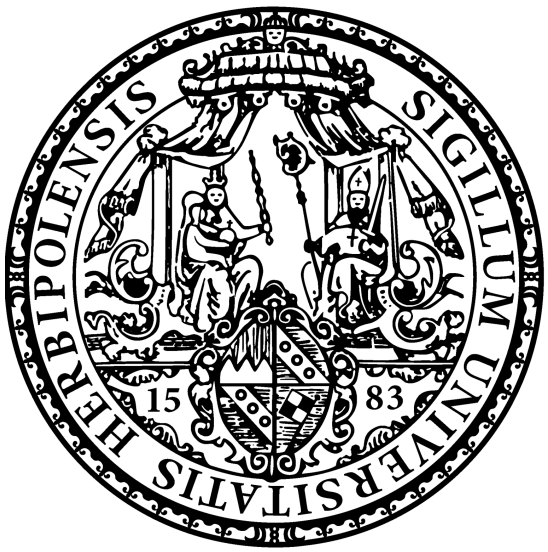

Interaction of mycobacteria with myeloid-derived suppressor cells

Wechselwirkung von Mykobakterien mit myeloiden Suppressorzellen

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg,

Section Infection and Immunity

Submitted by

Vini John

from

Puthur, Kerala, India

Würzburg, 2019

Submitted on: …….

Office stamp

Members of the PhD Thesis Committee

Chairperson: Prof. Dr.G. Gasteiger

Primary Supervisor: Prof. Dr.Manfred Lutz

Supervisor (Second): Dr.Martin Fraunholz

Supervisor (Third): Prof. Dr.Andreas Beilhack

Date of Public Defense: ……..……

Date of Receipt of Certificates: …….

Affidavit

I hereby confirm that my thesis entitled ‘Interaction of mycobacteria with myeloid-derived suppressor cells’ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 30.01.2019

Place, Date **Signature**

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation ‘Wechselwirkung von Mykobakterien mit myeloiden Suppressorzellen’ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 30.01.2019

Ort, Datum **Unterschrift**

Table of Contents Page no.

1.Introduction

1.1 Tuberculosis

Tuberculosis (TB) is an airborne infectious disease which is still one of the major causes of death around the globe. In 2017, nearly 10.4 million people fell ill with TB and 1.3 million died of it ('WHO | Global tuberculosis report 2017', 2017) (Figure.1). TB is caused by an intracellular pathogen *Mycobacterium tuberculosis* (Mtb) which can be transmitted through the aerosol route. Robert Koch described Mtb more than 100 years ago and has since then a topic of extensive research. Most of the humans and experimental animals develop proper immune responses after Mtb infection. However, these immune responses are still inefficient to completely clear the infection because of which Mtb adopts a latent state of infection. Mtb can then be reactivated and reversed to the active stage of TB at any later time in life. Unfortunately, despite of extensive research, we still do not have a clear understanding of mechanisms underlying TB immunity, pathogenesis and most importantly reinfection (Ernst, 2012).

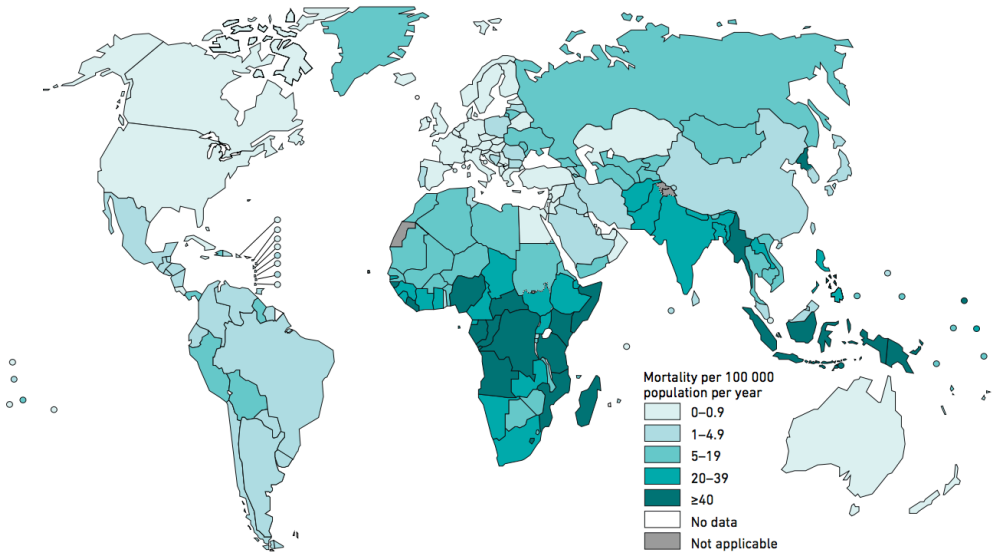

Figure 1: TB mortality rates excluding TB deaths among HIV-positive people. Shaded regions represent uncertainty intervals among countries, ranging from less than one TB death per 100,000 population in many high-income countries, to 40 or more deaths per 100,000 population. (*GLOBAL TUBERCULOSIS REPORT 2018*, 2018)

It is challenging to detect extrapulmonary TB and TB in children or TB in people diagnosed with AIDS (Walzl *et al.*, 2018). There is no proper test to detect the presence or absence of Mtb in asymptomatic individuals. For treatment of active TB, multidrug therapy is recommended by WHO (under DOTS program). However, the emergence of Multi-Drug Resistance (MDR) strains of Mtb made the current treatment even more difficult. Due to the long duration of therapy and rising cases

of MDR or Extremely Drug Resistance (XDR) strains of Mtb has called for renewed efforts towards improved therapeutic strategies. There is an urgent need of biomarkers to distinguish between latent infection and an active TB disease (Walzl *et al.*, 2018). This will help in predicting the reactivation risk after the disease has been cured. To this goal, the End TB strategy by the WHO targets to end the global TB infections by 2035 and aim for the development of vaccines with greater efficacy (Pai *et al.*, 2016).

1.2 BCG vaccine against TB

Bacillus Calmette-Guérin (BCG) is a vaccine against TB which consists of the live attenuated strain of *Mycobacterium bovis* developed nearly 100 years ago. In 1908, while working at the Institute Pasteur de Lille (Lille, France), Albert Calmette and Camille Guérin used BCG as a vaccine after continuous *in vitro* sub-culturing for 13 years (Davenne and McShane, 2016). BCG vaccine was first used in humans in 1921. Despite its widespread use in new-born babies, BCG does not satisfactorily prevent adult pulmonary disease and therefore, has not reduced the global burden of TB. The reasons for the varying efficacy of BCG in protection against pulmonary TB are not fully understood. Potential explanations include interference with the immune response to BCG caused by previous exposure to environmental mycobacteria (Andersen and Doherty, 2005). Other possible factors include differences among BCG vaccine sub-strains and phenotypic changes in the vaccine during passage from the original cultures to the final cultures during the manufacturing process (Andersen and Doherty, 2005). BCG fails to stimulate adequate, balanced anti-mycobacterial $CD4^+$ and $CD8^+$ T-cell responses (Andersen and Doherty, 2005). Variability in dose, route of administration, the age of recipient during administration and genetic differences among recipients (Copin *et al.*, 2014) or even lyophilization of the vaccine (Brewer and Colditz, 1995) are some of the possible reasons for the failure of the vaccine. BCG might restrain Mtb over a period, but Mtb can persist and might reactivate later in life. This is due to an insufficient BCG induced immune responses mounted to a suboptimal T cell activation.

1.3 Recognition of mycobacteria by immune cells

Once Mtb enter the lungs, alveolar macrophages are the primary cell type which takes up the pathogen. Apart from alveolar macrophages, Mtb can also be taken up by dendritic cells (DCs), neutrophils and myeloid-derived suppressor cells (MDSCs). These phagocytic cells express several receptors such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), scavenger receptors (SRs), complement receptors (CRs) and NOD-like receptors which help in recognizing mycobacteria for uptake and inflammatory response (Stamm, Collins and Shiloh, 2015). However, due to the complex structure of the mycobacteria, it is very challenging to determine the role of individual receptors for mycobacterial recognition. The receptors that have been shown to be involved in mycobacterial recognition are discussed below in details (Figure .2).

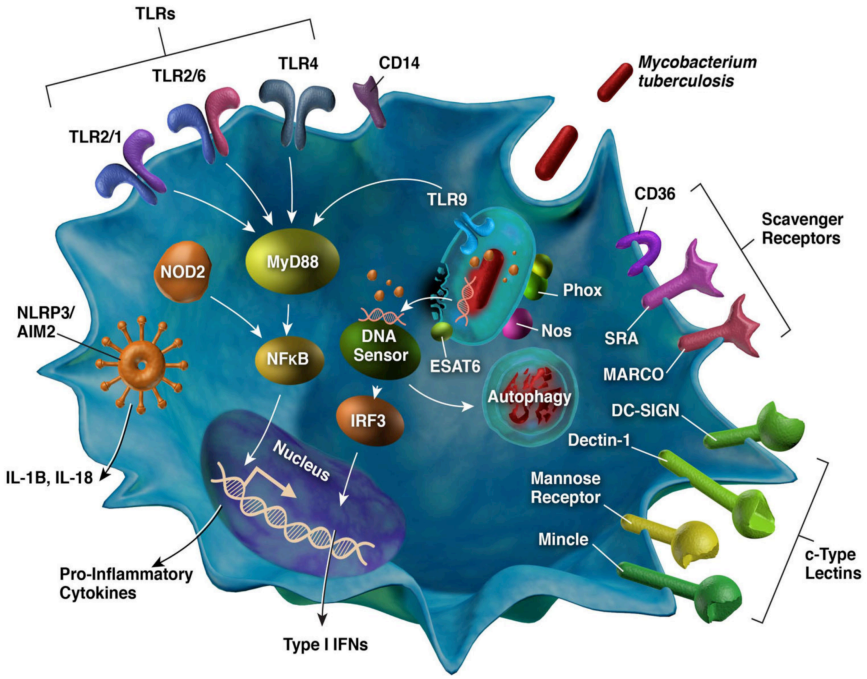

Figure 2: Pattern recognition receptors (PRRs) involved in Mtb recognition. The above figure shows receptors expressed on innate immune cells which are crucial for mycobacterial cell wall recognition. PRRs on the cell surface include scavenger receptors, TLRs and C-type lectin receptors. Cytoplasmic receptors respond to mycobacterial components such as DNA and secreted proteins. TLRs can recognize conserved molecular patterns of Mtb cell wall. TLRs such as TLR2, TLR4 and TLR9 recognize Mtb and start an immune response to activate NF-KB and secrete inflammatory cytokines. Other signalling pathways include IRF3 dependent cytokine production, inflammasome-mediated IL-18 and IL-1β secretion. (Stamm, Collins and Shiloh, 2015)

1.3.1 Toll-like receptors (TLRs)

TLRs belong to the evolutionary conserved innate pattern recognition receptor (PRR) family which are expressed in immune cells such as DCs, macrophages, neutrophils and MDSCs. Mycobacteria or their products activate TLRs and start the cascade of events necessary for mounting inflammatory immune responses. The Mtb cell wall has several antigens for different TLRs for instance, CpG DNA sequences are recognized by TLR9 and certain heat-sensitive mycobacterial proteins are recognized by TLR4. The Mtb genome encodes 99 lipoproteins which are potent TLR2 agonist (Sutcliffe and Harrington, 2004). Additionally, mycobacterial ligands such as mannosylated phosphatidylinositol (PIM), lipopeptides, lipoarabinomannan (LAM) can activate TLR2 (Quesniaux *et al.*, 2004).

TLR signaling is needed for effector functions during mycobacterial infections including inflammatory cytokine secretion, production of ROS and reactive nitrogen intermediates. TLR activation also triggers the maturation of phagosomes and autophagy, a central process in mycobacterial killing (Underhill *et al.*, 1999)·(Doz *et al.*, 2007). Mtb recognition by TLRs is followed by recruitment of the adaptor protein MyD88 to initiate inflammatory signaling cascades (Muzio *et al.*, 1997). The MyD88 adapter protein links members of the toll-like receptor (TLR) and interleukin-

1 receptor (IL-1R) superfamily to the downstream activation of nuclear factor-κB (NF- κB) and mitogen-activated protein kinases (MAPK) (Janssens and Beyaert, 2002). Mice lacking the adaptor protein MyD88 are more susceptible to Mtb than are TLR2/TLR9 double-knockout mice (Philips and Ernst, 2012a). Moreover, TLR4 can also induce the MyD88-independent TIR-domain holding adapter-inducing interferon-β (TRIF) pathway. TRIF induces IFN-β secretion by upregulating IRF3 which is essential for Mtb pathogenesis (Manzanillo *et al.*, 2012)(Stanley *et al.*, 2007). TLR activation in DCs shape the adaptive immune response by priming the development of either a Th1, Th2 or Th17 type of immune response. TLR–MyD88 signaling pathway induced NF-κB activation lead to the expression and secretion of several Th1-promoting cytokines (e.g. IL-12).

1.3.2 C-type lectin receptors

C-type lectin receptors are another family of PRRs which recognize conserved carbohydrate moieties on the cell wall of pathogens (Cambi, Koopman and Figdor, 2005). These receptors can act as both pathogen recognition receptors as well as cell adhesion molecules during the immune response to the pathogen (Cambi and Figdor, 2003). The C-type lectin receptor family includes DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), mannose receptor (MR), Mincle and Dectin-1 (Killick *et al.*, 2013). DC-SIGN receptor is found on human DCs and macrophages and recognize Man-LAM on the mycobacterial surface and induces serine/threonine kinase Raf-1 signaling to secrete IL-10 (Gringhuis *et al.*, 2007). It is difficult to study the role of DC-SIGN in mouse models since there is no clear ortholog and eight genetic homologs of human DC-SIGN in mice (Garcia-Vallejo and van Kooyk, 2013). Human macrophages mainly phagocytose through MRs. Activation of MRs results in the production of the anti-inflammatory cytokines IL-10, IL-1R antagonist and inhibits the secretion of IL-12 (Chieppa *et al.*, 2003). Another CLR, Mincle is found on macrophages which recognize the most abundant glycolipid in the Mtb cell wall known as trehalose dimycolate (TDM) (Ishikawa *et al.*, 2009). In a Mincle dependent manner, TDM triggers macrophages to secrete inflammatory cytokines and reactive nitrogen intermediates (Schoenen *et al.*, 2010). Reports about the role of Mincle in Mtb infection is controversial. In one report, macrophages from Mincle deficient mice secreted less G-CSF and TNF upon BCG and Mtb infection. However, Mincle knockout mice had no defect in the granulomatous response or in controlling the mycobacterial infection (Heitmann *et al.*, 2013). In another report, Mincle KO mice infected with BCG had reduced pro-inflammatory cytokine levels in their BAL fluid. Also, bacterial load in the lungs, spleen and draining lymph nodes of Mincle deficient mice were higher compared to wild type mice (Behler *et al.*, 2012). The Dectin-1 receptor is present on the surface of DCs, macrophages and neutrophils. The Dectin-1 receptor recognizes β-glucan on fungal pathogens, but the mycobacterial ligand is not characterized yet. However, Dectin-1 deficient bone marrow-derived macrophages (BMDMs) had impaired secretion of TNF-α or ROS upon *M. bovis* infection (Das *et al.*, 2013) possibly via influencing TLR2 activation in macrophages (Yadav and Schorey, 2006).

1.3.3 NOD2 and NOD-like receptors

Another cytoplasmic PRR which respond to peptidoglycan and muramyl dipeptide from mycobacterial cell wall is NOD2 (Killick *et al.*, 2013). It is important for cytokine secretion in response to the mycobacteria. NOD2 deficient mice are susceptible to Mtb infection and also show higher bacterial burden (Divangahi *et al.*, 2008). In contrast to this, others have shown that NOD2-deficient mice could control the Mtb infection like wild type mice. Rather, DCs and macrophages derived from NOD2 knock out mice had defect only in cytokine secretion upon Mtb infection (Gandotra *et al.*, 2007). However, due to contradictory reports, the exact role of NOD2 in Mtb pathogenesis is still not clear.

1.4 Adaptive immune response to Mtb

Most studies focus on T cell-mediated adaptive immunity in TB infection rather than B cells. T cells are important for controlling the Mtb infection. Both $CD4^+$ and $CD8^+$ T cells contribute to Mtb infection in mice (Ernst, 2018) (Figure.3). In humans, the essential role of $CD4^+$ T cells is revealed by HIV-mediated T cell reduction and decreased TB immunity (Kwan and Ernst, 2011). HIV infection resulted in increased chances of progression from latent to the active TB by 10-fold. One of the main features of adaptive immunity in TB is delayed initiation of Mtb specific $CD4^+$ T cell responses in both humans and mice compared to other pathogens (Ernst, 2018). This delay is because of the long gap between the initial infection and migration of Mtb infected DCs to the lymph nodes from the lungs (Wolf *et al.*, 2008). This allows the bacteria to replicate tremendously and get an advantage over the host to evade host immunity. The role of $CD8^+$ T cells is not clear yet, although depletion of CD8+ T cells resulted in a worsened outcome of TB in non-human primates. IFNγ production by T cells is considered crucial for TB immunity. However, in mice, $CD4^+$ cells can control the Mtb infection without expressing IFNγ (Sakai *et al.*, 2016). In rhesus macaques also, aerosol infection with Ag85A increased IFNγ producing $CD4^+$ and $CD8^+$ T cells did not confer protection against Mtb (Darrah *et al.*, 2014). Thus, although IFNγ is important for Mtb control, it is not the only effector molecule for protective immunity in TB. In fact, evidence suggests that Th17 cells which produce IL-17 is also essential for controlling Mtb infection (Khader *et al.*, 2007). Their role in TB pathogenesis is still not clearly defined and further investigations are needed to understand whether Th17 and IL-17 cells are involved in immunopathology or protective immunity in TB. Another type of T cell implicated in TB pathogenesis is regulatory T cells (Tregs). High frequencies of Tregs are found in humans with active TB disease (Chen *et al.*, 2007). Inhibiting Tregs in mice results in reduced bacterial burden in the lungs (Philips and Ernst, 2012b) but they are also important to prevent exacerbated inflammation. Therefore, understanding Treg expansion and regulation are crucial for maintaining a balance between pathology and protection in TB.

