell line were treated with M1 condition LPS or IFNγ and IL-4 or IL-13 for 6, 24, 48, and 72 h, respectively, which were analyzed to detect protein expression of constitutive proteasome a1-7 and immunoproteasome subunits LMP2 and LMP7 during polarization of primary alveolar macrophages from C57BL/6 mice. Results are representative for two independent experiments; the control (c) is the unstimulated 24 h control.

In accordance with our expression data, immuno- and standard proteasome activities increased as well after 48 and 72 h in both M1 and M2 polarized AMs (Fig.3.23). These results clearly show IL-4-induced formation of active immunoproteasomes during M2 polarization of alveolar macrophages. In contrast to IFNγ-mediated transcriptional induction of immunoproteasomes, this may involve the posttranscriptional mechanisms of protein stabilization. Our kinetic data also indicate that elevated immunoproteasome

expression and activity are not the driving force for M1 and M2 marker gene expression but rather a consequence of the distinct polarization states.

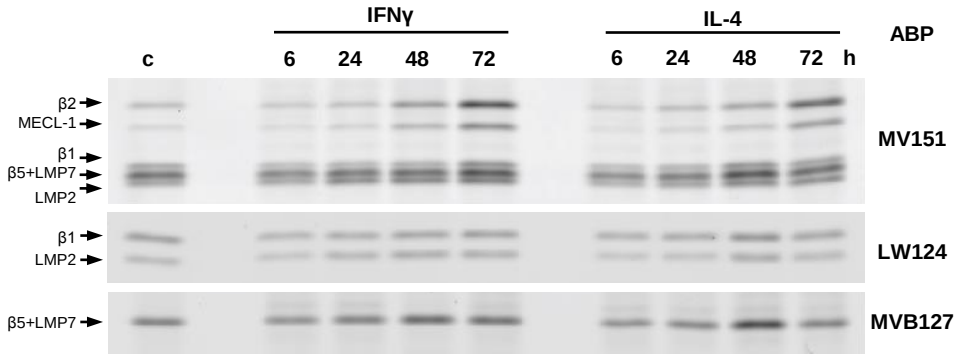

Figure 3.23: Proteasome activity analysis during primary alveolar macrophage polarization. Proteasome activity in polarized alveolar macrophages from C57BL/6 mice detected by ABPs MV151 (labeling all catalytically active β-subunits), LW124 (β1 and LMP2) or MVB127 (β5 and LMP7). Data are representative for three independent experiments; the control (c) is the unstimulated 24 h control. Picture provided courtesy of Oliver Vosyka.

3.3.4. Deficiency of LMP2 and LMP7 immunoproteasome subunits does not affect M1 but enhance M2 alveolar macrophage polarization

To investigate, whether immunoproteasome activity and expression in AMs is of any functional relevance for macrophage polarization, we polarized primary AMs from LMP2$^{-/-}$ and LMP7$^{-/-}$ mice towards M1 or M2 phenotypes, respectively. As a first step, we performed the Water soluble Tetrazolium (WST) salt cell viability assay to exclude any unspecific effect after LMPs knock out and polarization. After 24 h of M1 and M2 polarization, both the AMs from wt and LMPs$^{-/-}$ showed no treatment or genotype depending effect on cell viability (Fig. 3.24).

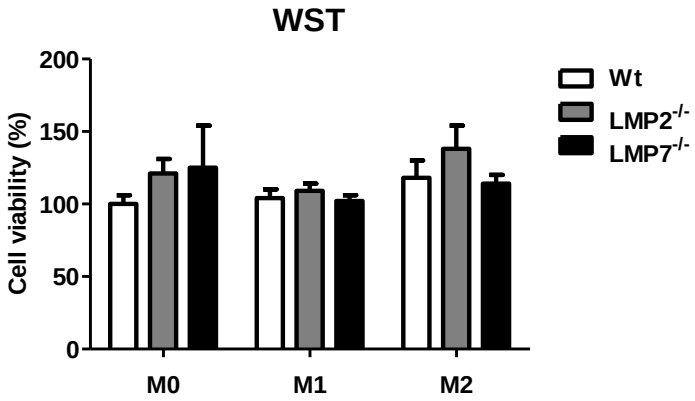

Figure 3.24: WST cell viability assay. WST assay of primary alveolar macrophages from C57BL/6 Wt, LMP2$^{-/-}$ or LMP7$^{-/-}$ mice polarized towards M1 phenotype (LPS and IFNγ for 24 h). Unpolarized cells (M0) were set to 100 %. Data are combined from measurements of 3 individual mice.

At M1 polarizing conditions, mRNA expression of M1 markers (Nos2, Tnf, Il1b and Il12b) was clearly increased in AMs of wt and LMP2$^{-/-}$, and LMP7$^{-/-}$ mice (Fig. 3.25). Nevertheless, there was no M1 consistent alteration in marker expression, although the extent of mRNA induction was different between genotypes: e.g. LMP7$^{-/-}$ AMs exhibited a reduced induction of Nos2 and Tnf; LMP2$^{-/-}$ AMs had increased levels of Nos2 and Il12b but a decreased level of Tnf compared to wt mice, while there was no difference in Il1b expression.

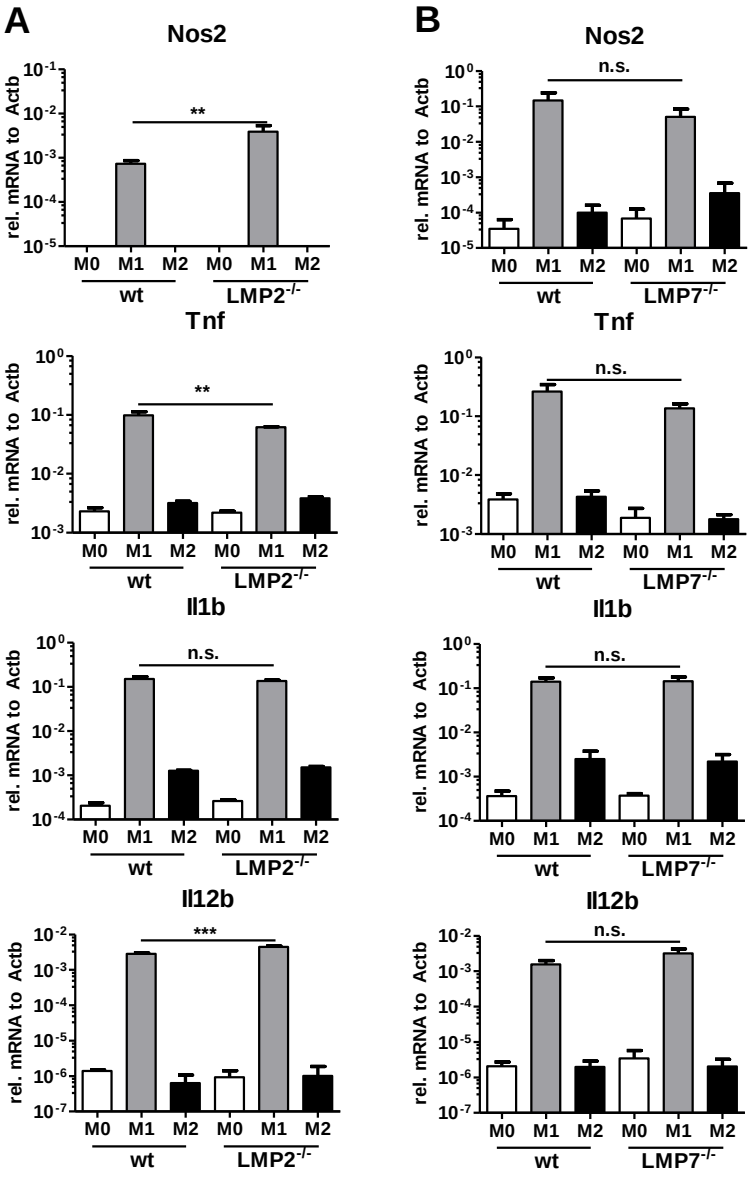

Figure 3.25: Deficiency of LMP2 and LMP7 immunoproteasome subunits does not affect M1 alveolar macrophage polarization (A, B) M1 marker gene expression analysis of primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice polarized towards M1 phenotype (LPS and IFNγ for 24 h): expression of Nos2 (iNos), Tnf, Il1b, Il12b is shown relative to Actb. Results are representative for three independent experiments.

Interestingly, our heat map array analysis revealed that 21 and 43 M2 signature genes expression were significantly enhanced in LMP2-/- and in LMP7-/- M2 cell respectively,

compared to wt cells (Fig. 3.26). Among these genes, the major M2 marker Arg1, and Retnla as well as the Th2 cytokine Ccl17 were increased in immunoproteasome deficient alveolar macrophages.

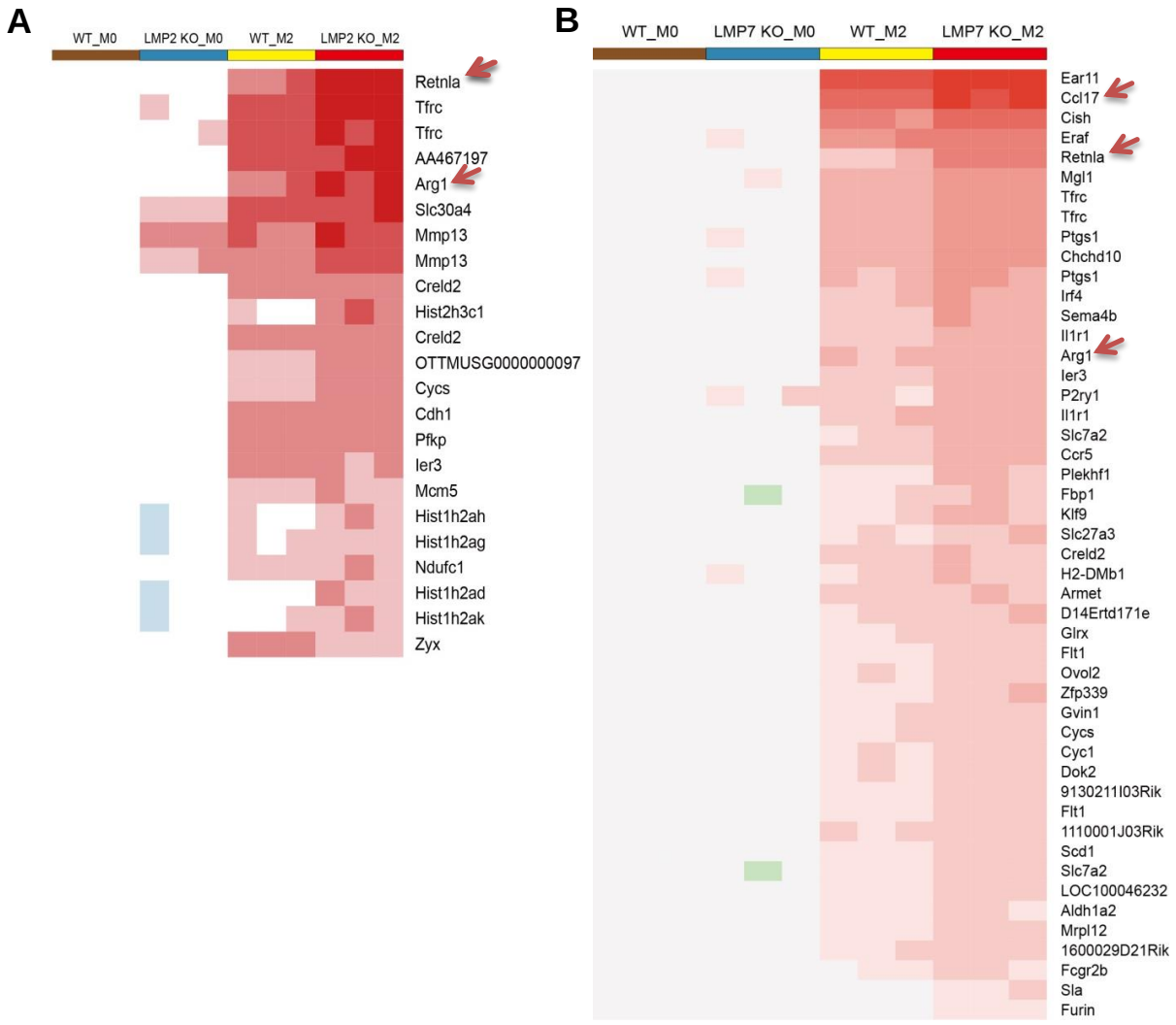

Figure 3.26: Heat map analysis of M2 related genes expression altered in immunoproteasome deficient alveolar macrophages. (A) The left map includes 21 genes which expression levels were significantly enhanced in LMP2-/- M2 cell, compared to wt counterpart. (B) The right map includes 43 genes whose expression levels were significantly enhanced in LMP7-/- M2 cell, compared to wt controls. Pink in the heat maps indicates up-regulation. The genes with $P<0.05$ were displayed. Results were normalized to wt-M0, and were from three individual C57BL/6 wt or LMP2-/- or LMP7-/- mice.

Further qPCR experiments confirmed the results from our microarray analysis, as gene expression of the major M2 markers Arg1, Retnla and Ccl17 was slightly increased in LMP2-/- and clearly upregulated in LMP7-/-AMs, compared to wt cells (Fig. 3.27).

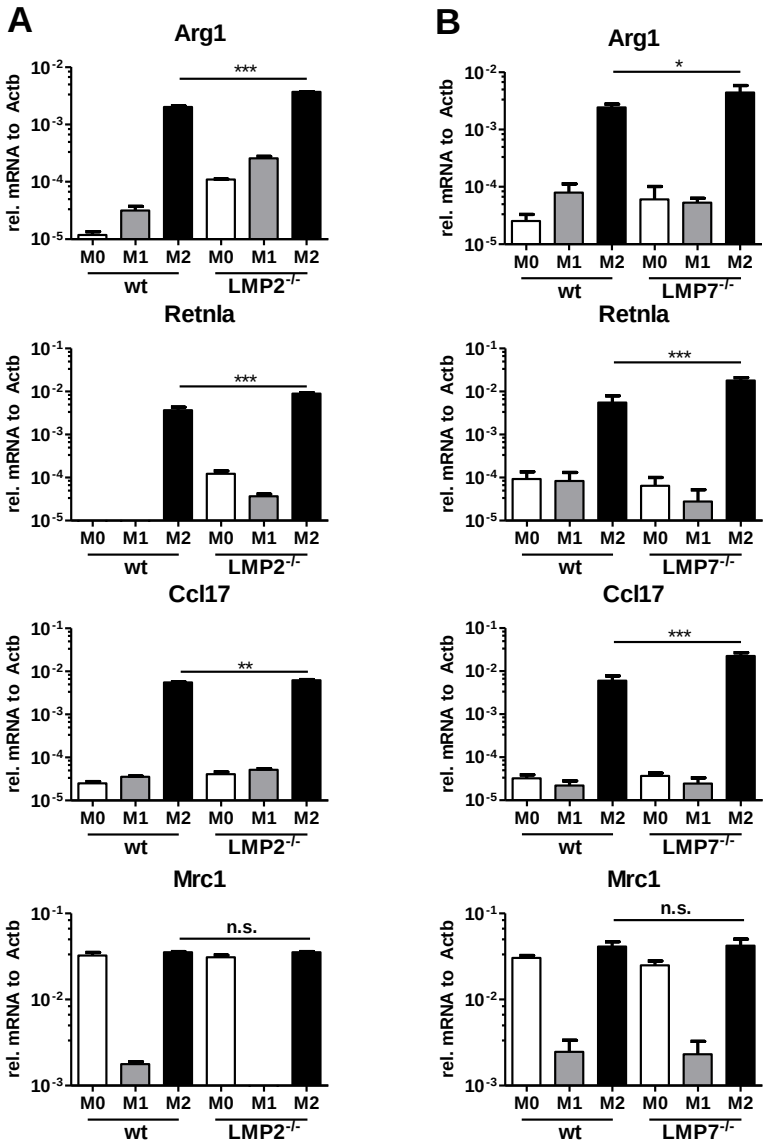

Figure 3.27: Deficiency of LMP2 and LMP7 immunoproteasome subunits affects M2 alveolar macrophage polarization. (A, B) M2 marker gene expression analysis of primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice polarized towards M2 phenotype (IL-4 for 24 h): expression of Arg1 (Arginase1), Ccl17, Retnla (Fizz-1), Mrc1 (mannose receptor 1) relative to Actb expression. Results are representative for three independent experiments.

