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Role of Myeloid Derived Suppressor Cells in Mycobacterial Infection

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Dedicated to my wife Biftu, and my son Tokuma
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Abbreviations and Acronyms

\(\mu g\) .............................................. Microgram
\(\mu l\) ............................................... Microliter
AIDS .......................................... Acquired immune deficiency syndrome
APC ........................................... Allophycocyanin
APC ........................................... Antigen presenting cells
Arg1 ........................................... Arginase 1
ATCC ........................................ American Type Culture Collection
BD ............................................. Becton Dickinson
BSA ........................................... Bovine serum albumin
cDC ........................................... Convectional dendritic cells
cDNA ......................................... Complimentary deoxyribonucleic acid
CFU ........................................... Colony forming unit
Cy .............................................. Cyanine
DAPI .......................................... 4’,6-diamidino-2-phenylindole
DH\(_2\)O ......................................... Double distilled water
dNTP ......................................... Deoxynucleotide triphosphates
D-PBS ....................................... Dulbecco’s phosphate-buffered saline
DSM .......................................... German collection of microorganisms
EDTA .......................................... Ethylenediaminetetraacetic acid
et al. .......................................... and others
FcR ............................................ Fc receptor
FCS ........................................... Fetal calf serum
FITC .......................................... Fluorescein isothiocyanate
H and E ..................................... Hematoxylin and eosin
HIV ............................................ Human immunodeficiency virus
hr ............................................... Hour
HRP ........................................... Horse radish peroxidase
i.p .............................................. Intraperitoneal
i.v .............................................. Intravenous
IFN-\(\gamma\) ........................................ Interferon gamma
IgG ............................................ Immunoglobulin G
IL ............................................. Interleukin
IMDM ......................................... Iscove’s Modified Dulbecco’s Medium
iNOS .......................................... Inducible nitric oxide synthase
IRF ............................................ Interferon regulatory factor
LPS ........................................... Lipopolysaccharide
Ly6C .......................................... Lymphocytes antigen 6 complex
MAA ........................................... *Mycobacterium avium* ssp. *avium*
MAC ........................................... *Mycobacterium avium* complex
MAH ........................................... *Mycobacterium avium* ssp. *hominissuis*
MDSC ........................................ Myeloid derived suppressor cells
MHC-II ....................................... Major histocompatibility complex class two
MIP ............................................ Macrophage inflammatory protein
mM ............................................ Millimolar
M-MDSC ................................... Monocytic myeloid derived suppressor cells
G-MDSC ................................... Granulocytic myeloid derived suppressor cells
MTBC ........................................ *Mycobacterium tuberculosis* complex
MyD88 ................................. Myeloid differentiation primary response gene 88
n.s ............................................. not significant
NaCl .......................................... Sodium chloride
ND ............................................. Not detectable
NF ............................................. Nuclear factor
NH₄Cl ........................................ Ammonium chloride
NK ............................................. Natural killer
NO ............................................. Nitric oxide
NTM .......................................... Nontuberculous mycobacteria
OAGC ........................................ Oleic albumin glucose catalase
°C ............................................. Degree Celsius
OD ............................................. Optical density
p.i ............................................. Post infection
PAMPs ...................................... Pathogen-associated molecular patterns
PBS ........................................... Phosphate buffered saline
PCR ........................................... Polymerase chain reaction
PE ............................................. Phycoerythrin
PRR ........................................... Pattern recognition receptor
qRT-PCR ................................... Quantitative-real-time PCR
RNA ........................................... Ribonucleic acid
rpm ............................................ Revolution per minute
Stat ............................................ Signal transducer and activator of transcription
TB ............................................ Tuberculosis
TCRζ ......................................... T cell receptor zeta
TLR ........................................... Toll-like receptor
TLR2 ......................................... Toll like receptor 2
TMC .......................................... Trudeau Mycobacterial Culture Collection
TNF ........................................... Tumor Necrosis Factor
ZN ............................................. Ziehl-Neelsen
Summary

Ketema Abdissa Merga

Role of myeloid derived suppressor cells in mycobacterial infection

Non-tuberculous mycobacteria (NTM) are receiving growing attention as causative agents for infections in humans and animals. The most common NTM species associated with disease is *Mycobacterium (M)*. *avium*. Despite its importance there is little known regarding the host immune response to *M. avium* infection and in particular about the contribution of myeloid derived suppressor cells (MDSC). MDSC represent a heterogeneous population of immature myeloid cells able to suppress innate and adaptive immunity. They are known to have beneficial and detrimental effects on the immune responses during cancer, inflammation and infection. The role of MDSC during mycobacterial infection is still not completely resolved. In the present study we compared two *M. avium* subspecies (ssp.) - *M. avium* ssp. *avium* (MAA) and *M. avium* ssp. *hominissuis* (MAH) - in an infection model for disseminated mycobacterial infection. Intraperitoneal infection of mice with MAA resulted in severe disease with progressive histiocytic infiltration in the spleen and immune granuloma with low numbers of lymphocytes in the liver. In contrast, infection with MAH caused less severe disease and lower splenic histiocytic infiltration, combined with classical granuloma harboring mononuclear cells and peripheral lymphocytes. Spleen of MAA infected mice exhibited high numbers of mycobacteria infected, nitric oxide (NO) producing Gr-1<sup>lo</sup>CD11b<sup>+</sup> cells that had mostly a Gr-1<sup>lo</sup>CD11b<sup>+</sup>CD11c<sup>int</sup>Ly6C<sup>+</sup>Ly6G<sup>-</sup> monocytic MDSC (M-MDSC) like phenotype. Presence of these M-MDSC in spleen was associated with a specific NO dependent ablation of CD4 T cells. Functional characterization of the M-MDSC revealed a dendritic cell phenotype but M-MDSC were unable to present antigens to CD4 T cells. However, by NO, M-MDSC were able to suppress CD4 T cell proliferation as well as protein processing and presentation by splenic conventional dendritic cells *ex vivo*. Specific inhibition of proliferation of adoptively transferred CD4 T cells was observed in MAA infected mice *in vivo*. Interestingly, in mice lacking iNOS, arginase 1 (Arg1) was upregulated and likely compensated the loss of iNOS, ultimately inhibiting T cell proliferation. Overall we show that induction of M-MDSC is a particular feature of highly virulent
mycobacteria in mice. M-MDSC operate to suppress local immune responses by alternatively using either NO or Arg1. Hence, this phenomenon enhances uncontrolled pathogen expansion.
Zusammenfassung

Ketema Abdissa Merga

Die Bedeutung myeloider Suppressorzellen bei mykobakteriellen Infektionen

wurden auch in MAA infizierten iNOS defizienten Mäusen induziert. Auch in diesen Mäusen wurde eine spezifische Hemmung der Proliferation von adoptiv transferierten CD4 T-Zellen \textit{in vivo} beobachtet. Offensichtlich wird die T-Zell-inhibitorische Wirkung der M-MDSC in der Abwesenheit von iNOS, durch eine erhöhte Arginase 1 Expression kompensiert.

Zusammenfassend zeigen die Arbeiten der These, dass die Fähigkeit von Mykobakterien M-MDSC zu induzieren ein wesentliches Virulenzmerkmal ist. Nach Induktion können M-MDSC kontextunabhängig durch die alternative Nutzung von NO oder Arginase1 ihr T-Zell-inhibitorische Wirkung entfalten.
1 Introduction

1.1 Genus Mycobacterium

Mycobacteria are aerobic, acid fast actinomycetes usually curved or straight non motile rods (Hartmans, de Bont et al. 2006). The genus *Mycobacterium* contains over 150 species (Gonzalez-Perez, Marino-Ramirez et al. 2013). Mycobacteria contain a high density of lipids in their cell wall which makes them unique from Gram positive and Gram negative bacteria. The outer cell wall layer contributes to resistance of mycobacteria to hazardous environments (Brennan 2003). The outer layer is made up of lipids and proteins. The cell wall lipids, mostly mycolic acids, are linked to polysaccharides such as arabinomannan (Hett and Rubin 2008, Kieser and Rubin 2014). As shown in Figure 1.1, the structural layer is made of peptidoglycan, arabinogalactan, mycolic acids and capsule. Due to the assembly of such complex cell wall structure, the division rate of pathogenic mycobacteria is generally slow (Kieser and Rubin 2014).

Due to the presence of long chain fatty acids in their cell wall, mycobacteria are not stainable by Gram’s Method; however, they are classified as Gram positive bacteria. They are resistant to acid decolorization hence commonly identified by acid fast staining method. As stated above, the complex nature of mycobacterial cell wall renders them resistant to commonly used disinfectants and antibiotics (Jarlier and Nikaido 1994). This is due to the impermeability of the cell wall and only hydrophobic antibiotics can cross the cell wall barrier (Russell 1999, Lambert 2002). Moreover, such a complex cell wall also attributes to the spore like properties of mycobacteria (Marrakchi, Laneelle et al. 2014).

Unlike to Gram positive and Gram negative bacteria, toxins are not the main virulence factor for mycobacteria. Rather cell wall lipids serve as main mycobacterial virulence factors. For example, lipoarabinomannan acts as an immune modulatory molecule. It acts as a chemoattractant and can induce pro-inflammatory response in macrophages and dendritic cells (DC) (Bernardo, Billingslea et al. 1998). Mannose capped lipoarabinomannan inhibits T cell migration (Richmond, Lee et al. 2012). The
mycobacterial cell wall outer layer also acts as anti-phagocytic capsule, limiting the contact between macrophages and the pathogen (Stokes, Norris-Jones et al. 2004).

**Figure 1.1. The mycobacterial cell envelope.** The mycobacterial cell envelope is composed of mycolic acid, arabinogalactan and peptidoglycan. The outer layer of cell envelope is covered by non-covalently linked proteins and polysaccharides. The lipoarabinomannan (LAM) containing long polymer of mannose is intercalated in the cell wall. The polymers of peptidoglycan, N-acetyl glucosamine and N-acetyl muramic acid, are interlinked by peptide bridges (Modified from (Kieser and Rubin 2014)).

1.2 Classification

Generally, mycobacteria are divided into *M. tuberculosis* complex (tuberculosis mycobacteria), *M. leprae* and mycobacteria other than TB (MOTT, also denominated atypical mycobacteria or as non tuberculous mycobacteria, NTM). It is believed that all mycobacterial species have developed from a common ancestor via DNA insertions and deletions (**Figure 1.2**).
1.3 Tuberculosis mycobacteria

*M. tuberculosis* complex (MTBC) is composed of 7 closely related subspecies causing human and animal tuberculosis (TB). These subspecies include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. microti*, *M. canettii* and *M. pinnipedii* (Huard, Lazzarini et al. 2003, Huard, Fabre et al. 2006). The members of the MTBC are facultative intracellular bacteria and obligate host pathogens as their replication in the environment is not reported (de Jong, Antonio et al. 2010). Thus, transmission is taking place from human to human, animal to human or vice versa (Ocepek, Pate et al. 2005). TB was declared as global emergency in 1993 and remains of public health importance particularly in the developing world. Today, TB is the leading infectious cause of death. Indeed according to the world health organization (WHO) report in 2016, TB accounts for 10.4 million new cases and 1.4 million deaths (WHO 2016).
1.4 Non tuberculous mycobacteria (NTM)

Standard classification of NTM has been made in the 1950s by Timple and Runyon (Table 1.1). According to their classification, pigment production, colony morphology and growth characteristics have been used to classify into four categories. Accordingly, Group I or atypical mycobacteria produce pigmented colonies in the presence of light and are slow growers (> 7 days). Under group II or NTM, pigment producers regardless of light and slow growers are included. Group III or mycobacteria other than tuberculi bacilli are known by lack of pigmentation and slow growing characteristics. Group IV (MOTT) or potentially pathogenic environmental mycobacteria are those lacking pigmentation with fast growing characteristics (Society 1997, Koh, Kwon et al. 2002).

Table 1.1. Runyon classification of selected NTM species

<table>
<thead>
<tr>
<th>Group</th>
<th>Characteristics</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>slow growers</td>
<td><em>M. kansasii</em>, <em>M. marinum</em>, <em>M. simie</em></td>
</tr>
<tr>
<td></td>
<td>photo-chromogenic colony</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>slow growers</td>
<td><em>M. xenopi</em>, <em>M. scrofulaceum</em>, <em>M. szulgai</em>,</td>
</tr>
<tr>
<td></td>
<td>scoto-chromogenic colony</td>
<td><em>M. gordonae</em>, and <em>M. flavescens</em></td>
</tr>
<tr>
<td>III</td>
<td>slow growers</td>
<td><em>M. avium</em>, <em>M. intracellulare</em>, <em>M. chimera</em>,</td>
</tr>
<tr>
<td></td>
<td>non-chromogenic colony</td>
<td><em>M. colombiense</em></td>
</tr>
<tr>
<td>IV</td>
<td>fast growers</td>
<td><em>M. abscessus</em>, <em>M. fortuitum</em>, <em>M. cheloneae</em>,</td>
</tr>
<tr>
<td></td>
<td>non-chromogenic colony</td>
<td><em>M. smegmatis</em></td>
</tr>
</tbody>
</table>

Unlike MTBC, NTM are free-living and ubiquitous in the environment. They can be isolated from water, soil, domestic and wild animals, milk and other food products (Wolinsky and Rynearson 1968, Chapman 1971, Goslee and Wolinsky 1976). For long time, NTM were not considered as a major health concern and were assumed to be of very low virulence potential. Their clinical importance was recognized after the appearance of HIV infection. Subsequently, frequent NTM infection among elderly and immune comprised was recognized. Today, NTM infections are
considered as emerging human and animal pathogens in both immune compromised and immune competent hosts (Bodle, Cunningham et al. 2008, Thomson, Centre et al. 2010).