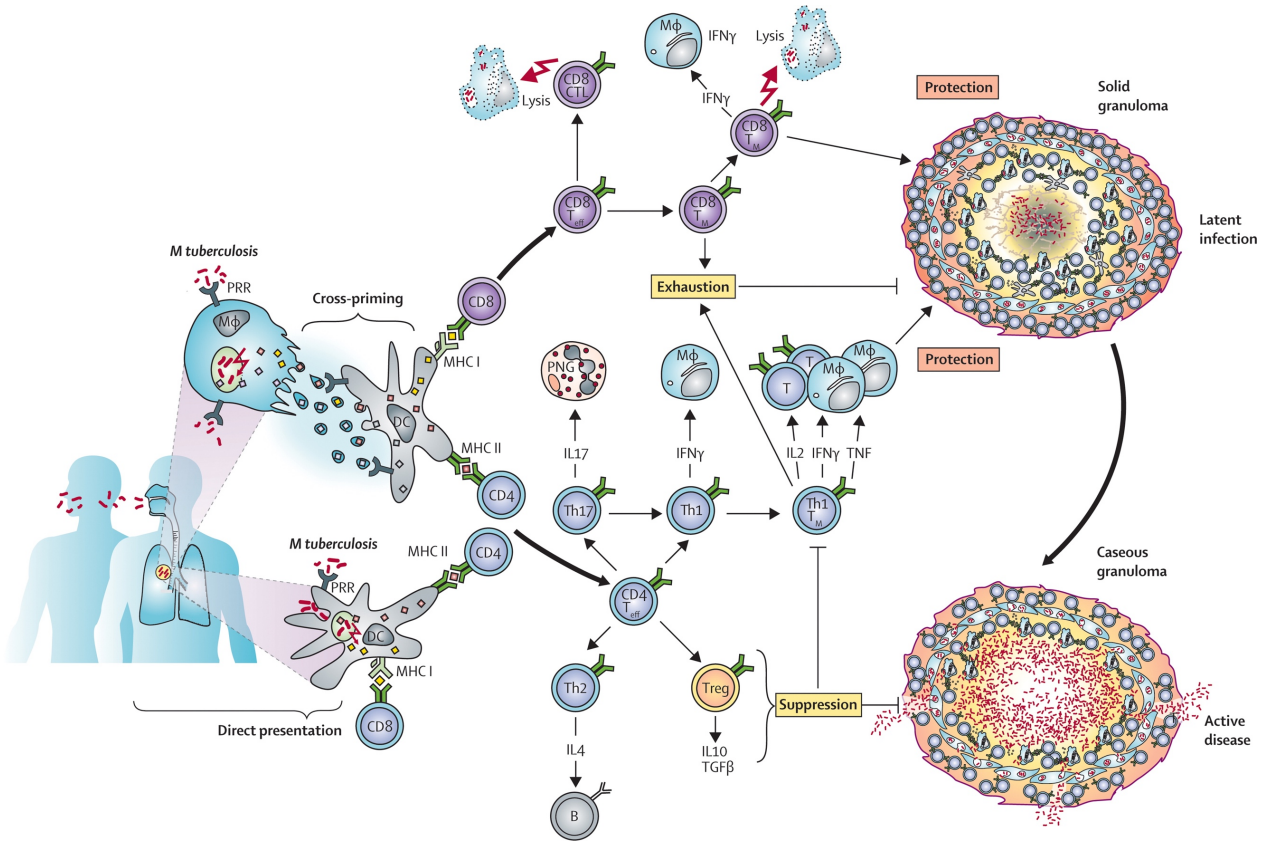

Figure 3: Immune response in TB. The collaborative teamwork of innate immune cells and T cells results in the control of Mtb. Mycobacterial peptides are presented to CD4 T cells or CD8 T cells.CD4 T helper cells can polarize into Th1, Th2 and Th17 subsets. IL-2 is secreted by Th1 cells for T-cell activation, TNF or IFN-γ for activating macrophages. Th17 cells contribute to protective immunity in the lung after vaccination by activating granulocytes. Th2 and Tregs regulate Th1-mediated protection by secreting IL-4, TGF-β or IL-10. CD8 T cells secrete TNF and IFN-γ to activate macrophages and secrete granulysin or perforins to kill Mtb. (Kaufmann, Hussey and Lambert, 2010)

1.5 Myeloid-derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSCs) are one of the major regulatory cells which are induced in pathological conditions. In normal physiological conditions, growth factors such as GM-CSF, M-CSF drives myelopoiesis and induces the differentiation of granulocytes, DCs and macrophages, respectively. In pathological conditions, such as cancer, infection or inflammation, these factors favor the generation of MDSCs where they have the immunosuppressive function (Gabrilovich and Nagaraj, 2009). These MDSCs are a heterogeneous population which consists of two subsets: granulocytic or polymorphonuclear MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). G-MDSCs resemble phenotypically and morphologically neutrophils while M-MDSCs appear similarly to monocytes. G-MDSC and M-MDSC have relatively low phagocytic activity compared to DCs and macrophages but they have increased levels of reactive oxygen species (ROS), nitric oxide (NO) production, arginase expression, PGE2 and a number of anti-inflammatory cytokines (Kumar, Patel, Tcyganov and Dmitry I. Gabrilovich, 2016).

1.5.1 Phenotypic features of MDSCs

One of the main problems with MDSCs is the difficulty in distinguishing these cells from neutrophils and monocytes as the surface markers expressed by these G-MDSCs and M-MDSCs are similar to neutrophils and monocytes, respectively. In mice, G-MDSCs can be identified as $CD11b^{+}$ $Ly6G^{hi}$ $Ly6C^{lo}$. M-MDSCs are identified as $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{hi}$ cells with low side scatter in mice. M-MDSCs lack surface markers of monocytes such as CD11c and MHC class II. Some other markers such as CD115, CCR2 and CD49d (VLA4) are also expressed by M-MDSCs (Veglia, Perego and Gabrilovich, 2018a).

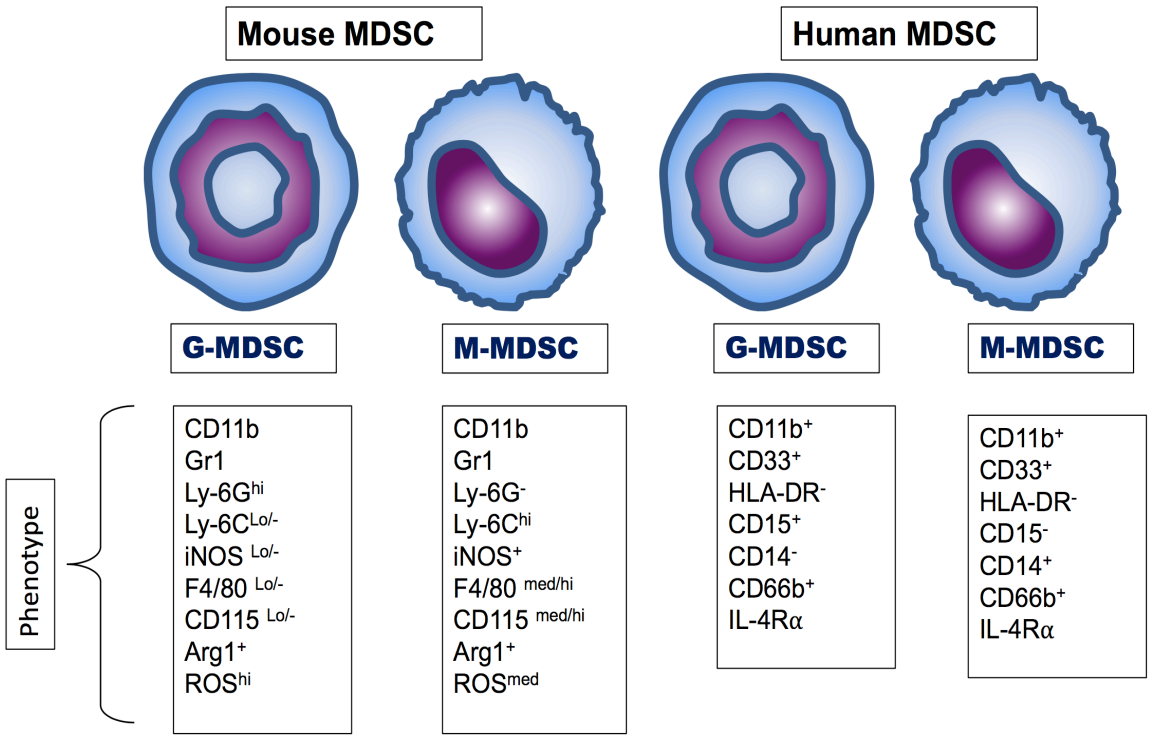

Figure 4: Phenotypical features of MDSCs. The figure shows the classical phenotype expression of several markers on G-MDSCs and M-MDSCs in mouse and human.G-MDSCs and M-MDSCs can also be distinguished on the basis their nucleus shape.G-MDSCs have polymorphic or ring shaped nucleus whereas M-MDSCs have kidney shaped nucleus. (Figure credit : Prof. Manfred Lutz)

In humans, also G-MDSCs and neutrophils have a similar phenotype: $CD11b^{+}$ $CD15^{+}$ $CD33^{+}$ $CD14^{-}$. However, different density gradients allow identification of G-MDSC and neutrophils. G-MDSC are isolated in the low-density ficoll-gradient of peripheral blood mononuclear cells (PBMCs) while neutrophils are found in the high-density fraction. Recently, $LOX\text{-}1^{+}$ (lectin-type oxidized LDL receptor 1) immunosuppressive cells have been identified to distinguish human G-MDSCs and neutrophils (Condamine *et al.*, 2016). M-MDSCs in the humans are defined as $CD11b^{+}$ $CD14^{+}$ $CD33^{+}CD15^{-}$ $HLA\text{-}DR^{-/Lo}$ while monocytes are $HLA\text{-}DR^{hi}$ (Figure.4).

1.5.2 Immunosuppressive mechanisms of MDSCs

The distinguishing feature of MDSCs compared to other innate immune cells such as dendritic cells or macrophages is their immune suppressive activity. G-MDSCs and M-MDSCs have different mechanisms by which they suppress immune responses. Some of the most prominent mechanisms are introduced below:

1.5.2.1 Reactive oxygen and nitrogen intermediates

One of the major mechanisms widely studied for MDSC suppression includes the production of nitric oxide (NO), reactive oxygen species (ROS) and arginase1 (Arg-1). M-MDSC are known to suppress T cells by secreting NO whereas G-MDSC produces large amount O^{2-}, H_2O_2 and peroxynitrite (PNT) (Veglia, Perego and Gabrilovich, 2018b). G-MDSCs suppress T-cells by cell-cell contact as ROS has a very short half-life (Corzo *et al.*, 2009). M-MDSC suppress by secreting immune suppressive molecules such as NO, Arg1 which have higher half-life than ROS (Kumar, Patel, Tcyganov and Dmitry I Gabrilovich, 2016). Furthermore, M-MDSCs suppress better than G-MDSCs (Kumar, Patel, Tcyganov and Dmitry I Gabrilovich, 2016). Previous reports have shown that H_2O_2 produced by the expanded pool of circulating, low-density granulocytes from the advanced cancer patients was associated with functional impairment and suppression of CD3 expression by T cells (Gabrilovich and Nagaraj, 2009). Deficiency in arginine inhibits T-cell proliferation by removing CD3 ζ-chain which prevents upregulation of the expression of cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 (Rodriguez, Quiceno and Ochoa, 2007) (Figure.5). Ligation of integrins, expressed on MDSCs, also promote increased ROS production after MDSC-T cell interaction (Corzo *et al.*, 2009). MDSCs suppressive activity is also associated with the L-arginine metabolism. L-arginine is a substrate of two enzymes namely, iNOS and arginase 1 (Rodriguez, Quiceno and Ochoa, 2007). iNOS is an enzyme which generates NO while Arg1 converts L-arginine to urea and ornithine. MDSCs have high levels of both Arg1 and iNOS both of which are involved in the inhibiting T-cell function. Depletion of L- arginine (via Arg1) by MDSCs from the microenvironment results in T-cell suppression (Rodriguez, Quiceno and Ochoa, 2007). NO suppress T-cell function by inducing T-cell apoptosis or blocking JAK3 and STAT5 function in T cells (Gabrilovich and Nagaraj, 2009). Peroxynitrite ($ONOO^-$) is also one of the soluble mediators formed by the chemical reaction between NO and superoxide anion which is involved in the suppression of T-cells (Pacher, Beckman and Liaudet, 2007). Peroxynitrites acts as intra and intercellular messengers as they can promote post-translational modifications by nitrating tyrosine residues in the proteins (Monteiro, Arai and Travassos, 2008). Tyrosine nitration can influence different biological activities such as cell differentiation and cell proliferation. Apart from tyrosine, they also induce nitration and nitrosylation of other amino acids such as tryptophan, cysteine and methionine (Goedegebuure *et al.*, 2011). Peroxynitrite levels are increased at the site where MDSCs accumulate in case of inflammation or

infection in the body. Peroxynitrite production by MDSCs during direct contact with T cells leads to nitration of the T-cell receptor and CD8 molecules and thereby, altering the specific peptide binding of the T cells (Lindau *et al.*, 2013). Peroxynitrites have a role in inducing apoptosis of antigen-activated T cells by downregulating intracellular levels of the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) (Kumar, Patel, Tcyganov and Dmitry I. Gabrilovich, 2016)

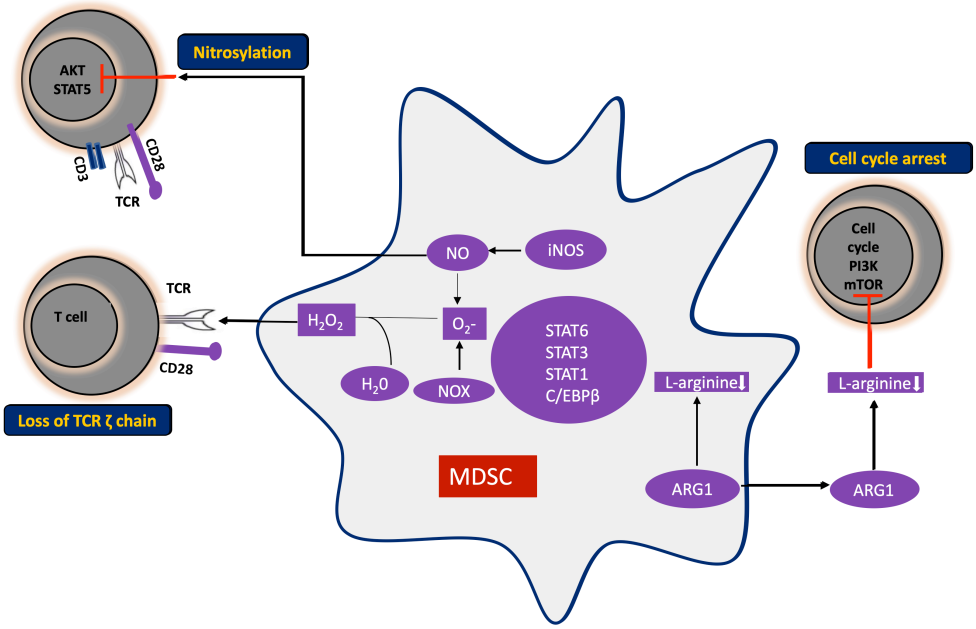

Figure 5: Mechanisms of MDSC-mediated immune suppression. MDSCs suppress T cells by several mechanisms. Some of them are depicted in the above figure. MDSCs deprive T cells of amino acids required for T-cell growth and differentiation which results in cell cycle arrest. MDSCs secrete hydrogen peroxide which causes loss of TCR ζ-chain. MDSCs can also secrete peroxynitrite which leads to nitration and nitrosylation of the TCR signaling complex.

1.6 Signaling pathways in MDSC

To understand MDSC biology, it is important to understand how MDSC expansion is regulated. MDSC generation and expansion include several signaling pathways such as Janus Kinase and signal transducers (Jak/STAT), phosphoinositol-3-OH-Kinase (PI3K), Rat sarcoma (Ras) and the transforming growth factor-β (TGFβ) (Trikha, Carson and III, 2014) (Figure.6). In the plasma membrane, the enzyme PI3K catalyzes the phosphorylation of inositol phospholipids. PI3K/AKT signaling regulates cell survival, migration, cell growth and metabolism. In resting conditions, these proteins are present in the cytoplasm and translocate to the cell membrane upon lipid phosphorylation. In myeloid cells, PI3K is activated by the several cytokines such as GM-CSF, IL-6 and interferons (Trikha, Carson and III, 2014). Also, MDSC function is regulated by PI3K/AKT signaling in aging mice (Enioutina, Bareyan and Daynes, 2011). Jak/STAT pathway is essential for mediating inflammatory response. Moreover, Jak/STAT signaling can be activated by IFNα/β and IFNγ. In the absence of STAT-1, MDSCs fail to suppress T cell proliferation due to reduced iNOS

and Arg1 activities (Mundy-Bosse *et al.*, 2011). Another report showed that splenic $Gr1^+$ $CD11b^+$ MDSCs had reduced phosphorylation of STAT-1 upon IFN-γ stimulation (Mundy-Bosse *et al.*, 2011). In addition, STAT-3 also functions as a regulator of MDSCs suppressive function by regulating Arg1 activity in head and neck cancer patients (Vasquez-Dunddel *et al.*, 2013). Activation of STAT-3 influences downstream S100A8 and S100A9 which are pro-inflammatory proteins secreted by MDSCs (Sinha *et al.*, 2008). Moreover, STAT-5 has a role in MDSC survival. Conditioning of MDSCs with GM-CSF promoted STAT-5 and inhibited STAT-3 activation (Ko *et al.*, 2010). Thus, these findings suggest that STAT-1, STAT-5 and STAT-3 are the key factors for MDSC expansion, activation and their immune suppressive function.

In MDSCs, TLRs have a key role in NF-kB activation through the MyD88 pathway. MyD88 is required for MDSC accumulation in a model of sepsis (Delano *et al.*, 2007). Furthermore, MDSC function can be mediated by TLR4 signaling during inflammation and infection (Bunt *et al.*, 2009). M-MDSCs accumulate at the infected site and require MyD88-dependent BCG-specific signals to evade the infection site (Martino *et al.*, 2010). MDSCs can also be activated by IL-1β *in vitro* and *in vivo* through NF- κB pathways (Tu *et al.*, 2008). These reports suggest that NF-κB is also involved in the expansion of MDSCs and their immune suppressive function.