Because CCL17 is thought to play a crucial role as an M2 effector cytokine for Th2-related lung diseases, we validated the Ccl17 expression data by analyzing CCL17 protein release in supernatants from polarized AMs using a specific ELISA. As shown in Figure (Fig. 3.28), CCL17 release was induced in IL-4 treated M2 polarized wt cells. It is important to note that secretion of CCL17 was significantly enhanced in M2 polarized AMs from LMP2-/- and LMP7-/- mice compared to wt cells. Taken in combination, our results clearly indicate that expression and activity of distinct immunoproteasome subunits is of functional relevance for the plasticity of alveolar macrophages with subsequent release of effector cytokines.

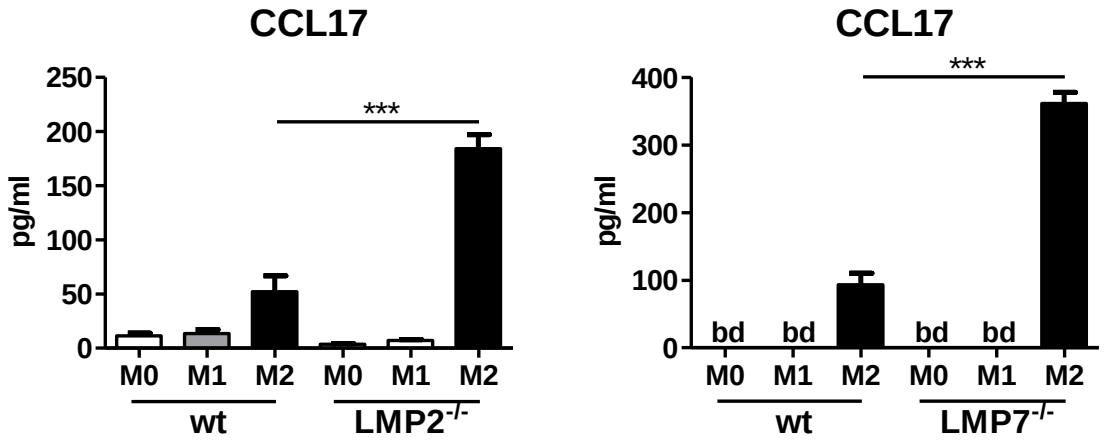

Figure 3.28: Deficiency of LMP2 and LMP7 immunoproteasome subunits enhances the release of Th2 chemokine CCL17 from M2 alveolar macrophage polarization. CCL17 secretion measured in supernatants of polarized primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice (M1: LPS and IFNγ; M2 IL-4 for 24 h). Results show the mean ± SEM for 4 replicates and are representative for 2 independent experiments. bd, below detection.

It has been well described that the effects of IL-13 on immune cells are similar to those of the closely related cytokine IL-4 due to their sequence similarity and similar structure (165). In addition, both of them can induce the phosphorylation of STAT6 as they share

a common receptor IL-4Ra (166). Therefore, it seems reasonable to investigate the role of immunoproteasome in IL-13 induced M2 alveolar macrophage polarization, as shown in Figure (Fig. 3.29)

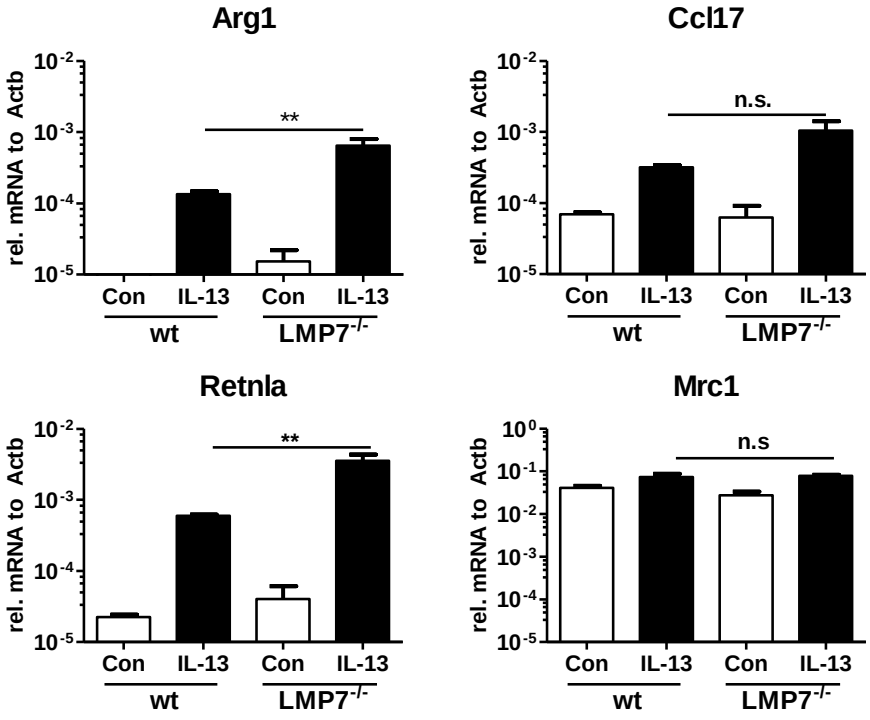

Figure 3.29: Deficiency of the LMP7 immunoproteasome subunit enhances IL-13 induced M2 alveolar macrophage polarization. M2 marker gene expression analysis of primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice polarized towards M2 phenotype (IL-13 for 24 h): expression of Arg1 (Arginase1), Ccl17, Retnla (Fizz-1), Mrc1 (mannose receptor 1) relative to Actb expression. Results are from three individual C57BL/6 wt or LMP7-/- mice.

3.3.5. No alteration of M1 transcription factors in LMPs deficient macrophages

To study the molecular mechanism of dysregulated AM polarization, we further analyzed transcription factors that are involved in the transcriptional activation of M1 or M2 macrophage polarization, such as NF-κB subunits NF-κB 1 (p105/p50) and Rela (p65),

as well as Irf-4 and -5 (60). We found no genotype-dependent changes for RNA expression of the NF-κB genes Rela and NF-κB 1, or Irf-5 in wt, LMP2-/- or LMP7-/- AMs (Fig.3.30).

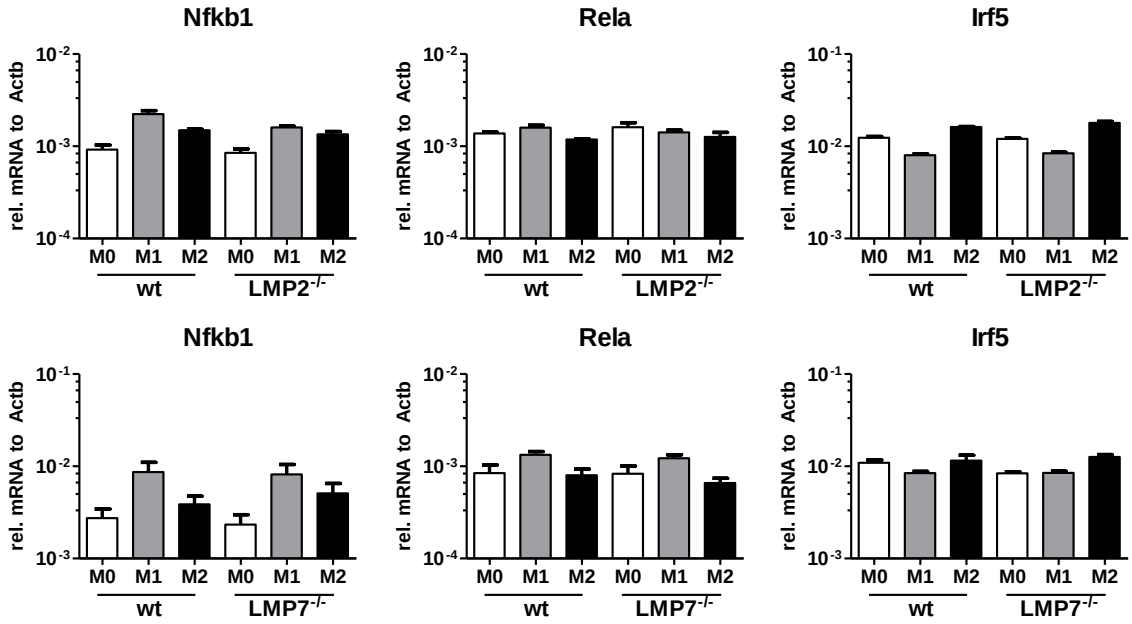

Figure 3.30: Deficiency of LMP2 and LMP7 has no impact on NF-κB 1, Rela and Irf-5 expression in M2 alveolar macrophages. NF-κB 1, Rela and Irf-5 gene expression relative to Actb in primary alveolar macrophages from C57BL/6 wt, LMP2$^{-/-}$ or LMP7$^{-/-}$ mice, polarized towards M1 (LPS and IFNγ) or M2 (IL-4) phenotype for 24 h. Results are from three independent experiments.

Irf-4 has been described as a key regulator of M2 macrophage polarization in bone marrow-derived macrophages (53), but not yet for alveolar macrophages. We thus confirmed its M2-specific mRNA induction in different primary AMs from C57BL/6 or BALB/c mice and in the MH-S cell line (Fig. 3.31 A). On the protein level IRF-4 transiently increased with highest levels after 48 and 72 h of IL-4 stimulation in primary C57BL/6 AMs (Fig. 3.31 B) and MH-S cells.

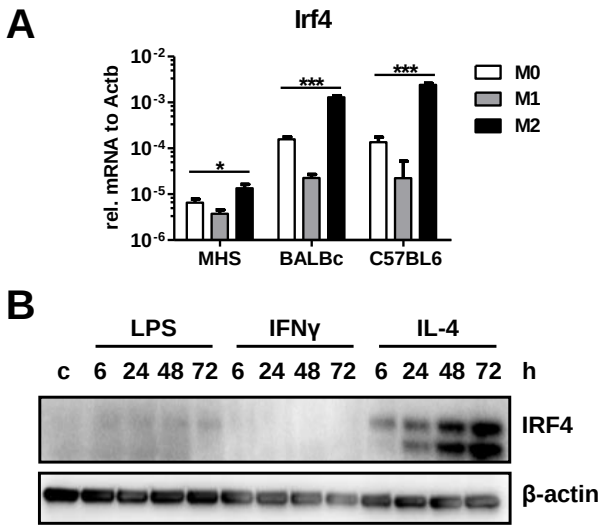

Figure 3.31 Irf-4 expression in primary alveolar macrophages is induced in M2 polarized alveolar macrophages (A) Irf4 gene expression relative to Actb in MH-S cells or primary alveolar macrophages from BALB/c or C57BL/6 mice polarized for 24 h. (B) Time course of IRF-4 protein expression in primary alveolar macrophages from C57BL/6 mice treated with LPS, IFNγ or IL-4 for up to 72 h. Results are from two independent experiments.

We next determined whether IRF-4 protein was induced by IL-13 which also induces M2 alveolar macrophage polarization. We found that IL-13 showed a similar pattern in induction of IRF-4 during polarization: IRF-4 also transiently increased to highest levels after 48 and 72 h of IL-13 stimulation in primary C57BL/6 AMs (Fig. 3.32). In addition, we observed that phosphorylation of STAT6 was induced during polarization.

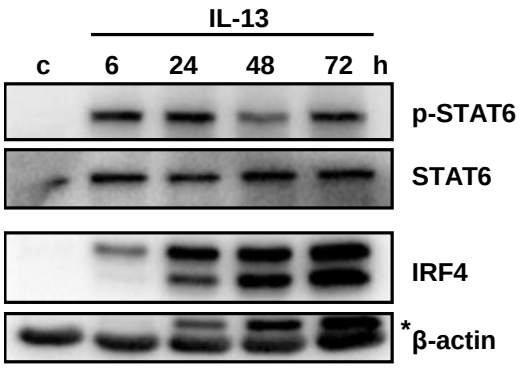

Figure 3.32 Irf-4 expression in primary alveolar macrophages is induced in IL-13 polarized alveolar macrophages. Time course of LMP2, LMP/, and IRF-4 protein expression and phosphorylation of STAT6 in primary alveolar macrophages from C57BL/6 mice treated with IL-13 for up to 72 h. Results are from two independent experiments.

As shown in above data, the kinetics of IRF-4 upregulation was similar in both IL-4 and IL-13 treatment. Therefore, we further investigated the kinetics of Irf4 expression in LMP7 deficient AMs. As shown in (Fig. 3.33), Irf4 was rapidly induced within 6 h and stayed elevated till day 3 after IL-4 treatment in wt AMs, but was markedly amplified in LMP7 deficient AMs.

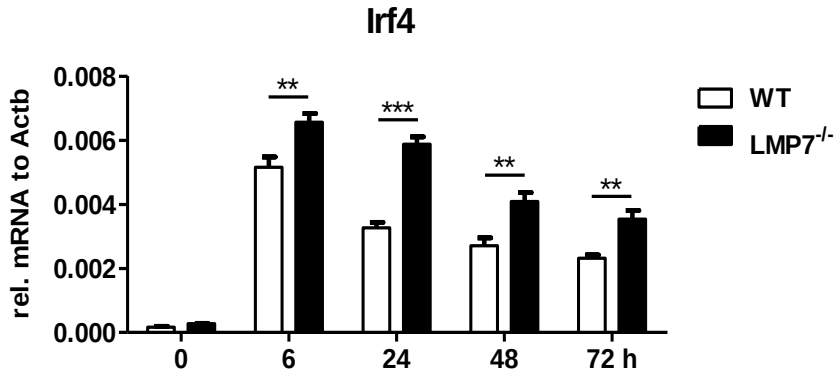

Figure 3.33: Deficiency of the LMP7 immunoproteasome subunit enhances Irf4 expression during M2 alveolar macrophages polarization. Irf4 gene expression in primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice treated with IL-4 for up to 72 h to polarize them towards the M2 phenotype. Results are combined data from three experiments.

As shown above, Irf4 gene expression was enhanced in LMP7-/- AMs, thus it is necessary to confirm this finding on the protein level. As shown in Figure 3.34, IRF-4 protein was also induced within 3 h in wt AMs after IL-4 treatment. n Both, LMP2-/- and LMP7-/- AMs, showed comparatively higher IRF-4 levels, in particular at later time points after IL-4 treatment.