While the prevalence of MTBC continues to decline in developed countries, the prevalence and incidence of NTM are increasing (McGrath, McCabe et al. 2008). Even though, worldwide consolidated data are missing some countries report up to 21% NTM infection among HIV patients (Bjerrum, Oliver-Commey et al. 2016). Clinical syndromes caused by NTM are diverse. These include lung (Johnson and Odell 2014), cutaneous (Touma, Haddad et al. 2013), systemic (Chetchotisakd, Kiertiburanakul et al. 2007), nervous system (Jacob, Henein et al. 1993), bone (Bi, Hu et al. 2015) and intestinal infections (Yamazaki, Mori et al. 2010). Until now there are no convincing data regarding animal to human or human to human transmission of NTM. The chance of human or animal exposure to NTM is high as they are widely distributed in the environment including drinking water (Kim, Greenberg et al. 2008, Wu and Holland 2015). NTM infection poses several challenges due the lack of effective standard treatment and high resistance against antibiotics (Novosad, Henkle et al. 2015).

Amongst all NTM, infections due to M. avium complex are the most prevalent. M. avium complex (MAC) is the second largest mycobacterial complex of medical importance. For example, in Europe out of all NTM lung infections, 79% was due to MAC infection (Wagner, van Ingen et al. 2014). Among several predisposing factors, organ transplantation, previous history of lung disease, cystic fibrosis, HIV infection, IL-12/IFN-γ axis genetic defect and occupational exposure are the most common (Marras and Daley 2002).

MAC includes the species M. avium and M. intracellulare. M. avium comprises four genetically closely related subspecies with different host and tissue tropism. These subspecies include M. avium ssp. avium (MAA), M. avium ssp. hominissuis (MAH), M. avium ssp. silvaticum (MAS), M. avium ssp. paratuberculosis (Rindi and Garzelli 2014). While MAH is usually associated with human infection, MAA is less frequently isolated from humans (Pavlik, Svastova et al. 2000). Moreover, MAH seems more pathogenic than MAA in humans. Domestic animals, as well as environmental
sources such as soil, water, compost could be sources of infection (Kaevska, Slana et al. 2011). Horses can be infected with MAH. (Zakhashvili, Tsertsvadze et al. 2010). Both, MAA and MAH can infect pigs and birds (Mijs, de Haas et al. 2002, Rindi and Garzelli 2014).

1.5 Natural history of mycobacterial infection

TB can be demonstrated in humans and other mammals (MTBC and MAC) (Davies 2006), birds (MAC and MTBC) (Dhama, Mahendran et al. 2011), and as well as fish (M. marinum) (Akram and Bhimji 2017) with similar pathophysiology even though different organs are involved. After mycobacterial uptake, the outcome of infection in the described hosts varies according to the host immune status. Thus, effective clearance, primary disease, latent infection or reactivation might occur (Cambier, Falkow et al. 2014). Innate immune cells can clear the pathogen even before development of adaptive immune cells. Primary infection occurs if the pathogen continues to proliferate and the bacilli seed the draining lymph nodes. This situation may be followed by dissemination to other organs via hematogenous route leading to disseminated infection. Latent subclinical infection develops if the host attempts to clear the pathogen fail, however, the pathogen proliferation is under control by the concerted effort of innate and adaptive immune cells by walling off the pathogen inside the granuloma. This phenomenon leads towards latent infection which may sustain throughout life. If the immune system fails in life, the pathogen continues to expand post latency period, reactivation of the pathogen leads to reactivation tuberculosis (Rook, Dheda et al. 2005, Hunter 2016). Over all, factors contributing to containment of infection or progression to disease are not well understood.

1.6 Role of myeloid cells in mycobacterial infection

While myeloid cells are becoming versatile due to the emerging powerful techniques of phenotyping, little is known about the role of such diverse myeloid cells in mycobacteria and in particular NTM infection. Myeloid cells are cells of the immune system developing from hematopoietic stem cells (HSC) for repopulating the peripheral blood and tissue leukocyte pool. Myeloid cells include monocytes, macrophages, dendritic cells, neutrophils, eosinophils and basophils and their
progenitors (Geissmann, Manz et al. 2010). Under physiologic conditions, HSC renew themselves and develop towards terminally differentiated cells up on proper lineage specific colony stimulating factors (CSFs) (Iwasaki and Akashi 2008, Metcalf 2008).

Cells of myeloid lineage express germline encoded pattern recognition receptors (PRRs) to recognize conserved foreign stimuli (Nagai, Garrett et al. 2006). Among these, Toll like receptors (TLR), TLR2 particularly plays a dominant role in mycobacterial infection. Other receptors include C-type lectin receptors and cytosolic nucleotide binding oligomerization domain like receptors. TLR2 polymorphism has been reported to be associated with TB and NTM lung disease (Rahman, Sobia et al. 2014). NTM infected patients have impaired expression of TLR2 (Ryu, Kim et al. 2007). Mice lacking MyD88 adaptor molecules are even more susceptible to *M. avium* infection compared to TLR2⁻/⁻ mice as shown by lower survival rates and higher CFU in organs (Feng, Scanga et al. 2003).

Monocytes, macrophages and DC are the major target cells for pathogenic mycobacteria (Srivastava, Ernst et al. 2014). During mycobacterial lung infection, the pathogen is immediately engulfed by alveolar macrophages and dendritic cells. Such infected cells either undergo necrosis or migrate to other organs via lymphatic and hematogenous route (Guirado, Schlesinger et al. 2013). During infection via intestinal route after crossing epithelial barrier, *M. avium* and *M. tuberculosis* parasitize intestinal macrophages (Smith, Smythies et al. 2011, Bannantine and Bermudez 2013). Macrophages serve as initial niche of mycobacterial replication as depletion of alveolar macrophages was found to be protective after aerosol infection in mice (Leemans, Juffermans et al. 2001). Once inside the macrophages, inflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), interleukin 1β (IL-1β) and chemokines are released by the infected cell (Flynn, Chan et al. 2011, Verrall, Netea et al. 2014). DC are of major importance for initiating adaptive immune response by ingesting and presenting pathogens and pathogen derived antigens to T cells via molecules of histocompatibility complex (MHC). This has been shown by depletion of CD11c expressing cells (Tian, Woodworth et al. 2005). However, the pathogen takes advantage of a delay in antigen presentation to
T cells in the lymphoid organs which takes between 12 to 21 days after infection in mice (Chackerian, Alt et al. 2002).

### 1.7 Role of lymphoid cells in mycobacterial infection

The role of T cells in mycobacterial infection is long known from adoptive transfer of T cells from immunized mice to T cell deficient mice (Orme 1987). More detailed role of T cells was recognized in mice lacking αβ T cells. While wild type mice controlled *M. tuberculosis* infection after 20 days, αβ T cells lacking mice could not control the infection and died at 48 days post infection (Mogues, Goodrich et al. 2001). As shown in CD4 T cells deficient mice, protective granuloma formation is impaired in the absence of CD4 T cells even in the presence of IFN-γ (Saunders, Frank et al. 2002). In human context, the role of T cells has been recognized as the susceptibility of HIV positive patients to mycobacterial infection was related to loss of CD4 T cells. The role of B cells in control of mycobacteria is less appreciated. However, B cells also participate in granuloma formation. They can also present mycobacterial antigens (Chan, Mehta et al. 2016).

The critical importance of IFN-γ produced by T cells in mycobacterial infection is well established. Even if IFN-γ can be produced by different cells of the immune system, IFN-γ produced by CD4 T cells is required for optimal control of infection (Green, Difazio et al. 2013). Moreover, CD4 T cell dependent, IFN-γ independent (yet less clear) mechanisms operate in control of mycobacterial infection (Cowley and Elkins 2003). Another related finding shows that CD4 T cells contribute to *in vivo* control of *M. tuberculosis* even if they are not able to produce effector cytokine IFN-γ (Gallegos, Pamer et al. 2008). Other works show that while IFN-γ is an important mediator in TB control, the absence of CD4 T cells exacerbates the infection (Green, Difazio et al. 2013).

Recently it has been described that direct contact between CD4 T cells and mycobacteria infected myeloid cells is required for optimal control of intracellular mycobacteria. This has been proven by chimera of mycobacteria infected myeloid cells with or without MHC-II. MHC-II⁺ cells harbored significantly lower numbers of mycobacteria compared to MHC-II⁻ cells *in vivo*. In addition, depletion of CD4 T cells
did not affect bacterial burden in MHC-II\(^{-}\) cells (Srivastava and Ernst 2013). This phenomenon is in contrast to other intracellular pathogens like *Leishmania* where bystander CD4 cytokines enhance pathogen control (Muller, Filipe-Santos et al. 2012). MHC-II\(^{-}\) mice are more susceptible to mycobacterial infection than CD4\(^{-}\) T cells regardless of IFN-\(\gamma\) secretion (Caruso, Serbina et al. 1999).

1.8 The granuloma: aggregate of myeloid and lymphoid cells

The hallmark of mycobacterial infection is development of granuloma in the affected host tissues. Granuloma is an organized aggregate of innate and adaptive immune cells which is formed due to chronic antigen stimulation (*Figure 1.3*). In this structure, infected macrophages are located at the center surrounded by primed lymphocytes. In addition, monocytes, dendritic cells, neutrophils and NK cells are also found. Macrophages play a leading role in granuloma formation. The macrophage phenotype could be either pro or anti-inflammatory or both. Macrophages can fuse together forming multinucleated giant cells or differentiate towards lipid rich foamy macrophages (Silva Miranda, Breiman et al. 2012). As mycobacteria are facultative intracellular pathogens, they can reside either inside the cell or freely in the granuloma. DCs composing granuloma migrate out to present bacterial antigen to T cells in secondary lymphoid organs while DCs can also activate lymphoid cells in inducible tertiary lymphoid organs like inducible bronchial associated lymphoid tissue (Schreiber and Sandor 2010).

Granuloma type and formation depends on a dynamic process influenced by host immune response and pathogen interaction. The initial stage of mycobacterial interaction with monocyte/macrophages induces release of chemokines. Such chemokines induce further recruitment of immune cells like neutrophils, NK cells, monocytes and DCs. Next, DCs ingest and process pathogen related antigen and present it to CD4 T cells. Moreover, DCs release IL-12 which further augments T cell activation. Activated T cells migrate to the site of infection and release IFN-\(\gamma\) which in turn intensifies the macrophage activation via the IFN-\(\gamma\) receptor. Such a loop potentiates macrophages to kill the ingested pathogen (Flynn, Chan et al. 2011). The different immune reactions take place in distinct parts of granuloma. The center of the granuloma shows inflammatory reaction associated with anti-microbial peptides,
reactive oxygen species and anti-inflammatory reaction at the periphery of the granuloma (Marakalala, Raju et al. 2016).

Several types of granuloma are formed. A protective granuloma is characterized by controlled growth of the bacteria which leads to fibrotic transformation, calcification and possibly elimination of the pathogen. This type of granuloma is indicative of successful immune response. A homeostatic granuloma is a stage where the immune balance is maintained and this leads to a latency stage of infection where the pathogen enters into the stage of dormancy and metabolic adaptation (Ehlers and Schaible 2012). It is also characterized by the presence of epithelioid macrophages surrounding mostly an acellular necrotic area. The worst case of granuloma is characterized by necrosis, extensive replication and further seeding of the pathogen to other tissue sites or organs (Ehlers and Schaible 2012). Necrotic granuloma is composed of macrophages and few lymphocytes (Flynn, Chan et al. 2011).

![Figure 1.3. Structure of granuloma and associated immune cells (Ramakrishnan 2012).](image)
Whether granuloma formation is advantageous for the host is topic of debate (Bold and Ernst 2009). It is well documented that a granuloma contains mycobacteria and inhibits further seeding and dissemination. This has been demonstrated in the TNF-α deficient host. TNF-α not only protects granuloma architecture but it also initiates formation of granuloma. TNF-α deficiency leads to poor granuloma formation which is accompanied by exacerbated infection (Algood, Lin et al. 2005). TNF-α plays a main role in leukocyte attraction to the site of infection by inducing chemokines production (Algood, Lin et al. 2005). On the other hand, the granuloma isolates the bacteria and restricts the accessibility to host immune cells. For example, inhibition of E-cadherin dependent epithelial transition of macrophages leads to disorganized granuloma accompanied by increased access to immune cells, hence decreased bacterial load and enhanced host survival. This indicates that granuloma formation might have mutual benefits for host and pathogen (Cronan, Beerman et al. 2016).

1.9 Role of nitric oxide: in and outside of the granuloma

Nitric oxide (NO) is a simple gaseous molecule whose biological relevance was first discovered in 1980. NO was named molecule of the year in 1992 (Koshland 1992). NO is a bioactive molecule with a short half-life (<30 seconds). It has neutral charge and readily diffuse in to host and microbial cells (Kelm 1999) and develops bioactivity by reacting with thiol group and iron harboring proteins (Rosselli, Keller et al. 1998).

NO has several physiologic, anti-microbial, anti-viral, anti-parasitic, anti-tumor, and immune modulatory functions (Bogdan 2001). Its physiologic function includes its effect of relaxation of blood vessel and in various reproductive processes (Rosselli, Keller et al. 1998). It also regulates smooth muscle cell tone, platelet adhesion, cell growth and apoptosis (Rosselli, Keller et al. 1998).

In eukaryotic cells three nitric oxide synthase isoforms, inducible nitric oxide synthase (iNOS also designated as NOS2), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), can produce NO. All isoforms share 50-60% amino acid sequence similarity (Rosselli, Keller et al. 1998). All isoforms use L-arginine as substrate (Moncada 1999). While eNOS and nNOS are constitutively expressed, iNOS is only found in activated myeloid immune cells, particularly
monocytes and macrophages (Kleinert, Schwarz et al. 2003). In general inducible NO can be produced by monocyte, macrophage, microglia, Kupfer cells, eosinophils, neutrophils, endothelial cells and epithelial cells (Bogdan 2001). iNOS expression is enhanced by lipopolysaccharide (LPS), IL-1β, IL-6, TNF-α, type I interferons and related cytokines (Lin, Chang et al. 1996). Moreover iNOS can undergo self-regulation via its product, NO (Kleinert, Schwarz et al. 2003, Bogdan 2015). Several iNOS promoter binding transcription factors have been reported in several species. These include STAT-1, STAT-2, IRF-9, IRF-1, NF-κB, AP-1, CREB, NFIL-6, Oct-1 and SRF (Lowenstein, Alley et al. 1993, Kleinert, Schwarz et al. 2003, Guo, Mi et al. 2008). The iNOS regulating pathways differ in different cells and several species (Kleinert, Schwarz et al. 2003). In addition, iNOS expression is regulated by the level of arginine supply and uptake, and related intracellular co-factors like tetrahydrobiopterin (BH$_4$) (Kleinert, Schwarz et al. 2003).