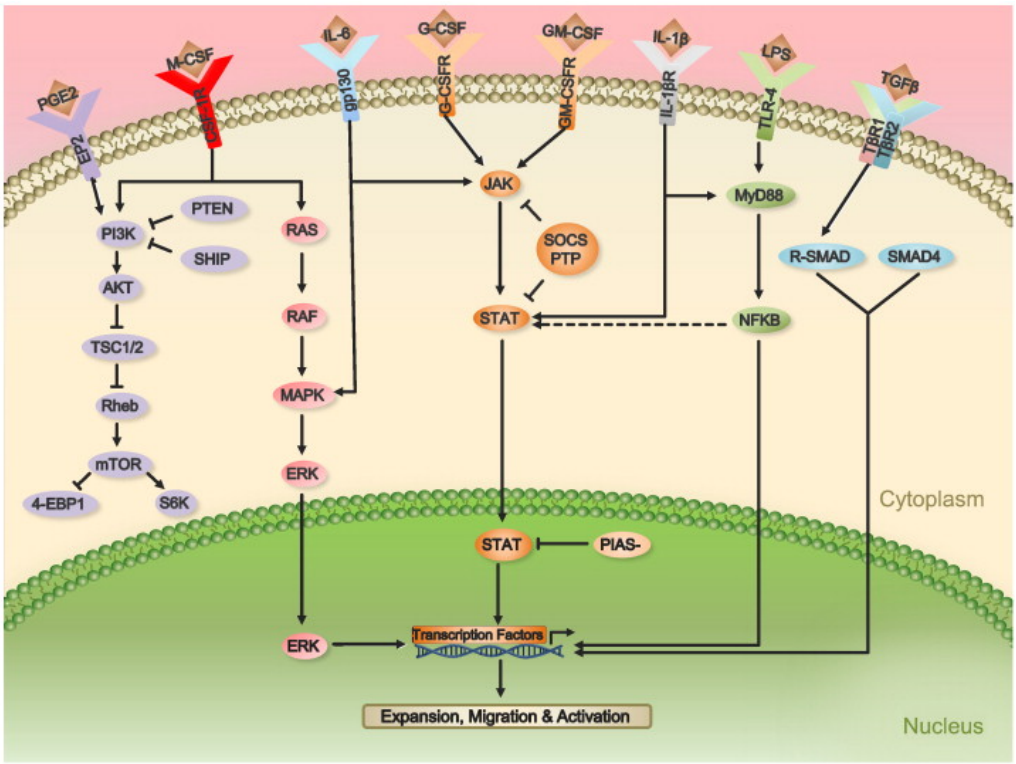

Figure 6: Signaling pathways in the MDSC regulation and function. Cytokines secreted by hematopoietic cells bind to their respective receptors and activate Ras/MAPK, PI3K, Jak/STAT and TGF-β pathways. This leads to the activation of transcription factors which binds to their putative sites on the gene promoters and thereby, activating genes involved in survival, proliferation and migration of MDSCs. (Trikha, Carson and III, 2014)

1.7 MDSCs in TB

MDSCs have been extensively studied in cancer but their role in chronic infections has only recently gathered attention such as in TB research. Although the report on MDSCs and BCG was published around 40 years ago, where they found that systemic delivery of BCG activates natural suppressor cells in the bone marrow and spleen (Bennett, Rao and Mitchell, 1978), although by that time MDSCs was not described. Later, MDSCs were found in the pleural effusions of patients diagnosed with active TB (du Plessis *et al.*, 2013). Also, in the murine model of TB, MDSCs phagocytose Mtb and secrete IL-10, IL-6 and IL-1α (Knaul *et al.*, 2014). Furthermore, MDSCs were also observed in the bone marrow of Mtb infected mice (Tsiganov *et al.*, 2014a). Higher frequency of MDSCs was associated with higher levels of IL-4α and targeted depletion of MDSC by anti-Gr-1 antibodies or all-trans-retinoic acid (ATRA) resulted in the better outcome of the disease (Knaul *et al.*, 2014). Recruitment of $CD11b^+$ $Gr1^+$ cells occurs in the lung of both TB-susceptible and-resistant mice (Knaul *et al.*, 2014). Additionally, expansion of MDSCs in the lung and blood of TB patients correlate with enhanced L-arginine catabolism and NO production. After successful TB chemotherapy, the frequency of MDSCs are reduced in the TB patients (du Plessis *et al.*, 2013), (El Daker *et al.*, 2015). Both monocytic and granulocytic subsets are recruited at the infection site as well as in the blood depending on the severity of disease and other factors (du Plessis *et al.*, 2013), (El Daker *et al.*, 2015). Increased frequencies of MDSCs worsen disease pathology. Recently, M-MDSCs have been described *in vitro* granuloma model of TB. They found that MDSCs promoted bacterial growth in the granulomas by secreting IL-10. Furthermore, M-MDSCs also had increased PD-L1 expression and suppressed T-cell proliferation (Agrawal *et al.*, 2018). MDSCs are considered as one of the cellular targets for host-directed therapies against active TB disease (du Plessis *et al.*, 2018). However, MDSCs in Mtb field awaits further investigations about the interaction of this deadly pathogen with these myeloid regulatory cells and underlying mechanisms. In this work, the role of lipid-rich surfaces in MDSC activation and immune response to mycobacterial infections has been explored.

1.8 Lipid rafts in immune signaling

Lipid rafts are dynamic regions in the outer leaflet of plasma membrane enriched in cholesterol and glycosphingolipids (Shaw, 2006). In addition, they also contain glycosylphosphatidylinositol (GPI)-anchored proteins and proteins involved in signal transduction (Boscher and Nabi, 2012). Lipid raft domains are resistant to detergents and are potent due to their ability to assemble and disassemble. Modulation of the lipid rafts is associated with the pathogenesis of various human diseases (Varshney, Yadav and Saini, 2016). Therefore, in this work, we studied the interaction of mycobacteria with lipid-rich platforms of the plasma-membrane of MDSCs.

1.8.1 Caveolae

George Palade first identified the morphology of plasma membrane invaginations in 1953 from electron micrographs of endothelial cells (Bruns and Palade, 1968). As they looked like little caves Yamada named them as caveolae in 1955 (YAMADA, 1955). In 1989, caveolin was first described as a ~22-kDa tyrosine-phosphorylated protein *in vivo* in Rous sarcoma virus-transformed chick fibroblasts (Glenney, 1989). Later, caveolin was reported as a protein component of caveolae involved in the intracellular transport of molecules (Karen G. Rothberg *et al.*, 1992). Caveolae are highly hydrophobic membrane domains which are detergent resistant and consists of cholesterol and sphingolipids. It is now quite clear that Caveolin-1(Cav-1) and another protein called cavins are needed to form the caveola structure and function. Caveolae are defined as bulb-shaped pits of 60-80 nm diameter on the plasma membrane having oligomeric caveolins and cavins. One flask shaped caveola contains 140-150 Cav-1 molecules (Pelkmans and Zerial, 2005). The mechanism of endocytosis by caveolae mediated pathways has been debated for many years as there are mixed reports of how it occurs. Caveolae with Cav-1 and cholesterol traffic to the early endosomal compartment and then recycle back to the cell surface. This to and from shuttling of caveola maintains the caveolar density. The cholesterol depletion results in the disruption of caveola formation (K G Rothberg *et al.*, 1992). One of the cargos that are transported through the caveolar pathways is ganglioside GM1. Caveolae are abundantly expressed in some cell types such as, smooth-muscle cells, endothelial cells, adipocytes and fibroblasts (Parton and Howes, 2010)

1.8.1.2 Caveolin proteins

Caveolin proteins are considered as the key components for caveola formation. There are three isoforms of caveolins termed as caveolin-1 (Cav-1), caveolin-2 (Cav-2), caveolin-3 (Cav-3). All three of them have palmitoylation sites, a cytoplasmic N and C terminal and a scaffolding domain which is involved in the signal transduction (Chidlow and Sessa, 2010) (Figure.7). Cav-1 and Cav-2 are all integral membrane proteins with a hairpin like domain structure. Both amino and the carboxyl terminus are facing towards the cytoplasmic side of the membrane. Loss of Cav-1 and Cav-3 results in the caveola disruption whereas deficiency of Cav-2 alone do not affect caveola formation (Parton and Simons, 2007). Caveolins are synthesized in the endoplasmic reticulum (ER) and then transported to the Golgi complex from where they are transported to the plasma membrane (Tagawa *et al.*, 2005). Caveolin, GM1 along with the glycosylphosphatidylinositol (GPI)-anchored proteins are delivered to the plasma membrane by syntaxin-6 (Choudhury *et al.*, 2006).

1.8.1.3 Functions of caveolin-1

Cav-1 is an integral membrane protein holding hydrophobic amino acids embedded into the inner leaflet of the membrane bilayer. Cav-1 protein is present on the inner leaflet of the membrane as well as on the cytoplasm. The scaffolding domain (N-terminal) of this protein is involved in the binding to the sphingolipid and cholesterol-rich membrane domains and can also bind to the signaling

molecules (Kiss and Botos, 2009).

Endocytosis and membrane trafficking: One of the major functions of Cav-1 is endocytosis. Ligands such as autocrine motility factor (Benlimame, Le and Nabi, 1998), folic acid (Nichols, 2003), lactosyl ceramides and pathogens such as SV40 virus (Pietiäinen *et al.*, 2004), polyoma virus (Richterova *et al.*, 2001), echovirus (Pietiäinen *et al.*, 2004), respiratory synctia virus (Werling *et al.*, 1999), certain FimH-expressing bacteria(Shin, Gao and Abraham, 2000) are shown to be endocytosed by caveolae-mediated pathways. Endocytic caveolar carriers fuse with caveosome in a Rab5-independent or Rab-dependent manner.

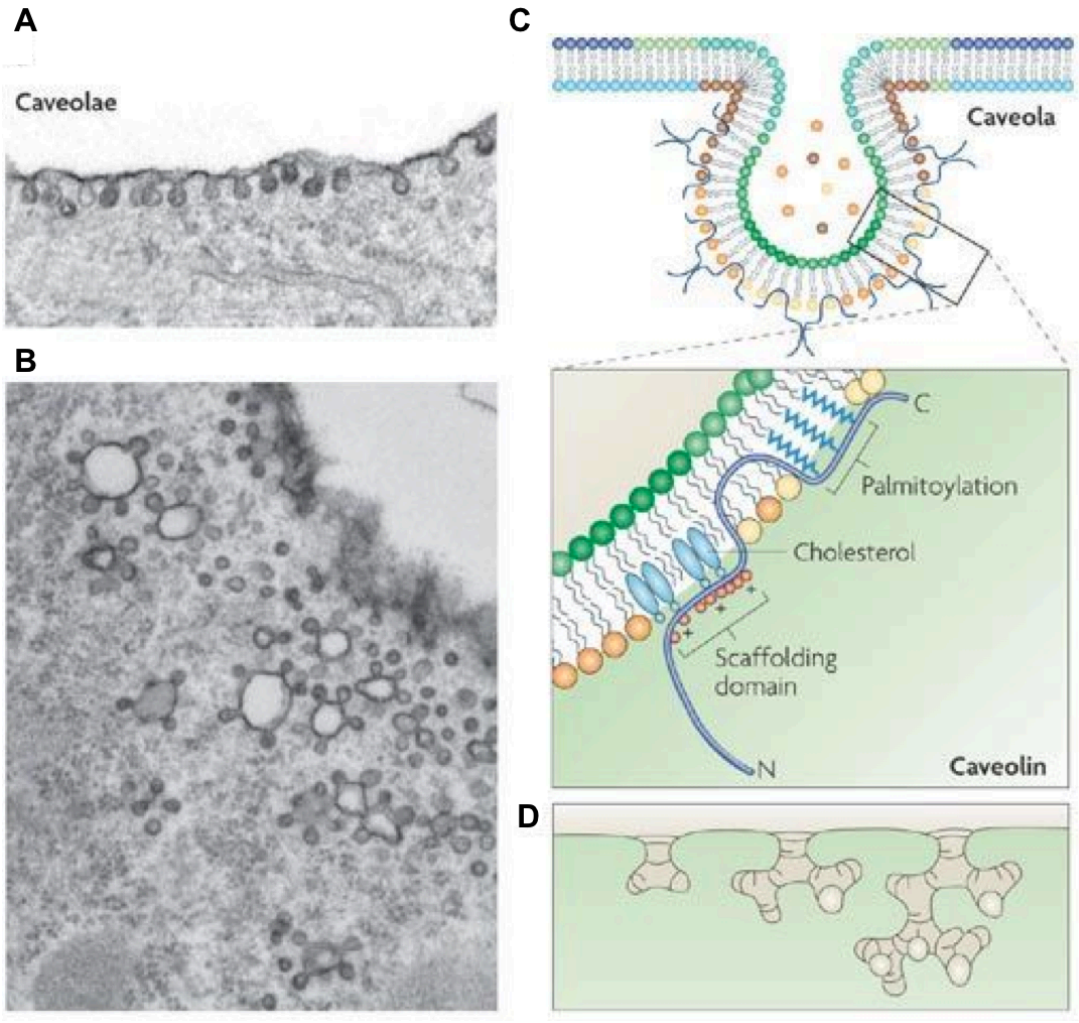

Figure 7: Structure of caveolae and caveolins. A&B) Microscope image refers to caveolae in adipocytes which can be seen as flask shaped or bulb shaped pits. **C&D)** Caveolins are inserted into the caveolar membrane. N and C terminal face the cytoplasm and the hairpin structure domain is embedded within the membrane bilayer. Cholesterol binds to the scaffolding domain of caveolin which a highly conserved region of caveolin is. The C-terminal domain is close to the intramembrane domain and modified by palmitoyl groups that are inserted into the lipid bilayer. Adapted from (Parton and Simons, 2007).

Signal transduction: Cav-1 is dynamically associated with a number of signaling molecules such as endothelial nitric oxide synthase, Src family tyrosine kinase, PI3K, heme oxygenase-1 GTPases, components of MAPK pathway etc., by forming complexes (Li, Couet and Lisanti, 1996)·(Feng *et al.*, 2013)·(Engelman *et al.*, 1998). Cav-1 protein motifs can recruit several proteins and lipids to the caveolae to help intracellular trafficking of molecules or pathogens and regulate signaling pathways. Cav-1 has been shown to inhibit signaling pathways by dampening the associated proteins such as H-Ras, c-Src, eNOS and MAP kinase (Engelman *et al.*, 1998),(Mirza *et al.*, 2010). *Cav-1*$^{-/-}$ mice had a significantly increased endothelial NO synthase (eNOS) expression in their lung and showed impaired of NF-KB activity (Garrean *et al.*, 2006a). Moreover, Cav-1 also regulates TLR signaling by altering eNOS activity (Feng *et al.*, 2013). Another study has shown that Cav-1 regulates CD36, CD14 and MyD88 protein expression in macrophages in addition to TLR4 signaling (Tsai *et al.*, 2011a). These studies suggest that TLR recruitment in lipid rafts is dependent on Cav-1. Thus, Cav-1 plays multiple roles including plasma membrane organization.

Lipid transport: Cav-1 is also involved in the import and export of cholesterol. It can bind long- chain unsaturated fatty acids with high affinity (Trigatti, Anderson and Gerber, 1999) and interacts with GM1 gangliosides in the caveolae (Choudhury *et al.*, 2006). Cav-1 can traffic cholesterol in a lipoprotein chaperone cluster containing cyclophilin A, cyclophilin 40, HSP56 and Cav-1 through the cytoplasm to the cell membrane (Igbavboa *et al.*, 2009). Others have shown that Cav-1 transports newly synthesized cholesterol from the ER to the cell membrane (Figure.8). Thus, Cav-1 is always shuttling between plasma membrane, Golgi and ER.

1.8.1.4 Caveolin-1 knockout mice

To study the role of Cav-1 several groups have used Cav-1 knockout mouse models. Although these mice are viable but they have a shortened life-span (Razani *et al.*, 2001)·(David S. Park *et al.*, 2003). Cav-1 knock out mice has aberrant pulmonary and vascular phenotypes which include increased cellularity in the heart and lungs (Razani *et al.*, 2001)·. Moreover, they are also reported to have insulin resistance and increased proliferation of adipose tissue (Razani *et al.*, 2002). *Cav1*$^{-/-}$ mice have lungs with thickened parenchyma and alveolar septae leading from hyperproliferation of bronchial and alveolar epithelial cells (Murata *et al.*, 2007) and they also display pulmonary fibrosis (Drab *et al.*, 2001). Re-expression of Cav-1 in endothelial cells in these knockout animals restored their pulmonary and vascular defects (Murata *et al.*, 2007). In cardiac tissue, these mice displayed increased eNOS expression (Cohen *et al.*, 2003). Furthermore, there are also reports with increased p42/44 MAPK (ERK1/2) activation in cardiac fibroblasts and increased Akt activation in the lung vasculature of Cav-1 deficient mice (Cohen *et al.*, 2003),(Murata *et al.*, 2007). Expression of Cav-1 is upregulated in senescent cells and old animals (Volonte *et al.*, 2002). Mice lacking Cav-1 are more resistant to LPS-induced inflammatory injury in the lung (Garrean *et al.*, 2006b). In contrast to this, Cav-1 deficient mice had a significantly reduced survival chance in response to *Salmonella*

typhimurium infection. They also have an increased secretion of inflammatory chemokines, cytokines and NO (Medina *et al.*, 2006). In *Pseudomonas aeruginosa* infection, *Cav1*$^{-/-}$ mice had an increased mortality rate, bacterial burdens and inflammatory response (Gadjeva *et al.*, 2010).

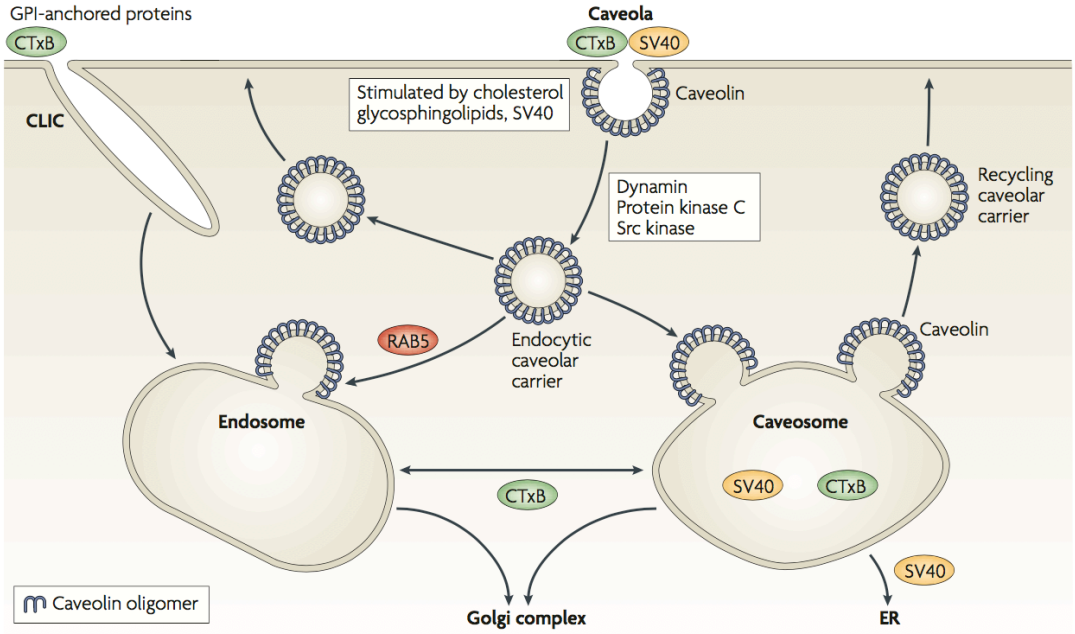

Figure 8: Endocytosis of caveola. The figure shows budding and binding of caveolae at the cell membrane to cholera toxin and SV40. This is mediated by dynamin, protein kinase C and tyrosine kinase such as Src kinases. Caveolae fuse with caveosomes or with early endosomes. SV40 is then transported to the ER. Endocytic carriers are then carried back to the cell membrane. Cholera toxin is transported to the Golgi complex through early endosomes (Parton and Simons, 2007).

1.8.2 Acid Sphingomyelinase (ASM)

Plasma membrane consists of sphingolipids which have the tendency to associate with each other through hydrogen bonds (Simons and Ikonen, 1997). The most abundant sphingolipid in the eukaryotic plasma membrane is sphingomyelin (SM). Acid sphingomyelinase(ASM) is present in the lysosomes and generates ceramide by hydrolyzing SM on the membrane (Truman *et al.*, 2011) (Figure.9). Therefore, it plays a role in metabolic functions and transmembrane signaling. Based on the pH three different SMases have been characterized: neutral, alkaline and acid SMases (Truman *et al.*, 2011). ASMase is the most studied sphingomyelinase but its regulation is still unclear.

ASMase can be of two types: lysosomal form (L-ASMase) or secretory protein(S-ASMase) (Truman *et al.*, 2011). These enzymes can be present on the inner leaflet of endosomes, lysosomes and phagosomes. Lysosomal stability in fibroblasts also depends on ASMase (Kirkegaard *et al.*, 2010). Depending on the location of ASM in the cell, it can have different functions. ASMase is important for the exocytosis of cytotoxic effector molecules such as perforin and granules in $CD8^+$ T-cells (Herz *et al.*, 2009). ASM also controls T lymphocyte migration by regulating ICAM-1 function in brain endothelial cells (Lopes Pinheiro *et al.*, 2016). ASM-mediated ceramide generation is crucial for cell proliferation, apoptosis and immune modulation in cancer cells. The activity of ASM is increased in the lung tumor environment and blood of patients with lung cancer (Kachler *et al.*, 2017).

ASM deficiency in the murine model of adenocarcinoma resulted in reduced tumor development correlating with increased Th1 and cytotoxic T-cell mediated anti-tumor immunity (Kachler *et al.*, 2017).