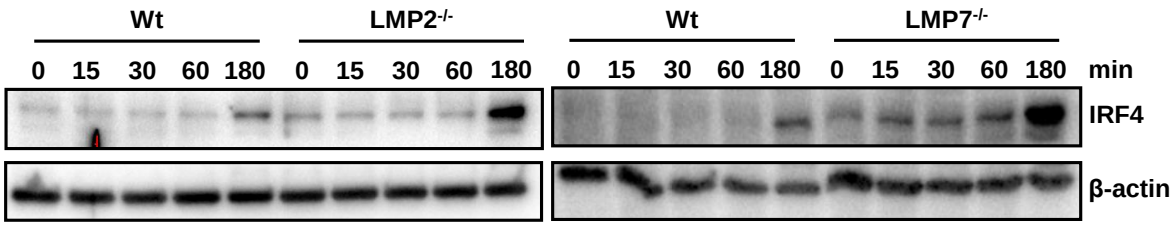

Figure 3.34: Deficiency of LMP2 or LMP7 immunoproteasome subunits enhances IRF-4 protein expression during M2 alveolar macrophages polarization. Time course of IRF-4 protein expression within 180 min after IL-4 treatment in primary alveolar macrophages from C57BL/6 wt, LMP2-/-, or LMP7-/- mice. Results are representative for two independent experiments.

3.3.6. Deficiency of LMP2 and LMP7 immunoproteasome subunits alters signaling towards alveolar macrophage M2 polarization

Elevated IRF-4 levels in LMP2 and LMP7 -/- AMS are indicative of altered M2 polarization upon immunoproteasome deficiency. We thus investigated whether LMP2-/- and LMP7-/- alveolar macrophages have altered signaling properties in response to IL-4 treatment by assaying the phosphorylation status of STAT6. Independent of the macrophage genotype, we observed distinct activation of STAT6 in M2-polarized AMs. To gain insight into early IL-4 signaling, a more immediate time window of 0 - 180 min was chosen to monitor STAT6 and also AKT activation. While STAT6 was steadily phosphorylated in wt cells from 15 min to 180 min peaking at 60 minutes after IL-4 stimulation (Fig. 3.35), STAT6 phosphorylation occurred with a similar time kinetic but at

higher levels in LMP2-/- alveolar macrophages (Fig. 3.35). AKT phosphorylation was steadily increased in wt cells from 15 min to 180 min but with no obvious change in LMP2-/- cells (Fig. 3.35). In contrast, activation of both STAT6 and AKT was enhanced in LMP7-/- AMs compared to wild type cells, particularly at later time points (Fig. 3.35). This signaling data further corroborates that LMP2-/- and LMP7-/- AMs have intrinsic similarity that govern a signaling response to IL-4-mediated signaling towards M2 macrophage polarization.

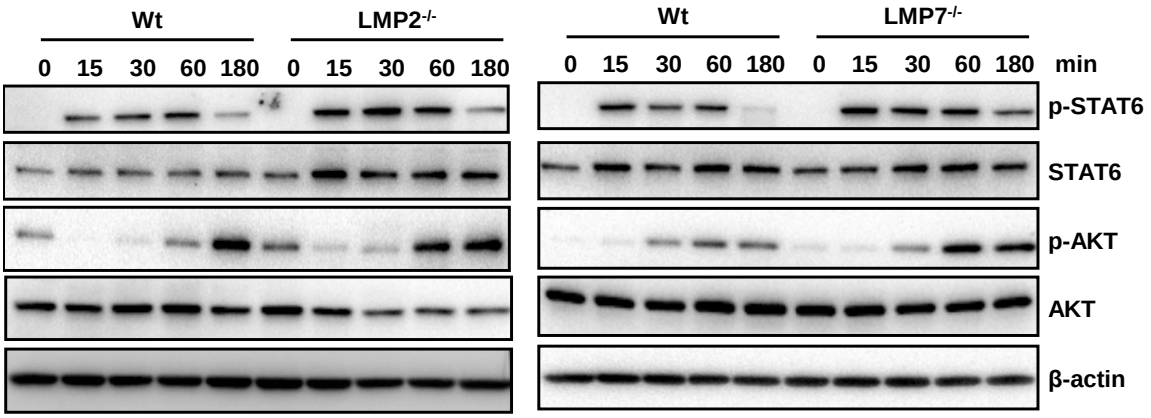

Figure 3.35: Deficiency of LMP2 and LMP7 affects M2 signaling. Time course of STAT6 ($pTyr^{641}$) and AKT ($pSer^{473}$) pathway activation within 180 min after IL-4 treatment in primary alveolar macrophages from C57BL/6 wt, $LMP2^{-/-}$ or $LMP7^{-/-}$ mice. Results are representative for two independent experiments.

3.3.7. LMP2 and LMP7 immunoproteasome deficiency affects IL-4Ra protein expression.

Since the phosphorylation status of STAT6 and AKT was altered in LMP2 and LMP7 deficient AMs, this indicated an alteration upstream of these IL-4 signaling mediators. As it is well known that the immunoproteasome presents as important machinery regulating the protein turnover in the cells, we decided to test the possibility that the IL-4Ra might

be degraded by immunoproteasome subunits. To test whether IL4Ra is differentially expressed in wt and immunoproteasome deficient AMs, western blotting was performed to detect the IL-4Ra protein level in wt, LMP2-/- or LMP7-/- AMs. As shown in Figure (Fig. 3.36), the overall IL-4Ra protein level was present at a higher level in LMP2-/- and LMP7-/- AMs compared with wt cells.

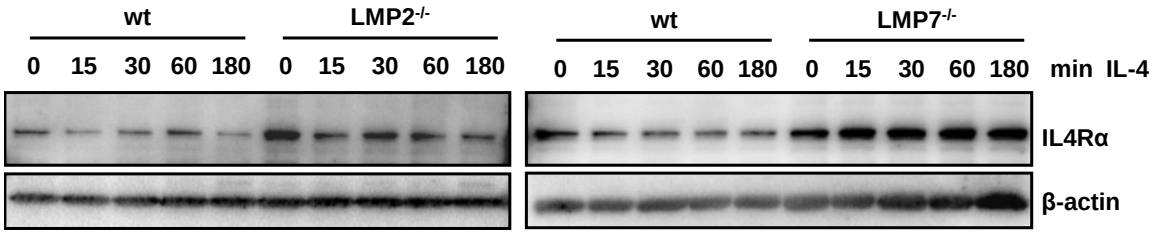

Figure 3.36 Deficiency of LMP2 and LMP7 immunoproteasome increases IL-4Ra protein expression. IL-4Ra protein expressions within 0-180 min after IL-4 treatment in primary alveolar macrophages from C57BL/6 wt, LMP2-/- or LMP7-/- mice were detected by western blotting. Results are representative for two independent experiments.

To determine if the increased IL-4Ra protein expression in LMP2$^{-/-}$ or LMP7$^{-/-}$ cells is dependent on mRNA level changes, we further investigated the IL-4Ra mRNA expression in polarized AMs from wt, LMP2$^{-/-}$ or LMP7$^{-/-}$ mice. As shown in (Figure 3.37), there are no significant differences for the IL-4Ra mRNA expression in the AMs from wt, LMP2$^{-/-}$ or LMP7$^{-/-}$ mice, which indicate that the accumulative IL-4Ra protein in LMP2$^{-/-}$ or LMP7$^{-/-}$ AMs is due to post-transcriptional effects such as deficient protein degradation due to immunoproteasome deficiency.

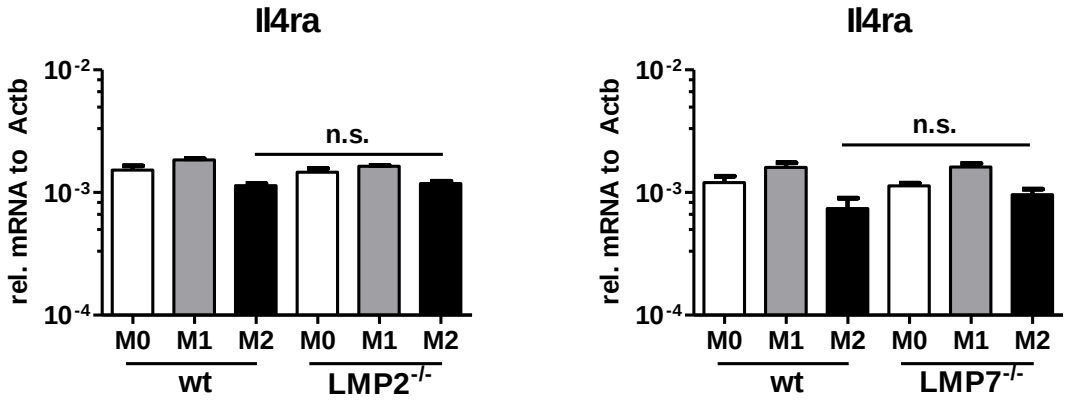

Fig. 3.37: No change in Il4ra expression level between polarized alveolar macrophages from wt, LMP2-/- and LMP7-/- mice. Il4ra expression analysis of primary alveolar macrophages from C57BL/6 wt, LMP2-/- and LMP7-/- mice polarized for 24 h with IL-4, relative to Actb expression. Results are combined data from three experiments.

3.3.8. The Iimunoproteasome subunit LMP7 specific inhibitor ONX 0914 enhances M2 alveolar macrophage polarization

Since we have demonstrated the critical role of immunoproteasome in M2 AMs polarization by using LMP2 and LMP7 knock out mice, it will be particularly meaningful to test whether a similar effect will be achieved by using an immunoproteasome specific inhibitor. Onyx pharmaceuticals developed ONX 0914 to be an inhibitor of the immunoproteasome subunit LMP7, with minimal cross-reactivity for the constitutive proteasome. One recent study has reported that the LMP7-specific inhibitor ONX 0914 is able to prevent collagen antibody–induced arthritis progression (167). Another newly published study has proven that inhibition of the LMP7 subunit prevented lupus disease progression by targeting two critical pathways of its disease pathogenesis, i.e. initiation of type I IFN activation and autoantibody production by plasma cells (168). We thus investigated the possibility of altering the M2 macrophage polarization via inhibition of immunoproteasome subunit LMP7 with the specific inhibitor ONX 0914.

Firstly, in order to exclude any unspecific toxic side effects of ONX 0914 on M2 polarization, WST cell viability assay were performed to identify the appropriate dose and time point of giving ONX 0914 to AMs. As shown in (Figure 3.38), we investigated cell viability at 6 and 24 h time points with a dosage range from 0.1-50 µM, and found that ONX 0914 did not cause the cell death from dose range 0.1 to 1 µM at the 6 h time point, whereas more than 25% cells died at a dose range of 5 to 50 µM. However, we observed that ONX0914 causes a significant dose dependent cell death at the 24 h point starting with a dose of 0.2 µM.

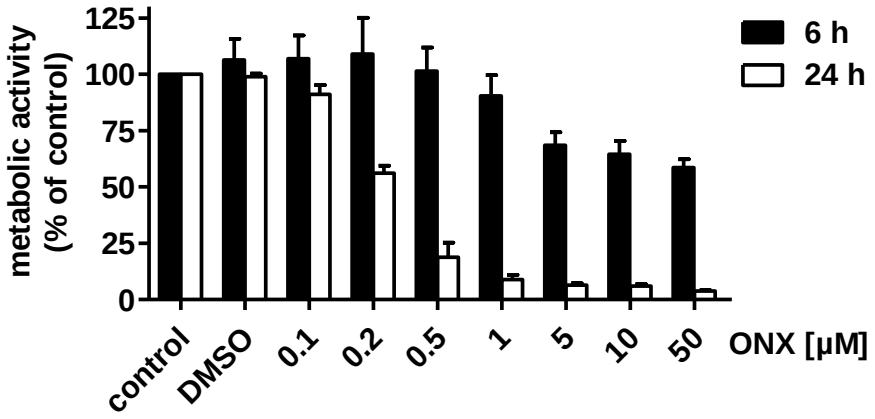

Figure 3.38: WST cell viability assay of ONX 0914 on cell line MH-S The alveolar macrophage cell line MH-S was treated with ONX 0914 at a dose range from 0.1-50 µM respectively at 6 or 24 h time points. The 10% cell proliferation reagent WST-1 was added to the cell to measure the cell viability. Results are mean + SEM from 4-7 individual experiments.

To investigate if the ONX 0914 can enhance the M2 signaling pathway and its upstream receptor IL-4Rα protein expressions, MH-S cells were pretreated without or with 0.2 or 1 µM ONX 0914 for 2-4 h, and IL-4 was added for 0 min to 180 min, ONX 0914 blocked the degradation of IL-4Rα from 30 to 180 min, resulting in enhanced phosphorylation of STAT6 (Fig. 3.40).

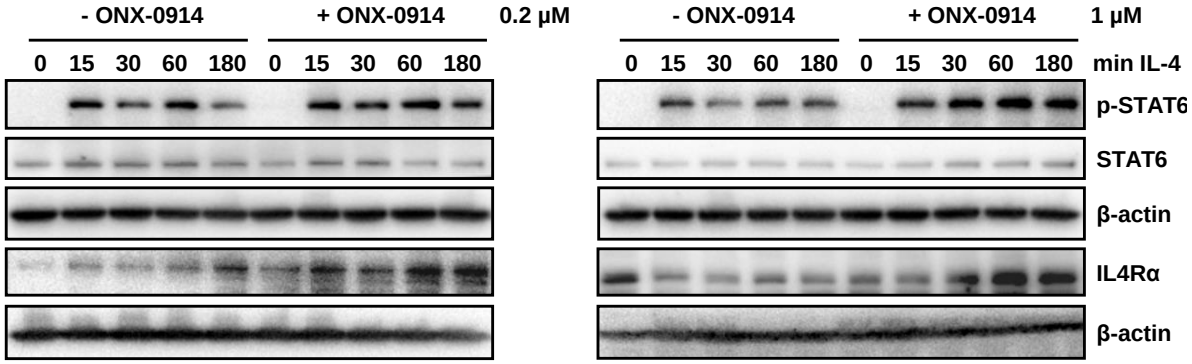

Figure 3.40: M2 alveolar macrophage polarization signaling is enhanced by LMP7 inhibition. MH-S cells were pretreated with or without 0.2 and 1 µM ONX 0914 for 2-4 h, and then treated together with IL-4 from 0 min to 180 min. IL-4Rα protein level and time course of STAT6 (pTyr641) and pathway activation within 180 min after IL-4 treatment in MH-S cells were analyzed by western blotting. Results are representative for three independent experiments.

To further determine if the LMP7 inhibition alters M2 macrophage polarization Arg1, Mrc1 and Irf4 mRNA expressions were analyzed in primary alveolar macrophages and MH-S cells that had been pretreated with 0.2 or 1 µM ONX 0914 for 2 h and then treated with IL-4 for 6 h. We chose this treatment scheme to prevent cytotoxic side-effects of ONX-0914. As shown in (Fig. 3.41), the Arg1 gene expression was significantly enhanced by ONX 0914 in MH-S cells at both 0.2 and 1 µM ONX 0914 treatment doses compared to untreated cells, whereas it was only significantly enhanced in primary AMs at a dose of 1 µM ONX 0914. Expression of Mrc1 and Irf4, were significantly enhanced by ONX 0914 at both 0.2 and 1 µM ONX 0914 treatment in both MH-S and primary AMs.