Differential cell and species specific iNOS expression is controversially reported. For example, in contrast to mouse macrophages, human alveolar macrophages iNOS expression is hyporesponsive to LPS and IFN-γ due to the difference in methylation of iNOS promoter sites (Gross, Kremens et al. 2014). In humans, intestinal macrophages do not express iNOS while mouse macrophages do (Cipriani, Gibbons et al. 2016). The same difference was reported between human and mouse endothelia cells (Chan, Fish et al. 2005, Dreger, Ludwig et al. 2016). However, there are reports of iNOS expression in human alveolar epithelial cells lines (Roy, Sharma et al. 2004). More importantly, 65% of alveolar macrophages from TB patients were iNOS positive compared to 10% in apparently health subjects (Nicholson, Bonecini-Almeida Mda et al. 1996). Human monocyte derived macrophages also express iNOS up on stimulation with *M. bovis* BCG (Jung, Madan-Lala et al. 2013). Differences in bovine and caprine iNOS expression are also reported (Adler, Adler et al. 1996).

The role of NO/iNOS in immune response is either suppressive or stimulatory (Bogdan 2001, Bogdan 2015). The suppressive effects of NO/iNOS pathway include inhibition of T and B cell proliferation and antibody production. T cells at different stages of development are differentially susceptible to NO. For instance, the killing
effect of NO on double positive T cells in the thymus is more exaggerated when compared to single positive T cells (Tai, Toyo-oka et al. 1997, Moulian, Truffault et al. 2001). Depletion of L-arginine from macrophages via iNOS impairs the intracellular inflammatory signaling cascade (Pekarova and Lojek 2015). NO produced by DCs inhibits effective DC differentiation (Si, Zhang et al. 2016). The recently described myeloid derived suppressor cells (MDSC) inhibit anti-tumor and anti-microbial immune response via iNOS expression and NO production (Gabrilovich and Nagaraj 2009).

As shown in Figure 1.4, the effect of NO on microbial pathogens is either direct or indirect (Bogdan 2001). The direct way includes its interaction with DNA, proteins, lipids, thiol groups and such interaction leads to functional and physical alterations. While pathogenic mycobacteria avoid fusion between phagosome and lysosome, NO can readily diffuse into the phagosome (Bogdan 2001). Exposure of the highly prominent human pathogen *M. tuberculosis* to NO leads towards expression of dormancy promoting genes without killing the bacterium (Voskuil, Bartek et al. 2011). For instance, *M. tuberculosis* has been shown to be resistant to *in vitro* NO treatment even at high concentration (5 mM) with only growth inhibition (Voskuil, Schnappinger et al. 2003). Of course, not all pathogens are susceptible to NO. Some bacterial pathogens have devised mechanisms to detoxify NO. For example, *Neisseria meningitidis* expresses nitric oxide reductase to convert NO to a non-toxic product. *Salmonella enterica* and *Escherichia coli* express flavohemoglobins to scavenge NO (Stevanin, Moir et al. 2005, Laver, Stevanin et al. 2009).

The indirect anti-microbial effect of NO includes host cell apoptosis of pathogen harboring cells. For example during mycobacterial infection, IFN-γ activated macrophages undergo apoptosis via NO dependent pathway (Herbst, Schaible et al. 2011). Impaired phagosome maturation is reversed by NO during *M. tuberculosis* infection (Axelrod, Oschkinat et al. 2008). NO enhances intracellular iron export via up-regulation of ferroportin-1, thereby limiting availability of intracellular iron (Nairz, Schleicher et al. 2013). NO inhibits production of pathogen virulence factors such as toxin and adhesins. For example *E. coli* shiga toxin synthesis is inhibited by NO (Vareille, de Sablet et al. 2007).
In the context of granuloma, activated macrophage iNOS expression increases the NO output into the granuloma (Mattila, Ojo et al. 2013). NO has been shown to inhibit collagen deposition in the granuloma during *M. avium* infection. Moreover, the presence of iNOS expression has been shown to correlate with reduced T cell cuff in *M. avium* granuloma (Lousada, Florido et al. 2006). However, the nature of NO producing cells and its consequence in granuloma maintenance remains less clear.

**Figure 1.4.** Direct and indirect mechanisms of NO anti-microbial effect (Bogdan 2015).

### 1.10 Immune escape strategies of mycobacteria

Virulent mycobacteria have several sophisticated host immune evasions strategies. These range from avoiding the recognition by immune cell PRRs to immune suppression. Current hypotheses were, however, largely deduced from *in vitro* analyses. Pathogenic mycobacteria evade recognition by host PRR via surface modification. For example, the presence of mannose capped lipoarabinomannan limits the immunogenicity in contrast to avirulent mycobacteria without capped
lipoarabinomannan (Briken, Porcelli et al. 2004). The presence of surface capsule limits the phagocytosis of mycobacteria (Stokes, Norris-Jones et al. 2004).

Once inside the cell, a well characterized immune evasion mechanism of virulent mycobacteria is blocking fusion of phagosome and lysosome (Mwandumba, Russell et al. 2004, Singh, Moulton et al. 2006, Russell 2011). Moreover, the release of bacterial tyrosine phosphatase (PtpA) inhibits vacuolar H\(^+\)-ATPase and ultimately escapes the degradation by lysosomal acid hydrolases (Wong, Bach et al. 2011). In addition, virulent mycobacteria can escape the endosome to the cytosol where it can freely replicate (McDonough, Kress et al. 1993). Recently \(M.\) tuberculosis serine/threonine kinase G (pknG) was identified as an effective inhibitor of phagosome and lysosome fusion. However, recent animal studies indicate that \(M.\) bovis BCG lacking pknG can survive in the lysosome \textit{in vivo} (Sundaramurthy, Korf et al. 2017). Those studies confirmed that virulent mycobacteria can resist the acidic environment of the phagolysosome (Levitte, Adams et al. 2016).

Mycobacteria can interact with and impair the function of antigen presenting cells. This includes impairing of DC maturation under \textit{in vitro} infection as shown by impaired MHC-II expression. Poorly coordinated antigen presentation is also suggested. This occurs due to induction of rapid maturation of DCs and subsequent quick surface MHC-II expression preceding antigen availability. Thus, further coupling of the antigen and MHC-II presentation is blocked (Hava, van der Wel et al. 2008).

Mycobacteria also target adaptive immune responses to further enhance its survival. Expansion of regulatory T cell (Tregs) was shown to enhance mycobacterial replication by limiting protective Th1 activity (Kursar, Koch et al. 2007). Moreover, mycobacterial infection induces less efficient CD4 T cell subsets with reduced effector functions. For instance, CD4 T cells expressing killer cell lectin like receptor G1 (KLRG1) highly expand during infection exhibit self-renewal efficiency (Reiley, Shafiani et al. 2010).

Mycobacterial species have devised several mechanisms to escape the effect of NO. For example, \(M.\) tuberculosis inhibits recruitment of iNOS towards bacteria
phagosome. This inhibits effective production and delivery of NO in the vicinity of the bacteria (Miller, Fratti et al. 2004). Such mechanism is dependent on the binding activity of Ezrin/radixin/moesin (ERM)-binding phosphoprotein 50 (EBP50) to iNOS which binds to cytoskeleton rather than phagosome (Mwandumba, Russell et al. 2004, Davis, Vergne et al. 2007).

To date, knowledge on mycobacterial immune evasion mechanisms under in vivo conditions is still rather scarce. For example, inhibition of fusion between phagosome and lysosome is an established immune evasion mechanism of virulent mycobacteria in vitro. However, mycobacteria can also survive in phagolysosomes in vivo (Levitte, Adams et al. 2016). Inhibition of MHC-II expression by antigen presenting cells is another strategy used by mycobacteria to inhibit T cell activation in vitro. Yet in vivo, DCs have impaired antigen presentation capacities despite expressing high levels of MHC-II (Wolf, Linas et al. 2007). In addition, in vivo matured DCs present M. tuberculosis antigen less efficiently than antigens from the attenuated M. bovis BCG. Thus, virulent mycobacteria impair antigen presentation in vivo via mechanisms that are still unclear (Grace and Ernst 2016).

Recently, a defined role of suppressor cells of myeloid origin has been described for bacterial including mycobacterial infection (du Plessis, Loebenberg et al. 2013, Yang, Wang et al. 2014, El Daker, Sacchi et al. 2015, Ost, Singh et al. 2016). Thus, for human tuberculosis accumulation of immature monocytic and granulocytic myeloid cells expressing Gr-1 and CD11b are supposed to be biomarkers for the lethal outcome of infection (Tsiganov, Verbina et al. 2014).

1.11 Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSC) were first noticed in the tumor micro-environment (Strober 1984). Due to the lack of markers of mature monocyte/macrophages, B cells, T cells or NK cells, they were termed as null cells. Years later their functional properties showed that they can suppress T cell response and hence were renamed as natural suppressor cells. Recently their origin of development was included into the nomenclature and named as myeloid derived suppressor cells (Gabrilovich, Velders et al. 2001, Talmadge and Gabrilovich 2013).
Today, MDSC represent a heterogeneous group of cell populations with diverse phenotypic markers. Identification markers in mice include Gr-1 and CD11b expression. Due to the expression of Ly6G or Ly6C, MDSC are classified as granulocytic (G-MDSC) or monocytic MDCS (M-MDSC) respectively. The peripheral frequency of MDSC under normal circumstances is ≤1% and increase in frequency upon chronic or acute inflammatory conditions (du Plessis, Loebenberg et al. 2013). MDSC play a role in limiting immune induced pathology during chronic inflammation. However, they have detrimental effect in suppressing immune response in infection and cancer.

The mechanism of MDSC development are only partly resolved. Some factors involved in either expansion or activation have been explored. These include peroxisome proliferator-activated receptor gamma (PPARγ), vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF), and IL-6 (Dilek, Vuillefroy de Silly et al. 2012). Further activation of intracellular signaling molecules leads to activation of transcription factor Stat-3 enhancing MDSC expansion. Activation of MDSC requires stimulators like IFN-γ, IL-1β, IL-4, or S100A8/9 which finally lead to activation of transcription factors Stat-1 and Sta-6 (Ost, Singh et al. 2016). Activation of G-MDSC and M-MDSC leads to divergent response. Both subsets exhibit comparable level of arginase I (Arg1) expression. M-MDSC produce more iNOS dependent NO while G-MDSC produce more reactive oxygen species (ROS) (Movahedi, Guilliams et al. 2008).

The role of MDSC in cancer is under extensive investigation. As MDSC expand in tumor microenvironment, they enhance tumor cells to escape from immune cells. Thus, they are being considered as potential targets of cancer treatment to boost host immune response against cancer cells. Recently, it was recognized that MDSC expand under inflammatory conditions of various causes. One main cause of expansion is infection of bacterial (Ost, Singh et al. 2016), viral (Seddiki, Brezar et al. 2014), parasitic or (Rieber, Singh et al. 2015) fungal origin (Van Ginderachter, Beschin et al. 2010). Myeloid cells with such suppressive properties are recruited during various infectious conditions. For example in tularemia infection, there is an
increased accumulation of Gr-1 myeloid cells is seen (Periasamy, Avram et al. 2016).

MDSC suppress immune response by expressing plethora of suppressor cytokines, enzymes, receptors and ligands. The two well-known enzymes associated with MDSC immune suppression are Arg1 and iNOS. Both enzymes compete for the same amino acid substrate L-arginine. As already stated above, iNOS converts L-arginine to nitric oxide (NO) and citrulline while Arg1 converts it to urea and ornithine (Rath, Muller et al. 2014).

MDSC induced Arg1 or iNOS suppresses immune responses via dual mechanisms: depletion of L-arginine and the toxic effect of metabolic product NO. Both NO accumulation and arginine depletion have negative consequence on T cell proliferation. Particularly NO inhibits Stat-5 phosphorylation in T cells. Moreover, arginine depletion down regulates T cell receptor zeta chain (CD3ζ) (Taheri, Ochoa et al. 2001). NO induces expansion of CD4+CD25+ T cells from CD4+CD25-T cells (Niedbala, Cai et al. 2006). Higher levels of memory T cells were found in the absence of iNOS expression (Vig, Srivastava et al. 2004). In general MDSC suppress T cell responses in antigen specific and nonspecific manner (Solito, Bronte et al. 2011). A recent report shows that not only T cells but also B cells are modulated by MDSC (Lelis, Jaufmann et al. 2017).

Immature MDSC expressing Gr1<sup>low</sup>CD11b<sup>+</sup> have been shown to be an indicator of severity of mycobacterial infection in mice (Tsiganov, Verbina et al. 2014). In humans, granulocytic MDSC expand during pulmonary TB infection (El Daker, Sacchi et al. 2015). Monocytic and granulocytic MDSC in human TB have been shown to impair CD4 and CD8 T cell cytokine secretion and trafficking (du Plessis, Loebenberg et al. 2013). Overall, our knowledge about the role of MDSC in mycobacterial infection is at its infancy stage. More importantly no report has been shown the role of MDSC in NTM infection.
1.12 Animal models for mycobacterial infection

The lack of appropriate animal models hampered the progress in the field of tuberculosis research towards development of effective vaccine and drug treatment. Mice are becoming a powerful tool to investigate disease mechanisms and intervention approaches of communicable and non-communicable diseases. Among several mouse strains being used in field of research, C57B6/J is ideal due to the availability of several inbred targeted genes knockouts and transgenic strains and reagents on the market. However, the C57B6/J murine model of TB infection is limited due to the lack of lesion characteristics of TB. Mice are not natural hosts of *M. tuberculosis*. Due to this, they can harbor large amounts of *M. tuberculosis* without presenting symptoms of the disease. Compared to rabbits, guinea pigs and humans, mice are quite resistant. In addition, neither caseating nor necrotic granuloma is typically found in the murine TB models. To mimic the latent infection model which is typical for tuberculosis, the Cornell approach has been extensively used. In this model, after infection with virulent mycobacteria, mice are treated with anti-mycobacterial drugs for several months until the pathogens become uncultivable. Spontaneous or induced reactivation occurs which is considered typical for reactivating TB (Scanga, Mohan et al. 1999).