Activation of ASMase is crucial for inducing apoptosis through signaling mediated by Fas/CD95 and TNF-α (Grassmé *et al.*, 2001)·(Schütze *et al.*, 1992) . Patients with clinical depression have an increased ASM activity in peripheral blood mononuclear cells (Kornhuber *et al.*, 2005). Drugs for the treatment of depression such as desipramine and imipramine have been shown to reduce ASM activity (Albouz *et al.*, 1986). However, the involvement of ASMase in depressive disorders is not clearly understood. Blocking ASMase activity results in the suppression of LPS induced TNF-α secretion by macrophages and also reduce the outcome of the inflammatory bowel disease (Sakata *et al.*, 2007a). During the phagocytosis of Listeria monocytogenes, ASMase is essential for fusion of lysosomes with the phagosome (Schramm *et al.*, 2008). Cells lacking ASM have decreased ability to ingest pathogens such as *Staphylococcus aureus* and *Neisseria gonorrhoeae* (Esen *et al.*, 2001)·(C R Hauck *et al.*, 2000).This also implies that a defect in ASM helps the infection to survive in the cell. Despite a number of studies on ASM in cellular processes, many key questions regarding the role of ASM remains unanswered. It is important to understand the biochemical and cellular regulation of ASM function to assess the impact of ASM and ceramide generation on the cell surface. The role of ASM in mycobacterial infection in MDSCs has also not been explored.

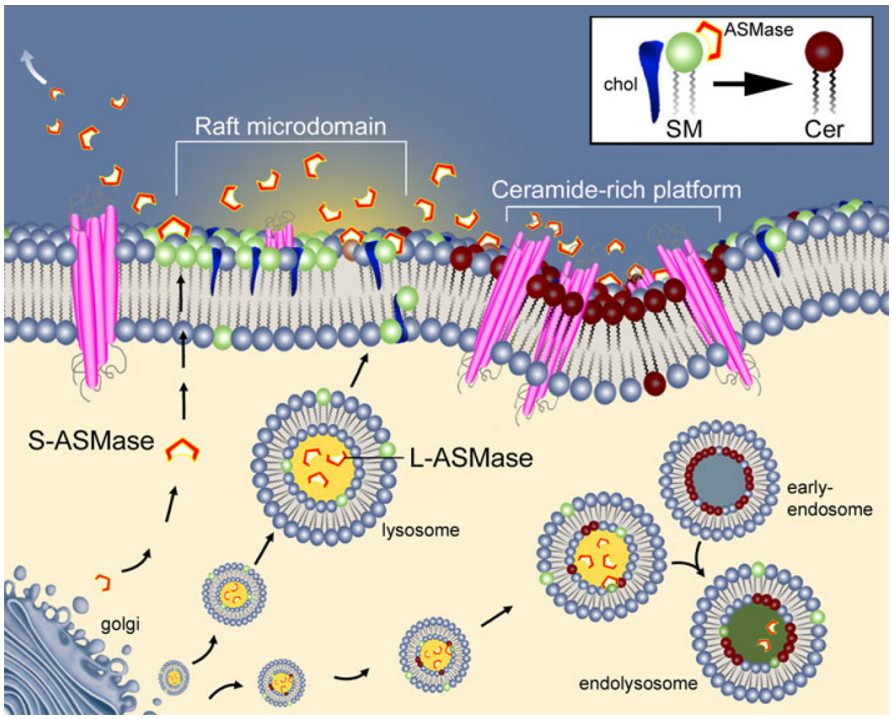

Figure 9: Acid sphingomyelinase. ASM is located in the lysosomes and fuse to the cell membrane containing sphingomyelin. This causes hydrolysis of sphingomyelin to ceramide-rich platforms which mediate transmembrane signaling. Adapted from (Truman *et al.*, 2011)

1.8.3 Asialo-GM1

Gangliosides consist of a common hydrophobic moiety and an oligosaccharide chain containing one or more sialic acid residues which are hydrophilic (Huwiler *et al.*, 2000). Most of the gangliosides are on the outer leaflet of the cell membrane which maintains membrane structure and organization (Zeller and Marchase, 1992). 10% gangliosides are localized in endoplasmic reticulum and mitochondria. Gangliosides can also segregate and form clusters with cholesterol or lipid rafts or caveolae (Mori *et al.*, 2012). Additionally, they are also involved in cell growth regulation, recognition and adhesion and signal transduction (d'Azzo, Tessitore and Sano, 2006). GM1 is one of the most commonly known markers of lipid rafts and also accumulates in the immunological synapses (Thomas *et al.*, 2004). Alveolar, lung interstitial macrophages, blood monocytes and spleen macrophages are positive for asialoGM1 (Riser, Laybourn and Varani, 1988). Another report showed that MDSCs also express asialoGM1 marker on their cell surface (Rößner *et al.*, 2005a). AsialoGM1 is expressed by NK cells and T cells and further upregulated in viral infection (Moore *et al.*, 2008). Asialo-GM1 was found to co-localize in the lipid raft structures in $CD8^+$ T cell and NK cell from the lungs of virus-infected mice. Depletion of asialo-GM1 results in delayed viral clearance and reduced IFNγ levels (Moore *et al.*, 2008). Asialo-GM1 expression can vary according to the cell type.

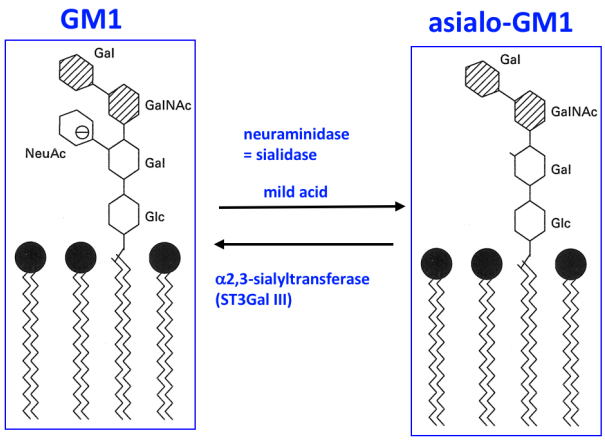

Figure 10: Structure of GM1 and asialo-GM1. GM1 is a glycosphingolipid consisting of sialic acid (n-acetylneuraminic acid) linked to sugar chains. The letter G refers to ganglioside and subscript M indicate that molecule contains mono-sialic acid. The number 1,2,3 indicates carbohydrate sequence that is attached to the ceramide. Asialo-GM1 refers to the ganglioside GM1 without sialic acid.

Expression of asialo-GM1 on the cell surface correlates with the cancer progression and is important to regulate cancer metastatic potential by affecting migration, adhesion and invasion (Van Slambrouck *et al.*, 2009). Also, asialo-GM1 is a membrane receptor for *Pseudomonas aeruginosa*

expressed at the surface of respiratory epithelial cells(de Bentzmann *et al.*, 1996). Bacterial flagellin interacts with TLR5 and asialo-GM1 to start an immune response (Figure. 11). Flagellin-induced release of ATP activated Ca2+ mobilization and Erk1/2 phosphorylation is mediated by asialo-GM1 and TLR5 cooperation (McNamara *et al.*, 2006). In the past decades, several reports investigated the role of gangliosides for signaling pathways and immune responses. However, asialo-GM1 has not been investigated so far for Mtb infections.

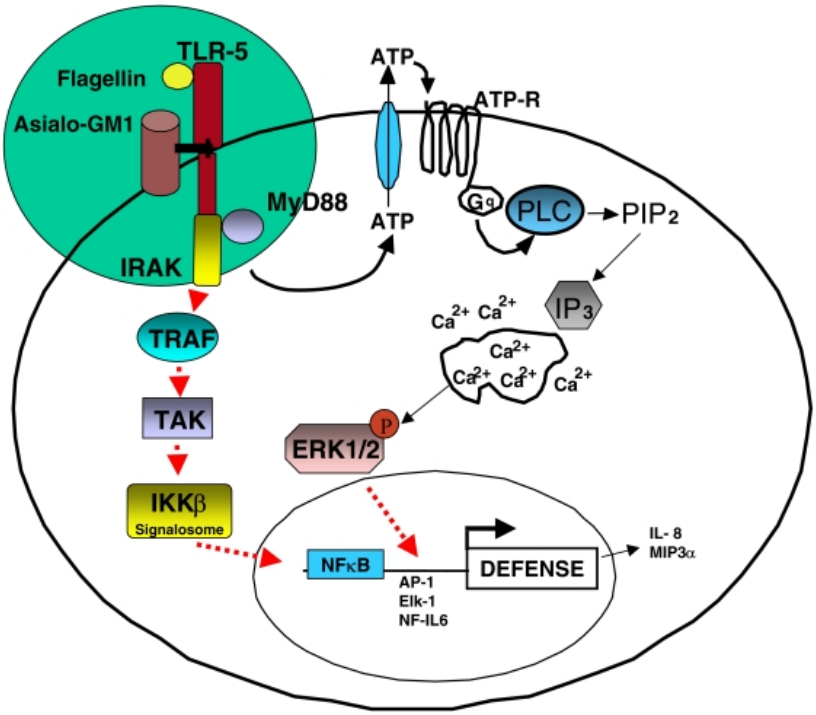

Figure 11: Cooperation of TLR5 and Asialo-GM1. Flagellin leads to an enhanced association between asialo-GM1 and TLR5 and results in the autocrine release of ATP which binds and activates a G-protein coupled receptor on the cell membrane. This further activates Ca^{2+} mobilization and Erk1/2 phosphorylation. (McNamara *et al.*, 2006)

1.9 Aim of the thesis

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells massively induced during TB infection. MDSCs are considered one of the cellular targets for host-directed therapies against active TB disease as depletion of MDSCs is associated with the improved condition in TB patients. However, MDSCs in Mtb field awaits further investigations about the interaction of this deadly pathogen with these myeloid regulatory cells and underlying mechanisms. Lipid-rich areas of the cell surfaces have been shown to constitute major entry sites for microbes, including mycobacteria and are associated with immune activation, signal transduction and bacterial persistence. Therefore, in this study role of lipid raft components in mycobacteria activated MDSCs was investigated.

The main aims of this thesis were:

1. To evaluate the functional role of Caveolin-1 in BCG-activated MDSCs
2. To investigate the role of Acid Sphingomyelinase in mycobacteria-infected MDSCs
3. To examine the role of asialo-GM1 on BCG-stimulated MDSCs

2.Materials

2.1 Mice

For this study, C57BL/6 mice were used to generate bone-marrow-derived MDSCs (Rößner *et al.*, 2005a),DCs (Lutz *et al.*, 1999) and macrophages. C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and bred under specific pathogen-free conditions in our animal facility at the Institute of Virology and Immunobiology, Wuerzburg, Germany. The following mice strains were also used to generate bone marrow-derived MDSCs, DCs and macrophages: *Cav1*$^{-/-}$ mice (B6. Cg-Cav^{tm1Mls}/J, JAX mice) were kindly provided by Elke Burgermeister. ASM$^{-/-}$ Mice were kindly provided by Sibylle Schneider-Schaulies.

2.2 Reagents

2.2.1 Chemical reagents

Product	Company
Agarose	Roth (Karlsruhe, Germany)
Ammonium per sulphate	Applichem (Darmstadt, Germany)
Amitryptiline	Sigma-Aldrich (Germany)
Asialo-GM1	Sigma-Aldrich (Germany)
β-mercaptoethanol	Sigma-Aldrich (Germany)
β-Cyclodextrine	Sigma-Aldrich (Germany)
BSA	Roth (Karlsruhe, Germany)
Catalase	Sigma-Aldrich (Germany)
Cytochalasin D	Sigma-Aldrich (Germany)
DAPI (4',6-diamidino-2-phenylindole)	eBioscience
Ethidium Bromide	Roth (Karlsruhe, Germany)
FCS	PAA laboratories (Austria)
Formaldehyde (37%)	Roth (Karlsruhe, Germany)
Hygromycin	Sigma-Aldrich (Germany)
IC Fixation buffer	Affymetrix eBioscience
Kanamycin	Sigma-Aldrich (Germany)
L-Glutamine	PAA Laboratories (Pasching, Austria)
LPS (*E. coli* 0127: B8)	Sigma-Aldrich (Germany)
Methanol	Applichem (Darmstadt, Germany)
Middlebrook 7H9 broth	BD Difco
Mycobacterium tuberculosis H37R	Difco Laboratories (Detroit, USA)

Pam_3CSK_4	Sigma-Aldrich (Germany)
Phosflow perm buffer III	BD Biosciences (Germany)
Sodium azide (NaN_3	Roth (Karlsruhe, Germany)
TMB (3,3',5,5'-Tetramethylbenzidine)	eBioscience (Frankfurt, Germany), BD
Tris	Applichem
Triton X-100	Sigma-Aldrich (Germany)
Trypan blue	Sigma-Aldrich (Germany)

2.2.2 Antibodies

2.2.2.1 Primary antibodies

(a) For FACS and microscopy following primary antibodies were used:

Antigen	Clone	Dilution (fold)	Company
Arginase	A1exF5	1:100	BD/invitrogen
Asialo-GM1	Poly 21460	1:100	BioLegend
Caveolin-1	D48G3	1:100	CST
CD3	145-2C11	1µg/ml	House-made
CD4	GK1.5	1:200	BioLegend
CD8α	53-6.7	1:200	BioLegend
CD11b	M1/70	1:200	BioLegend
CD11c	N418	1:200	BioLegend
CD28	E18	1µg/ml	House-made
CD40	3/23	1:100	BioLegend
CD69	H1.2F3	1:100	BioLegend
CD86(B7.2)	GL1	1:100	BioLegend
DC-SIGN	5H10	1:100	eBioscience
E-Cadherin	DECMA1	1:100	BioLegend
F4/80	BM8	1:100	BioLegend
IL-6	MP5-20F3	1:100	BioLegend
IL-12p40	C15.6	1:100	BioLegend

Ly-6C	HK1.4	1:200	BioLegend
Ly-6G	1A8	1:200	BioLegend
Nos2	CXNFT	1:100	BioLegend
PD-L1	10F.9G 2	1:100	BioLegend
Phospho-AKT	SDRNR	1:100(FACS)	eBioscience
Phospho-p38 MAPK	4NIT4KK	1:100	eBioscience
TLR2	T2.5	1:100	BioLegend
TLR4	MTS510	1:100	BioLegend

(b) For western blot analysis following primary antibodies or reagents were used:

Antigen	Dilution	Company
Phospho-p38 MAPK	1:1000	CST (#9211)
Total p38 MAPK	1:1000	CST (#9212)
Phospho-ERK1/2	1:1000	CST (#9101)
Total ERK1/2	1:1000	CST (#4695)
Phospho-NFKB p65	1:1000	CST #3033
Total NFKB p65	1:1000	CST (#8242)
Phospho-AKT	1:2000	CST (#4060)
Total AKT	1:2000	CST (#4691)

2.2.2.2 Secondary antibodies

For FACS and microscopy analysis following secondary antibodies were used:

Antigen	Host	Conjugate	Dilution(fold)	Company
F(ab')$_2$ anti rabbit IgG (H+L)	Donkey	DyLight 649	300	Jackson Immuno Research (JIR)

F(ab')$_2$ anti rabbit IgG (H +L)	Goat	Cy3	400	JIR
Streptavidin	X	DyLight 549	300	JIR
Streptavidin	X	PE or APC or PE-Cy7 or Cy3	300	BioLegend

2.3 Buffers, media and solutions

For the preparation of buffers and solutions,ultrapure Mili-Q water was obtained from Mili-Q water purification systems(Millipore, Schwalbach/Ts, Germany).

Buffers	Composition
PBS buffer (phosphate-buffered saline), pH7.4	0.2g KCl 8.0 g NaCl 1.15 g KH_2PO_4 1.15 g Na_2HPO_4 Fill up to 1l Milli-Q water
RPMI 1640 complete medium	500ml RPMI 1640 (PAA Paching Austria) 10% heat-inactivated sterile filtered FCS(PAA) 100U/ml penincillin (PAA) 100µg/ml streptomycin (PAA) 2mM L-glutamine (PAA) 50mM β-mercaptoethanol (Sigma-Adrich)
FACS Buffer	500 ml PBS 0.1% BSA (Roth) 0.1% NaN3 (Roth)
2% FA	35ml PBS 2ml FA (37%) Roth
Perm buffer	PBS 0.1% BSA (Roth) 0.1% NaN3 (Roth) 0.5% Saponin (Sigma-Aldrich)

ELISA wash buffer	0.05% Tween 20 (Applichem) in PBS
ELISA assay diluent	10% FCS (PAA) in PBS
TBST wash buffer	20mM Tris-HCl (pH 7.6) 150mM NaCl 0.05% Tween 20 (Applichem)
BCG media	4.70g Middle brook 7H9 in 900ml ddH_2O 0.05% Tween 80 500µl glycerol
Albumin dextrose catalase	5g BSA in 100ml ddH_2O 2g Dextrose 0.004g Catalase 0.850g NaCl
PBST	10% FCS (PAA) in PBS
Protein gel buffer	Glycine 1.44 % (w/v) Tris base 0.303% (w/v) SDS 0.1% (w/v) Dissolve in dH_2O
Laemilli buffer	10% - SDS 250 mM - Tris.HCl pH 8.0 50%-Glycerol 500 mM- Dithiothreitol (DTT) 0.25%-Bromophenol Blue (BPB) Dissolve in dH_2O
Transfer Buffer	25 mM Tris 192 mM Glycine 20% Methanol
Blocking buffer for Western Blot	5%-Non-fat dry milk powder 0.05%-Tween 20 Dissolve in PBS 1 liter
Resolving gel	37.5% (v/v) 1.5 M Tris.Cl pH 8.7 30% (v/v)4KBisacrylamide 0.2% (v/v) Ammonium per sulfate (APS) 1% (w/v) Sodium dodecyl sulfate (SDS) 0.25% (v/v) TEMED 22.25%(v/v) dH2O
Stacking gel	12.4% (v/v) 1.5 M Tris.Cl pH 8.7

	12.4% (v/v) 4K Bisacrylamid 0.2% (v/v) Ammonium per sulfate (APS) 1% (w/v) Sodium dodecyl sulfate (SDS) TEMED 0.25% (v/v) 74.95%(v/v) dH2O
Stripping buffer	90 ml - Tris. HCl pH 6.8 20%- SDS 700µl - Beta-Mercaptoethanol Dissolve in dH_2O, Total volume 100
Griess Reagent A	l %Naphthylethylenediamine dihydrochloride (Sigma) in dH_2O. Stored at 4°C; away from light.
Griess Reagent B	l % Sulfanilamide (Sigma) in 5 % H_3PO_4/dH_2O. Stored at 4°C

3. Methods

3.1 Mycobacterial cultures

BCG-GFP and BCG-dsRed were cultured in Middlebrook 7H9 broth (BD Difco) supplemented with glycerol (0.05% for BCG), 0.05% tween 80 and 10% albumin-dextrose-catalase enrichment at 35^{o}C. BCG was cultured in standing condition with intermittent manual shaking. Bacilli were harvested at log growth phase by centrifugation at 1000g for 10 min, washed with PBS-3 (PBS supplemented with 3% FCS) and resuspended in PBS-3. Heat-killed BCG was prepared by heating the BCG suspension for 60 mins at 80^{o}c in the water bath. BCG-GFP or BCG-dsRed grown at 35^{o}C in the presence of 30µg/ml kanamycin or 30µg/ml hygromycin respectively.

3.2 Primary cell techniques

3.2.1 Generation of GM-CSF cell supernatant

For the generation of GM-CSF supernatants, the culture supernatant was sampled from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line kindly provided by B. Stockinger (London, UK) (Zal, Volkmann and Stockinger, 1994). Briefly, GM-CSF transfected cell line was thawed according to standard procedure and left for 2 days in a T75 cell culture flask (Greiner Bio-One, Frickenhausen, Germany). Then, 10^7 cells were harvested and transferred to a T182 cell culture flask (Greiner Bio-One) in ca. 90ml complete RPMI 1640 (PAA, Pasching, Austria). After 3-4 days, 90% of the cells were confluent in T182 cell culture flask after which culture supernatants were harvested and centrifuged by 1000rpm for 10 mins.