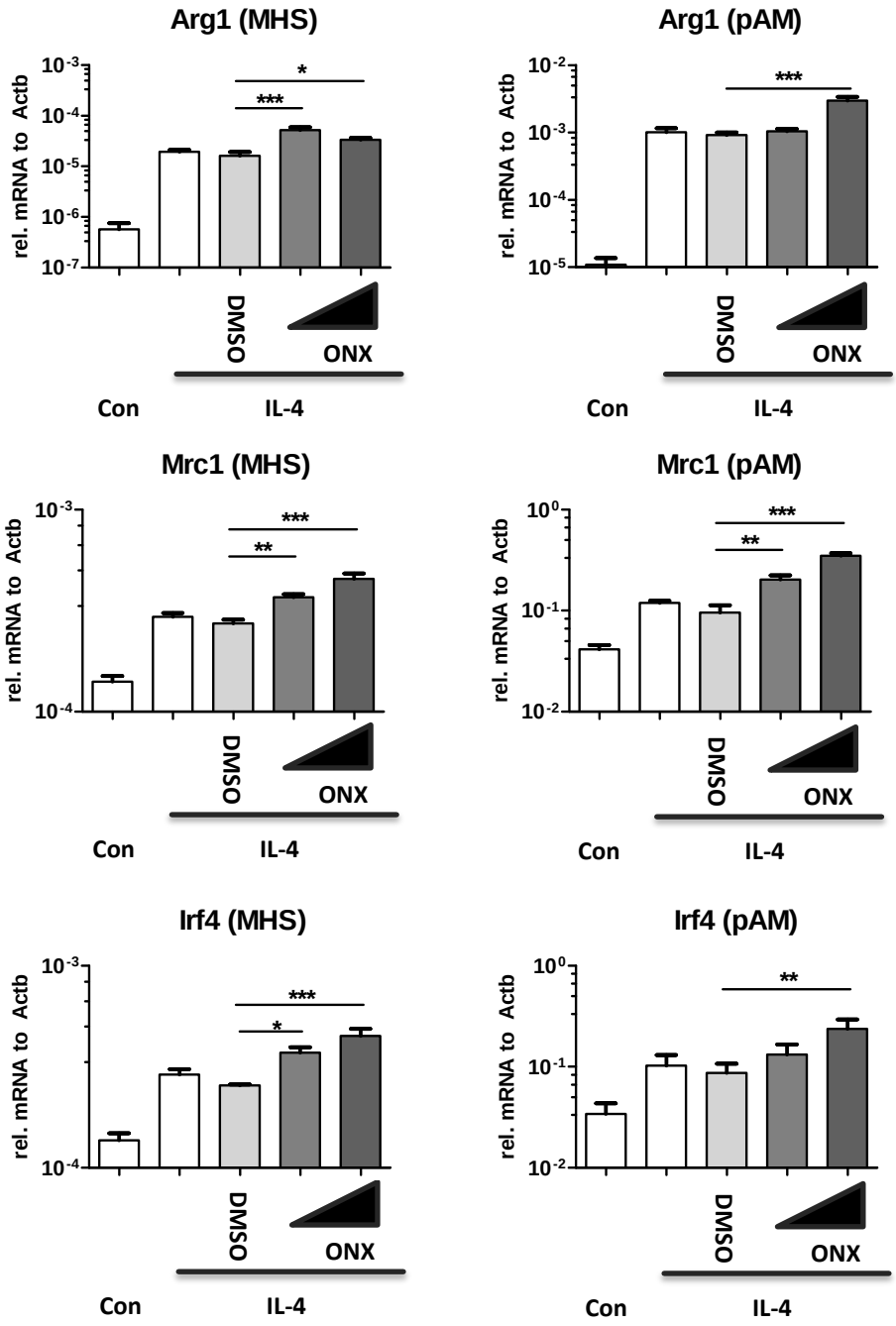

Figure 3.41: M2 maker gene expression was enhanced by LMP7 inhibition. M2 marker gene expression (Arg1, Mrc1 and Irf4) in MH-S cells or primary alveolar macrophages (pAM) from C57BL/6 mice: Cells were pretreated with DMSO or ONX-0914 (0.2 or 1 μM) for 2 h, afterwards IL-4 was added for another 4 h. Untreated cells and cells treated only with IL-4 served as controls. Results are the mean + SEM of three individual experiments (MH-S) or 3-6 individual mice (pAM).

3.4. Discussion

The conversion of human monocyte-derived macrophages by the Th2 cytokine IL-4 into a special activation state of inhibited respiratory burst and increased MHC II expression was first recognized by Abramson and Gallin in 1990 (169). Siamon Gordon's lab subsequently proposed the concept of an alternative IL-4/IL-13-activated macrophage phenotype (now also known as M2), characterized by the up-regulated macrophage mannose receptor (MRC1) expression coupled with enhanced MHC II and reduced pro-inflammatory cytokine levels (170). Since then, numerous studies have investigated polarization of mainly bone marrow- and monocyte-derived macrophages. The finding that tissue macrophages are of different origin and self-renew throughout life (23, 171, 172), however, has stimulated research on the polarization capability of tissue resident AMs (17).

3.4.1. Polarization capability of alveolar macrophages

Here we profiled global gens expression in polarized M1 and M2 alveolar macrophages using Illumina microarray system, and demonstrated that the expression of 162 genes were significantly induced in M1 AMs and 35 genes were induced in M2 AMs. Within these genes, the expression of well described M1 marker genes (Tnf, Il1b, and Il12b) and M2 marker genes (Arg1, Relnla and Ccl17) were also shown to be respectively induced in M1 or M2 AMs, which was further validated by qPCR analysis. While, no induction of Nos2 in M1 and Mcr1 in M2 AMs was detected in microarray analysis, the increased expression of these two important markers were confirmed by qPCR for mouse primary AMs of two different mouse strains and the MH-S cell line. Therefore, our

results demonstrate that alveolar macrophages can be polarized in vitro to classically, pro-inflammatory activated M1, or alternatively activated M2 macrophages. In addition, we showed that murine alveolar macrophages (AM) of different sources can be polarized *in vitro* to M1 or M2 macrophages. The M1 polarization profile of AMs from the MH-S cell line (BALB/c derived) matched well with that of primary cells derived from BALB/c and C57BL/6 mice. Obvious differences, however, were observed for some of the investigated M2-like gene expression markers. For example, CCL17 is a small cytokine belonging to the CC chemokine family that is also known as thymus and activation regulated chemokine (TARC). A number of studies have identified CCL17 as a marker for M2 or tolerance macrophages which are related processes orchestrated by a p50 nuclear factor κB, which suggest the crucial role of M2 in chemoattractant of Th2 and promotion of Th2 type of immune responses (173). Our unexpected results showed that CCL17 was not induced by IL-4 in the MHS cell line, which indicated some difference of macrophage biology between cell line and primary macrophages. Thus people should consider this factor when using a cell line alveolar macrophage in their study model.

Polarization of AMs has been described for various chronic inflammatory conditions of the lungs, for example during infection (86, 174) and allergic asthma (175), upon inhalation of sterile irritants such as toxic chemicals (86), or insoluble particles (176), and also in response to tumor growth (177). Pulmonary IFNγ and IL-4 production coincided with altered polarization of alveolar macrophages and are associated with inflammation, resolution, and tissue remodeling (86). They are thus considered to be relevant stimuli for AM polarization *ex vivo*. Impaired polarization of tissue macrophages

may be the cause or consequence for development of chronic diseases (66). Cigarette smoke-dependent reprogramming of AMs has been considered to contribute to the pathogenesis of COPD, as AM transcriptomes of COPD smokers showed a partially M2-shifted profile compared to healthy smokers and non-smokers (178). Also, idiopathic pulmonary fibrosis (IPF), a fatal fibrotic disease of the lung, has been associated with alternative AM activation (179) and M2-related production of CCL17 and CCL22 was proposed as a new marker for IPF(180). Hence, several signals characteristically expressed by M2 macrophages are known for their pro-fibrotic activity and suggest that these cells act as master regulators of fibrosis (120). Accordingly, restoring the M1/M2 balance by serum amyloid P has been effective in reducing fibrosis and remodeling caused by bleomycin application in mice (181). Targeted overexpression of TGF-β1 in the lungs of mice which is used as a model system for IPF, also demonstrated the significance of the M1/M2 balance with severe pulmonary inflammation followed by subsequent accumulation of alternatively activated BAL macrophages, while clodronate mediated AM depletion ameliorated the TGF-β1 driven fibrotic phenotype (182). Altogether, accumulating evidence suggests that excessive M2 activation of AMs contributes to the development of chronic lung disease.

3.4.2. Expression and activity of immunoproteasomes in polarized alveolar macrophages

It has been well described that IFNγ induces the expression of the immunoproteasome subunit in a variety of cell types in a STAT1 dependent manner. As we used LPS plus IFNγ to polarize alveolar macrophages into M1 in vitro, we firstly analyzed the expression of the immunoproteasome subunits in microarray data, and then investigated

the expression of the immunoproteasome subunit in polarized macrophages. Our results showed that in murine AMs, formation of active immunoproteasomes is not only induced by Th1 related stimuli such as IFNγ/LPS but also by Th2 cytokines, such as IL-4. In M1 polarization, LMP2 and -7 but also MECL-1 gene expression was concertedly upregulated by LPS/IFNγ. Expression of immunoproteasome subunits steadily increased during M1 polarization up to 72 h, reaching a plateau 24 h after IFNγ treatment, a time point where the Tnf expression had already markedly declined thereby indicating different pathways of transcriptional regulation. Induction of immunoproteasomes during M1 polarization was confirmed at the protein level for LMP2 and -7 with a marked increase after 24 h, further increasing up to day 3. Importantly, expressional changes of immunoproteasomes were translated into enhanced formation of active immunoproteasomes after 24 h as assessed by a specific set of activity-based probes. Previous studies have suggested that immunoproteasome formation can be induced by LPS stimulation in RAW 264.7 ascites tumor macrophages, with this interaction being critical for NO production but not for the TNF-α expression(160). Although, there is no study to describe the transcriptional mechanism for LPS induced immunoproteasome expression, we assumed that LPS induced immunoproteasome expression is dependent on NF-kB which is the most crucial transcriptional factor downstream of LPS In addition, we found the NF-kB binding site in the gene promotor area of LMP2 and LMP7 using a public promotor searching software (data not sown). Another indirect mechanism involves IFNβ, which has been shown to induce immunoproteasome expression and is known to be induced by LPS in macrophages (183). Therefore, it is not surprising to see induction of immunoproteasomes by both LPS and IFNγ.

Upon M2 polarization, mRNA expression of the immunoproteasome subunits LMP2 and -7 was not altered in IL-4-stimulated AMs while MECL-1 expression was slightly induced, Both LMP2 and -7 protein expression, however, were significantly elevated by IL-4 after 24 h and remained increased for 3 days. Even more important, immunoproteasome activity was clearly stimulated during M2 polarization after 48 h of IL-4 treatment. Similar to M1 polarization, expression and activity of standard proteasomes were also increased. The functional relevance of this regulation, however, remains to be determined. Furthermore, we observed that constitutive proteasomes were also induced in both M1 and M2 conditions. This is a novel finding and may indicate an important role for regulation of constitutive proteasome activity in macrophage biology that is worth investigating further.

So far only little information is available on the role of the immunoproteasome in macrophage biology. For the more prominent class of professional antigen-presenting cells, i.e. dendritic cells (DC), it has been shown that IFNγ-stimulated DCs upregulate the immunoproteasome, whereas IL-4 matured DCs co-express both standard (β1, β2, and β5) and immunoproteasome subunits (184). Similar as suggested for DCs, enhanced immunoproteasome expressions in M1 and M2 polarized alveolar macrophages might also play a role in increased microbicide activity of IFNγ/Th1, or improved antigen-presentation at IL-4/Th2 conditions, respectively. Of note, the observed posttranscriptional regulation of LMP2 and LMP7 by IL-4 identifies a novel regulatory mechanism for immunoproteasome regulation beyond its well-established transcriptional activation by IFNγ and TNF-α (185). The kinetics of immunoproteasome activation upon M1 or M2 polarization of AM suggest, that up-regulation of proteasome

and immunoproteasome activity is a consequence of the altered activation state of macrophages, as also suggested for DC differentiation.

3.4.3. Immunoproteasome function alters macrophage polarization

Our data reveal a novel role for the immunoproteasome in the innate immune cell function, namely macrophage polarization, and thus adds another immune-modulatory function to the enigmatic immunoproteasomes beyond their role in adaptive immune responses.

We showed that the absence of specific immunoproteasome subunits, namely LMP2 or LMP7, modulates the ability of AMs to polarize towards the M2 phenotype. Such disturbance of AM plasticity most likely has important consequences for homeostasis and responses to environmental stimuli of the pulmonary tissue. M1 polarization of AMs was less affected by LMP2 or LMP7 depletion, and overall marker genes of classical activation (Nos2, Tnf, Il1b and Il12b) were largely regulated independently of the genotype upon IFNγ/LPS stimulation. This finding is supported by a consistent M1 profile of well-known pro-inflammatory transcription factors of classical macrophage activation which was not altered by immunoproteasome subunit depletion. We conclude that LMP2 and -7 are not required for NF-κB1 (p50/p65) signaling during M1 polarization. The role of immunoproteasomes for canonical NF-κB1 signaling has been controversially discussed but can be ruled out for M1 alveolar macrophage differentiation (151, 159, 186, 187).

Remarkably, M2 polarization of AMs was disturbed by immunoproteasome deficiency. Our heat map analysis showed that the expression of 21 and 43 M2 signature genes

were respectively enhanced, in LMP2$^{-/-}$ or LMP7$^{-/-}$ cells, compared to wt cells. Among these genes, 4 well described M2 marker genes (Arg1, Retnla, Ccl17 and Mcr1) were selected for the further qPCR validation for their expression, which confirmed the enhancement of M2 marker gene expression and thus M2 polarization in LMP2-/- and LMP7$^{-/-}$ AMs. Most notably, the IL-4 dependent Th2 chemokine CCL17 was released in high amounts from LMP7 and LMP2 deficient AMs. Our findings that LMP2 and -7 similarly affect M2 polarization were further corroborated by analysis of early signaling kinetics upon IL-4 stimulation. In particular, expression of the M2 specific transcription factor Irf4 - a key transcription factor that controls M2 macrophage polarization (80) was disproportionately higher in M2 polarized LMP2$^{-/-}$ and LMP7$^{-/-}$ cells and its expression exceeded wt levels particularly in the first 48 h after IL-4 induction. Similar results were obtained for STAT6 and AKT activation, all hallmarks of IL-4 mediated signaling towards M2 polarization (83, 188): Both STAT6 and AKT phosphorylation were enhanced in LMP2$^{-/-}$ and LMP7$^{-/-}$ cells, respectively. In addition, we are the first to find that IRF4 expression was induced in M2 but suppressed in M1 AMs, which fits well the literature. Previous studies have proven that IRF4 is the downstream target of STAT6 activation, and in addition, there is a protein-protein interaction between IRF4 and STAT6. Hence, we believe that the IRF4 service is a positive loop to cooperate with STAT6 to derive M2 markers gene expression.