Recently, the zebra fish model has shed new light on the pathomechanisms of *M. marinum* infection, the closet of *M. tuberculosis* (Cronan and Tobin 2014). The transparency, fast replication rate and ease of use are some of the advantages of the fish model (Meijer and Spaink 2011). More hope has been put on this model as it is also relatively easy to manipulate genes of interest but of course the lung is absent. Rabbit, macaques and guinea pig models are also other tools as the infection induces caseating lesion and latent infection which is typical for human tuberculosis (Converse, Dannenberg et al. 1996, Capuano, Croix et al. 2003, Li, Chen et al. 2010). However, studies in these animals are limited due to ethical concerns and lack of gene manipulated strains.

*M. avium* infection as mouse tuberculosis model has unique advantages. *M. avium* infects several host species including humans, birds, pigs, dogs, monkeys (Gangadharam 1995, Bermudez, Danelishvili et al. 2006). *M. avium* causes
progressive infection in WT C57B6/J mice. It induces and grows in necrotic and hypoxic lesion typical of human tuberculosis which is not induced in \textit{M. tuberculosis} infected mice (Aly, Wagner et al. 2006). \textit{M. avium} infection inoculum as low as 200 CFU can cause progressive infection in mice (Florido, Cooper et al. 2002). Overall, \textit{M. avium} infections of WT C57B6/J mice have been suggested as alternative surrogate model for mycobacterial infection (Kondratieva, Evstifeev et al. 2007, Apt and Kramnik 2009). Besides, \textit{M. avium} has been used as a model to study the effect of chronic infection on hematopoietic stem cell depletion (Baldridge, King et al. 2010, Matatall, Jeong et al. 2016). \textit{M. avium} reference strains 104, TMC724 and ATCC25291 have been widely studied in mice.

\subsection*{1.13 Aim of the study}

A balanced interaction between myeloid and T cells is important to elicit pathogen tailored immune response which enhances the clearance of the invader while limiting excessive inflammation. Factors limiting such well-coordinated interaction during mycobacterial infection under \textit{in vivo} condition are still not completely understood. Hence, this study focused on understanding the nature of \textit{in vivo} targeted myeloid cells using \textit{M. avium} mouse infection model. Moreover, the specific factors enabling mycobacteria to persist and/or replicate in myeloid cells have been addressed. In addition, factors hampering the interaction of myeloid and T cells \textit{in vivo} have been characterized. Over all, the aim of this study was to understand the phenotype, function and interaction of granuloma composing innate and adaptive immune cells.
2 Materials and Methods

2.1 Mice

Wild type (WT) female C57BL6/J mice of 6-7 weeks old were purchased from Janvier (La Genest-saint-Isle, France) and maintained under specific pathogen (SPF) free conditions at the animal facility of the Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany. CD90.1 (Thy1.1) OVA albumin transgenic II (OT-II) C57BL6/J mice were kindly provided by Dr. Markus Gereke, HZI. Female iNOS<sup>−/−</sup> C57B6/L/J mice were kindly provided by Dr. Ulrike Schleicher, Mikrobiologisches Institut – Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen. Mice were infected at the age of 8-12 weeks. In all experiments, mice were placed on ad-libitum feeding and unlimited access to water. All animal experiments were conducted in accordance with the german law for animal protection (Tierschutzgesetz, §16 Abs.1 Satz–5). Approval of the study was granted from research ethics committee of local authority LAVES in Lower Saxony (permission Nr. 33.12-42502-14). All efforts were made to minimize the number of animals used and their suffering. All mice were humanely euthanized by CO₂ asphyxiation.

2.2 Growth of mycobacterial strains and infection

Mycobacterial strains (M. avium DSM 44156 and M. avium 104 (a kind gift from Dr. Otto Holst, Borstel, Germany)) were grown in Middlebrook (MB) 7H9 broth (Difco™ BD, Heidelberg, Germany) supplemented with 0.5% glycerol, 10% OAGC (0.06% oleic acid, 5% albumin, 2% glucose monohydrate, 0.003% catalase enrichment (Carl Roth)). To attain logarithmic phase of bacterial growth, initial inoculum OD₆₀₀ was adjusted to 0.2 and grown at 37°C under steering condition until it reached a final OD₆₀₀ of ~1. The bacterial culture was washed 3 times with Dulbecco’s phosphate buffered saline (PBS) at 3000 rpm for 10 minutes at 4°C. To avoid bacterial clumping, the suspension was briefly vortexed in the presence of 3mm glass beads. Bacterial suspension was adjusted to OD₆₀₀ of 5 in PBS. Mice were given intraperitoneal (i.p) injection of 200µl (~10<sup>8</sup> colony forming unit (CFU)) of
the bacterial suspension. Body weight was monitored on a weekly basis. Infected mice were maintained for a maximum of 5 weeks.

### 2.3 Organ collection and plating

Liver and spleen were collected aseptically and weighed. Liver (≈ 300 mg) was homogenized in final volume of 1 ml in sterile PBS containing 0.1% Triton-X100 with sterile 3 mm glass beads by beating 3 times for 20 seconds with 5 minutes cooling interval using homogenizer FastPrep-24 (MP Biomedicals). Homogenous samples were serially diluted until $10^{-9}$ and 20 μl of $10^{-5}$ to $10^{-9}$ dilutions were plated on OAGC supplemented MB agar (Difco™ BD). Colonies of mycobacteria were counted after 2-6 weeks. The bacterial load was calculated as CFU/organ.

### 2.4 Flow cytometry and single cell sorting

Spleen cells suspension were prepared by gently flushing in cool complete medium (Iscove’s Modified Dulbecco’s Medium (IMDM (Gibco, Invitrogen)), 10% heat inactivated fetal calf serum (FCS), penicillin (100U/ml), streptomycin (100μg/ml), 2mM L-glutamine, 50μM 2-mercaptoethanol). Then cells were filtered with 70μm and later with 50μm diameter cell strainer (cell Trics®). Red blood cells were removed by erythrocyte lysis buffer (14.2mM sodium hydrogen carbonate (NaHCO₃), 155mM ammonium chloride (NH₄Cl), 0.1mM EDTA, at final pH of 7.3) at room temperature (RT) for 3-5 minutes. Cells were centrifuged and re-suspended in 1ml FACS buffer (2% sterile FCS and 2mM EDTA in PBS). Viable cells were counted by mixing cell suspension with trypan blue (1:1) and counted with Neubauer counting chamber. About $10^6$ cells were blocked with 100μl FACS buffer containing 1μg/ml FcR block (rat anti-mouse CD16/CD32, BD Pharmigen)) for 5-10 minutes on 4°C. Then, cells were washed once with FACS buffer and antibodies diluted in FACS buffer were added to the cells and incubated for 15 minutes at 4°C. Cells were washed twice with FACS buffer and the pellets were re-suspended in 100μl FACS buffer. Finally, 50μl of DAPI (10μg/ml) was added to discriminate live and dead cells immediately before acquiring samples. For single cell sorting, spleen cells from 3-5 mice were pooled together and stained with antibodies in 3ml FACS buffer. Cells were washed twice in 10 ml FACS buffer and finally resuspended in 3 ml FACS buffer for sorting. All
antibodies used, with their respective clones are provided in **Table 2.1**. Data were acquired on LSR II analyzer (BD Biosciences, NJ, USA). Data analysis was done using FACSDiva software (BD Biosciences) or FlowJo (TreeStar). Cell sorting was done on FACSAria-II (BD Biosciences). Re-analysis of sorted cells was done for purity check.

**Table 2.1. Antibodies used for flow cytometry**

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**2.5 Intracellular staining**

Spleen cells \((10^7)\) were incubated in 200µl complete IMDM containing 5µg/ml brefeldin A (BioLegend) for 2 hours at 37°C. Cells were stained for surface markers using standard staining protocol. Then cells were fixed in BD™ fix/perm buffer (BD
Biosciences) and stained for intracellular antigens in BD™ perm/wash buffer (BD) and analyzed using flow cytometry.

2.6 Ex vivo antigen dependent T cell proliferation

Spleen conventional dendritic cells, CD11chighCD11b+/− (cDC) were sorted and pulsed with EndoGrade® ovalbumin protein (Hyglos, bioMeréieux, Germany) (100µg/ml) and ovalbumin peptide 323-339 (1µg/ml) for 1 hour at 37°C in complete IMDM medium. CD4 T cells were isolated from OT-II mice spleens using Dynabeads® Untouched™ Mouse CD4 Cells Kit (Invitrogen, life technologies, Norway). Isolated cells were stained with 5µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) and incubated for 10 minutes at 37°C in dark condition and washed 3 times in complete medium. Finally, viable 3x10⁴ cDC were co-cultured with viable 3x10⁵ CD4 T cells in complete medium in round bottom 96 well plates (Greiner CELLSTAR®). Proliferation was measured after 3 days for peptide or 4 days for protein.

2.7 iNOS dependent ex vivo T cell inhibition assay

One day before the assay, round bottom 96 well plates were coated with anti-CD3 antibody (clone 145-2C11, eBioscience) at a final concentration of 5µg/ml in PBS and kept overnight at 4°C. The next day, spleen CD11bhighCD11cint cells were sorted and co-cultured with untouched, CFSE labeled CD4 T cells in the presence of immobilized anti-CD3. Different ratios of T cells and CD11bhighCD11cint cells were co-cultured in the presence or absence of iNOS inhibitor, L-N6-(1-Iminoethyl) lysine dihydrochloride (L-NIL) (Cayman chemicals, Biomol, Germany) at a final concentration of 40µM. T cells proliferation was measured after 4 days in culture.

2.8 In vivo T cell proliferation

Naïve CFSE labeled Thy1.1 OT-II CD4 T cells (2x10⁶) were injected intravenously via tail vein. After 24 hours, 200µg of ovalbumin protein was injected intraperitoneally. Three days after immunization, mice were sacrificed and spleen cells were prepared for FACS. In vivo proliferation of Thy1.1 expressing CD4 T cells was measured by CFSE dilution using flow cytometry.
2.9 Analysis of OVA protein antigen uptake and processing

A standard protocol for antigen uptake using OVA-cy5 (endocytosis) and processing by DQ-OVA (life technologies) degradation was followed (Ziętara, Łyszkiewicz et al. 2013). Briefly, cDC were sorted from spleen and incubated with 100µg/ml OVA-cy5 or 62.5µg/ml DQ-OVA for 90 minutes at 37°C and analyzed using flow cytometry. To control nonspecific adherence of the antigens, cells were incubated with the respective antigens at 4°C. Finally, net mean fluorescence intensity (MFI) was calculated after subtracting nonspecific fluorescence.

2.10 Localization of endocytosed DQ OVA using confocal microscopy

To determine the rate and compartment in which degradation of DQ-OVA takes place in cDC, spleen cDC were sorted with anti-CD11c-PE-Cy7 and anti-CD11b-PE antibodies. Sorted cells were seeded on Poly-L-lysine (Life science, Sigma Aldrich) coated coverslip at a density of 2x10^5 cells per well overnight. After changing to new complete medium, cells were pulsed with 62.5µg/ml DQ-OVA and incubated for 45 minutes at 37°C. After 3x washing, cells were further incubated for 2 hours at 37°C. Then cells were washed and fixed in 20% eBioscience™ fixation buffer. Fixed cells were stained with early endosomal antigen 1(EEA-1) and lysosome associated membrane protein 1 (LAMP-1) antibodies (BD Biosciences). Finally, images were analyzed by confocal fluorescent microscopy.

2.11 Splenocytes nitrite assay

Spleen single cell suspension was prepared following the standard protocol described above. A total of 10^7 cells per well were seeded in 2ml complete IMDM in 6 well plates and incubated for 48 hours. Cell supernatant was collected and analyzed for nitrite production using Griess reaaction (Promega).