The adherent cells were provided with fresh complete RPMI 1640 for 2 days prior to de novo supernatant harvesting. The procedure was repeated 3-4 times before cell line were discarded according to standard GMO-guidelines. The harvested culture supernatant was sterile-filtered with Minisart syringe filters (Sartorius Stedim Biotech, Goettingen, Germany) before using them for the generation of bone-marrow derived DC or MDSC.

3.2.2 Isolation of bone marrow (BM) cells

Briefly, tibiae or femurs were removed from 4 to 12-week-old mice and intact bones were soaked for 1-2mins in sterile 10cm petri dishes filled with an ethanol-propanol solution (Terralin liquid) for disinfection. A minimal fraction of both ends of the tibiae or femurs were cut by scissors and bone marrow was flushed out with a PBS-filled sterile 10ml tuberculin syringe using a Neoject 27G or 0.40mm diameter needle. Bone marrow was washed once by centrifugation at 1000rpm for 10 mins and clusters of the BM cell suspension was disrupted by vigorous pipetting. About 5 to 7 x 10^7 BM cells could be obtained from one mouse.

3.2.3 Generation of GM-CSF derived BM-MDSC and BM-DC

BM-DCs were generated as previously described (Lutz *et al.*, 1999). At day 0, bone marrow cells were seeded at 3 x 10^6 in sterile 10cm petri dishes containing 10ml complete RPMI 1640 medium supplemented with 10% culture supernatant from a murine GM-CSF transfected X63-Ag8.653 myeloma cell line as described in the previous section.

a) For MDSCs (Rößner *et al.*, 2005a) : At day 3, cells were harvested, washed with RPMI 1640 medium + 10% FCS (R10) and then were used for *in vitro* stimulation assays.

b) For DCs: Fresh RPMI medium containing 10% GM-CSF was added on day 3. Then at day 6, BM cells were fed by gently replacing 9ml old RPMI 1640 medium with 10ml R10 containing 10% GM-CSF culture supernatants. Cells were harvested at day 7 and were subsequently used for experiments. The procedure typically yields 60-80% $CD11c^{hi}$ expressing cells as determined by flow cytometry.

3.2.4 Generation of M-CSF derived Macrophages

At day 0, Bone marrow cells were seeded at 3 x 10^6 in sterile 10cm petri dishes containing 10ml R10 with 20% M-CSF supernatant (Shibata *et al.*, 1994). At day 3, another 10ml R10 containing 20% L929 culture supernatant was added to the plates. At day 5, BM cells were fed by gently replacing 10ml old R10 with 10ml fresh R10 medium containing 20% M-CSF supernatant. The medium was changed on day 3, 5 and 7 and the cells were ready on day 7. To harvest adherent macrophages, the medium was discarded, and the cell layer was washed lightly with PBS. 5 ml-EDTA solution was added to the plate and the cells were incubated at 37°C for 10 mins. The plate was tapped gently but thoroughly to dislodge all cells. The detached cells were added to a 50 ml Falcon tube. The cells were washed to remove all EDTA. Cells were then used for *in vitro* stimulation assays in complete R10 medium.

3.2.5 Generation of single cell suspension from spleen and lymph nodes

Organs were isolated from 4-8-week-old mice under sterile conditions and transferred in 5 cm petri dishes containing ice-cold sterile PBS. A single cell suspension was obtained by mashing spleen/lymph nodes with a tuberculin syringe. Then, the cell suspension was filtered through a 0.70µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) positioned on a 5cm petri plate. Cells were then washed by centrifugation on 1000 rpm for 5 mins at 25°C. For spleen cell suspension, erythrocyte lysis was performed by resuspending cell pellet in 1:1 solution of PBS and 1.67% ammonium chloride (NH_4Cl) buffer followed by incubation for 3 mins in 37°C pre-warmed water bath. To remove ammonium chloride, splenocytes were washed and centrifuged for 5 mins at 1200 rpm at 4°C before determining the cell count.

3.3 Standard immunological/biochemistry techniques

3.3.1 Cytokine detection by ELISA

MDSC, DC and Macrophage culture supernatants were analyzed for their cytokine levels by commercially available ELISA kits for IL-1β , IL-6, IL-10, IL-12p40, IL-12p70 BioLegend, San Diego, CA, according to the instructions provided by the manufacturer. 96-well Costar plates (#3690, Corning Life Sciences, Amsterdam, The Netherlands) were used for coating of capture antibodies in respective coating buffer according to the manufacturer's instructions. All washing steps were conducted by using an automated 96-plate washer (Tecan Group, Maennedorf, Switzerland) with ELISA wash buffer or TBST. Samples were stored at - 20°C before performing ELISA assay. Captured cytokines were detected by cytokine-specific biotinylated detection antibodies and streptavidin-horseradish peroxidase conjugates followed by the substrate 3,3',5,5'-Tetramethylbenzidine (TMB), derived as TMB substrate solution (eBioscience) or freshly prepared by mixing equal volumes of substrate reagent A and substrate reagent B (BD Biosciences). Absorbance was detected at 450nm using a Vmax kinetic microplate reader (Molecular Devices) and analyzed by SOFTmax PRO 3.0 Software (Molecular Devices, Ismaning, Germany).

3.3.2 Flow cytometry

3.3.2.1 Surface staining

Cells were stained in FACS buffer containing ice-cold PBS supplemented with 0.1% BSA and 0.1% sodium azide. Typically, 1-5 x 10^5 cells were stained in 50µl FACS buffer supplemented with antibodies directed against surface markers for 20-30 mins at 4°C in the dark. To remove unbound antibodies, cells were washed by centrifugation at 1200 rpm for 5 mins at 4°C. Samples were stored in 100µl of a 1:1 solution containing 1-part FACS buffer and 1 part 2% FA until samples were acquired at FACS LSR II flow cytometer (BD Biosciences, Heidelberg, Germany) provided with CellQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, USA).

3.3.2.2 Intracellular cytokine staining

For intracellular cytokine detection, cells were stained for 20 mins with surface markers as described prior to fixation in a 2% FA solution for 20-30 mins at 4°C. Then, cells were washed with FACS buffer by centrifugation at 1200 rpm for 5 mins at 4°C followed by permeabilization in perm wash buffer (BioLegend) for 20 mins at 4°C. Staining of intracellular cytokines was performed in 50µl Perm buffer for 30 mins at 4°C followed by washing with Perm buffer to remove unbound antibodies. Cells were then acquired at FACS LSR II flow cytometer (BD Biosciences, Heidelberg, Germany) provided with CellQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, USA).

3.3.2.3 Phospho-protein staining

For phoshpho detection, cells were stained for 20 mins with surface markers as described prior to fixation in a 2% FA solution for 20-30 mins at 4°C. Cells were then incubated in IC fixation buffer

(eBioscience) for 30 mins followed. Cells were then washed and incubated with ice-cold methanol. After washing the cells twice, cells were incubated with respective phospho markers for 60 mins. Cells were resuspended in FACS buffer and acquired by flow cytometry. Cells were then acquired at FACS LSR II flow cytometer (BD Biosciences, Heidelberg, Germany) provided with CellQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, USA).

3.3.3 Immunofluorescence staining

Immunofluorescence microscopy combines light microscopy with fluorescence allowing the visualization of the dynamics of cells. After the *in vitro* experiments, cells were then centrifuged onto a glass slide by cytospin at 600*g* for 10 mins. Cells were washed gently with cold PBS and fixed with 4% PFA for 20 mins at RT. Cells were washed thrice and then permeabilized with 0.1% Triton X 100 for 10 min at 4°C. Blocking of non- specific antigens was done with 5% BSA for 30 mins. Next day, cells were washed thrice with cold PBS and stained with primary antibody diluted in PBS with 1% BSA at 4°C overnight. After washing thrice with PBS, secondary antibody diluted in PBS with 1% BSA was added and incubated at RT for 1 hr. Nuclear staining was done using DAPI. Slides were briefly dried before adding Fluoromount-G and carefully coverslip was placed on the slides. Samples were then analyzed by Zeiss LSM 780 confocal microscope.

3.3.4 Western Blotting

3.3.4.1 Protein lysate preparation

To analyze specific proteins from cells, western blotting was used to separate and identify proteins. Cellular proteins were extracted to detect the protein of interest. For protein extraction lysis of cells, Radio immunoprecipitation assay (RIPA) buffer extraction method was performed.

Cells were washed twice with ice-cold PBS. The cells pellet was then re-suspended in RIPA buffer containing 1:100 dilution of protease inhibitor cocktail II (Sigma) and 1µM DTT. The cells were then lysed on a shaker for 1 hr at 4°C. The lysate was then centrifuged at 10,000 rpm for 5 mins at 4°C in Biofuge. Supernatants were carefully removed and transferred in pre-cooled micro-centrifuge tubes. Protein concentration in these lysates was quantified using BCA and samples were stored at -20°C till further use.

For the detection of phosphorylated proteins, cell lysates were prepared by a different method to avoid any loss or degradation of phospho proteins. All the steps were carried out on ice. Cells were washed once with ice-cold PBS and then directly lysed in 4X Laemmeli buffer. Samples were mixed in Laemmeli buffer and incubated at -80°C overnight. Samples were then sonicated 3 times for 5 mins followed by boiling at 95°C for 10 mins. These samples were used for SDS PAGE analysis or stored at -20°C till further use.

3.3.4.2 Quantification of proteins by BCA

To determine the effect of the various experimental procedure on the expression of proteins in SDS PAGE, an equal amount of proteins is loaded for separation. Either an equal number of cells were lysed in an equal amount of lysis buffer and equal volume was loaded on the gel or the protein

concentration was determined using BCA protein quantification method. Peptide bonds in proteins reduce Cu^{2+} ions from $CuSO_4$. The highly alkaline solution of Bicinchoninic acid (BCA) chelates Cu^{+} ions to form a purple colour complex at higher temperatures (60°C). The intensity of the colour complex is directly proportional to the protein concentration of the sample. This purple complex can be measured using absorbance at 562 nm in the colorimeter. To determine protein concentration, 1ml of BCA was aliquoted in microcentrifuge tubes followed by addition of 20µl of $CuSO_4$ and 5 µl of sample/DPEC H_2O/standard. Solutions were mixed by gentle vortexing and then heated at 60°C for 15 mins. Samples were then transferred in cuvettes for measurement of protein concentration. The colorimetric readings were acquired in Eppendorf Photometer using manufacturer's instructions.

3.3.4.3 SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins based on their molecular weight in the electric field.

All the gels used were 10 or 12% polyacrylamide. 20-50 µg of protein lysates were mixed with 4X Laemmeli buffer and heated at 95°C for 5 mins for denaturation. These samples were then stored at -20°C. The protein detection by SDS PAGE was carried out in following steps.

1. Assembly and preparation of gel for electrophoresis: Two glass plates were assembled in gel apparatus separated by a spacer. The assembly was checked for any leakage and resolving gel was prepared. The components were poured between the glass plates. The gel was layered with 1 ml of isopropanol till the gel was polymerized. The isopropanol was removed and stacking gel was poured on top and comb was inserted in stacking gel.

2. Electrophoresis: The electrophoresis unit was then filled with protein gel buffer. The comb was removed carefully from stacking gel and the wells were gently flushed with protein gel buffer to remove gel residues. Samples and pre-stained protein marker (6µl) were then loaded in the wells and separated at 90V for 2 hrs or till the samples resolved completely.

3. Electrophoretic transfer: To detect target protein, they were immobilized by electrotransfer from gel on a solid membrane support. For all experiments, nitrocellulose (NC) membranes were used. The NC membrane and the gel were placed together with Whatman filter paper between two electrodes. The transfer set up was carefully placed in transfer assembly and any air bubbles were removed by gentle rolling. The transfer was carried out at 10 V for one hour. Due to the electrophoretic field generated between the electrodes, the proteins were transferred from the gel onto the NC membrane. The transfer was checked by successful transfer of all bands of a protein marker. The membranes were then probed with specific antibodies as described.

3.3.4.4 Detection of proteins on nitrocellulose membrane

Following the transfer of protein on NC membrane, the unoccupied binding sites on the membrane were blocked to avoid non-specific binding of antibodies. The membranes were blocked at room temperature for 30 mins in 5% non-fat dry milk or BSA in PBS-T. The blocked membranes

were then probed with specific primary antibody dilution (1:500 – 1:2000) in 5% milk or BSA in PBS-T overnight at 4°C on a gentle shaker. The membrane was then washed thrice for 5 mins each with PBS-T and incubated with HRP or fluorescent labeled secondary antibody dilution (1:10,000-1:15,000) in 5% milk for 1 hour at room temperature on a gentle shaker. The membranes were then washed thrice with PBS-T. Images were acquired in Li-cor Odyssey Fc Imaging system. For HRP labeled secondary antibodies, Chemiluminescent Femto MaxTM Super Sensitive HRP Substrate (Rockland) was added on membranes before Imaging and then acquired in Chemi channel whereas, fluorescent labeled secondary antibodies were imaged in 700 or 800 channels directly depending on the fluorescent antibody used.

3.3.4.5 Stripping of nitrocellulose membrane

To remove primary and secondary antibodies bound on the membrane, the NC membranes were incubated in stripping buffer for 30 mins at 50°C (stripping buffer was always prepared fresh). Membranes were then washed 5 times thoroughly in PBS-T for 10 mins each. The membranes were blocked with 5% milk or BSA at room temperature for 20-30 mins and then probed with next primary antibody dilution.

3.3.5 Griess assay for NO detection

Nitric oxide production was determined by measuring its stable end product nitrite, using the standard Griess reagent (Rößner *et al.*, 2005a). Briefly, 50µl of supernatant were added to 96 well plate, followed by 50 µl of Griess reagent (A+B) A= 0.1% sulphanilamide and B=0.1% N-1-napthylethylenediamine dihydrochloride (NED). Absorbance at 492 nm was measured by microplate reader and standard curves were created based on the $NaNO_2$ optical density (OD) readings. From this standard curve, samples concentrations were calculated**.**

3.3.6 *In vitro* cellular assays

3.3.6.1 *In vitro* stimulation of MDSCs with BCG

MDSCs from WT or *Cav1*$^{-/-}$ were added in a 24-well-plate ($1.5x10^6$ cells per well). BCG was added to cultures at an indicated multiplicity of infection (MOI). Cells were harvested after 16 hours and analyzed for the surface expression of TLR2, TLR4, Cav-1, PD-L1, E-Cadherin, CD40, CD69 or intracellular expression of TLR2, TLR4, iNOS and arginase1. To analyze the production of various cytokines and NO, MDSCs were stimulated with BCG at 2,5,10 MOI and culture supernatants were collected after 16 hours.

3.3.6.2 T cell suppressor assay

MDSCs from WT or *Cav1*$^{-/-}$ mice were pre-activated with BCG for 1 hour. T cells from Spleen and lymph nodes of a syngeneic mouse (as described in 3.2.5) were then labeled with the proliferation dye CellTrace Violet (Thermo Fisher Scientific). T cells were stimulated with soluble anti-CD3(1µg/ml) and anti-CD28 (1µg/ml). Stimulated T cells alone without MDSCs was used as a control. MDSCs activated with BCG were co-cultured with T cells in different ratios. Co-cultures were

analyzed for T-cell proliferation after 3 days. Cells were harvested and stained for CD4 and CD8 and analyzed by flow cytometry to detect T cell suppression. Culture supernatants were used for detecting NO by Griess assay.

3.4 Statistical analysis

All data obtained were analyzed using GraphPad Prism 6.0 software (USA). Data are represented as mean data ± SD. The unpaired, two-tailed, student ‘s t-test was used. The data was considered significant if ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5.

4.Results

4.1 Myeloid-derived suppressor cell subsets and their interaction with mycobacteria

4.1.1 Identification of MDSC subsets by FACS and microscopy

In this work, murine MDSCs were used to study the interaction of MDSCs with mycobacteria by using an *in vitro* infection model. BM-MDSCs were obtained by culturing mouse bone marrow in the presence of 10% GM-CSF for 3 days (Rößner *et al.*, 2005a). Murine MDSCs consists of two subsets, granulocytic (G-MDSCs) and monocytic (M-MDSCs). Murine G-MDSCs and M-MDSC were identified by their differential expression of CD11b, Ly-6G and Ly-6C by flow cytometry (Figure.12A).

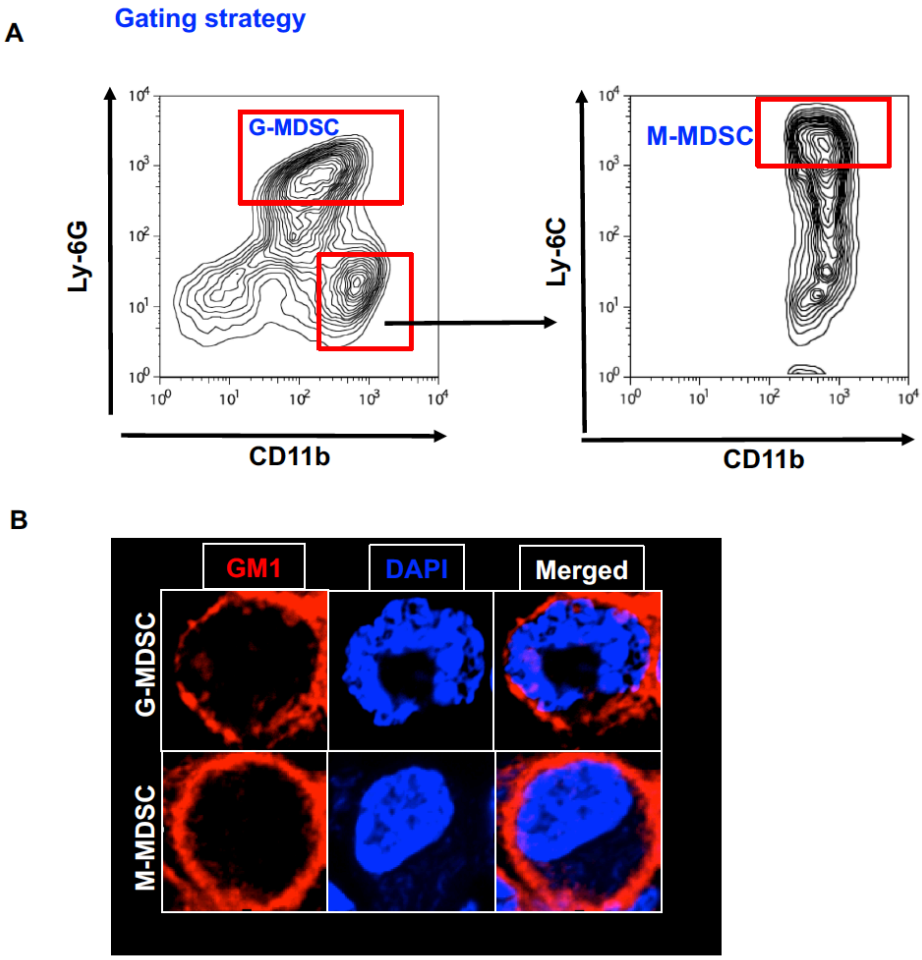

Figure12: Identification of G-MDSCs and M-MDSCs by flow cytometry and confocal microscopy. BM-MDSCs were differentiated from mouse bone marrow cells and cultured *in vitro* in the presence of 10% GM-CSF for 3 days. **(A)** Cells were then harvested and stained for CD11b, Ly-6C and Ly-6G surface markers and analyzed by flow Cytometry. Figure shows the gating strategy to define G-MDSCs and M-MDSCs after FSC/SSC live gating and doublet exclusion by expression of CD11b, Ly-6C and Ly-6G. **(B)** BM-MDSCs were cytospinned and stained for cholera toxin B (Red) and DAPI. Stained cells were acquired by confocal microscopy

Furthermore, MDSCs were stained with cholera toxin B subunit and DAPI and images were acquired by confocal microcopy. GM1 was used here as a cell surface marker. Cholera toxin B binds to GM1 (ganglioside) on the cell surface (Aman *et al.*, 2001). G-MDSC and M-MDSC subsets can be distinguished on the basis of DAPI staining. G-MDSCs have ring or polymorphic shaped nuclei whereas M-MDSCs have kidney or round shaped nuclei (Figure.12B). Hence, MDSC subsets can be identified and distinguished by flow cytometry and confocal microcopy.