These findings clearly reveal that immunoproteasome-deficient AMs have increased responsiveness towards IL-4 mediated signaling. Of note, as neither basal nor M1- or M2-dependent expression levels of the interleukin 4 receptor alpha (IL-4 ra) genes were affected by the LMP genotype, these differences are most probably not due to an altered

mRNA expression of the IL-4 receptor but rather due to the altered protein turnover of IL-4 receptor. We were thus tempted to speculate that LMP2 and LMP7 affect specific substrate degradation in alveolar macrophages, thereby contributing to altered M2 activation. The proteasome is the cellular machinery which is responsible for cellular protein degradation. They are not only responsible for degradation of damaged and misfolded proteins during cellular stress, but also for proteins involved in the signaling pathway. Thus, the proteasome plays a crucial role in activation of the signaling pathways, such as IκBα is degraded by the proteasome and leads to the activation of the NF-κB pathway (189). Khalid W. Kalim has reported that in the differentiation of Th17 cells, immunoproteasome inhibition blocked phosphorylation of STAT3, whereas in Tregs SMAD phosphorylation was enhanced. Additionally, LMP7 inhibition led to reduced STAT1 phosphorylation and Th1 differentiation (155). Although a number of studies have indicated that immunoproteasomes are involved in many immune signaling pathways, none of these studies identified specific substrates for immunoproteasome-dependent protein degradation, thus the underlying molecular mechanisms of the effect on the above mentioned pathways are largely unclear. We are the first to report that IL-4Ra is a possible substrate for LMP7-dependent degradation as the deficiency and inhibition of LMP7 caused the accumulation of the IL-4Ra protein contributing to the observed enhancement of downstream STAT6 and AKT activation. As IL-4Ra is also shared by lL-13 to trigger STAT6 dependent M2 polarization, it is not surprising to observe enhanced M2 marker gene expression in LMP7 deficient AMs after IL-13 stimulation. To make sure the increased IL-4Ra protein in LMP7 and LMP2 deficient AMs is not due to the adaption effect of the knock out mice strains, we applied the

specific LMP7 inhibitor ONX 0914 to the AMs, and found that STAT6 phosphorylation was enhanced in ONX 0914 treated AMs, which further lead to the up-regulation of M2 markers Arg1 and Mrc1. In addition, in order to exclude any unspecific effects of ONX 0914 on M2 polarization, an ABP-based pulldown assay was performed to confirm the specificity of the given dose of ONX 0914 to AMs, and this experiment were done by our collaborators (Ilona Keller and Oliver Vosyka) from lab of Silke Meiners of Comprehensive Pneumology Center (CPC) of Helmholtz München. Their experiments suggested about 50% inhibition of LMP7 with 200 nM and close to 80% inhibition with 1 µM ONX0914, while β5 was only partially inhibited by about 30% with high doses of ONX 0914 (Shanze Chen, Ilona Keller and Oliver Vosyka; Submitted results to Journal of Immunology). Thus our inhibitor experiments confirmed that IL-4Ra is regulated by the catalytic activity of the immunoproteasome subunit LMP7. Therefore it is worthwhile figuring out more substrates of immunoproteasome by using a profiling technique such as protein microarray and mass spectrometry, which could help to better understand the role of immunoproteasome in disease conditions. For example, it has been well described that the Th2 cytokines IL-4 and IL-13 play a crucial role in initiation and development of a chronic allergic inflammatory disease asthma by interacting with related receptor complexes (190). We have also demonstrated that LMP7 deficiency enhances the IL-4 receptor signaling pathway, therefore, theoretically we should able to observe aggravated asthma in LMP7 deficient mice. However, Anton Volkov, et al. have reported that LMP7 deficiency leads to a reduced Th2 response in the OVA induced acute asthma model (191). In this study, the authors were not able to offer an explanation for the observed effect due to the complexity of the disease. It is well

possible that this effect may be due to the suppression of Th1 and Th17 differentiation after LMP7 deficiency. The underlying mechanism for these responses remains to be determined, but is certainly related to alter IL-4 receptor signaling in the LMP7 knockout cells. In spite of an opposite effect of LMP7 deficiency in the asthma model, it is possible to apply our findings to other chronic inflammatory lung disease models. We have recently collected data (unpublished), which showed that immunoproteasome subunits LMP2 and LMP7 protein levels are induced in whole lung tissue samples from IPF patients and BAL fluid the CCL17 protein level is higher in LMP7 deficient mice compared with wt mice in a bleomycin induced lung fibrosis model, which may indicate the critical role of immunoproteasome in IPF pathogenesis (Shanze Chen and Ilona keller, unpublished results). In addition to IPF, our findings may also be applicable to COPD as it has been suggested that IL-4/IL-13 signaling contributes to the pathogenesis of COPD. IL-4 and IL-13 are known for their capacity to promote mucus production from bronchial epithelial cells (192). A transgenic mouse model has revealed that over-expression of IL-13 in the mouse lung causes emphysema (193). M2 macrophages play a crucial role in resolution of inflammation via phagocytosing apoptotic neutrophils (194). IL-4 and IL-4Ra were shown to be essential for the resolution of sterile inflammation (195) due to enhanced M2 polarization in LMPs deficient macrophages, thereby we might observe an enhanced resolution of inflammation in LMPs deficient mice.

In conclusion, our study demonstrates that LMP2 and LMP7 ablation enhances M2 polarization of alveolar macrophages, while not impacting M1 polarization. These results indicate a crucial role of immunoproteasome in alveolar macrophages biology and

suggest the novel potential therapeutic intervention of innate immunity in the lungs by inhibition of individual immunoproteasome subunits.

4. Chapter 2 : Polarized alveolar macrophage-epithelial cell communication

4.1. Introduction

4.1.1. Pulmonary alveolus

An alveolus is a form of a hollow cavity located in the lung parenchyma, representing the terminal end of the respiratory tree and the site of gas exchange with the blood. (196).

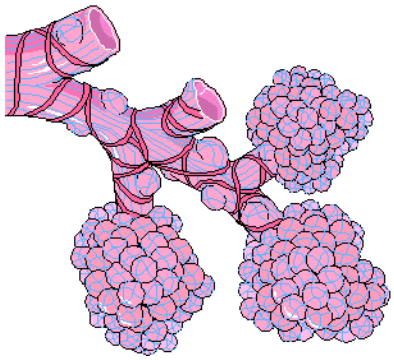

Fig 4.1: Scheme of pulmonary alveolus. Copied from http://medicalterms.info/anatomy/Alveoli/

There are three major cell types in the alveolar wall (pneumocytes): type I alveolar epithelial cells (AEC I), type II alveolar epithelial cells (AEC II) and alveolar macrophages (AM). AEC I are squamous, large and thin cells which occupy 90 to 95% of the alveolar surface (197). These cells are so thin that they can facilitate the gas exchange between the alveoli and the blood (197). AEC I are able to fight against microbes and thus initiate the immune responses (198). Currently it is still not clear how AEC I are regenerated in the normal lung, but evidence indicates that AEC I are transdifferentiated from AEC II during the alveolar epithelial wound repair(199). AEC II are cuboidal cells that constitute around 15% of total lung cells and cover about 7% of the total alveolar surface (200). They are responsible for the secretion of surfactant

which reduces the alveolar surface tension to increase the gas exchange. In addition to the secretion of surfactant, AEC II can also sense the invasion of pathogens and produce antimicrobial products such as complement, lysozyme, and antibacterial peptide. In addition they can amplify the inflammatory response by secretion of cytokine and chemokines (197). As we have introduced in chapter 1, AM is a type of tissue macrophage found in the pulmonary alveolus, which has close contact with its neighbours AEC I and AEC II. They are one of the key cell types for initiating inflammatory and immune responses in the lung.

4.1.2. Macrophage-epithelial communication

Cell-to-cell communication is the sharing of information between cells, which serves as the basis for functional coordination between cells in multicellular organisms and plays a crucial role in cell growth, cell differentiation and tissue homeostasis (201-204). Communication between immune and epithelial cells has been suggested to be crucial for the fight against the invasion of pathogens to the epithelium (205). In the alveolus, one of the critical functions of AEC is to keep the integrity of the epithelial barrier during infection and injury. Therefore, the crosstalk between AEC with its neighbor AM is required to initiate an appropriate response to invaders, which involves not only the killing of microbes but also regulation of tissue repair and resolution of inflammation (206).

There are two ways of communication: contact and non-contact cell-to-cell communication. One of the direct contact communications is the gap junction. Close adherence of alveolar macrophages to alveolar epithelial cells facilitates sharing of the

information through the gap junctions such as connexins (201). Using real-time alveolar imaging in situ, Kristin Westphalen et al. showed that the gap junctions called connexin 43 (Cx43) mediated intercommunication between AMs and alveolar epithelial cells (207). These were immunosuppressive signals to reduce endotoxin-induced lung inflammation, which involved Ca2+-dependent activation of AKT (207). In contrast to the direct contact communication, the non-contact communication has been well investigated involving the secreted mediators such as cytokines and chemokines. One example is that microbial infections induce M1 polarization of AM and further lead to high production of pro-inflammatory cytokines IL-1ß, TNF-a, IL-6 and IL-12 from macrophages. As early response cytokines, TNF-α and IL-1ß further induce the release of chemokines from epithelial cells, such as chemokine (C-X-C motif) ligand 1/5 (CXCL1/5) in order to attract neutrophils and chemokine (C-C motif) ligand 2 (CCL2) to attract monocytes (208-210). Apart from inducing the production of chemoattractant, AM derived TNF-alpha release can induce CSF2 (GM-CSF, Granulocyte-macrophage colony-stimulating factor) expression in AEC, which in turn initiates AEC proliferation and contributes to alveolar barrier integrity (206). In addition, it has been suggested that communication of AM with AEC plays a key role in hypoxia-induced lung inflammation affecting the IL-8 release (211). Additionally, it has been proven that the macrophage pro-inflammatory cytokine IL-1ß augments in vitro alveolar epithelial repair by inducing TGF-α and EGF production in epithelial cells (212). Hence defective communication in response to damage represents a pathophysiological mechanism, which contributes to the development of infectious diseases, chronic inflammatory diseases, and cancer.

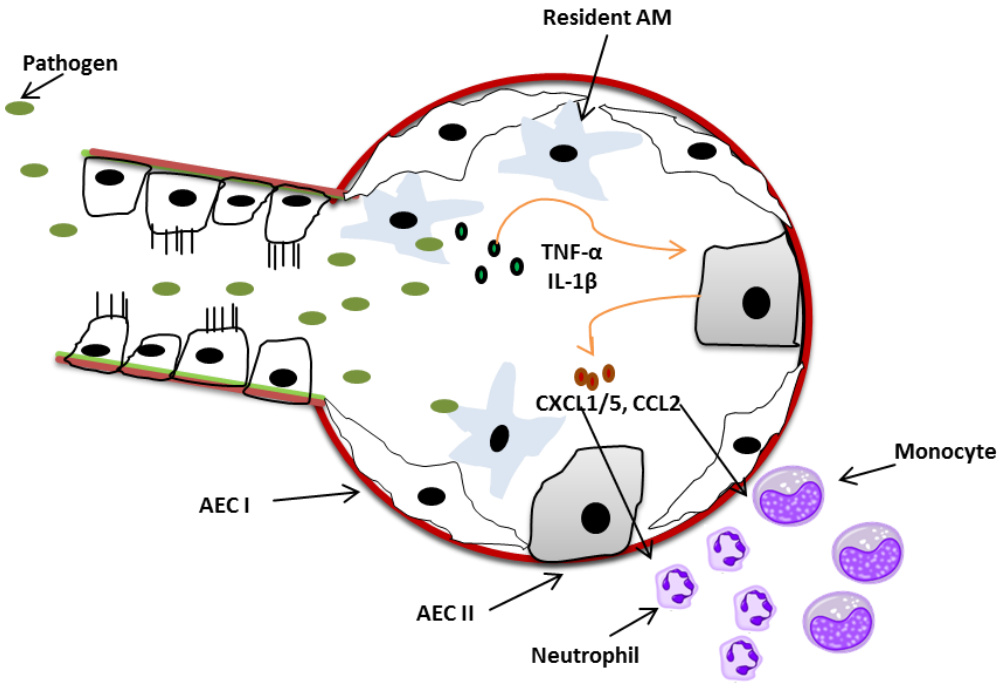

Fig 4.2: The communication of alveolar macrophage with alveolar epithelial cell during pathogen infection in the alveolus. As the first line of defense, AM is the first cell type in the alveolus to react to the pathogen invasion, afterwards, AM secrete TNF-a and IL-1β, which stimulate the production of chemokines from AEC such as CXCL1/5 and CCL2, following that, these chemokines recruit neutrophils and monocytes. Adapted from "Fig. 1 of Lidija Cakarova's thesis: Macrophage-Epithelial Crosstalk during Alveolar Epithelial Repair following Pathogen-induced Pulmonary Inflammation".

A group of cytokines and chemokines have been reported to be expressed by activated epithelial cell.

IL-6: Interleukin 6 is an interleukin that acts as an inflammatory cytokine via binding to IL-6R and activation of STAT3 (213). IL-6 is released by epithelial and macrophages to amplify the immune response during inflammation, thereby playing a role in many inflammatory diseases (214).

GM-CSF: Granulocyte-macrophage colony-stimulating factor, also referred to as colony stimulating factor 2 (CSF2). It is a monomeric glycoprotein that functions as a white

blood cell growth factor, and is secreted by macrophages, T cells, endothelial cells and epithelial cells (215). GM-CSF plays the curial role in macrophage biology. Upon stimulation of GM-CSF, newly recruited monocytes at the site of inflammation can mature into macrophages (216). Bone marrow derived macrophages (BMDM) differentiated with GM-CSF display characteristic M1polarization phenotype (217).

TGF-β: The transforming growth factor beta is a polypeptide cytokine that acts on TGFB1R to activate SMADs (218). It is a secreted multifunctional protein that can control cell growth, cell proliferation, cell differentiation and apoptosis (219). In addition, TGF-β induces trans-differentiation of epithelial cells into mesenchymal cells, a process called EMT (220). It has been suggested that TGF-β plays a role in inflammatory lung diseases, including lung fibrosis, asthma, COPD (221-223).

CX_3CL1: Chemokine (C-X3-C motif) ligand 1, also known as fractalkine, is constitutively expressed by alveolar epithelial cells (224). There is a soluble form of CX3CL1 which can potently attract dendritic cells (DC) and monocytes via its receptor CX3CR1, while the cell-bound form is responsible for adhesion of leukocytes to activated epithelial cells (225, 226). It has been shown that the CX3CL1/CX3CR1 axis is associated with inflammatory lung diseases and involved in the recruitment ofCX3CR1+ macrophages in the lungs contributing to the development of COPD (226).

CCL2: chemokine (C-C motif) ligand 2 (CCL2), also called monocyte chemotactic protein 1 (MCP1), is the CC family chemokine which attracts monocytes to the sites of inflammation via its receptor CCR2 (227). CCL2 is expressed by a variety of cell types such as macrophages, epithelial cells and endothelial cells (228). CCL2 is thought to be

involved in the pathogenesis of several inflammatory diseases characterized by monocyte infiltration (228). The increased concentration of CCL2 in bronchoalveolar lavage (BAL) from COPD patients contributes to the recruitment of monocytes that can differentiate into macrophages (229).

LCN2: Lipocalin-2 also known as neutrophil gelatinase-associated lipocalin (NGAL), was initially found to be expressed by neutrophils, and is also reported to be induced in numerous epithelial cell types in a TLR dependent manner (230). LCN2 can fight against the bacterial infection by sequestering iron-containing siderophores (231). In addition, LCN2 was shown to promote neutrophil recruitment and trigger G-CSF and CXCL1 in alveolar macrophages (232). LCN2 induced macrophage IL-10 formation, skewing STAT3 dependent macrophage polarization (233).