2.12 Quantitative real time PCR (qRT-PCR)

After spleen cells sorting, cells were kept in 500µl (1X concentrate) DNA/RNA Shield™ (ZYMO RESEARCH, Epigenetics, USA). Further RNA extraction was processed using Direct-zol™ RNA miniprep kit (ZYMO RESEARCH, Epigenetics,
Reverse transcription of RNA was performed using M-MLV-reverse transcriptase (Promega, Mannheim, Germany). DNase treated RNA (500ng-2μg) was mixed with 1 or 2μl (depending on 20 or 40μl reaction volume) of 100 pmole/μl oligo(T)12-18 primers (Carl Roth, Karlsruhe, Germany) and incubated for 10 minutes at 70°C. The reaction mix was then chilled on ice for 2 minutes and reverse transcriptase buffer, deoxynucleotide triphosphates (dNTP) mix (0.5 mM), and M-MLV reverse transcriptase enzyme (100 U) was added. The final volume of each sample was adjusted with RNase-free water to 40μl. The reaction mixture was incubated for 60 minutes at 42°C. After inactivation of the enzyme by incubation at 85°C for 5 minutes, the reaction was adjusted to the final concentration of 10 ng/μl in RNase-free water. A 2.5 μl aliquot of cDNA was used as a template for qRT-PCR reaction containing specific forward and reverse primers of target genes, using the fluorescent DNA binding dye SYBR Green (Qiagen, Hilden, Germany) or TaqMan® Gene Expression Assays (Applied Biosystems, ThermoFisher). SYBR Green PCR was performed in a real-time thermal cycler (Stratagene, Mx3005P) with the following program: 95°C initial denaturation for 20 minutes, followed by 40-45 cycles of 95°C denaturation for 20 seconds, 58°C primer annealing for 30 seconds, and an extension at 72°C for 20 seconds. After the 45 cycles, dissociation curves of the PCR products were obtained by one cycle of 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 seconds. For TaqMan® Gene Expression Assays, with comparative quantitation (calibrator) program, a cycle of 95°C for 10 minutes followed by 95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute for 45 cycles were used. The results were analyzed using a real-time PCR automated method (Mx-Pro Software, Stratagene) and agarose gel electrophoresis. First, difference in cycle threshold (ΔCT) was calculated as: ΔCT = CT value of gene of interest – CT value of Rps9. Relative amount of RNA compared to Rps9 was calculated as $2^{-\Delta CT}$. 
Materials and Methods

Table 2.2 List of primers used for SYBR Quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5’-3’)</th>
<th>Reverse primers (5’-3’)</th>
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<tbody>
<tr>
<td>Tnf</td>
<td>ATGAGCACAGAAAGCATGATC</td>
<td>TACAGGCTTGTCACTCGAATT</td>
</tr>
<tr>
<td>Il1b</td>
<td>TTGACGGACCACAAAAGATG</td>
<td>AGAAGGTGCTCATGTCCCTCA</td>
</tr>
<tr>
<td>MMP3</td>
<td>ATGGAGCTGCAAGGGGTGAG</td>
<td>CCCGTACCTCCAATCCAAG</td>
</tr>
<tr>
<td>MMP13</td>
<td>GGTCCCAAACGAACCTAACTTACA</td>
<td>CCTTGAAACGTATCTACAGGAAGC</td>
</tr>
<tr>
<td>Nos2</td>
<td>CCCAGCACAAGGGCTCAA</td>
<td>GCACCTGGAACAGCAGCTCTC</td>
</tr>
<tr>
<td>Rps9</td>
<td>CTGGACCGAGGCAAGATGAAGC</td>
<td>TGACGTGGGCGGATGAGCACA</td>
</tr>
<tr>
<td>Arg1</td>
<td>GATGTCCCTAATGACAGCTCC</td>
<td>AGCACCAACACTGACTCTTCC</td>
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Table 2.3. TaqMan® Gene Expression Assays ID

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<th>Gene Assay ID</th>
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<tr>
<td>Ifg</td>
<td>Mm01168134_m1</td>
</tr>
<tr>
<td>Il10</td>
<td>Mm00439616_m1</td>
</tr>
<tr>
<td>IL6</td>
<td>Mm00446190_m1</td>
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2.13 PCR for comparative intracellular mycobacteria quantitation

Whole cell (eukaryotic and bacterial) DNA was extracted from sorted, 3% paraformaldehyde fixed spleen cells. Briefly, zirconium beads (0.1mm diameter) were added and cells were disrupted using a tissue homogenizer. The homogenate was sonicated using Branson sonifier 450. Supernatant (120µl) was collected after centrifugation. After adding an equal volume of TE buffer, RNA was removed by adding 10µl RNase A (Roche) followed by 1 hour incubation at 37°C. To reverse the cross link, 15µl of 4M NaCl was added and incubated for 5 hours at 65°C. Finally, DNA was extracted using standard phenol chloroform extraction method. Bacterial DNA PCR was performed using the IS901 specific primers and normalized against eukaryotic CXCL2 (MIP-2) promoter. For PCR, 95°C initial denaturation for 20 minutes, followed by 45 cycles of 95°C denaturation for 20 seconds, 58°C primer annealing for 30 seconds, and an extension at 72°C for 20 seconds. After the 45 cycles, dissociation curves of the PCR products were obtained by one cycle of 95°C for 1 minute, 55°C for 30 seconds and 95°C for other 30 seconds. The results were analyzed using a real-time PCR automated method (Mx-Pro Software, Stratagene).
and agarose gel electrophoresis. Relative amount of bacterial DNA was calculated by normalizing against the eukaryotic gene.

Table 2.4. List of primers used for SYBR Quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5'-3')</th>
<th>Reverse primers(5'-3')</th>
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</thead>
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<tr>
<td>IS901</td>
<td>GTGATCAAGCACCTTCGGAA</td>
<td>GCTGCGAGTTGCTTGATGAG</td>
</tr>
<tr>
<td>MIP2α</td>
<td>GAAGGGCAGGGCAGTAGAAT</td>
<td>ATGGCGCTAGGCTGAAGTGT</td>
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</tbody>
</table>

2.14 Enzyme linked immunosorbent assay (ELISA)

Blood was collected via cardiac puncture in 500 Serum-Gel tubes (Sarstedt). Serum was separated by centrifugation at 10,000 rpm for 4 minutes at room temperature. Serum was analyzed for IFN-γ using coating (clone A.N 18) and detection (clone R46A2) antibodies. In brief, rat anti-mouse IFN-γ was incubated in coating buffer (50µl) in 96 well plates (MaxiSorb TM Immunoplates, Nunc) over night. The 96 well plates were then blocked for 1 hour with 3% BSA in 0.05% Tween 20. Diluted sera were added to the wells and incubated for 2 hours at room temperature. Biotinylated anti-mouse IFN-γ was added and incubated for 1 hour. Then horseradish peroxidase (HRP) conjugated streptavidin (BD) was added and incubated for 30 minutes and the bound HRP was detected with o-phenylenediamine (OPD) substrate in terms of absorbance at 490 nm using ELISA reader XFluor software (Tecan SUNRISE).

2.15 Histopathology

Preparation of histology sections was performed in the Mouse Pathology Platform at HZI in Braunschweig. Spleen and liver organs were processed for microscopic examination by fixing in 4% (v/v) para-formaldehyde and kept between 24-48 hours at 4°C, then dehydrated with 70% ethanol, and embedded in paraffin. Approximately 3-5µm thick sections were cut and stained with hematoxylin/eosin according to standard laboratory procedures. Immuno-histo-chemical staining was performed using the following antibody: rabbit anti-mouse -Ki-67 (Neo Makers, RM-9106-S), and developed using DAB (3,3'-Diaminobenzidine Zytomed Systems DAB530) as chromogen. Hematoxylin was used for counterstaining. Caspase-3 staining of
spleen sections was done at Institute for Pathology, University of Veterinary Medicine Hannover, following standard protocol.

2.16 Immunofluorescence staining of paraffin embedded tissue sections

For immunofluorescence staining, tissue slides were incubated overnight at 70°C to remove the mounted wax. Sections were then rehydrated in 3 x in 100 % Xylene for 10 minutes and in gradient concentration of ethanol (2 x in 100 % Ethanol for 5 minutes, 2 x in 95 % Ethanol for 5 minutes, 2 x in 70 % Ethanol for 5 minutes and finally 2 x in ddH₂O for 5 minutes). To enhance antigenic epitope retrieval, sections were cooked for 10 minutes in 10 mM sodium citrate, pH 6 in a pressure cooker. Slides were kept for 30 minutes in the 10 mM sodium citrate pH 6 for cooling and washed 1 x in 1 x PBS. Sections were blocked in blocking buffer (1 x PBS + 5% horse serum + 0.3 % Triton X-100) for 1 hour at room temperature. Then the sections were stained with anti-Arg1 antibody (polyclonal goat anti-human, SC-18351, Santa Cruz Biotechnology, Dallas, USA), at final dilution of 1:50 (diluted in 1 x PBS + 1% BSA + 0.3 % Triton X-100) overnight at 4°C in dark humid temperature. The sections were washed 3 times with 1 x PBS for 5 minutes and stained with Alexa fluor 488 conjugated anti-goat IgG (Thermo A21467) at final dilution of 1:500 for 1 hour at room temperature. To localize mycobacteria in the tissue, 1:250 dilutions of anti-HBHA rabbit serum (self-made) was added for 1 hour at room temperature and stained with 1:500 dilution of Alexa fluor 568 conjugated goat anti-rabbit antibodies (Thermo A11011) in dilution buffer for 1 hour. Antibodies used for iNOS and MAC-2 were anti-mouse iNOS (eBioscience, 12-5920), MAC-2 (Biozol diagnostics). Alexa Fluor anti-mouse 488 and Alexa Fluor anti-rabbit 594 were used. Finally, slides were washed 3 times with 1 x PBS for 5 minutes and stained with 50µl DAPI for 1 hour. Slides were preserved by adding 3µl ProLong Gold and kept overnight at room temperature for curing. Sections were analyzed by confocal fluorescence microscopy blinded to the experimental groups.

2.17 Ziehl-Neelsen (ZN) staining

Sorted cells suspensions were fixed in 3% paraformaldehyde on ice for 10 minutes. Fixed cells were suspended in FACS buffer. Of this, 50µl of cell suspension was
added to CytoSep funnels attached with frosted glass slides and centrifuged at 800 rpm for 5 minutes by using Cytospin centrifuge. Slides were briefly heat fixed and stained with hot carbolfuscsin for 5 minutes, washed and decolorized with 3% acid alcohol solution for 1 minute. After second washing step, slides were counterstained with methylene blue solution for 1 minute. Finally, the slides were rinsed with water and air dried before examination.

2.18 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.03 (GraphPad, San Diego, CA, USA). Mean±standard error of the mean (M±SEM) was used for data description. Statistical test between two groups was determined using Student’s t-test. Difference between more than two groups was determined either with one way or two-way analysis of variance (ANOVA) using Dunnett's multiple comparisons against the control group. Cut off P-value of <0.05 was considered as statistically significant difference.
3 Results

3.1 *M. avium* subspecies induce infection with different degrees of severity in mice

Infection of mice with the highly virulent *M. avium* ssp. *avium* strain ATCC 25291 (DSM 44156) also named TMC724 (following designated as MAA) causes progressive chronic infection which resembles systemic tuberculosis in humans (Appelberg, Castro et al. 1994, Ehlers, Benini et al. 2001, Kondratieva, Evstifeev et al. 2007). To gain more insight into the role of innate immune cells during such progressive *M. avium* infection, we infected mice with MAA and strain 104 of the genetically closely related but less virulent subspecies, *M. avium* ssp. *hominissuis* (following designated as MAH). Approximately $10^8$ CFU was injected intraperitoneally. As controls, mice received PBS only. Body weight as general indicator of health was measured at regular time intervals. All infected mice were followed for maximum of 5 weeks and results showed represent 5 weeks unless stated. Mice infected with either of the strains lost body weight until 14 days post infection. After 14 days, mice infected with MAH started to gain body weight while MAA infected mice persisted with low body weights and did not show any sign of body weight recovery (Figure 3.1A). There was a significant increase in spleen weight in both infected groups compared to PBS control, however, spleen weights were higher in MAA infected mice compared to MAH (Figure 3.1B).

![Figure 3.1](image-url)

**Figure 3.1. Severity of infection upon *M. avium* subspecies infection.** A. shows the differences in body weight gain between MAH and MAA compared to PBS (***, p value <0.001, * p value <0.05** (two-way ANOVA)). B. shows differences in spleen weight compared to PBS control (***, p value <0.001, ** p value <0.005**, one-way ANOVA- Dunnett's multiple comparison test)). At least two independent experiments were included.
3.2 Differential growth of MAA and MAH in the liver

To compare the difference, in *in vivo* growth of both strains, bacterial plating was performed from the liver. As shown in Figure 3.2, large numbers of bacteria were recovered, reaching above 10 and 12 logs of CFU per liver. However, the number of bacteria in MAA infected mice was highly increased showing log of 2.6 (~400x) higher compared to MAH infected mice (Figure 3.2A). This indicates the difference in severity of infection shown in body weight could be attributed to differences in bacterial load.

![Graph showing bacterial growth in liver](image)

*Figure 3.2. Differential growth of MAA and MAH in the liver 5 weeks post infection.* The figure indicates both strains grow in mice. However, MAA growth is more extensive than MAH.

3.3 MAA and MAH induces different types of granulomatous inflammation in spleen and liver

Histopathology of paraffin embedded and stained spleen and liver sections of MAA or MAH infected mice were monitored for pathological changes. Interestingly granuloma formation involving epithelioid like macrophages was observed in the spleens of MAH infected mice. In contrast, diffuse histiocytic granulomatous inflammation with destruction of lymphoid follicles was observed in the spleen of MAA infected mice (Figure 3.3 upper panel). In addition, classical granuloma harboring mononuclear cells and peripheral lymphocytes was observed in MAH infected mice while granuloma with higher number of mononuclear cells and low number of lymphocytes were found in the liver of MAA infected mice (Figure 3.3 lower panel).
Results

Figure 3.3. Differences in type of granulomatous inflammation formed in MAA and MAH infected mice at five weeks post infection. H and E staining of granulomatous inflammation in spleen (upper panel) and liver (lower panel). Black arrows indicate the presence of lymphocytes at the rim of the granuloma.

3.4 MAA but not MAH infection induces CD4 T cells with regulatory phenotype in the spleen

Regulatory CD4 T cells have the ability to suppress conventional T helper 1 (Th1) response (Corthay 2009). It has been reported in different studies that regulatory T cells are predominantly found in the CD4⁺CD25⁺CD45RBlow T cell subpopulations (Sakaguchi, Sakaguchi et al. 1995, Maloy and Powrie 2001). It has been shown during mycobacterial infection that inactivation of CD4⁺CD25⁺ T cells is associated with increased cytokine production (Quinn, McHugh et al. 2006). Since the difference in accumulation of lymphocytes in the granulomatous inflammation of MAA and MAH infected mice in spleen and liver tissues was observed, CD4 T cells phenotype in the spleen were characterized by flow cytometry (Gating strategy Figure 3.4A). It was found that the frequency of CD4 T cells expressing CD25 (Figure 3.4B) and low levels of CD45RB (Figure 3.4C) were significantly increased. Interestingly frequency of CD4⁺CD25⁺ and CD4⁺CD25⁺CD45RBlow T cells in MAH infected mice was not significantly different from control mice while MAA infected mice showed a significant
increase in these cells. Such differential induction of regulatory T cells between MAA and MAH infected mice might be attributed to their differential virulence.