4.1.2 MDSCs phagocytose mycobacteria, secrete soluble mediators upon mycobacterial infection and gain immune suppressive function

MDSCs have been reported to uptake Mtb and release pro-inflammatory and immunomodulatory cytokines while maintaining their suppressive feature (Knaul *et al.*, 2014). We wanted to confirm whether MDSC subsets obtained from bone marrow were able to phagocytose mycobacteria and retain their function. To test this, we stimulated BM-MDSCs with BCG live, BCG killed and Mtb killed and analyzed for their uptake, cytokine production and NO secretion. We observed that all mycobacterial preparations were internalized by both subsets of G-MDSCs and M-MDSCs (Figure.13A). However, M-MDSC internalized Mtb better compared to BCG live and BCG killed. We also evaluated the efficiency of MDSCs to uptake BCG by confocal microscopy (Figure.13B). MDSCs infected all mycobacterial preparations secreted NO, IL-10 and IL-6 (Figure.13 D, E and F). Interestingly, MDSCs stimulated with Mtb killed had significantly higher NO and IL-10 production as compared to BCG live (Figure.13 D and E). Although BCG killed was endocytosed similar to BCG live, MDSCs stimulated with BCG killed had significantly lower amount of IL-6, IL-10 and NO release. BCG-infected MDSCs stained positive for iNOS (Figure.13C).

In Mtb infection, MDSCs accumulate in the lung of mice and suppress T-cell proliferation and IFNγ production in NO dependent manner (Tsiganov *et al.*, 2014b). To analyze the functionality of these BM-MDSCs, we performed *in vitro* T cell suppression assay where BCG-infected BM-MDSCs were added at different ratios to CD3/CD28 antibody activated T cells. MDSCs suppressed both $CD4^+$ and $CD8^+$ T cell in a dose dependent manner (Figure. 13 G, H and I). At lower MDSC: T cell ratios (1:30), MDSCs were not suppressive. Together, these results confirm that MDSCs take up mycobacteria and secrete NO (iNOS) and cytokine upon mycobacterial stimulation. Moreover, these MDSCs also have the potential to suppress T-cell proliferation.

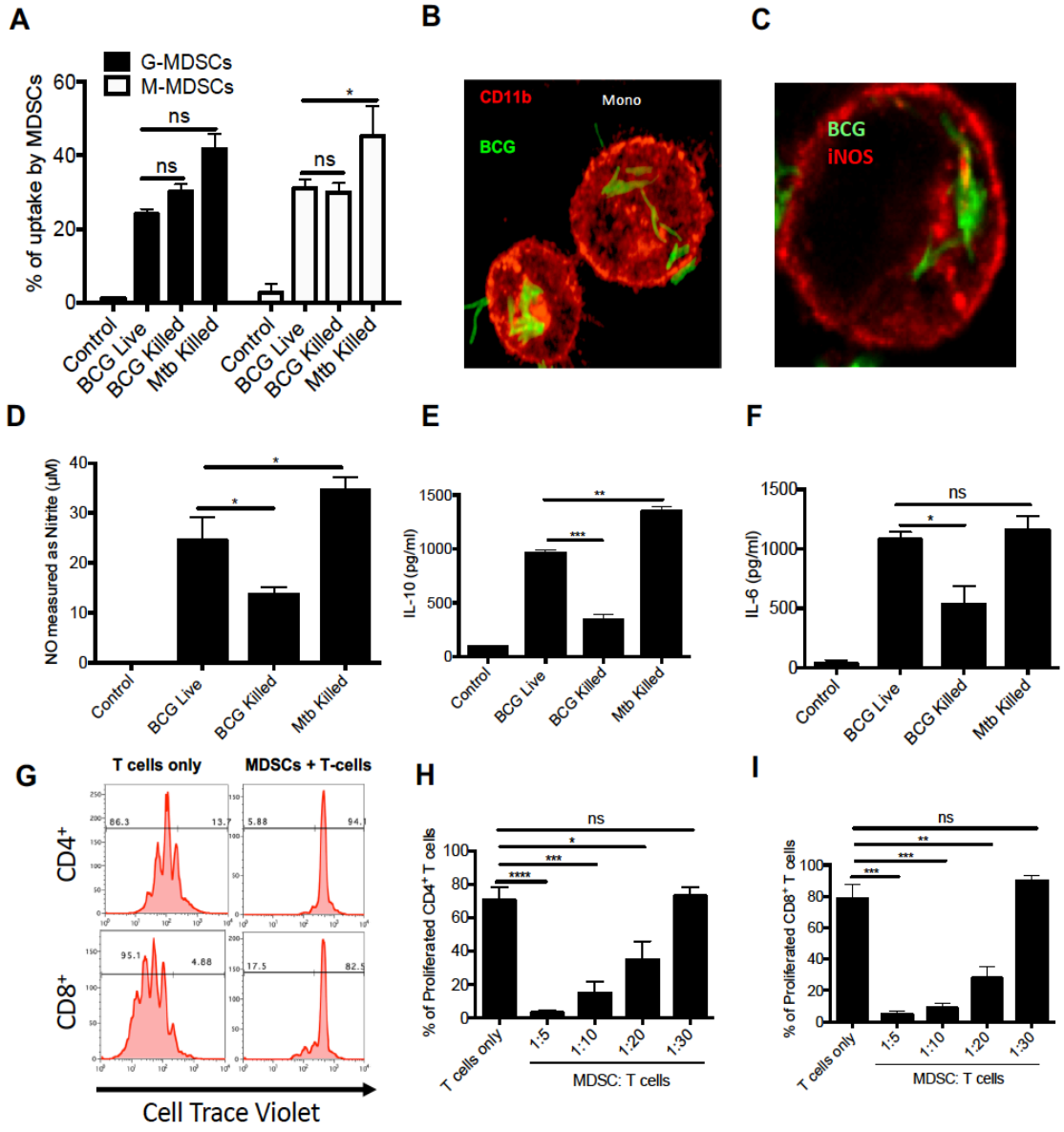

Figure13: MDSCs phagocytose mycobacteria, secrete soluble mediators in response to mycobacteria and gain immune suppressive function. (A) BM-MDSCs were stimulated with BCG-live, BCG killed and Mtb killed for 8 hours. Cells were harvested and stained for Ly-6C and Ly-6G surface markers and analyzed for the of GFP positive cells of G-MDSC and M-MDSC uptake by flow cytometry. **(B)** BM-MDSCs stimulated with BCG-GFP were stained for CD11b (Red) or **(C)** iNOS (Red). Cell supernatants from experimental settings from (A) were also measured for **(D)** NO production by Griess assay and **(E)** IL-10, **(F)** IL-6. **(G-I)** T cell suppressor assay. Syngeneic lymph node and spleen cells as a source of T cells were labelled with the proliferation dye Cell Trace Violet and then stimulated with anti-CD3 and anti-CD28. Then 1h BCG pre-activated BM-MDSCs were added or, as a control, T cells remained without MDSCs. Co-cultures were analyzed after 3 days. Cells were harvested and stained for CD4 and CD8 and analyzed by FACS to detect T cell proliferation **(G)**. Quantification of proliferated $CD4^+$ T cells **(H)** and $CD8^+$ **(I).** Data represent for n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5 by unpaired, two-tailed, student's t-test.

4.2 Role of Caveolin-1 in MDSCs during mycobacterial infection

4.2.1 Cav-1 is expressed on the surface of both subsets of MDSCs

Caveolin-1(Cav-1) is reported to be expressed on immune cell types such as dendritic cells, macrophages, neutrophils, T cells, B cells (Harris *et al.*, 2002)·(Vargas *et al.*, 2002). Here, we first confirmed the expression of Cav-1 on BM-MDSCs by flow cytometry, confocal microscopy and western blot. By flow cytometry, we observed that M-MDSCs had more expression of Cav-1 as compared to G-MDSCs (Figure.14A). These observations were also validated by confocal microcopy (Figure.14B). G-MDSC and M-MDSC subsets can be distinguished on the basis of DAPI staining. G-MDSCs have ring or polymorphic shaped nuclei whereas M-MDSCs have kidney or round shaped nuclei. Also, here, we noted more expression of Cav-1 on M-MDSCs than G-MDSCs. Cav-1 was expressed on the cell surface as well as intracellularly. Next, we confirmed these findings by western blot. Cav-1 was expressed on D3-MDSCs and *Cav-1*$^{-/-}$ MDSCs were used as a control (Figure.14C). However, these western blot results were from pooled D3-MDSCs and not from the sorted G-MDSCs or M-MDSCs These results confirm the expression of Cav-1 on both the G-MDSCs and M-MDSCs subsets.

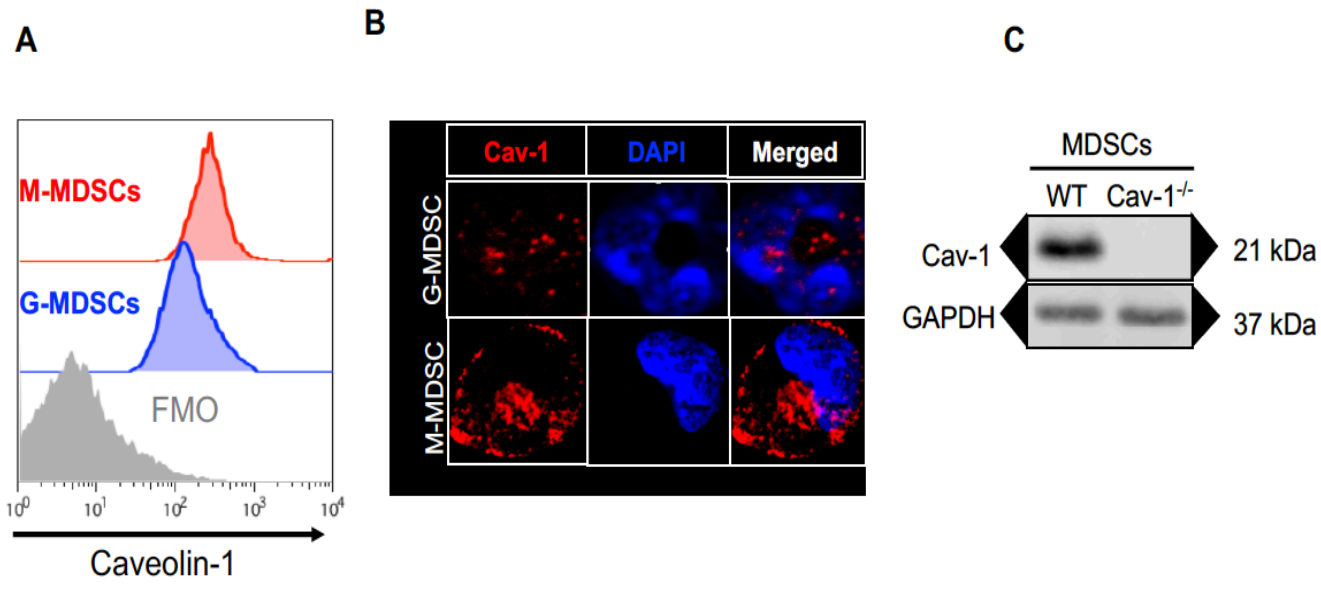

Figure 14: Caveolin-1 is expressed on the surface of both granulocytic and monocytic subsets. (A) BM-MDSCs stained with Ly-6C, Ly-6G, CD11b and Cav-1 and then analyzed for the expression of caveolin-1 by flow cytometry. **(B)** Cytospins were stained for Caveolin-1 and DAPI and analyzed by confocal microscopy. G-MDSCs and M-MDSCs were identified on the basis of polymorphonuclear or mononuclear shape by DAPI staining. **(C)** Cell lysates of WT or *Cav1* $^{-/-}$ MDSCs were prepared and western blot was done to examine the expression of Cav-1 and the housekeeping protein GAPDH was used as a control.

4.2.2 Cav-1 is upregulated upon BCG infection but its deficiency does not affect TLR4 and TLR2 surface expression on MDSCs

Cav-1 has been demonstrated to be upregulated in macrophages upon HIV infection (Mergia, 2017). However, the functional role of Cav-1 has not been investigated so far. Therefore, we wanted to study the effect of BCG on MDSCs. For that, we stimulated MDSCs with BCG and analyzed for the expression of Cav-1 on G-MDSCs and M-MDSCs by flow cytometry. We observed upregulation of Cav-1 expression on both subsets of MDSCs (Figure.15A). On the other hand, M-MDSCs upon BCG infection had more increased expression compared to G-MDSCs.

Mycobacterial ligands are recognized by defined pattern recognition receptors (PRRs) such as TLR2 and TLR4 to induce immune responses by macrophages and dendritic cells (Sánchez *et al.*, 2010). In addition, MDSCs express TLRs but their activation induces immunosuppressive responses in them, a phenomenon that can be exploited for microbial immune evasion (Vendelova *et al.*, 2018). Lipid raft proteins are known to cooperate with TLRs for initiating signaling (Płóciennikowska *et al.*, 2015). Cav-1 is reported to be important for TLR4 expression and signaling (Xiao Mei Wang *et al.*, 2009). Hence, we wanted to examine if Cav-1 is required for the expression of mycobacterial PRRs such as TLR2 and TLR4. To examine this, we analyzed for the surface expression of TLR2 and TLR4 after BCG stimulation by flow cytometry. Murine MDSCs up-regulated TLR2 and TLR4 expression on the cell surface upon BCG infection with different MOIs or after exposure to their respective ligands for TLR2 (Pam_3CSK_4) or TLR4 (LPS) by flow cytometry (Figure.15 C-E), except that there was no significant difference in surface TLR4 expression between unstimulated M-MDSCs or BCG infected M-MDSCs. (Figure.15G). In all cases, we did not observe any significant difference between WT and *Cav1*$^{-/-}$ MDSCs (Figure.15B-G). Together, these data indicate that although Cav-1 is increased in murine G-MDSC and M-MDSC upon BCG infection, its genetic deficiency does not alter the surface expression of TLR2 and TLR4.

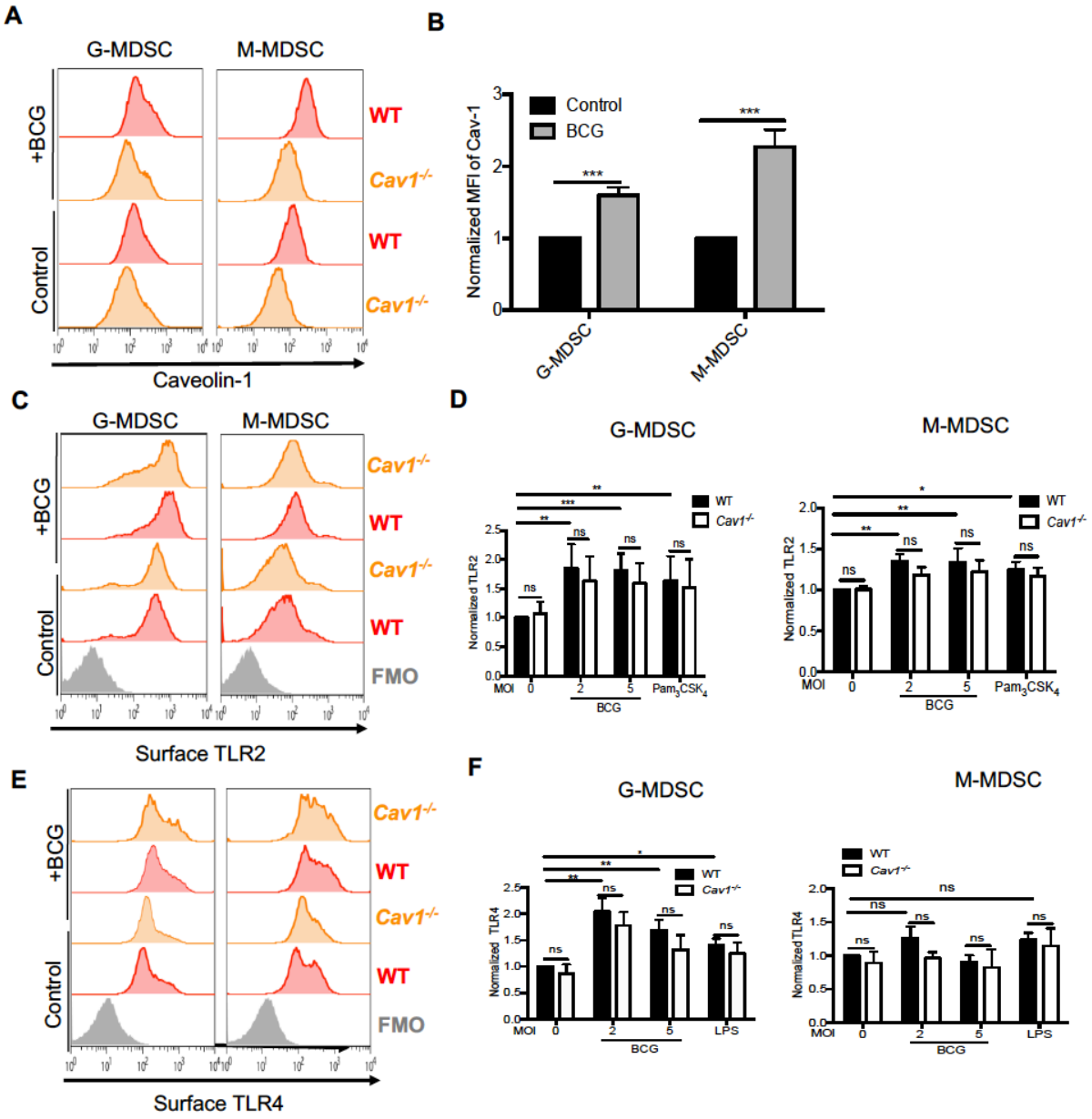

Figure 15: Caveolin-1 is upregulated upon BCG infection but its deficiency does not affect TLR4 and TLR2 surface expression on murine MDSCs. BM-MDSCs from WT and Cav-1$^{-/-}$ mice were stimulated with BCG at 5 MOI and after 16h analyzed for Cav-1 expression on G-MDSCs and M-MDSCs as exemplified **(A)** and quantified **(B)**. BM-MDSCs from WT or *Cav1*$^{-/-}$ mice were stimulated for 16h with BCG at 2 or 5 MOI or the TLR2 agonist Pam_3CSK_4 and analyzed for surface TLR2 expression by flow cytometry on G-MDSCs and M-MDSCs as exemplified **(C)** and quantified **(D)**; or stimulated additionally with the TLR4 agonist LPS and analyzed for surface TLR4 as exemplified **(E)** and quantified **(F)**. Data represent n=6 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5 by unpaired, two-tailed, student ‘s t-test.