4.2. Project aims

The communications between alveolar macrophages and epithelial cells has been suggested to play an important role in maintaining the functional integrity of the lung. As we have shown in chapter 1, alveolar macrophages can polarize into M1 and M2 phenotypes upon respective treatment of LPS/IFNγ or IL-4. Epithelial cells, a crucial part of the innate lung immunity, establish the local environment of AMs, can release chemokines such as CXCL1/5 and CCL2 under inflammatory conditions, and are responsible for the recruitment of monocytes and neutrophils in inflamed lung tissue. However, the extent of polarized alveolar macrophage-epithelial cell communication remains unclear. Therefore, using a trans-well co-culture and conditioned medium model depicted in Figure 4.3, we aimed to identify the cellular immune factors derived from alveolar macrophages to interact with alveolar epithelial cells.

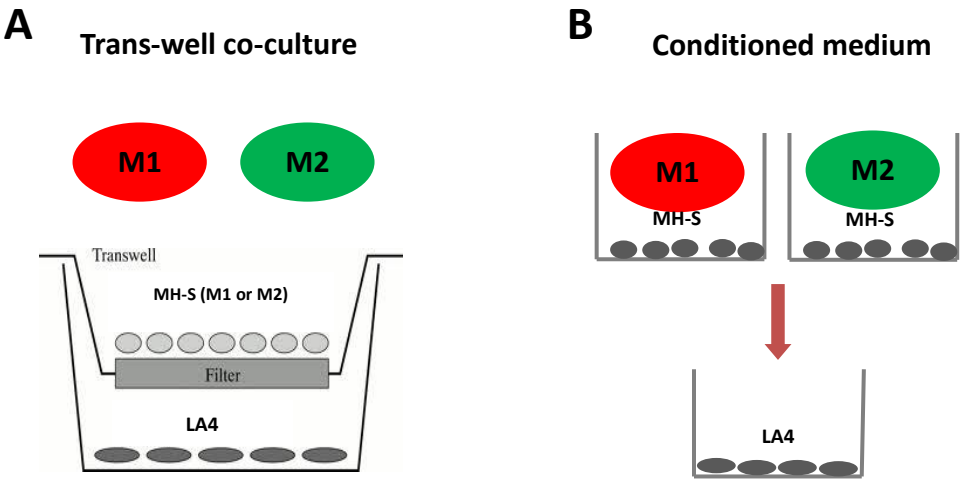

Fig 4.3: Scheme of trans-well co-culture and conditioned medium model.

4.3. Results

4.3.1. Profiling of polarization markers for the alveolar macrophage cell line MH-S

As we have shown in the chapter 1, the alveolar macrophages cell line MH-S can be polarized into respective M1 and M2 phenotypes upon treatment with either LPS and IFNγ or IL-4. Apart from the markers investigated in chapter 1, we found that the mRNA level of Cxcl1, Cxcl2, Cxcl9, Il6 and Lipocalin-2 (Lcn2) were also induced in M1 cells, while Pparg, Cd36, Cxcr2, Irf4 and Galectin3 (Lgals3) were induced in M2 cells (Fig 4.4).

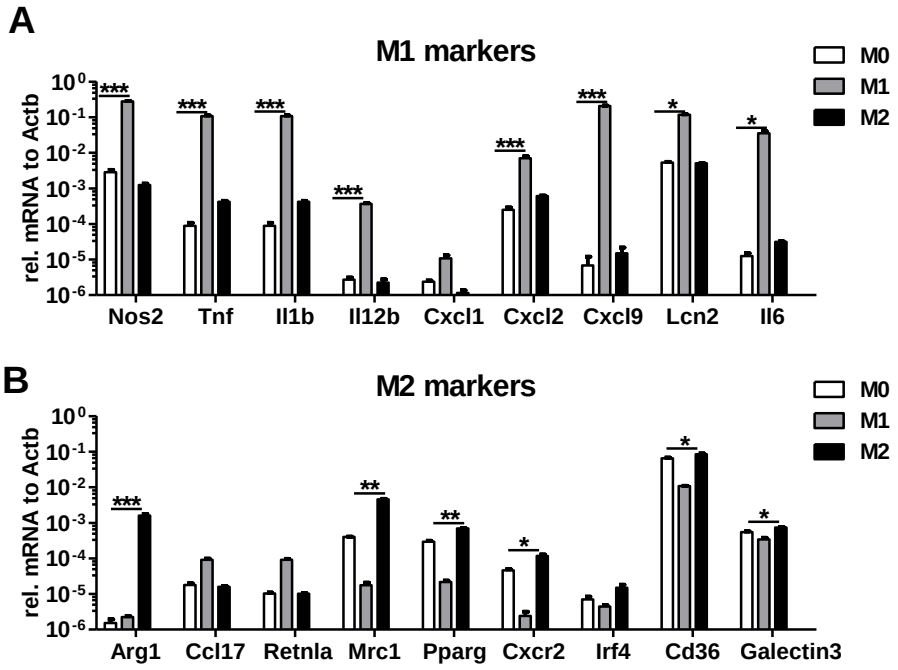

Fig 4.4: Alveolar macrophages cell line MH-S shows characteristics of M1 and M2 polarization by maker genes profiling. (A, B) M1 and M2 marker gene expression analysis of MH-S cells polarized for 24 h: Nos2, Tnf, Il1b, Il12b, Cxcl1, Cxcl2, Cxcl9, Il6 and Lcn2 relative to Actb (β-actin) expression for M1, and Arg1, Ccl17, Retnla, Mrc1, Pparg, Cd36, Cxcr2, Irf4 and Galectin3 relative to Actb expression for M2. Results are from three independent experiments.

4.3.2. Polarized M1 AMs activate the LA4 in a transwell co-culture system.

To determine if the polarized M1 or M2 alveolar macrophages can communicate with alveolar epithelial cells, we introduced a trans-well system to co-culture MH-S with the murine alveolar epithelial cell line LA4 separated by a membrane. LA4 were seeded on the lower side and MH-S cells on upper side of the trans-well. After 24 h co-culture with polarized or non-polarized MHS cell, LA4 cell was prepared for the qPCR profiling. As shown in Figure 4.5, we found that the mRNA level of Il6, Lcn2, Csf2, Ccl2 and Cx_3cl1 were induced by LPS/IFNγ not by IL-4 in LA4, while LPS/IFNγ derived expression of these cytokines were further enhanced by a co-cultured M1 macrophage. The mRNA expression of Tgfb1 was not impacted by either LPS/IFNγ or IL-4 treatment, however, its expression was induced in M1 co-cultured LA4 cell.

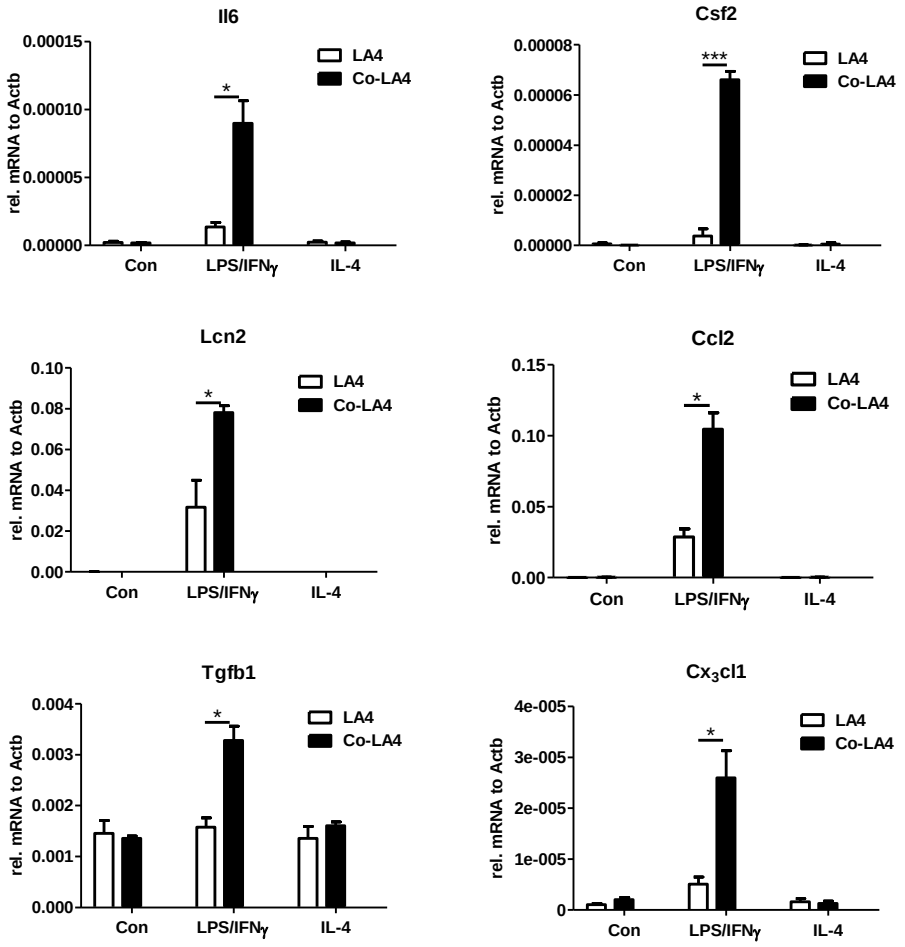

Figure 4.5: Polarized M1 AMs amplify gene expression from alveolar epithelial cell LA4. Gene expression analysis of LA4 co-cultured with polarized or non-polarized MHS cell for 24h: Cx3cl, Il6, Lcn2, Csf2, Tgfb1 and Ccl2 relative to Actb (β-actin) expression and Results are mean for three replicates.

4.3.3. Conditioned medium from polarized AMs activate LA4 cells.

As we wanted to identify secreted mediators and exclude the direct contact effect, we further investigated if the conditioned medium from polarized MH-S cell could alter mRNA expression of cytokines in LA4 cells. For that, the conditioned medium was collected from 24 h M0, M1 and M2 cells, and then added to the LA4 for 24 h. As shown in Figure 4.6, we found that the mRNA expression of Il6, Lcn2, Ccl2, and Cx3cl1 was

significantly induced by an M1 conditioned medium in LA4; in addition, the mRNA level of Cx3cl1 was also induced by an M0 and M2 conditioned medium, however, the expression of Csf2 and Tgfb1 were not impacted by any of the conditioned medium.

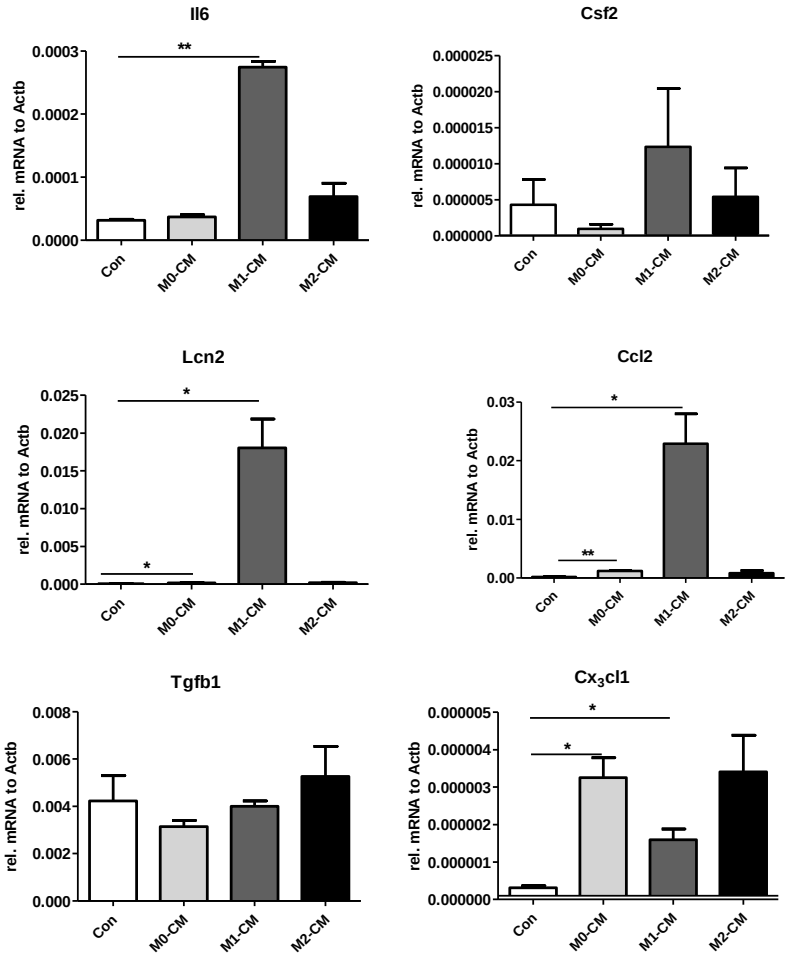

Figure 4.6: Conditioned medium from polarized alveolar macrophage amplifies the expression of several genes in alveolar epithelial cells. Gene expression analysis of LA4 cells treated with conditioned medium from M0, M1 and M2 cells for 24h: Cx3cl, Il6, Lcn2, Csf2, Tgfb1 and Ccl2 relative to Actb (β-actin) expression and Results are from three independent experiments.

4.3.4. The cytokines TNF-α, IL-1β, IFNγ and IL-17 activate LA4 cells.

To further determine which cytokines drive gene expression in alveolar epithelial cells in a similar manner as observed before in both trans-well co-culture and conditioned model, we applied the cytokines TNF-α, IL-1β, IFNγ and IL-17 at concentration of 20 ng/ml to LA4 cells for 24 h. As shown in Figure 4.5, we found that TNF-α strongly induced mRNA level of Ccl2, Cx3cl1 and Csf2 compared with other cytokines, whereas IL-1β preferentially induced the expression of Lcn2.

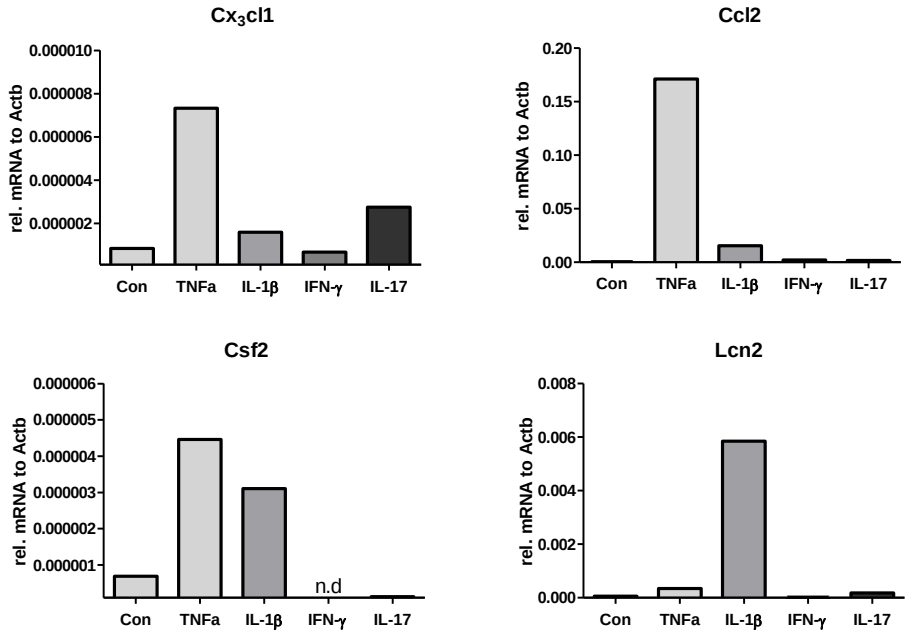

Figure 4.7: Macrophage derived pro-inflammatory cytokines TNF-α and IL-1β activate LA4 cell. Gene expression analysis of LA4 treated with 20ng/ml of TNF-α, IL-1β, IFNγ and IL-17 for 24h: Cx3cl, Il6, Lcn2, Csf2, Tgfb1 and Ccl2 relative to Actb (β-actin) expression and Results are mean for two replicates. n.d , no detection.