![Image of flow cytometry data](image.png)

**Figure 3.4.** MAA infection induces CD4 T cells with regulatory phenotype in spleen. A. shows gating strategy of CD4 T cells gated from live and spleen cells. B. shows frequency of CD4^+^CD25^+^ T cells. C. shows CD4^+^CD25^+^CD45RB^{low} T cells frequency (**< 0.0001 (t test), ns-non-significant).**

### 3.5 Chronic MAA infection induces accumulation of CD11b^{hi}CD11c^{int}Gr-1^{int} cells in the spleen

To delineate mechanisms of differential host reactions, we analyzed innate immune responses at the cellular level by flow cytometry. In spleen, MAA and MAH infections induced accumulation of Gr-1^{hi} inflammatory monocytes as well as Gr-1^{int} cells. Especially in spleens of MAA infected mice, Gr-1^{int}CD11b^{hi} were drastically increased compared to Gr-1^{hi}CD11b^{hi} cells (Figure 3.5A upper panel). Among these, an accumulation of CD11c positive myeloid cells, CD11b^{hi}CD11c^{int}Gr-1^{int} cells was
detected in spleens of mice infected by MAA but not in mice infected with MAH or treated with heat killed (HK) MAA (Figure 3.5A lower panel and Figure 3.5B).

Figure 3.5. Chronic MAA infection induces accumulation of CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup> cells in spleen. A. The upper panel shows frequency of Gr-1<sup>hi/low</sup> and CD11b<sup>high</sup> spleen myeloid cells gated from live cells. The lower panel shows frequency of CD11b<sup>high</sup>CD11c<sup>int</sup> cells gated from live cells. B. shows percentage of CD11b<sup>high</sup>CD11c<sup>int</sup> gated from live cells (**P<0.001 (one-way ANOVA-Dunnett’s multiple comparison test)). HK- heat killed bacteria.
3.6 CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> cells are monocytic myeloid derived suppressor (M-MDSC) like cells and heavily infected with MAA

To determine whether the accumulated splenic myeloid cells of MAA infected mice contribute to pathology, we sorted CD11b<sup>hi</sup>CD11c<sup>neg</sup> (P3) and CD11b<sup>hi</sup>CD11c<sup>int</sup> (P4) according to Gr-1 and Ly6C expression. By this, CD11b<sup>hi</sup>CD11c<sup>neg</sup> cells could be separated into CD11b<sup>hi</sup>CD11c<sup>neg</sup>Gr-1<sup>hi</sup>Ly6C<sup>int</sup> (P6) and CD11b<sup>hi</sup>CD11c<sup>neg</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> (P7) cells. Nearly all CD11b<sup>hi</sup>CD11c<sup>int</sup> (P4) were characterized as monocytic CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> (P5) (Figure 3.6A). Cytospins of such cells were ZN stained to analyze mycobacterial load. As shown in Figure 3.6B, MAA was almost exclusively found in the monocytic CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> cell population (P5). To corroborate differential mycobacterial loads, DNA was extracted from the sorted cells and bacterial content was evaluated by qRT-PCR using primers for the mycobacteria-specific insertion sequence 901 (IS901). Signals were normalized to the number of host cells using primers for the promoter of macrophage inflammatory protein 2 alpha (Mip2a). As expected, qRT-PCR confirmed the exclusive presence of MAA DNA in the monocytic CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> cell population (Figure 3.6C).
Figure 3.6. CD11b$^{\text{hi}}$CD11c$^{\text{int}}$Gr-1$^{\text{int}}$Ly6C$^{\text{hi}}$ cells are monocytic myeloid derived suppressor like cells and heavily infected with MAA. A. CD11b$^{\text{high}}$CD11c$^{\text{int}}$ are Ly6C$^+$Gr-1$^{\text{low}}$ while CD11b$^{\text{high}}$CD11c$^{\text{neg}}$ cells encompass Ly6C$^+$Gr-1$^{\text{low}}$ and Ly6$^-$Gr-1$^{\text{high}}$ subpopulations. B. Bacterial burden shown by ZN staining in CD11b$^{\text{high}}$CD11c$^{\text{int}}$ (P5), Ly6$^-$Gr-1$^{\text{high}}$ (P6) and Ly6$^+$Gr-1$^{\text{low}}$ (P7) sub-populations. C. Comparison of amount bacterial DNA (IS901) against MIP-2α promoter DNA.
### 3.7 Expression analysis of selected genes in CD11b$^{hi}$CD11c$^{int}$ and CD11b$^{hi}$CD11c$^{neg}$ cells

To characterize the MAA infected myeloid cells in more detail, RNA was extracted from sorted splenic CD11b$^{hi}$CD11c$^{int}$ and CD11b$^{hi}$CD11c$^{neg}$ cells (Figure 3.6A P4 and P3, respectively) and mRNA expression of selected genes was determined by qRT-PCR. Infected CD11b$^{hi}$CD11c$^{int}$ cells expressed little Il-1 (Figure 3.7) and no Il-12 (data not shown). mRNA expressions of other pro-inflammatory cytokines like Ifng, Tnf as well as Il-6 were considerably higher than in CD11b$^{hi}$CD11c$^{neg}$ cells from infected and control animals. On the other hand, mRNA expression of the anti-inflammatory cytokine Il10, as well as Arg1 and Nos2 (gene of iNOS) were markedly enhanced in CD11b$^{hi}$CD11c$^{int}$ cells as compared to uninfected control mice or in CD11b$^{hi}$CD11c$^{neg}$ cells from MAA infected mice (Figure 3.7). In addition matrix metalloproteinase 3 and 13 were upregulated in CD11b$^{hi}$CD11c$^{in}$ cells compared to CD11b$^{hi}$CD11c$^{neg}$ cells (data not shown).

![Figure 3.7](image)

**Figure 3.7. Characterization of M-MDSC like cells.** Spleen cells were sorted based on the expression level of CD11b and CD11c markers. Spleen CD11b$^{hi}$CD11c$^{neg}$ and CD11b$^{hi}$CD11c$^{int}$ cells were sorted from mice at 5 weeks post infection. RNA was extracted and qRT-PCR was done for selected genes shown. $2^{-\Delta\DeltaCT}$ value was calculated and displayed by using the respective cycle threshold (CT) value of Rps9.
3.8 Immune histologic confirmation of mycobacteria permissive cells in the spleen

The phenotype of CD11b$^{hi}$CD11c$^{int}$ cells could be confirmed by immuno-histology as such cells are mostly harboring the bacteria. MAA was present in macrophage surface glycoproteins binding to galectin-3 (MAC-2) expressing cells in spleen (Figure 3.8A) which apparently express iNOS (Figure 3.8B) and very low level of Arginase I (Arg1) expression (Figure 3.8C).

Figure 3.8. Confirmation of phenotype of infected cells by immuno-histology in the spleen paraffin section. A. shows expression of Mac-2 on mycobacteria infected cells. B. shows co-localization of iNOS and mycobacteria in mycobacteria infected cells. C. shows Arg1 expression in mycobacteria infected cells. DAPI staining shows cell nuclei.
3.9 CD11b<sup>hi</sup>CD11c<sup>int</sup> cells are the main source of nitric oxide in the spleen

Intracellular iNOS and TNF-α expression was confirmed for splenic CD11b<sup>hi</sup>CD11c<sup>int</sup> cells by flow cytometry. As shown in Figure 3.9A, CD11b<sup>hi</sup>CD11c<sup>int</sup> cells (P4) express high levels of iNOS and low levels of TNF-α as compared to the other spleen subpopulations. To confirm the activity of the highly expressed iNOS in the spleen, NO was measured in terms of nitrite in splenocyte conditioned medium. Splenocytes from MAA infected mice produced large amounts of NO while it was not detected in splenocytes of PBS injected mice as detected by Griess reaction (Figure 3.9B). Moreover CD11b<sup>hi</sup>CD11c<sup>int</sup> cells do not express Ly6G (data not shown). Overall the ambiguous, premature phenotype of the MAA infected monocytic CD11b<sup>hi</sup>CD11c<sup>int</sup>Gr-1<sup>int</sup>Ly6C<sup>hi</sup> cell population as well as the concomitant expression of low level TNF-α and high level of iNOS suggested that these cells might represent a monocytic myeloid derived suppressor cell (M-MDSC) population (Schmid, Wege et al. 2012).

Figure 3.9. CD11b<sup>hi</sup>CD11c<sup>int</sup> cells are the main source of nitric oxide in the spleen.
Flow cytometry analysis of iNOS and TNF-α expression among different spleen myeloid cell subpopulations (A). NO production measured in the form of nitrite in splenocytes from MAA infected mice (B). N.D- not detected.
3.10 Expansion of MAA during chronic infection and alterations of splenic architecture and cellular composition

A severe influence of MAA on the architecture of the spleen was observed during late stage of infection. To study this in more detail, H and E staining of the spleens of MAA infected mice were analyzed at different time points (Figure 3.10A). After 1 week, the infection had little effects on splenic structure. Lymphoid follicles were clearly visible. However, after 3 weeks the general architecture of the spleen had changed dramatically. Lymphoid follicles nearly completely disappeared. A progressive granulomatous inflammation became apparent with increasing numbers of monocytic cells (Figure 3.10A) bearing multiple mycobacteria as detected by ZN staining (Figure 3.10B). In fact, a $10^5$-fold increase in bacterial CFUs between 1 week and 3 weeks p.i. was detected by plating of the livers (Figure 3.10C). This correlated with the structural changes observed in spleen. The massive influx of monocytic cells was associated with dramatic changes in the lymphoid compartment. As shown in Figure 3.10D and E, at 5 weeks after MAA infection severe ablation of lymphoid cells occurred. B cells as well as CD4 and CD8 T cells were 3-4 folds reduced compared to spleens from uninfected mice. Despite such lymphocyte loss, serum level of IFN-γ was still high at 5 weeks p.i. as detected by ELISA(Figure 3.10F) suggesting the likely contribution of CD11b$^{hi}$CD11c$^{int}$ cells as source of IFN-γ (Figure 3.7).
Figure 3.10. Chronic MAA infection alters the architecture and cellular composition of the spleen. H and E (A, scale is 400µm) and ZN (B, scale is 20µm) staining at 1, 3, 4 and 5 weeks post infection. Kinetics of MAA proliferation in the liver (C). Total (D) and percentage (E) of lymphocytes gated on live cells. Serum level of IFN-γ as measured by ELISA at 5 weeks post infection (F) (*** p value<0.001 (t test)) ND- not detected.
3.11 Mycobacteria infected spleen shows reduced apoptosis

Apoptosis could be responsible for the lymphoid ablation shown in Figure 3.10. Therefore whether severe apoptosis would be detectable in the infected spleen was investigated. However, enhanced levels of activated caspase 3 indicative for early apoptosis could not be detected by immune-histology. Rather apoptotic events in the spleens of infected mice appeared to decrease in a time dependent manner (Figure 3.11).

Figure 3.11. Spleen caspase 3 staining at 1, 3, 4 and 5 weeks post infection (Scale is 40µm).

3.12 Lack of NO production by M-MDSC ameliorates chronic infection

NO can exert a direct pro-apoptotic effect in T cells (Serafini 2013). Even though apoptotic events were low at the tissue level, the question arose whether ablation of T and B cells in the spleen of MAA infected mice is due to the production of NO by M-MDSC (CD11b^{hi}CD11c^{int} monocytic cells). Therefore, iNOS knockout (iNOS^{-/-}) mice were infected with MAA. After 5 weeks, the mice were sacrificed and spleen and liver pathology were analyzed as well as myeloid and lymphoid content. Spleens of MAA infected WT (wild type) and iNOS^{-/-} mice showed a massive influx of histiocytic cells. In contrast to MAA infected WT mice, the livers of MAA infected iNOS^{-/-} mice exhibited mostly granuloma harboring lymphocytes (Figure 3.12A). At the cellular level, spleens of MAA infected iNOS^{-/-} mice exhibited CD11b^{hi}CD11c^{int} monocytic cells in numbers similar to the WT (Figure 3.12B). However, in contrast to WT mice CD11b^{hi}CD11c^{int} monocytic cells harbored considerably lower numbers of
bacteria (Figure 3.12B and C). Generally, lower numbers of MAA were detected under these conditions in iNOS<sup>−/−</sup> mice.

**Figure 3.12. Lack of NO production by M-MDSC ameliorated chronic infection.** Spleen and liver H and E staining from MAA infected iNOS<sup>−/−</sup> mice compared to WT (A, scale is 100µm). Frequency and bacterial load (as shown by ZN) of M-MDSC sorted from MAA infected WT and iNOS<sup>−/−</sup> mice (B, scale is 20µm). Comparison of amount of bacterial DNA (IS901) against MIP-2α promoter DNA of M-MDSC sorted from MAA infected WT and iNOS<sup>−/−</sup> mice (C).
Interestingly, ablation of B cells and CD8 T cells remained unaltered in iNOS$^{-/-}$ mice whereas the ablation of CD4 T cells was no longer observed. Thus, NO from M-MDSC in chronically MAA infected mice seriously and specifically affects the number of CD4 T cells but not B cells and CD8 T cells (Figure 3.13).

![Graph](image)

**Figure 3.13. NO production from M-MDSC specifically affected CD4 T cells.** Frequency and total numbers of spleen CD4, CD8 and B cells from MAA infected WT and iNOS$^{-/-}$ mice (**p value<0.001, **p value<0.005, ns-not significant (t test)).

### 3.13 NO produced by CD11b$^{hi}$CD11c$^{int}$ cells from MAA infected mice suppresses CD4 T cell responses *ex vivo*

As stated above, NO from M-MDSC is supposed to suppress directly T cell activation (Serafini 2013). To further characterize M-MDSC of MAA infected mice, we tested splenic CD11b$^{hi}$CD11c$^{int}$ M-MDSC NO dependent suppressive activity. CD4 T cells from uninfected mice were stimulated using plate bound anti-CD3 in the presence or absence of splenic CD11b$^{hi}$CD11c$^{int}$ cells from MAA infected mice. Strong proliferation was observed in the absence of M-MDSC (Figure 3.13A). In contrast, in the presence of M-MDSC, the proliferation was seriously inhibited. Inhibition was correlated with the ratio of MDSC like cells to T cells (Figure 3.14B). Importantly, inhibitory activity was completely abolished when the iNOS inhibitor L-N$^6$-(1-
Iminoethyl) lysine di-hydrochloride (L-NIL) was added to the co-cultures (Figure 3.14A and B). These data show that CD11b$^{hi}$CD11c$^{int}$ cells exhibit NO dependent inhibitory activity towards T cells ex vivo.