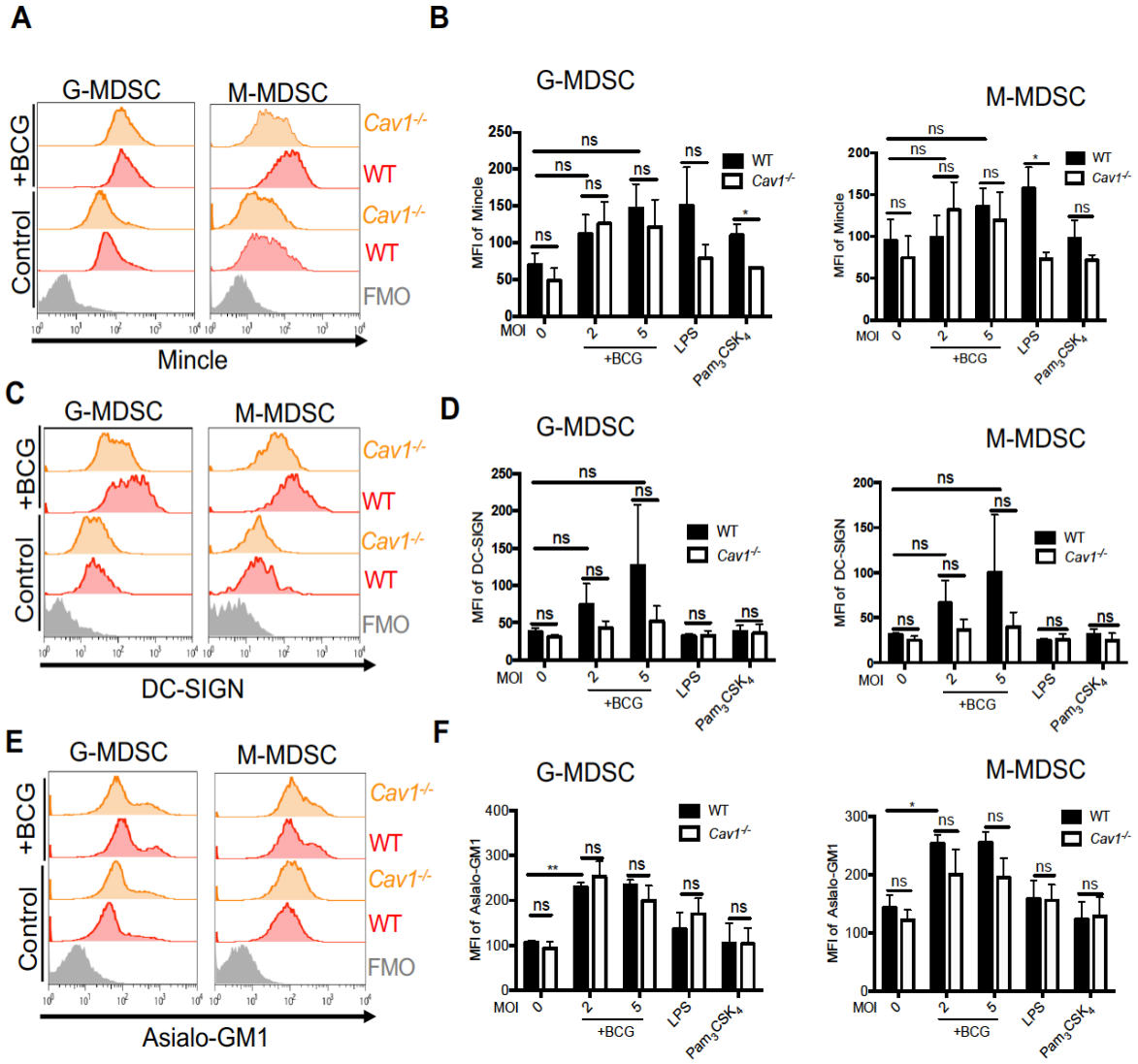

Figure 16: Lack of Cav-1 does not affect the surface expression of mycobacterial receptors on BCG-infected MDSCs. BM-MDSCs from WT or *Cav1*$^{-/-}$ mice were stimulated for 16h with BCG at 2 or 5 MOI or TLR4 agonist LPS or TLR2 agonist Pam_3CSK_4 and analyzed for surface expression of Mincle **(A&B)**, DC-SIGN **(C&D)** and asialo-GM1 **(E & F)** by flow cytometry on G-MDSCs and M-MDSCs. Data represent n=3 independent experiments. **P<0.01; *P<0.5; (ns) P>0.5 by unpaired, two-tailed, student 's t-test.

4.2.3 Cav-1 dispensable for the surface expression of mycobacterial receptors on BCG-infected MDSCs

Apart from TLRs, Mtb can be recognized by other receptors such as Mincle and DC-SIGN (Stamm, Collins and Shiloh, 2015). Since we did not observe any difference in the surface expression of TLRs between WT and *Cav1*$^{-/-}$ MDSCs. Thus, we asked whether expression of other PRRs is affected in the absence of Cav-1. Moreover, asialo-GM1 is a ganglioside which has been shown to act synergistically with TLR5 to activate phosphorylation of ERK signaling and further activate NF-κB signaling (McNamara *et al.*, 2006). Also,TLR2 in cooperation with asialo-GM1 acts as a signaling complex in epithelial cells to initiate the host response to *Staphylococcus aureus* (Soong *et al.*, 2004).

Therefore, we analyzed for the surface expression of Mincle, DC-SIGN and asialo-GM1. We did not observe any significant upregulation of Mincle and DC-SIGN on both WT and *Cav1*$^{-/-}$ G-MDSCs or M-MDSCs upon BCG infection (Figure.16 A-D). However, BCG-stimulated G-MDSCs and M-MDSCs had a significantly increased asialo-GM1 expression on the cell surface (Figure.16E, F). Furthermore, there were no significant differences in the expression levels of Mincle, DC-SIGN and asialo-GM1 between WT and Cav-1 deficient MDSCs upon BCG infection. Surprisingly, there was significantly reduced Mincle expression in BCG-infected *Cav1*$^{-/-}$ G-MDSCs and M-MDSCs (Figure 16. B). Hence, these results suggest that Cav-1 deficiency does not affect surface expression of other PRRs such as Mincle, DC-SIGN and asialo-GM1.

4.2.4 Cav-1 deficient M-MDSCs have reduced intracellular levels of TLR2 but not TLR4

Previous reports indicated that the TLR4 recycled between the Golgi apparatus and the cell membrane (Latz *et al.*, 2002); and in macrophages TLR2 localized around yeast containing phagosomes (David M. Underhill *et al.*, 1999). Since we did not observe any significant differences in the surface expression of TLRs between WT and *Cav1*$^{-/-}$ MDSCs, we hypothesized that Cav-1 might be involved in the recycling of PRRs. To address whether recycling of TLR2 and TLR4 is affected by Cav-1, we analyzed both intracellular TLR2 and TLR4 by confocal microscopy and flow cytometric analysis. Surprisingly, we observed that MDSCs from *Cav1*$^{-/-}$ mice had reduced cytoplasmic expression of TLR2 (Figure.17 A, C), but not of TLR4 by confocal microscopy (Figure.17 B, D). G-MDSCs and M-MDSCs were identified on the basis of polymorphonuclear and mononuclear shape, respectively. Quantification of TLR2 and TLR4 was done on M-MDSCs and not on G-MDSCs (Figure.17 C, D). This was further confirmed by intracellular staining of TLR2 and TLR4 expression in BCG-infected WT and *Cav-1*$^{-/-}$ MDSCs by flow cytometry. Also, here, TLR2 expression was diminished already in unstimulated *Cav-1*$^{-/-}$ M-MDSCs and not significantly increased after BCG infection (Figure.17 E, F). However, Pam_3CSK_4 stimulation to M-MDSCs had no significant difference between WT and *Cav1*$^{-/-}$ M-MDSCs (Figure.17F). TLR4 expression was unaffected in *Cav1*$^{-/-}$ G-MDSCs but upregulated in M-MDSCs from both WT and *Cav-1*$^{-/-}$ mice upon BCG infection (Figure.17G, H). Interestingly, WT and *Cav1*$^{-/-}$ G-MDSCs had no difference in the intracellular expression of TLR2 or TLR4 (Figure.17E-H). Together, our results indicate that Cav-1 deficiency does not affect surface expression of TLR2 and TLR4 but alters intracellular levels of TLR2. Moreover, BCG-induced upregulation of TLR2 is diminished in BCG-infected M-MDSCs but not in G-MDSCs.

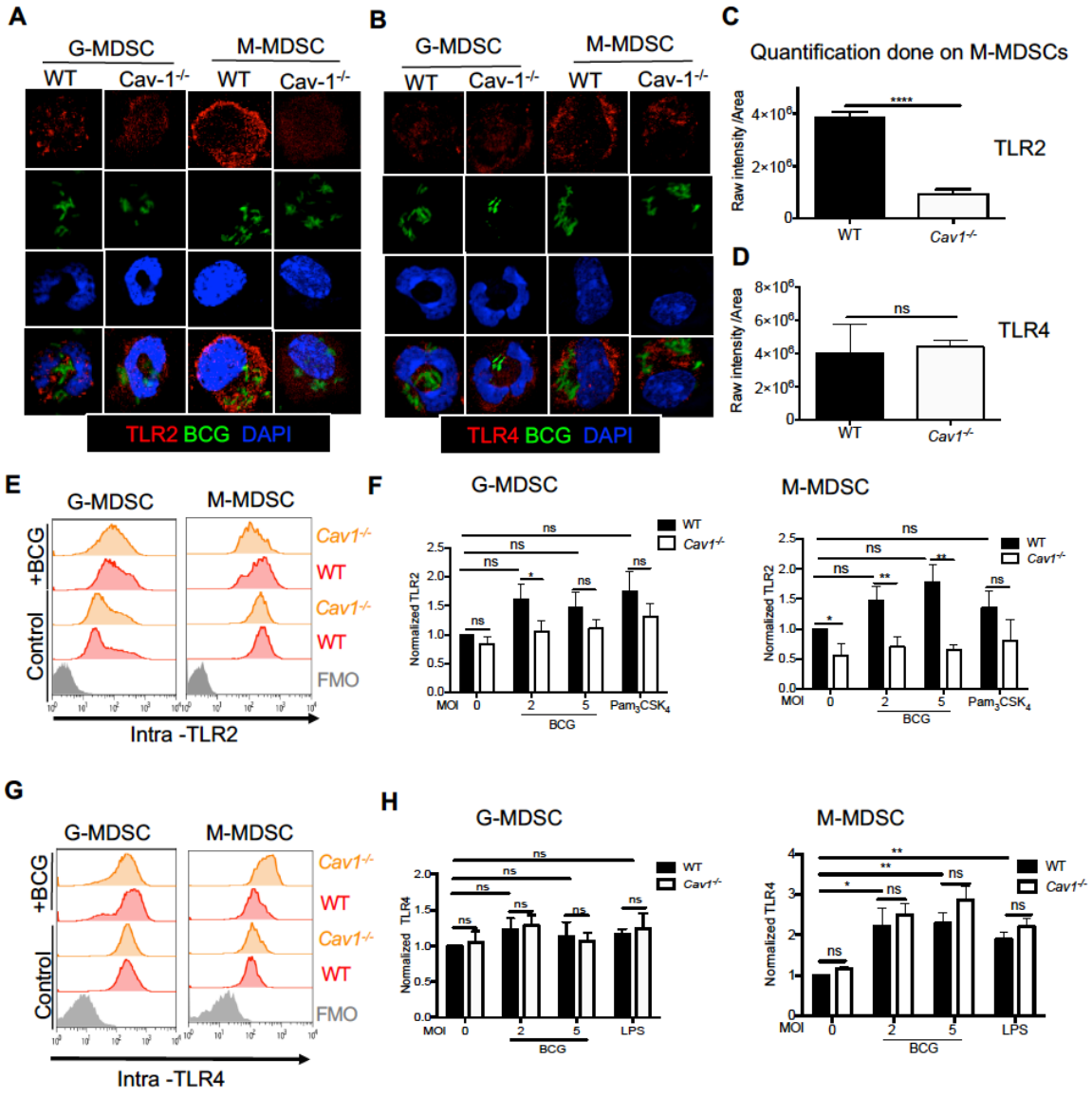

Figure 17: **Cav-1 deficient M-MDSCs have reduced intracellular TLR2 but not TLR4.** BM-MDSCs were stimulated with BCG-GFP for 16h at 1 MOI. Cytospins were stained for TLR2 **(A)** or TLR4**(B)** and analyzed by confocal microscopy. Quantified data of raw intensity/area for TLR2 **(C)** or TLR4 **(D)** of M-MDSCs from WT and *Cav1*$^{-/-}$ mice. MDSCs from WT or *Cav1*$^{-/-}$ mice were stimulated for 16h with BCG at 2 or 5 MOI or the TLR2 agonist Pam_3CSK_4 and analyzed for intracellular TLR2 expression by flow cytometry on G-MDSCs and M-MDSCs as exemplified **(E)** and quantified **(F)**; or stimulated with the TLR4 agonist LPS and analyzed for intracellular TLR4 as exemplified **(G)** and quantified **(H)**. Data are from n=5, independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5, unpaired, two-tailed, student's t-test.

4.2.5 Cav-1 inhibition or genetic deficiency does not impair BCG uptake into MDSCs

MDSCs have been shown to internalize mycobacteria in infected mice (Knaul *et al.*, 2014) and Cav-1 is involved in the uptake of several pathogens (Pietiäinen *et al.*, 2004)(Werling *et al.*, 1999)(Shin, Gao and Abraham, 2000)(Richterova *et al.*, 2001). Therefore, we examined whether Cav-1 is required for BCG uptake into MDSCs. Filipin III is a cholesterol binding drug which acts as

a caveolae disrupter (Peyron *et al.*, 2000)·(Peters *et al.*, 2003). In macrophages, Filipin III has been implicated as functionally important for caveolae-mediated endocytosis (Abraham *et al.*, 1997). Simvastatin and β-cyclodextrine are a lipid raft disrupter drugs which also influence caveolae functions, although in a less specific manner (Shin, Gao and Abraham, 2000). Cytochalasin-D was used as a positive control for blocking actin polymerization (Schliwa, 1982). MDSCs were treated with these inhibitors prior to BCG-GFP infection and then tested for uptake after 6 hours by flow cytometry. Pharmacological inhibition by filipin-III did not block the BCG uptake into G-MDSC or M-MDSC. However, inhibition with β-cyclodextrine reduced the uptake significantly in both G-MDSC and M-MDSC subsets, while simvastatin showed only a trend for reduction of uptake, but without statistical significance (Figure. 18A-B). To further address the role of Cav-1 in BCG uptake, we compared WT with *Cav1*$^{-/-}$ deficient MDSCs. As observed with the pharmacological inhibitors, also here, we did not find any significant difference in the uptake of BCG into WT and *Cav1*$^{-/-}$ G-MDSCs and M-MDSCs (Figure. 18C, D).

The formation of caveosomes has been described after mycobacterial uptake into a macrophage cell line J774 (Jayachandran *et al.*, 2008a). Therefore, we tested for the formation of caveosomes in BCG-stimulated MDSCs by confocal microscopy. G-MDSC were identified by their ring-shaped or polymorphic nuclei whereas M-MDSC has kidney shaped or round nuclei. Both MDSC subsets readily ingested BCG-GFP but showed no co-localization with Cav-1 (Figure. 16E). Thus, these results show that Cav-1 is dispensable for BCG uptake by BM-MDSCs and does not lead to caveosome formation in BM-MDSCs.

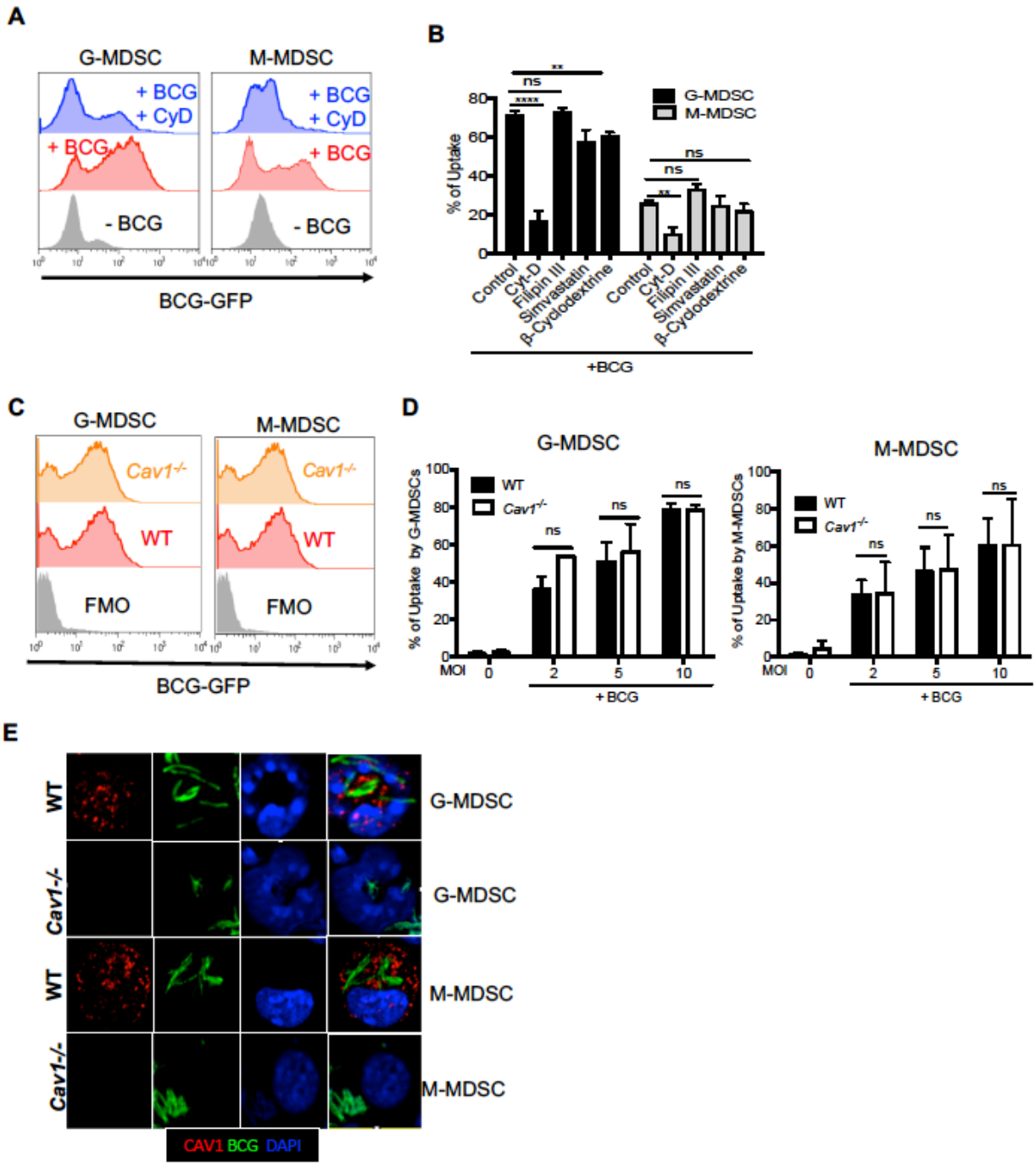

Figure 18: Pharmacological inhibition or genetic deficiency of Cav-1 do not impair BCG uptake by G-MDSC and M-MDSC. BM-MDSCs were incubated with cytochalasin-D, filipin III, simvastatin or β-cyclodextrine for 1h and then stimulated with BCG-GFP at MOI of 2, 5 or 10 for 6h. Cells were then analyzed by flow cytometry for BCG uptake by GFP detection in G-MDSC and M-MDSC subsets as exemplified **(A)** and quantified **(B)**. BM-MDSCs were generated from WT or *Cav1*$^{-/-}$ mice and tested for BCG uptake similar to the experiment shown in A/B as exemplified **(C)** and quantified **(D)**. BM-MDSCs were stimulated with BCG-GFP at 1 MOI for 16h. Cytospins were then stained for Cav-1 and DAPI and analyzed by confocal microcopy. G-MDSC and M-MDSCs were defined on the basis of their nuclear shape**(E)**.