4.4. Discussion

There are many studies using transwell co-cultures and conditions on medium models to investigate cytokine mediated communication between epithelial cells and macrophages. Using a transwell co-culture model, Jun-Li Ding`s Lab found that M2-polarized tumor-associated macrophages could promote epithelial–mesenchymal transition (EMT) in pancreatic cancer cells via TLR4/IL-10 signaling pathway (234). Olga D. Chuquimia, et al. found that the conditioned medium from LPS stimulated AEC modulates the activity of alveolar macrophages to control bacterial growth (197). Tiana V Curry-McCoy, et al. has suggested that communication between AECs and macrophages is involved in the alcohol-induced disruption of the epithelial barrier function via TGFβ1 (235). Therefore, epithelial-macrophage communication plays an important role in pathogenesis of cancer and inflammatory diseases.

Here, initially using a trans-well co-culture model, we found that M1 polarized alveolar macrophages could enhance the expression of Il6, Tgfb1, Lcn2, Csf2, Ccl2 and Cx_3cl1 in LA4 cells. Furthermore, we proved that conditioned medium from M1 AMs could induce the expression of Il6, Lcn2, Ccl2 and Cx_3cl1 in LA4 cells but not of Tgfb1 and Csf2. However, beyond our expectation, an M0 and M2 conditioned medium can also markedly induce the gene expression of Cx3cl1 in LA4 cells. TNF-α and IL-1β as master pro-inflammatory cytokines, which are supposed to be mainly derived from macrophages have been well described to act on alveolar epithelial cells during ~~the~~ acute inflammation. Hence, we used recombinant cytokines to stimulate LA4 cells to test whether these factors could be the mediators to induce the gene expression in LA4. We

found that the mRNA expression of Ccl2, Cx3cl1 and Csf2 were strongly induced by TNF-α, whereas Lcn2 was strongly induced by IL-1β.

Csf2, a monomeric glycoprotein, is secreted by macrophages, T cells, endothelial cells and fibroblasts, and is also expressed by alveolar epithelial cells at lower level (236). However, it has been reported that Csf2 expression could be induced in alveolar epithelial cells by alveolar macrophage derived TNF-α, and that this stimulation further leads to AEC proliferation and repair (206). In addition, expression of Csf2 in AECs is important for pulmonary surfactant homeostasis, and deficiency leads to impaired alveolar macrophage differentiation and alveolar proteinosis (237, 238). Similarly, we found that the Csf2 expression was enhanced in M1 co-cultured LA4 cells, further experiments confirmed the induction of Csf2 by TNF-α. However, we observed that IL-1β can induce the Csf2 expression in LA4 cells to the same extent as well as TNF-α, which does not completely align to Lidija Cakarova's findings. They suggested that it is TNF-α from LPS-activated alveolar macrophages that stimulate AECs to express Csf2 (206). LPS-activated alveolar macrophages have a higher release of IL-1β, but the contribution of IL-1β in inducing Csf2 was not investigated. Actually, we are the first to show the induction of Csf2 in alveolar epithelial cells by IL-1β. Robert Newton et al. have shown that the IL-1β induced Csf2 expression in A549 cell was repressed by glucocorticoids (239).

IL-6 is usually thought to be a pro-inflammatory cytokine involved in the regulation of the immune response and inflammation (240). IL-6 can be produced by numerous cell types such as macrophages, endothelial cells and epithelial cells (240). It has been reported

long ago that macrophage derived TNF-α and IL-1β can induce the gene expression of Il6 (241). Hence, we believe that the increased gene expression of Il6 in LA4 cells as shown in Figure 4.5 can be induced by TNF-α or IL-1β.

Both Ccl2 and Cx3cl1 are chemokines that recruit monocytes to the site of injury and inflammation by acting on the respective receptors CCR2 and CX3CR1. It is not surprising to observe that M1 cells could induce the Ccl2 up-regulation in LA4 cells. It has been demonstrated that silica-induced Ccl2 expressions in alveolar epithelial cells is mediated by TNF-α (242). Cx3cl1 is also defined as fractalkine, and was reported to be induced by TNF-α and IL-1β in alveolar epithelial cells and fibroblast (243, 244). We also observed that M1 cells were able to induce its expression in LA4 cells; however, M0 and M2 conditioned media also induced Cx3cl1 in LA4, but that cannot be explained by our observation of no induction of Cx3cl1 in LA4 cell co-culture with the M2 cell.

TGF-β is a secreted protein which is involved in many cellular processes, including cell migration, invasion, EMT, tissue remodeling and immune responses. We observed that M1 co-cultured LA4 cells showed an increased TGF-β expression that cannot be induced by an M1 conditioned medium. It has been reported that TNF-α promotes TGF-β expression in lung fibroblasts via AP-1 activation, therefore we believed that the increased TGF-β expression in co-culture models may also be induced by TNF-α. In terms of the well described role of TGF-β in EMT, as well as M2 cells, it is also possible for M1 cells to induce EMT of alveolar epithelial cell through the TGF-β signaling pathway.

Lipocalin 2 (LCN2), is an antibacterial peptide expressed by macrophage, neutrophils and epithelial cells following the microbe induced TLRs activation (245). LCN2 is involved in antimicrobial defense by sequestering iron (231). LCN2 is also recognized as a biomarker of kidney injury (246). It has been reported that Lcn2 expression can be induced by IL-1β in the human alveolar epithelial cell line A549 (247). Similarly, we also observed that M1 cells and IL-1β can significantly induce the Lcn2 expression in LA4. Interestingly, recent studies indicated an important role of Lcn2 in macrophage activation. It has been reported that Lcn2 deficient macrophages showed an enhanced M1 polarization via the NF-κB-STAT3 signaling pathway. As we unexpectedly showed that IL-1β remarkably induced Lcn2 in LA4 cells, we speculated that the IL-1β activated LA4 cell might on their part be able to inhibit M1 polarization by secreted LCN2. However to test this hypothesis further co-culture experiments would be required.

In conclusion, using a trans-well and conditioned medium model as well as several individually selected cytokines, we found that M1 polarized alveolar macrophages could potentially promote the activation of alveolar epithelial cells through secreted TNF-α and IL-1β, which was characterized by the gene expression of Il6, Tgfb1, Lcn2, Csf2, Ccl2 and Cx3cl1 in LA4 cells. Our findings essentially confirmed the former work from other laboratories.

5. Materials and methods

5.1. Materials

5.1.1. Mice

All mice were kept and bred at institute of Lung Biology and Disease (iLBD), Helmholtz Zentrum München, Neuherberg, according to the national and institutional guidelines. Wild type (WT) and LMP2 -/- and LMP7 Knockout mouse are all on a C57BL/6J genetic background. The generation of LMP2-/- ($Psmb9^{tm1Stl}$) or LMP7-/- ($Psmb8^{tm1Hjf}$) mice have been described in the part of materials & methods in the paper respectively from Susumu Tonegawa (248) and H von Boehmer (249). Mice were age (8-16 weeks) and gender matched was sacrificed for BAL cell preparation.

5.1.2. Commercial available kit

Kit Name	Company
Absolute qPCR SYBR® Green ROX Mix	Applied Biosystems
Superscript™ II Reverse Transcriptase kit	Invitrogen
GoTaq® Polymerase Green Master Mix	Promega
Douset ELISA kit (CCL17)	R&D Systems
Bio-Rad Protein Assay kit	Bio-Rad
RNeasy Mini Kit	Qiagen
High Pure RNA Isolation Kit	Roche
E.Z.N.A.® Viral RNA Kit	Omega
Giemsa and May Grünwald solutions kit	Sigma-Aldrich
Quick-RNA™ MicroPrep kit	ZYMO
RNaseOUT TM Recombinant Ribonuclease Inhibitor	Invitrogen

dNTP Mix (10mM each)	Fermentas
Activity-based probe assay (ABP MV151, ABP LW124 and MVB127)	Overkleeft Hermen S
ECL-Reagent	GE Health

5.1.3. Equipment

Equipment Name	Company
NanoDrop® ND-1000 spectrophotometer	Thermo Scientific, Wilmington, USA
Centrifuge: Eppendorf 5415D	Eppendorf, Hamburg, Germany
Centrifuge: Sigma 3K18	Sigma, Osterode am Harz, Germany
ABI PRISM® 7500 detection system	Applied Biosystems, Foster city, CA, USA
Shandon cytospin3 cytocentrifuge	Shandon, PA
4ml, 15 ml and 50 ml Tubes	BD Falcon, Heidelberg, Germany
0.2ml, 0.5ml, 1.5 ml and 2ml Tubes	Eppendorf, Hamburg, Germany
Vortexer	Scientific Industries, Karlsruhe, Germany
PCR- thermal cycler : PTC-225	MJ Research, Hamburg, Biozym, Germany
Pipetman (2µl, 10µl, 20µl, 200µl, 1ml)	Gilson, Limburg-Offheim, Germany
ABsolute ™ QPCR Seal (AB1170)	Thermo Scientific, Wilmington, USA
96 Wells qPCR plate	Thermo Scientific, Wilmington, USA
Western Blot system	Bio-Rad , USA

5.1.4. Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Phosphate buffered saline (DPBS), Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), RPMI Media 1640 and antibiotics were purchased from BioChrome (Berlin, Germany) and Invitrogen (Karlsruhe, Germany).

All chemicals were purchased from Invitrogen (Karlsruhe, Germany), Sigma-Aldrich (Deisenhofen, Roche (Mannheim, Germany), Germany), Bio-rad (Munich, Germany), Fluka (Deisenhofen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) unless otherwise specified.

5.1.5. Buffers and solutions

Name	Concentration	Compounds
Wash buffer (PBS-T)	1X	PBS
	0.05%	Tween-20
PBS buffer (10X)	137 mM	NaCl
	2.7 mM	KCl
	10 mM	Na_2HPO_4
	2 mM	KH_2PO_4
TBE buffer (10X)	890 mM	Tris Base
	890 mM	Boric Acid
	20 mM	EDTA (pH 8.0)
RPMI-1640 medium	1X	RPMI-1640 medium
	10%	Fetal bovine serum
	1%	penicillin/streptomycin
	2 mM	glutamine
	50 µM	ß-mercaptoethanol (for macrophage culture)
Dulbecco's Modified Eagle Medium (DMEM)	1X	Dulbecco's Modified Eagle Medium
	10%	Fetal bovine serum

	1%	penicillin/streptomycin
	2 mM	glutamine
	50 µM	ß-mercaptoethanol (for macrophage culture)
RIPA buffer (1X)	20 mM	Tris-HCl (pH 7.5)
	150 mM	NaCl
	1 mM	Na_2EDTA
	1 mM	EGTA
	1%	NP-40
	1%	sodium deoxycholate
	2.5 mM	sodium pyrophosphate
	1 mM	ß-glycerophosphate
	1 mM	Na_3VO_4
	1 µg/ml	leupeptin
loading buffer (2X)	100mM	Tris pH 6,8
	4%	SDS
	0,2%	Bromphenol blue
	20%	Glycero
Electrophoresis (5x)(running) buffer	15,1 g	Tris
	94 g	Glycine
	50 ml	10% SDS
Transfer buffer(1X)	3,02g	Tris
	14,4g	H20
	200ml	Methanol
10% PAGE (4 gels) Resolving	15,9 ml	H2O
	13,3 ml	30% Acrylamid
	10,0 ml	1.5M Tris pH 8,8
	400 µl	10% SDS
	400 µl	10% APS
	16 µl	TEMED
10% PAGE (4 gels) Stacking	13,6 ml	H2O
	3,4 ml	30% Acrylamid
	2,5 ml	1M Tris pH 6,8
	200 µl	10% SDS
	200 µl	10% APS
	20 µl	TEMED
Block buffer (ELISA)	1g	BSA

	100ml	1X PBS
Stop solution (ELISA)	0.18 M	H_2SO_4

5.1.6. Recombinant proteins and antibodies

Name	Company
Anti-p50, p65, Arginase1, IRF4	santa cruz biotechnology
Anti-LMP2, LMP7, a1-7, IL-4Ra	Abcam
Anti-STAT6, p-STAT6, AKT, p-AKT	Cell Signaling
Anti-iNOS	BD Transduction Laboratories
HRP-conjugated anti-β-actin	Sigma-Aldrich
Recombinant murine IFN-gamma	Immuno tool
Recombinant murine IL-4, IL-13	Immuno tool
Lipopolysaccharides (LPS) from *E.coli*	Sigma-Aldrich

5.2. Methods

5.2.1. Isolation of primary resident alveolar macrophages (AMs)

Animals were treated humanely and with regard for alleviation of suffering; all animal procedures were conducted with approval of the local ethics committee and the Bavarian Animal Research Authority of Germany. Mice were anesthetized by intraperitoneal injection of a mixture of xylazine (4.1 mg/kg body weight) and ketamine

(188.3 mg/kg body weight) and killed by exsanguination (250). Therefore blood was drawn from the retroorbital plexus by a capillary.

Primary alveolar macrophages (AMs) were isolated from the lungs of mice by bronchoalveolar lavage (BAL) with 8 washes of 1 ml PBS at room temperature. Cells were pelleted for 5 min at 1500 rpm and washed twice in complete RPMI-1640 medium. 1 x 105 - 5 x 105 cells were seeded in 12- or 24-well plates and were allowed to adhere for 1-3 hours. Non-adherent cells were removed by washing two times with PBS

5.2.2. Alveolar macrophage cell line

Murine alveolar macrophage cell line (MH-S, derived from BALB/c) was purchased from American Type Culture Collection. Cells were grown in complete RPMI-1640 medium supplemented with 10 % fetal bovine serum (Biochrom) and 0.05 mM β-mercaptoethanol and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco) at 37 °C and 5 % CO2. When MH-S cells reach confluence, they were washed once in 10 ml PBS, and then 5 ml pre-warmed Trypsin-EDTA was added to cover the cell layer. Cells were incubated at 37°C for 5 min, and then wait until the complete detachment from the flask. Fresh culture medium was added to stop the reaction of Trypsin-EDTA. After resuspension in culture medium, MH-S cells were collected in falcon tubes and centrifuged (1200 rpm, 5 min, RT). Cells were subsequently diluted at 1:3 ratios into new flasks or used in experiments. MH-S cells were splitted every 3 to 4 days. MH-S cells were plated on 6-, 12- or 24-well plates with a density of 3 x 105 - 6 x 105 cells/cm2 the day before treatment.

5.2.3. Polarization of alveolar macrophages into M1 or M2 phenotype

Primary alveolar macrophages and MH-S cells were polarized towards the M1 phenotype with LPS (1 µg/ml, Sigma) and/or IFNγ (20 ng/ml, Immunotool) or towards the M2 phenotype with IL-4 (20 ng/ml, Immunotool) treatment for up to 72 h. Unpolarized cells (M0) served as controls. Cell culture supernatants were collected for measurement of CCL17. Adherent AMs were washed twice with PBS and harvested for total RNA isolation or protein extraction.

5.2.4. Trans-well co-culture of alveolar epithelial cell and alveolar macrophage

For LA4/MH-S co-culture, we used a trans-well co-culture system from (Becton Dickinson Lab ware and Corning Incorporated). LA4 were plated seeded on the lower side of trans-well at a density of 5.0 x105/well. MH-S cells were seeded on upper side of trans-well at a density of 5.0 x105/well. Cells were grown in complete F-12 culture medium supplemented with 15 % fetal bovine serum (Biochrom) and 0.05 mM nonessential amino acids (NEAA) (Biochrom) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco) at 37 °C and 5 % CO2. After 24h treatment of LPS (1 µg/ml, Sigma) and IFNγ(20 ng/ml) or with IL-4(20 ng/ml), RNA from LA4 cells were isolated for qPCR profiling.