**Figure 3.14.** NO produced by CD11b$^{hi}$CD11c$^{int}$ cells from MAA infected mice suppress CD4 T cell responses ex vivo. CD11b$^{hi}$CD11c$^{int}$ cells inhibits ex vivo CD4 T cell proliferation via iNOS expression (A). The inhibitory effect was ratio (T: M-MDSC) dependent (B).
3.14 Antigen processing and presentation is impaired in MAA infected mice

To determine if a suppressive microenvironment likely induced by MDSC in MAA infected mice could influence cDC function, the capacity of cDC from MAA and MAH infected mice were tested for OVA protein and peptide presentation. Maturation markers of cDC from MAA and MAH infected mice were analyzed (Figure 3.15A). cDC from MAA infected mice showed strong upregulation of MHC-II and CD86. cDC were then sorted and tested for functional capacity (sorting gating shown in Figure 3.15B). cDC were sensitized with ovalbumin and co-incubated with OVA specific OTII CD4 T cells. Strong proliferation could be observed with cDC from PBS control and MAH infected mice whereas cDC from MAA infected mice were seriously hampered in their T cell stimulatory capacity (Figure 3.15C upper panel). cDC isolated from MAA but not from MAH infected mice were unable to induce CD4 T cells proliferation when stimulated with OVA protein. This indicated a defect in the antigen processing ability as OVA protein requires processing. Therefore, the experiment was repeated by using peptide as an antigen which does not require processing. Strong OTII T cell proliferation was elicited by both cDC populations (Figure 3.15C lower panel). This indicates that peptide based T cell stimulation is undisturbed in cDC from MAA infected mice but processing is altered. Even lower amount of peptide did not alter the results (Figure 3.15D). This was in agreement with the expression of MHC-II and the CD86 co-stimulatory molecules. Splenic cDC from MAA infected mice might be directly infected by the mycobacteria which might affect the processing compartments. This is suggested for infected macrophages (Chang, Linderman et al. 2005). However, cytopsins of sorted cDC from MAA infected mice revealed that hardy any cDC were found to bear mycobacteria (Figure 3.15E). Mycobacterial viability was required, to elicit impairment of antigen processing since administration of heat killed MAA did not result in impairment of T cell stimulation (data not shown).
Results

A

MHCI-IMFI

PBS     MAA     MAH

ns

CD86

PBS     MAA     MAH

ns

B

Pre-sorting

Post-sorting

C

PBS

MAH

MAA

OVA protein

40.6     59.4     39.8     60.2     5.9     94.1

OVA peptide

92.3     7.7     86.1     13.9     78.5     21.5

D

E

% proliferation

PBS     MAA

200     400     1000

20µm
Figure 3.15. Antigen processing and presentation is impaired MAA infected mice. 
A. shows mean fluorescence intensity of respective markers. cDC sorting strategy from PBS, MAA and MAH infected mice at 5 weeks post infection (B). Sorted cells were analyzed for OVA protein (upper panel) and OVA peptide (323-339) (lower panel) presentation (C). ZN stained cDCs from MAA infected mice (D) (\(*\*\*p< 0.001\), ns - non-significant (One way ANOVA Dunnet’s multiple comparison against PBS)). (3-5 mice were included per group and the Figure shows representative of two independent experiments performed).

3.15 MDSC express DC marker but do not present antigens

Upon phenotyping of CD11b\textsuperscript{hi}CD11c\textsuperscript{int} cells, expression of antigen presenting cell markers were observed (Figure 3.16A). In accordance, we analyzed if mMDSC might be involved in T cell stimulation in the spleens of MAA infected mice. Comparable levels of MHC-II and costimulatory CD86 were found for cDC from PBS control and M-MDSC from MAA infected mice. Both molecules were upregulated on cDC during MAA infection. Interestingly, high expression of program death ligand 1 (PD-L1) was found on M-MDSC of MAA infected mice which might be involved in T cell regulation (Figure 3.16. ). In contrast, M-MDSC were completely unable to directly stimulate OT-II T cells with either protein or peptide despite MHC II and CD86 expression (Figure 3.16. B and C).
Results

Figure 3.16. MDSC show DC phenotype but do not present antigen. Expression level of MHC-II, CD86, PD-L1 on MPM-MDSC compared to cDCs from MAA infected and PBS control mice (A) (t test). OVA protein (B) and OVA peptide (C) presenting function of MPM-MDSC compared to cDCs from PBS control and infected mice (**p< 0.01, ***p< 0.001, (One way ANOVA Dunnet's multiple comparison against PBS))

3.16 Impaired antigen uptake and faster degradation compromise cDC antigen processing and presentation

The impaired antigen processing capacity of cDC in MAA infected mice might be induced by NO produced by the M-MDSC. Therefore, splenic cDC were sorted from MAA infected WT and iNOS−/− mice and assessed for their antigen processing and presentation capacity. Clearly, cDC from infected iNOS−/− mice induced significantly higher OTII T-cell proliferation than cDC from infected WT mice (Figure 3.17A). More importantly cDC from WT infected mice had showed reduced antigen uptake when compared to cDC from uninfected and iNOS−/− mice as shown by OVA-cy5. However, there was no a significant difference in terms of antigen processing among the three group (Figure 3.17B). Moreover, antigen was mainly localized in the late
endosome in cDC from WT infected mice (**Figure 3.17C**). However was not possible to get representative picture as the cDC from uninfected mice were less adherent.

**Figure 3.17.** Impaired antigen uptake and faster degradation compromise cDC antigen processing and presentation. WT cDC induced lower level of T cell proliferation when stimulated with OVA protein as compared to iNOS\(^{-}\)/\(-\) cDC while peptide presentation was unaltered (**A**). While antigen uptake was lower in WT cDC, antigen processing was...
comparable to iNOS$^+$ cDC (B). Most of the processed antigen exclusively reside in the late endosomal compartment in cDC from WT infected mice (C) (*p< 0.05, t-(test)).

3.17 In vivo T cell inhibition in MAA infected mice

The above ex vivo data clearly demonstrated that M-MDSC hinder CD4 T cell proliferation as well as the function of cDC from the spleens of MAA infected mice. These effects are NO/iNOS dependent. Since the in vivo situation is significantly more complex, we also analyzed T cell activation in MAA infected mice. To this end, mice were infected with MAA. Then OVA specific OT-II T cells labeled with CFSE were adoptively transferred at 1, 3, 4 and 5 weeks p.i. according to the schedule displayed in Figure 3.18. Then, 24 hours after respective time points, antigen specific in vivo proliferation was induced by i.p. administration of OVA. Three days later proliferation of OT-II T cells isolated from spleen was evaluated based on CFSE dilution. Interestingly, in vivo CD4 T cell proliferation was not affected during the initial phase of infection. However, there was inhibitory effect on T cell proliferation after 3 weeks and clear inhibition was observed at 5 weeks post infection. This inhibition was only evident when viable bacteria were applied. Injection of heat inactivated MAA did not induce such a phenomenon (Figure 3.18B). Thus, T cell inhibitory activity positively correlated with severity of inflammation (Figure 3.10) and with the appearance of CD11b$^{hi}$CD11c$^{int}$ MDSC in the spleen of MAA infected mice (Figure 3.5).

To investigate whether inhibition of T cell activation was dependent on NO as observed ex vivo, OTII proliferation was tested in using MAA infected iNOS$^-\!^-$mice. Surprisingly, like in WT mice, inhibition of CD4$^+$ OT-II T cells proliferation was observed in iNOS$^-\!^-$ mice infected with MAA (Figure 3.18). This suggests that even though NO production by CD11b$^{hi}$CD11c$^{int}$ MDSC is abolished in iNOS$^-\!^-$ mice, the inhibitory ability is still active.
Results

**Figure 3.18. In vivo M-MDSC mediated T cell inhibition in MAA infected mice is NO independent.** Experimental scheme (A) and kinetics of in vivo T cells proliferation in WT mice at different time points (B). In vivo CD4 T cells proliferation in WT vs iNOS−/− 30 days post MAA infection (C) (t test *p <0.05, ***p <0.001).

### 3.18 Absence of iNOS exacerbates Arg1 expression

As the population of CD11b^hi^CD11c^int^ M-MDSC is still present in MAA infected iNOS−/− mice, most likely, they are still competent to inhibit T cell proliferation under in vivo conditions by compensatory mechanisms. Thus, we focused on cell specific Arg1 expression as this enzyme is known to be produced by MDSC and exhibit T cell inhibition. Our result indicates that a higher level of Arg1 expression is observed in the absence of iNOS in in the spleen tissue (Figure 3.19A). To further confirm Arg1 expression in M-MDSC at cellular level, M-MDSC were sorted from WT and iNOS−/− infected spleen. Our results confirm that M-MDSC lacking iNOS showed higher expression of Arg1 (>100x) compared to WT M-MDSC. However other cytokines were not differentially expressed, as shown for Tnf with only two fold difference and no difference in Il10 expression (Figure 3.19B). This indicates that M-MDSC do not concomitantly express iNOS and Arg1 but uses them alternatively to suppress T cell proliferation in vivo.
Figure 3.19 Absence of iNOS exacerbates Arg1 expression in spleen of MAA infected mice. **A.** Paraffin embedded spleen sections were stained for nuclei (DAPI) and MAA and arg1. **B.** M-MDSC were sorted from WT and iNOS−/− infected spleen. RNA was extracted and qRT-PCR was performed. Results were displayed as $2^{-\Delta CT}$ relative to Rps9. Results are shown for 30 days post infection.
4 Discussion

Despite the orchestrated action of the innate and adaptive immune responses against tuberculosis and nontuberculous mycobacterial infection, the immune factors contributing to disease exacerbation are still far from being understood (Cambier, Falkow et al. 2014). In the present study, by infecting mice with *M. avium*, it was revealed that monocytic myeloid derived suppressor cells (M-MDSC) considerably influence the outcome of the local innate and adaptive immune response, thereby facilitating exacerbation of mycobacterial disease. This study shows that the ability to induce M-MDSC is a virulence trait of pathogenic mycobacteria. The data reveal that in mycobacterial infection M-MDSC definitely inhibit local CD4 T cell proliferation *in vivo*. An alternative switches to arginase expression is observed in the absence of iNOS.

As a mycobacterial infection model in mice, *M. avium* holds some advantages due to its natural resistance to NO (Escuyer, Haddad et al. 1996, Pearl 2012). *M. avium* causes progressive infection in WT C57B6/J mice. It induces and grows in necrotic and hypoxic lesions typical of human tuberculosis which *M. tuberculosis* does not induce in mice (Aly, Wagner et al. 2006). In contrast, NO controls *M. tuberculosis* infection in WT C57BL/6 mice. Only in iNOS⁻/⁻ C57BL/6 mice, *M. tuberculosis* infection induces hypoxic granuloma; similar to human TB (Reece, Loddenkemper et al. 2010). Overall, *M. avium* infections of WT C57B6/J mice have been suggested as alternative surrogate model for mycobacterial infection (Kondratieva, Evstifeev et al. 2007, Apt and Kramnik 2009).

It has been reported that *M. avium* ATCC 25291 (TMC 724, DSM44156) infection leads to iNOS dependent thymic atrophy (Borges, Barreira-Silva et al. 2012), local CD4 T cell depletion (Florido, Pearl et al. 2005), iNOS dependent granuloma necrosis and improved infection control in iNOS gene disrupted mice (Gomes, Florido et al. 1999). However phenotypic and functional relevance of iNOS expressing innate immune cells involved in *M. avium* pathogenesis are not known.

From studies on bacterial survival and CD4 T cell responses after infection of mice with *M. avium*, it is known that the immune responses and outcome of infection
strongly depend on the strain type used and the route of infection (Saunders, Dane et al. 2002, Petrofsky and Bermudez 2005, Lousada, Florido et al. 2006). Therefore, to dissect the relevance of myeloid cells during chronic *M. avium* infection in more detail, the MAA strain 25291 and the MAH strain 104, known as of intermediate virulence were used (Agdestein, Johansen et al. 2012, Haug, Awuh et al. 2013) in C57B6/J mice. Intraperitoneal route of infection was selected as it results in fast systemic dissemination and reproducible chronic infection with granulomatous inflammation in the inner organs.

In the present model, five weeks after infection mice exhibited systemic mycobacterial infection regardless of the strain used. As expected, more severe disease and higher bacterial burden was observed in MAA infected mice. As battle between host immune cells and virulent mycobacteria takes place inside the granuloma the first focus was on characterizing the difference in type of granuloma induced by the two strains. MAH and MAA infection in mice induced granulomatous inflammation in liver and spleen. These findings were in agreement with the results of other groups on MAH and MAA mouse infections (Smith, Hansch et al. 1997, Saunders, Dane et al. 2002, Haug, Awuh et al. 2013). However, the quality of the granulomatous inflammation in both liver and spleen considerably differed between mice infected with MAA or MAH. In MAH infected mice, compact granuloma in spleen and liver containing central mononuclear cells and/or epithelioid like macrophages, surrounded by multiple T cells were observed. In contrast, granulomatous inflammation in MAA infected mice was characterized by the appearance of increasing numbers of mononuclear cells. In the liver, mononuclear cells were organized in granuloma like structures surrounded by only a limited number of T cells. This histiocytic type of granulomatous inflammation was particularly apparent in the spleen. Here the influx of high number of infected monocytic cells in the time course of infection nearly completely dissolved the physiological structure. Accordingly, phenotypic characterization of spleen cellularity revealed dramatically reduced number of CD4 and CD8 T cell as well as B cells. Ablation of the lymphoid cell population and loss of reactivity is commonly observed in *M. avium* infected mice (Florido, Pearl et al. 2005). However, as discussed below, the results from this study for the first time clearly emphasize the critical role of an immature monocytic cell population for the process.
Phenotypic characterization of spleen monocytic cells indicated that both strains induced an increased frequency of Gr-1 and CD11b expressing cells. However, MAA induced accumulation of significantly higher frequency of Gr-1\(^{lo}\) cells. Cells of this phenotype are recently described as myeloid derived suppressor cells (MDSC). MDSC have been widely described under tumor conditions (Ochando, Conde et al. 2015) and evidence for their existence during microbial infections is emerging (Van Ginderachter, Beschin et al. 2010, Goh, Narayanan et al. 2013, Ost, Singh et al. 2016). Studies have shown that M-MDSC can be found in high frequency in sepsis (Janols, Bergenfelz et al. 2014), biofilm formation (Heim, Vidlak et al. 2014), parasitic infection (Goni, Alcaide et al. 2002), traumatic stress (Makarenkova, Bansal et al. 2006) and acute and chronic microbial infection (Delano, Scumpia et al. 2007, Wojtasiak, Pickett et al. 2010).