5.2.5. Condition medium experiment

When MH-S cells reach confluence, cells were grown in F-12 culture medium, after the stimulation with LPS (1 µg/ml, Sigma) and/or IFNγ (20 ng/ml, Immunotool) toward M1or towards the M2 phenotype with IL-4 (20 ng/ml) for 6 h, the culture medium were aspirated, and then fresh medium were added again to the cell for another 24h.

Followed that, the supernatants from M0, M1and M2 cells were collected as conditioned medium, and it was added to the LA4 cell for 24 h.

5.2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatant of AMs was collected after treatment with LPS/IFNγ and IL-4 for M1 and M2 polarization and stored at -80 °C until analysis. CCL17 concentration was measured using a specific ELISA (Duoset Detection Kit; R&D Systems) according to the manufacturer's instruction. Concentrations were calculated with a standard curve (detection limit 31 pg/ml).

5.2.7. Transcriptome analysis

The samples were collected and prepared for RNA isolation by myself. Expressions profiling using the Illumina platform were done by Martin Irmler from the core unit from the Institute of Experimental Genetics at the Helmholtz Zentrum München. Heatmaps were generated with the help of Yuan De Tian from the Institute of Virology at the Helmholtz Zentrum München.

RNA isolation: Total RNA was isolated employing the RNeasy Mini (Qiagen) including RNase-Free DNase for digestion of remaining genomic DNA. The Agilent 2100 Bio-analyzer was used to assess RNA quality and only high quality RNA (RIN>7) was used for microarray analysis.

Expression profiling: Total RNA (about 10 ng) was amplified using the Ovation PicoSL WTA System V2 in combination with the Encore Biotin IL Module (Nugen). 1000 ng of amplified cDNA was hybridized to Mouse Ref-8 v2.0 Expression Bead Chips (Illumina,

San Diego, CA, USA). Staining and scanning were done according to the Illumina expression protocol. Data was processed using the GenomeStudioV2011.1 software (gene expression module version 1.9.0) in combination with the MouseRef-8_V2_0_R3_11278551_A.bgx annotation file. The background subtraction option was used and an offset to remove remaining negative expression values was introduced. CARMAweb was used for quantile normalization (251).

Statistical transcriptome analysis: Statistical analyses were performed by utilizing the statistical programming environment R (R Development Core Team). Genewise testing for differential expression was done employing the limma t-test ($p<0.05$). Heatmaps and GO enrichment analysis were done with R/Biocondcutor (www.bioconductor.org).

5.2.8. Water soluble Tetrazolium salt (WST) cell viability assay

MHS-cells and primary AMs isolated from Wt and LMPs-/- were seeded to 96 well palates at 30,000 per well. After 24h M1 and M2 polarization and serial dose of ONX0914 treatment for 6 and 24 h, 10% Roche WST reagent was added to the plate, and then wait for 15 min in a CO2-incubator at 37°C, followed that, the results were read by spectrophotometer at 490nm.

5.2.9. RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from AMs using Quick-RNA™ MicroPrep kit (ZYMO, Freiburg, Germany). 50-1000 ng total RNA was used for cDNA synthesis by Superscript™ II Reverse Transcriptase kit with the protocol described previously. To determine the expression of target genes relative to the actin housekeeper Actb, the ABsolute™ QPCR

SYBR® Green ROX Mix (Thermo Scientific, Wilmington, USA) was used on an ABI PRISM® 7000 detection system (Applied Biosystems, Foster city, CA, USA). Primer sequences are given in Supplementary Table I. Reaction mixture contained 1 µl cDNA (10 ng), 1 µl (5 µM) of each primer, 12.5 µl ROX mix and ddH2O up to a total volume of 25 µl. Following initial enzyme activation (one cycle at 50°C for 2min and 95 °C for 15 min), 40 cycles amplification (95 °C for 15 s, 60 °C for 1 min) were carried out, and then run a dissociation curve to detect nonspecific amplification. Relative expression of target genes and housekeeping gene Actb was calculated according to the 2-ΔCt method (49). We chose to show absolute values of a representative single experiment. Results of three independent replicates are provided in Table.

Table 2: Primer sequences and amplicon characteristics of genes of interest (GOI)

Target gene	Acc. No.	Forward primer (5'- 3')	Reverse primer (5'- 3')
Actb	NM_007393	TCCATCATGAAGTGTGACGT	GAGCAATGATCTTGATCTTCAT
Arg1	NM_007482	GGAACCCAGAGAGAGCATGA	TTTTTCCAGCAGACCAGCTT
Ccl17	NM_011332	TTGTGTTCGCCTGTAGTGCATA	CAGGAAGTTGGTGAGCTGGTAA
Il6	NM_031168	GCC AGA GTC CTT CAG AGA G	AGA CTC TCT CCC TTC TGA GC
Il1b	NM_008361	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il12b	NM_008352	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAAGG
Irf4	NM_013674	AAAGGCAAGTTCCGAGAAGGG	CTCGACCAATTCCTCAAAGTCA
Irf5	NM_012057	GCCACCTCAGCCGTACAAG	CTCCCAGAACGTAATCATCAGG
Mrc1	NM_008625	CATGAGGCTTCTCCTGCTTCT	TTGCCGTCTGAACTGAGATGG
Nfkb1	NM_008689.2	AGGAAGAAAATGGCGGAGTT	GCATAAGCTTCTGGCGTTTC
Nos2	NM_010927	CCTGTGAGACCTTTGATG	CCTATATTGCTGTGGCTC
Rela	NM_009045	CTTGGCAACAGCACAGACC	GAGAAGTCCATGTCCGCAAT
Retnla	NM_020509	CGAGTAAGCACAGGCAGT	CCAGCTAACTATCCCTCCAC
Tnf	NM_013693	CACCACGCTCTTCTGTCT	GGCTACAGGCTTGTCACTC

Il4ra	NM_001008700	TCTGCATCCCGTTGTTTTGC	GCACCTGTGCATCCTGAATG
Psmd11	NM_178616	GAATGGGCCAAATCAGAGAA	TGTACTTCCACCAAAAGGGC
Psme1	NM_011189	AGG CTT CCA CAC GCA GAT CT	ACC AGC TGC CGA TAG TCA CC
Psme2	NM_001029855	CCA GAT CCT CCA CCC AAG GA	CCG GGA GGT AGC CAC ACT TA
Psme3	NM_011192	TAGCCACGATGGACTGGATG	CACAAACACCTTGGTTCCTTGAA
Psma3	NM_011184.4	TGAAGAAGGCTCCAATAAACGTCT	AACGAGCATCTGCCAGCAA
Psmb5	NM_011186.1	TGCTCGCTAACATGGTGTATCAGTA	GGCCTCTCTTATCCCAGCCA
Psmb6	NM_008946.4	AGACGCTGTCACTTACCAACTTGG	AAGAGACTGGCGGCTGTGTG
Psmb7	NM_011187.1	TGCCTTATGTCACCATGGGTTC	TTCCTCCTCCATATCTGGCCTAA
Psmb8	NM_010724	TGCTTATGCTACCCACAGAGACAA	TTCACTTTCACCCAACCGTC
Psmb9	NM_013585	GTACCGTGAGGACTTGTTAGCGC	GGCTGTCGAATTAGCATCCCT
Psmb10	NM_013640	GAAGACCGGTTCCAGCCAA	CACTCAGGATCCCTGCTGTGAT
Csf-2	NM_009969	GCC ATC AAA GAA GCC CTG	GCG GGTCTGCAC ACA TGTTAAA
Lcn2	NM_008491	GAA GAA CCA AGG AGC TGT	TCA ATG CAT TGG TCG GTG
Tgfb	NM_001013025	TGA CGT CAC TGG AGT TGT ACG	GGT TCA TGT CAT GGA TGG TGC
Ccl2	NM_011331	CTT CTG GGC CTG CTG TTC A	CCA GCC TAC TCA TTG GGA TCA
Cx3cl1	NM_009142	GCGACAAGATGACCTCAC	CCAGGTGTCACATTGTCC
Cxcl1	NM_203320	CCG AAG TCA TAG CCA CAC	GTG CCA TCA GAG CAG TCT
Cxcl5	NM_002994	CCC TAC GGT GGA AGT CAT	CTT CAC TGG GGT CAG AGT
Cxcl2	NM_002089	TCCAGAGCTTGAGTGTGACG	TCCAGGTCAGTTAGCCTTGC
Cxcl9	NM_008599	GGAGTTCGAGGAACCCTA	GGGATTTGTAGTGGATCG
Pparg	NM_001127330	GTA GAA GCC GTG CAA GAG	GAG GAA CTC CCT GGT CAT
Cxcr2	NM_009909	AGCAAACACCTCTACTACCCTCTA	GGGCTGCATCAATTCAAATACCA
Cd36	NM_001159555	TGGAGATTACTTTTTCAGTGCAGAA	TCCAGCCAATGCCTTTGC
Galectin3	NM_001145953	GAG CTA CAC ATC CCT AGC C	CTC AGG AGG ATC TGA GAC TG

5.2.10. Cell lysis and Western blotting

For protein isolation, AMs were washed twice with ice cold PBS and lysed with RIPA buffer (50 mM Tris•HCl, pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1 % SDS). Samples were centrifuged to remove cell debris and

protein concentrations were determined using standard Bradford assays. 10 µg of protein lysates were separated on 10 % SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (162-0177, Bio-Rad,). The following antibodies were used: LMP2 (ab3328, Abcam), LMP7 (ab3329, Abcam), α1-7 (ab2267, Abcam); IL-4 Rα (ab157162, Abcam); AKT (4685, Cell Signaling), p-AKT (4060, pSer473, Cell Signaling), STAT6 (9362, Cell Signaling), p-STAT6 (9361, pTyr641, Cell Signaling); IRF4 (M17, Santa Cruz); Arginase1 (H-52, Santa Cruz); iNOS (610331, BD Transduction Laboratories); HRP-conjugated anti-β-actin (Sigma); HRP-conjugated anti-rabbit (Abcam) and anti-goat antibodies (Santa Cruz).

5.2.11. Activity-based probe labeling of proteasomes

The samples were collected and prepared for protein isolation by myself. Proteasome activity analysis using activity-based probe were done by our collaborator (Oliver Vosyka) from lab of Silke Meiners of Comprehensive Pneumology Center (CPC) at the Helmholtz Zentrum München.

Activity of the constitutive and immunoproteasome subunits was monitored by using a set of activity-based probes (ABP) (252). The pan-reactive proteasome ABP MV151 (163) was used for quantification of β-subunit activities. β1/LMP2 activities were quantified with the ABP LW124 while quantification of β5/LMP7 subunits was performed using the MVB127 ABP(164).

To obtain native lysates, cells were frozen in liquid nitrogen and thawed in a 37 °C water bath for five times in ddH2O supplemented with protease inhibitors. After removal of cellular debris, these hypoosmotic lysates were diluted to a total protein concentration of

0.5 µg/µl with reaction buffer (50 mM HEPES pH 7.4, 100 mM KCl, 10 mM MgCl2). 30 µl of sample was incubated with 0.5 µM MV151, 0.25 µM LW124 or 1 µM MVB127 for 1 h at 37 °C respectively and subsequently quenched by the addition of 6x Laemmli Buffer (50 % v/v glycerol, 300 mM Tris·HCl, pH 6.8, 6 % w/v SDS, 325 mM DTT, 0.1 % w/v bromophenol blue) to a final 1x concentration.

Samples were separated on a 15 % Tris-glycine SDS polyacrylamide gel and proteasome activity was visualized using a fluorescent scanner (Typhoon TRIO+; Amersham biosciences). Images were taken at 450 PMT (voltage of photo-multiplier tube) and 50 µm pixel resolution with fluorescence Cy3/TAMRA for ABPs MV151 and MVB127. The Cy2 florescent channel was used for LW124 and analyzed by using ImageJ software (http://imagej.nih.gov/ij/).

5.2.12. Statistical analysis

All values are showed as the mean ± SEM of at least five animals or 3 individual samples *in vitro*. We used analysis of variance (ANOVA), as calculated by GraphPad Prism 5, to establish the statistical significance of differences between the experimental groups. Individual inter-group comparisons were analyzed using the two-tailed unpaired t test with Welch's correction. Differences were considered significant at *, $p < 0.05$; **, $p<0.01$ and ***, $p<0.001$.

6. Reference

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7. Abbreviations

°C	Degrees Celsius
µl	Microliter
Ab	Antibody
Actb	Actin, beta
ALI	acute lung injury
AMs	Aveloar macrophages
BAL	Bronchoalveolar lavage
cDNA	Complementary DNA
COPD	chronic obstructive pulmonary disease
Ct	Threshold cycle
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethyldiaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
g	Gram
HRP	horseradish peroxidase
KO, -/-	Knock out
L	Liter
LPS	Lipopolysaccharides
M1 macrophages	classic activated macrophages
M2 macrophages	alternative activated macrophages
M-CSF	Macrophage colony-stimulating factor
ml	Milliliter
mm	Millimeter
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
PBS	Phosphate buffer saline
PBST	phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RIPA	radioimmunoprecipitation assay buffer
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription PCR

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SPF	Specific pathogen-free
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
WT	Wild type
HDL	high-density lipoprotein
IRF	interferon-regulatory factor
LMP	low molecular mass protein
MECL1	multicatalytic endopeptidase complex-like 1
ABP	activity-based probe
Tnf	Tumor necrosis factors
iNOS	inducible nitric oxide synthase
DC	dendritic cells
Il1b	Interleukin-1 beta
Il12b	interleukin 12 beta
IPF	Idiopathic pulmonary fibrosis
IL-4Ra	interleukin-4 receptor alpha chain
Arg1	arginase 1
Retnla	Resistin-like molecule alpha
Ccl17	Chemokine (C-C motif) ligand 17
Arg1	arginase 1
Mrc1	mannose receptor 1
WT	Wild type
Lcn2	Lipocalin-2
Tgfb	Transforming growth factor beta
Cxcl5	C-X-C motif chemokine 5
Il6	interleukin 6
Ccl2	chemokine (C-C motif) ligand 2
Csf-2	colony stimulating factor 2

8. Appendix

Lebenslauf

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   (Munich Lung Conference (MLC) 2012, Munich, Germany)

2. Poster 2: Immunoproteasome subunits LMP2 and LMP7 differentially contribute to alveolar macrophage polarization

   (Munich Lung Conference (MLC) 2013, Munich, Germany)

3. Poster 3: Who is the main producer of neutrophil-attracting chemokines initiating carbon nanoparticle induced acute lung inflammation

   (27th Annual EMDS Meeting, 10 – 12 October 2013, Erlangen, Germany)

4. Poster 4: Immunoproteasome Subunits LMP2 and LMP7 Differentially Contribute To Alveolar Macrophage Polarization

   (ATS 2014 International Conference, San Diego, USA)

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2. **Chen Shanze**, Renfu Yin, Kathrin Mutze, Oliver Eickelberg, Melanie Königshoff, Tobias Stoeger. (2015). Alveolar epithelial cells rather than macrophages initiate carbon nanoparticle-induced acute lung inflammation **(In preparation for Particle and Fibre Toxicology)**

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