Gr-1\(^{+}\)CD11b\(^{+}\) expressing cells have been reported in \textit{M. tuberculosis} infected iNOS\(^{-}\) WT C57BL/6 and I/St mice. However, they are absent or found at very low level in WT C57BL/6 mice (Obregón-Henao, Henao-Tamayo et al. 2013, Knaul, Jorg et al. 2014). In those susceptible mice strains, Gr-1\(^{lo}\)CD11b\(^{+}\) correlates with severity of infection. This shows that Gr-1 expressing myeloid cells are indicator of poor prognosis of tuberculosis in mice (Lyadova, Tsiganov et al. 2010). In the human context, active and recent TB infection has been associated with increased M-MDSC (du Plessis, Loebenberg et al. 2013).

\textit{M. avium} complex (MAC) is the second largest mycobacterial complex of medical importance preceded by \textit{M. tuberculosis} complex (Gonzalez-Perez, Murcia et al. 2016). Until now there is no report on existence and role of MDSC in \textit{M. avium} infection. In this study, MDSC with CD11b\(^{hi}\)CD11c\(^{int}\)Ly6G\(^{neg}\) phenotypes were identified which have not been described yet. This population is another example for to the heterogeneity of MDCS. Until now, MDSC are broadly classified as CD11b\(^{+}\)Ly6G\(^{+}\)Ly6C\(^{lo}\) (granulocytic) and CD11b\(^{+}\)Ly6G\(^{-}\)Ly6C\(^{hi}\) (monocytic) sub populations (Youn, Nagaraj et al. 2008). CD11b\(^{hi}\)CD11c\(^{int}\)Gr-1\(^{int}\)Ly6C\(^{hi}\) cells identified in this study fulfil the criteria of myeloid derived suppressor cells functionally and phenotypically (discussed below) (Gabrilovich and Nagaraj 2009, Bronte, Brandau et al. 2016) and here after named as monocytic-myeloid derived suppressor cells (M-MDSC).
The ability to suppress immune responses is an important characteristic of MDSC (Bronte, Brandau et al. 2016). The immune-regulatory activity of MDSC on T cells largely depends on the metabolic consumption of arginine by the activity of inducible enzymes iNOS and Arg1 (Gabrilovich and Nagaraj 2009, Bronte, Brandau et al. 2016). By this, MDSC create an arginine starved milieu which prevents T cell growth (Raber, Ochoa et al. 2012). In addition, iNOS expression and concomitant NO production is suggested to induce loss of the TCRζ chain thereby limiting the signaling ability (Nagy, Koncz et al. 2010). The immune-histology stains revealed that the mycobacteria containing M-MDSC in the spleens of MAA infected mice express high levels of iNOS and low levels of Arg1. Furthermore, it was found that CD4 T cell ablation observed in the spleens of MAA infected mice was restored in iNOS−/− mice. This pointed towards a NO/iNOS dominated immune suppressive activity of the M-MDSC. In agreement, the ex vivo analyses of M-MDSC from MAA infected mice showed that these cells are able to inhibit T cell proliferation in a NO/iNOS dependent manner since inhibition of proliferation could be restored by the iNOS inhibitor L-NIL.

The ex vivo data suggested that the immune suppressive activity of the M-MDSC is mediated via NO/iNOS. Since the in vivo situation is significantly more complex, T cell proliferation in MAA infected mice was analyzed. Studies show that M. avium infection influences T cell compartment in mice (Florido, Pearl et al. 2005, Borges, Barreira-Silva et al. 2012). Therefore, to analyze the relevance of the splenic monocytic population on T cell activation in vivo, the advantage of adoptive transfer of ovalbumin sensitive OT II cells was utilized, as this represents an elegant way to analyze T cell activity independently of the individual adaptive immune host response to the specific pathogen. These experiments clearly demonstrated that MAA infection of mice resulted in inhibition of OT II cell proliferation in the spleen. Furthermore, the extent of inhibition of OT II cell proliferation correlated with the extent of pathology in the spleens and number of the mycobacteria containing monocytic cells. This indicated that T cell inhibition in the spleen is independent of individual adaptive host response to the mycobacterial infection and that such effect is closely connected to the number of M-MDSC in the spleen. Using iNOS−/− mice, it was found that M-MDSC are induced even in the absence NO/iNOS. This is in contrast to studies on tumors where NO/iNOS was shown to control ex vivo induction of functional MDSC.
(Jayaraman, Parikh et al. 2012). The deletion of iNOS had a positive effect on the overall number of OT II cell emphasizing the above discussed detrimental effect of NO/iNOS on T cells. Surprisingly, inhibition of OT II cell proliferation was also observed in iNOS<sup>-/-</sup> mice. Thus it seems that MDSC exhibit an exchangeable T cell suppressive repertoire. Indeed, it was found that arginase I and iNOS expression are inversely correlated. Arg1 was highly expressed in iNOS<sup>-/-</sup> mice. This was confirmed at cellular level by qRT-PCR, enzyme activity and in situ at protein level. It has been shown that the products ofArg1 I and iNOS do cross regulate each other’s activity reciprocally. Thus, polyamines produced by Arg1 inhibit iNOS activity and nitrite ions produced by iNOS inhibits arginase activity (Rath, Muller et al. 2014). Furthermore, inhibitors of iNOS expression have been shown to upregulate Arg1 expression in murine macrophages (Sharda, Yu et al. 2011). Despite this, in agreement with other studies (Gomes, Florido et al. 1999) It was observed that iNOS<sup>-/-</sup> mice seemed to be more competent to control mycobacterial replication. Most plausibly, this effect is attributed to the unaffected T cell number and the absence of other detrimental effects on immune cells such as DC by NO in infected iNOS<sup>-/-</sup> mice. Thereby, establishment of classical granuloma guaranties reduction of bacterial spread during the course of infection even in the presence T cell suppressive M-MDSC activity.

M-MDSC identified in this study are target cells for *M. avium* infection and these cells were detected as early as one week post infection in the spleen and in the bone marrow at late stage of infection. Such cells with mycobacterial infection have not been reported yet in these two compartments. This confirms that in addition to the conventional macrophages, immature M-MDCS are particularly targeted. In agreement with in this study, Ly6C<sup>+</sup> monocytes expressing IL-10 has been shown to populate during the first 14 days in the lung of *M. tuberculosis* infected mice (Moreira-Teixeira, Redford et al. 2017). Recently the involvement of other mononuclear cells in mycobacterial infection have received more attention (Srivastava, Ernst et al. 2014). Therefore, while understanding of the ontogeny of myeloid cells expands, defining organ specific macrophages and other mononuclear cells under normal circumstances and pathologic conditions is an expanding complex research field. Even though cells with MDSC phenotype have been reported in *M. tuberculosis* infection, it is less clear if they are target of the pathogen. For instance in the lung of *M. tuberculosis* infected mice, the frequency of CD11c<sup>+</sup>CD11b<sup>+</sup> cells
have been increased, but they were not known whether they are infected or not (Skold and Behar 2008). A recent finding also indicated after poly-IC treatment increased infiltration of pathogen permissive CD11b+Gr-1int cells in the lung (Antonelli, Gigliotti Rothfuchs et al. 2010) but their function as suppressor cells was not tested.

As stated above, it seems that MDSC exhibit an exchangeable T cell suppressive repertoire. Recent findings show that MHC-II dependent direct interaction between mycobacteria infected cell and CD4 T cells enhance *M. tuberculosis* control *in vivo* (Srivastava and Ernst 2013) regardless of T cells derived cytokines (Gallegos, Pamer et al. 2008, Gallegos, van Heijst et al. 2011). Here it was found that the infected M-MDSC express MHC-II but also a multitude of factors that potentially inhibit the interaction with T cells. These include Arg1, iNOS, PD-L1 and IL-10 expression. The interaction between PD-L1 on M-MDSC and PD-1 on T cells can lead to T cell death (He, Hu et al. 2015). IL-10 produced by these cells might add to inhibition of T cell proliferation (Ye, Huang et al. 2007). Collectively these factors can inhibit local interaction between T cells and mycobacteria infected cells thereby exacerbating further expansion of the pathogen.

Mycobacteria infected cells are not able to present antigen to CD4 T cells (Baena and Porcelli 2009). This was also seen in the present findings. M-MDSC up-regulate CD11c expression together with MHC-II and CD86, however they were not able to present antigen. Thus, it seems that CD11c compliment receptor expressing myeloid lineages do not fully develop towards competent dendritic cells after mycobacterial infection.

During mycobacterial infection, DC do perform two functions: participating in granuloma formation and antigen presentation (Dreher and Nicod 2002). However, developmental and functional aspect of dendritic cells *in vivo* in the presence of infection and in particular infection induced MDSC have not yet been studied. This study could show that M-MDSC suppressive activity is not only limited to T cells but also to the splenic DC population. Thus, *ex vivo* analyses on splenic DC from MAA and MAH infected mice revealed that DC mediated inhibition of T cell proliferation was dependent on M-MDSC and iNOS since inhibition was observed only for DC
from MAA infected mice and restored in iNOS<sup>−/−</sup> mice. Obviously NO from M-MDSC seems to influence antigen processing and presentation since peptide presentation was intact. The results add to emerging number of reports on a direct or indirect influence of MDSC on other immune cells including DC. For instance, MDSC have been reported to impair DC functions in mouse tumor models by this enhancing tumor-induced immune suppression (Hu, Gan et al. 2011, Ostrand-Rosenberg, Sinha et al. 2012, Mondanelli, Bianchi et al. 2017).

The results from the present study help to resolve another yet unanswered question. Thus, it was not clear why <i>M. avium</i> infected SCID (severe combined immune deficient) mice still produced IFN-γ after depletion of NK cells indicating that IFN-γ source other than T cells exist (Appelberg, Castro et al. 1994, Smith, Hansch et al. 1997, Florido, Correia-Neves et al. 2003). It was found that high amounts of serum IFN-γ was measured even after severe T cell depletion. However, it has been shown that IFN-γ is not always beneficial to the host during <i>M. avium</i> infection. For example, <i>M. avium</i> infection induced IFN-γ dependent reduction of HSC self-renewal (Matatall, Jeong et al. 2016), IFN-γ dependent but IFN-γ receptor independent depletion of lymphocytes (Florido, Pearl et al. 2005), and NO dependent depletion of double negative thymocytes and mature T cells in the thymus & anemia (Mabbott and Sternberg 1995, Borges, Barreira-Silva et al. 2012). Hence, IFN-γ rather exerts a deleterious role possibly due to enhancing expansion and subsequent activation of M-MDSC NO/iNOS pathway (Greifenberg, Ribechini et al. 2009).

4.1 Conclusion

In conclusion, this study provides evidence that the overall ability of mycobacterial strains to induce M-MDSC is an important virulence feature. The monocytic nature of M-MDSC establishes them as target cells for mycobacterial replication. Their immature phenotype and their ambiguous response to infection considerably influences the outcome of the local innate and adaptive immune response, thereby facilitating exacerbation of mycobacterial disease. In addition, this study identified that M-MDSC use all alternatives at their disposal to inhibit T cell proliferation.
In summary, immature monocytes lineage cells recruited during MAA infection are particularly targeted by MAA which alters their development and rather drives them towards suppressor cells phenotype and function (Figure 4.1). Such MAA induced suppressor cells exert their suppressive effect on T cells and dendritic cells mainly via iNOS and enhanced Arg1 activity. It is plausible to speculate that M-MDSC induce B cell loss via arginase expression which needs to be confirmed. The mechanism how M-MDSC are attracted to the inflamed tissues awaits further studies.

Figure 4.1. *M. avium* infected immature monocytes develop towards suppressor cells (M-MDSC) and suppress innate and adaptive immune response. *M. avium* extensively replicate in M-MDSC inhibit T cell function via iNOS expression. M-MDSC also upregulate Arg1 I expression in the absence of iNOS. Moreover M-MDSC impair the function of cDC via NO/iNOS pathway.
4.2 Future directions

Understanding how iNOS expression regulates arginase at transcriptional level and why iNOS dominates Arg1 expression in WT mice after infection will need future work. Immunological relevance of such cross regulation is remaining to be understood. Exploring whether such phenomena exist in other pathogenic mycobacterial infection and targeting these cell populations may help to further understand pathogenesis of mycobacterial infection. It has been reported in previous studies that MAA infection induces thymic atrophy. The effect of MAA expanded M-MDSC described in this study on such pathologic conditions needs further investigation. It is also of interest to dissect why MAA and MAH, very closely related subspecies, are divergent in terms of M-MDSC induction. Last but not least, particular cytokine and chemokine profiles favoring the expansion of such M-MDSC needs to be elucidated.
5 References


References


6 Appendix

6.1 Supplementary figures for confocal microscopy pictures

WT Spleen PBS

WT Spleen infected
WT Spleen infected

iNOS⁻ Spleen infected
iNOS−/− Spleen infected

Figure 6.1. Confocal Microscopy pictures showing Arg1, iNOS, MAA staining in PBS control, WT infected and iNOS infected mice. Nuclei were stained with DAPI. Real fluorescence and autofluorescence has been shown in single channel (scale is 35µm).

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