4.2.6 Cav-1 deficiency impairs surface markers selectively in M-MDSCs upon BCG infection

MDSCs express typical surface markers such as CD40 and PD-L1 and are reported to be involved in immune suppression mechanisms of MDSCs (Lindau *et al.*, 2013). Previous report suggested that co-stimulator marker, CD40 is also important for MDSC-mediated immune suppression and Treg expansion (Pan *et al.*, 2010). Also, PD-L1 and CD40 is upregulated when activated by Mtb infection (McNab *et al.*, 2011)(Ma and Clark, 2009). Thus, we compared the expression of these inhibitory molecules CD40 and PD-L1 in BCG infected MDSCs of WT and *Cav1*$^{-/-}$ by flow cytometry. BCG infection resulted in significantly increased CD40 (Figure.19 A, B) and PD-L1 (Figure.

19C, D), expression in G-MDSCs and M-MDSCs from WT mice. However, *Cav1*$^{-/-}$ M-MDSCs had significantly reduced expression of CD40 and PD-L1 upon BCG infection while G-MDSCs remained unaffected (Figure.19 A-D). These data suggest that Cav-1 deficiency affects surface markers such as PD-L1 and CD40 in BCG-infected M-MDSCs but not in G-MDSCs.

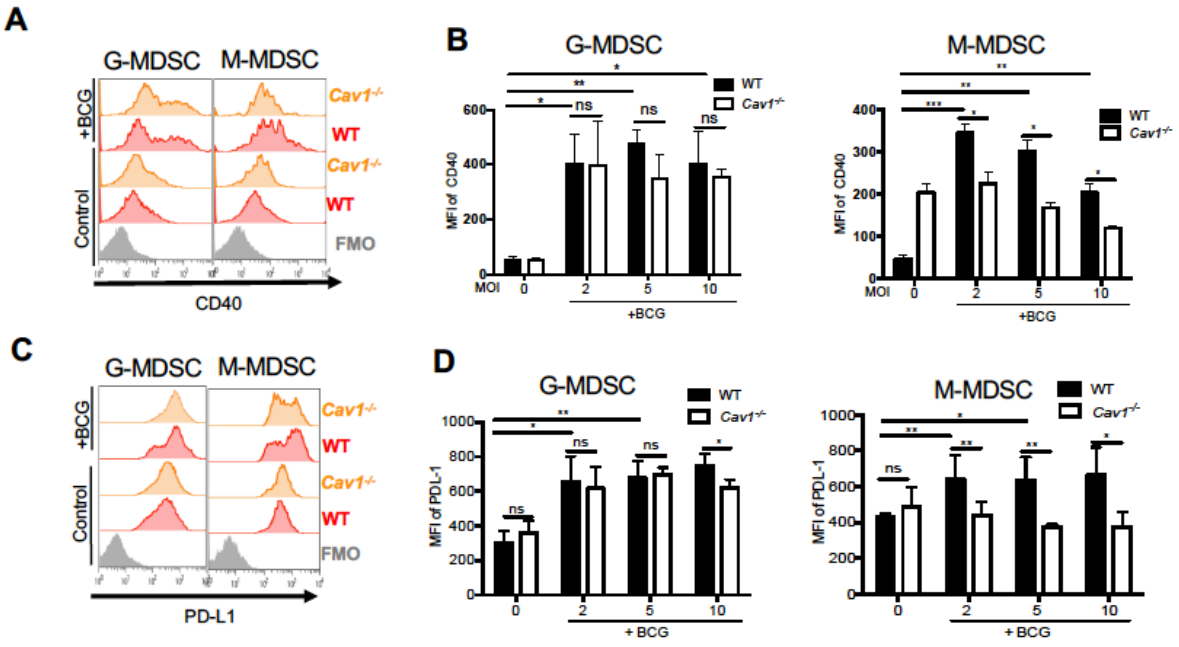

Figure 19: Cav-1 deficiency impairs the surface markers selectively in M-MDSCs upon BCG infection. BM-MDSCs from WT or *Cav1* $^{-/-}$ mice were stimulated with BCG for 16h at 2, 5, or 10 MOI. Cells were then harvested and G-MDSCs and M-MDSCs were separately analyzed by flow cytometry for CD40 **(A, B** quantified**)** and PD-L1 **(C, D** quantified**)**. Data shown are from n=4 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5;(ns) P>0.5 unpaired, two-tailed, student's t-test.

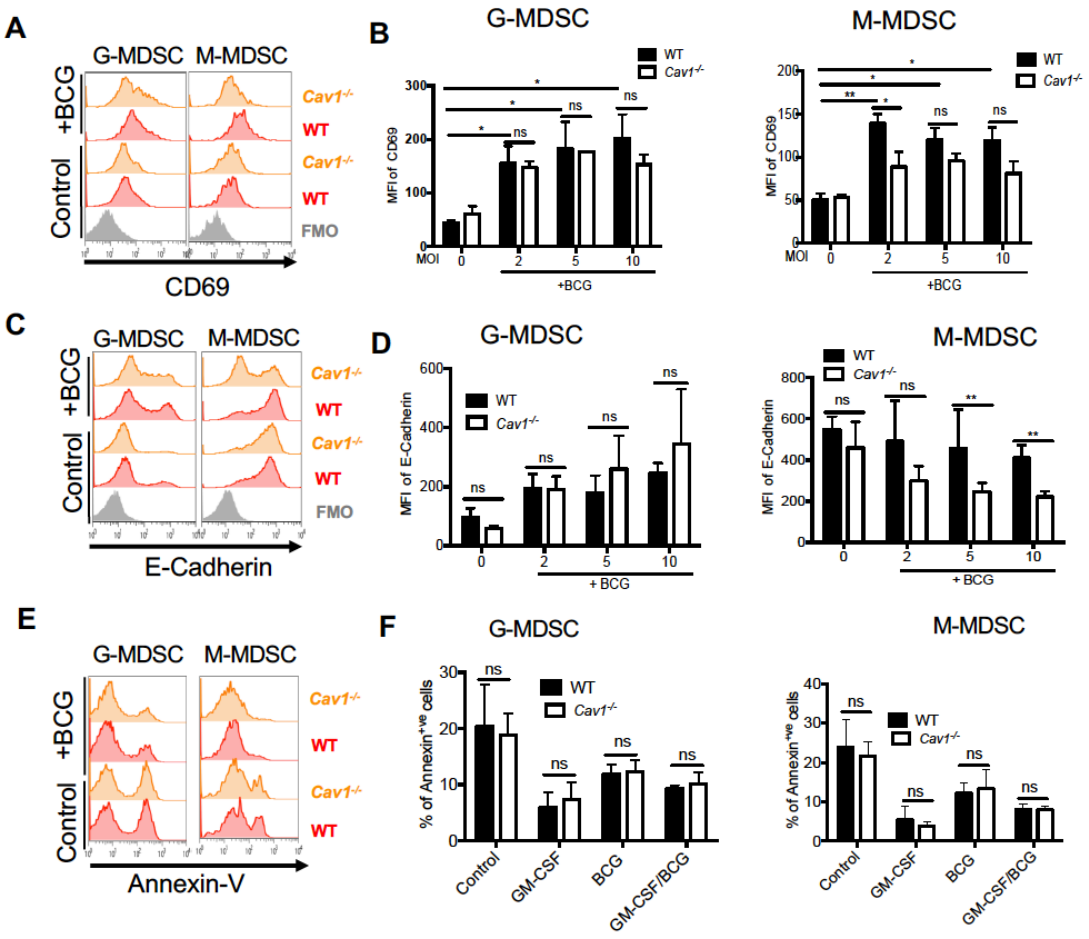

Figure 20: Deficiency of Cav-1 alters surface markers but do not affect MDSCs survival upon BCG infection. BM-MDSCs from WT or *Cav1* $^{-/-}$ mice were stimulated with BCG for 16h at 2, 5, or 10 MOI. Cells were then harvested and G-MDSCs and M-MDSCs were separately analyzed by flow cytometry for CD69 **(A, B** quantified), E-Cadherin **(C, D** quantified). D3-MDSCs were stimulated with GM-CSF, BCG or BCG/GM-CSF at 2 MOI (Multiplicity of infection). Cells were then harvested and stained for CD11b, Ly-6C and Ly-6G surface Markers and then analyzed for the Annexin-V positive G-MDSC and M-MDSC by flow cytometry after 48 hours. Representative histograms **(E)** of Annexin-V positive cells of G-MDSC and M-MDSC subsets **(F)** Graphical representation of quantified data of G-MDSCs and M-MDSCs. Data shown are from n=3-7 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5;(ns) P>0.5 unpaired, two-tailed, student's t-test.

4.2.7 Deficiency of Cav-1 alters surface markers but do not affect MDSC survival upon BCG infection

Next, we compared the activation status of WT and *Cav1*$^{-/-}$ MDSCs. We measured the early activation marker CD69 in both G-MDSCs and M-MDSCs upon BCG stimulation. BCG infection resulted significant upregulation of CD-69 in both G-MDSCs and M-MDSCs (Figure.17 A, B). Only *Cav1*$^{-/-}$ M-MDSCs had significantly decreased CD69 compared to WT. Moreover, there was no difference in CD69 expression of G-MDSC from WT and *Cav1*$^{-/-}$ mice. E-cadherin is a transmembrane protein expressed on DCs, macrophages, NK cells and associated with catenins to modulate several signaling pathways (Van den Bossche *et al.*, 2012). Additionally, deficiency of E-Cadherin has been shown to compromise the ability of Cav-1 to downregulate the inhibitor of

apoptosis protein survivin (Torres *et al.*, 2007). We found that MDSCs expressed E-Cadherin on both the subsets (Figure. 17C, D). Also, here we observed reduced E-Cadherin expression on *Cav1*$^{-/-}$ M-MDSCs but not in G-MDSCs.

Cav-1 has been previously implicated in regulating cell survival pathways (Han *et al.*, 2015). Therefore, we evaluated if Cav-1 has any role in MDSC cell survival. To rule out this, BCG-stimulated MDSCs from WT and *Cav1*$^{-/-}$ cells were analyzed for apoptosis by annexin-V staining after 48 hours. We observed BCG stimulation increased MDSC survival in both subsets of WT and Cav1$^{-/-}$ MDSCs (Figure 20E, F). However, there was no significant difference between BCG-stimulated MDSCs from WT or Cav1$^{-/-}$ MDSCs. Together, these results suggest that Cav-1 impairs surface markers such as PD-L1, CD40, CD69 and E-cadherin expression specifically on BCG-infected M-MDSCs and not on G-MDSCs. In addition, lack of Cav-1 does not affect the survival of M-MDSCs or G-MDSCs upon BCG infection.

4.2.8 Genetic deficiency of Cav-1 influences selected cytokine production in M-MDSCs upon BCG infection

MDSCs secrete an array of soluble molecules such as cytokines, chemokines, reactive oxygen and nitrogen intermediates upon mycobacterial activation (Knaul *et al.*, 2014). MDSC stimulated with BCG upregulated CD40 expression (Figure 19. B). In addition, CD40 ligation is associated with cytokine production by dendritic cells (Cella *et al.*, 1996). Since we observed reduced CD40 expression on Cav-1$^{-/-}$ M-MDSCs we further evaluated the role of Cav-1 in cytokine production in response to BCG infection. We stimulated WT and *Cav1*$^{-/-}$ MDSCs with BCG at increasing MOIs and analyzed for the cytokine production after 16 hours by ELISA. Cav-1 deficiency affected the secretion of IL-6, IL-12p40, IL-10, and TNF-α in response to BCG infection (Figure. 21 A-D). No significant difference was observed between BCG-infected WT and *Cav1*$^{-/-}$ MDSCs for IL-1β secretion (Figure. 21K). As these results were obtained from pooled MDSCs, we also wanted to know if both the subsets in *Cav1*$^{-/-}$ MDSCs had reduced cytokine secretion. For that, we carried out intracellular staining of IL-12p40 and IL-6 in BCG-infected MDSCs and analyzed by flow cytometry. M-MDSCs stimulated with BCG had increased IL-12p40 and IL-6 intracellularly (Figure.21F, G). G-MDSCs had no expression of IL-6 after BCG infection (Figure. 21 G). Moreover, *Cav1*$^{-/-}$ M-MDSCs had decreased IL-12p40 and IL-6 upon BCG infection. Together, these data imply that Cav-1 deficiency results in impaired cytokine secretion in BCG-infected M-MDSCs.

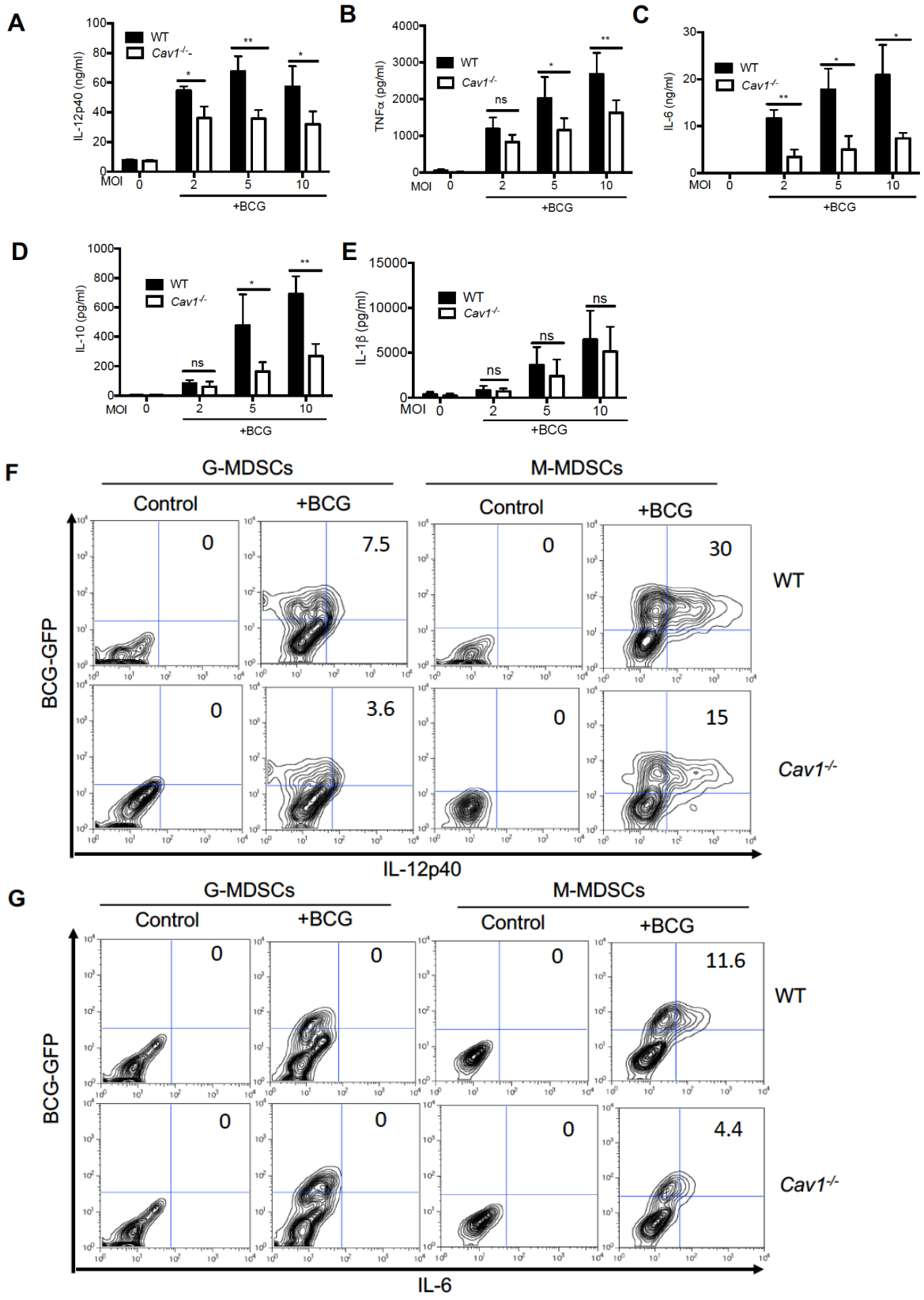

Figure 21: Genetic deficiency of Cav-1 influences cytokine production in M-MDSCs upon BCG. BM-MDSCs from WT or *Cav1* $^{-/-}$ mice were stimulated with BCG for 16h at 2, 5, or 10 MOI. Cell supernatants were measured for IL-12p40 **(A)**, TNFα **(B)**, IL-6 **(C)**, IL-10 **(D)**, IL-1β **(E)** by ELISA. Cells were harvested and G-MDSCs and M-MDSCs were separately analyzed by flow cytometry for intracellular IL-12p40 **(F)** and IL-6 **(G)**. Data shown are from n=3-7 independent experiments(A-E); n=1 (F&G). ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5;(ns) P>0.5 unpaired, two-tailed, student's t-test.

4.2.9 Cav-1 is essential for inducible nitric oxide synthase expression required for suppressive function in BCG-activated MDSCs

MDSCs secrete NO, ROS and Arg1 for suppressing T-cell proliferation (Kumar, Patel, Tcyganov and Dmitry I Gabrilovich, 2016). Hence, we also compared intracellular iNOS and arginase (Arg1) in BCG-activated WT and *Cav1*$^{-/-}$ MDSCs by flow cytometry. Interestingly, both G-MDSCs and M-MDSC had impaired inducible nitric oxide synthase upon BCG infection in the absence of Cav-1 (Figure. 22 A, B). However, level of iNOS was more in G-MDSCs as compared to M-MDSCs. These data are concordant with the finding that BM-MDSCs from *Cav1*$^{-/-}$ mice also showed massively reduced NO secretion upon BCG infection as compared to WT BM-MDSCs (Figure. 22E). Surprisingly, we did not observe any significant difference in the intracellular Arg-1 expression in WT or *Cav1*$^{-/-}$ G-MDSCs (Figure.22C, D). *Cav1*$^{-/-}$ M-MDSCs showed reduced Arg-1 expression upon BCG infection but this was not statistically significant (Figure. 22C, D). M-MDSCs had increased Arg-1 expression upon BCG stimulation but this was not the case for G-MDSCs.

As we observed impaired surface marker, iNOS expression as well as a reduced cytokine and NO secretion from *Cav1*$^{-/-}$ MDSCs in response to BCG infection, we hypothesized that MDSCs from *Cav1*$^{-/-}$ mice might also be impaired in their T cell suppression capacity. To test this, we performed *in vitro* T cell suppression assay where BCG-infected BM-MDSCs were added at different ratios to CD3/CD28 antibody activated T cells. MDSCs from *Cav1*$^{-/-}$ mice appeared with a reduced $CD4^+$ and $CD8^+$ T cell suppression capacity as compared to WT MDSCs (Figure. 22G). Furthermore, supernatants obtained from T cell suppressor assays contained less NO compared to WT MDSCs (Figure. 22 H). Since we have shown several times before that NO secretion by the M-MDSC subset among our BM-MDSCs is the major mechanism of T cell suppression (Rößner *et al.*, 2005a) (Ribechini *et al.*, 2017) (Greifenberg *et al.*, 2009), we conclude that also here, reduced NO production is functionally responsible for the reduced T cell suppression. Taken together, these results show that Cav-1 deficiency in MDSCs impairs iNOS and NO secretion that was associated with reduced T cell suppression.

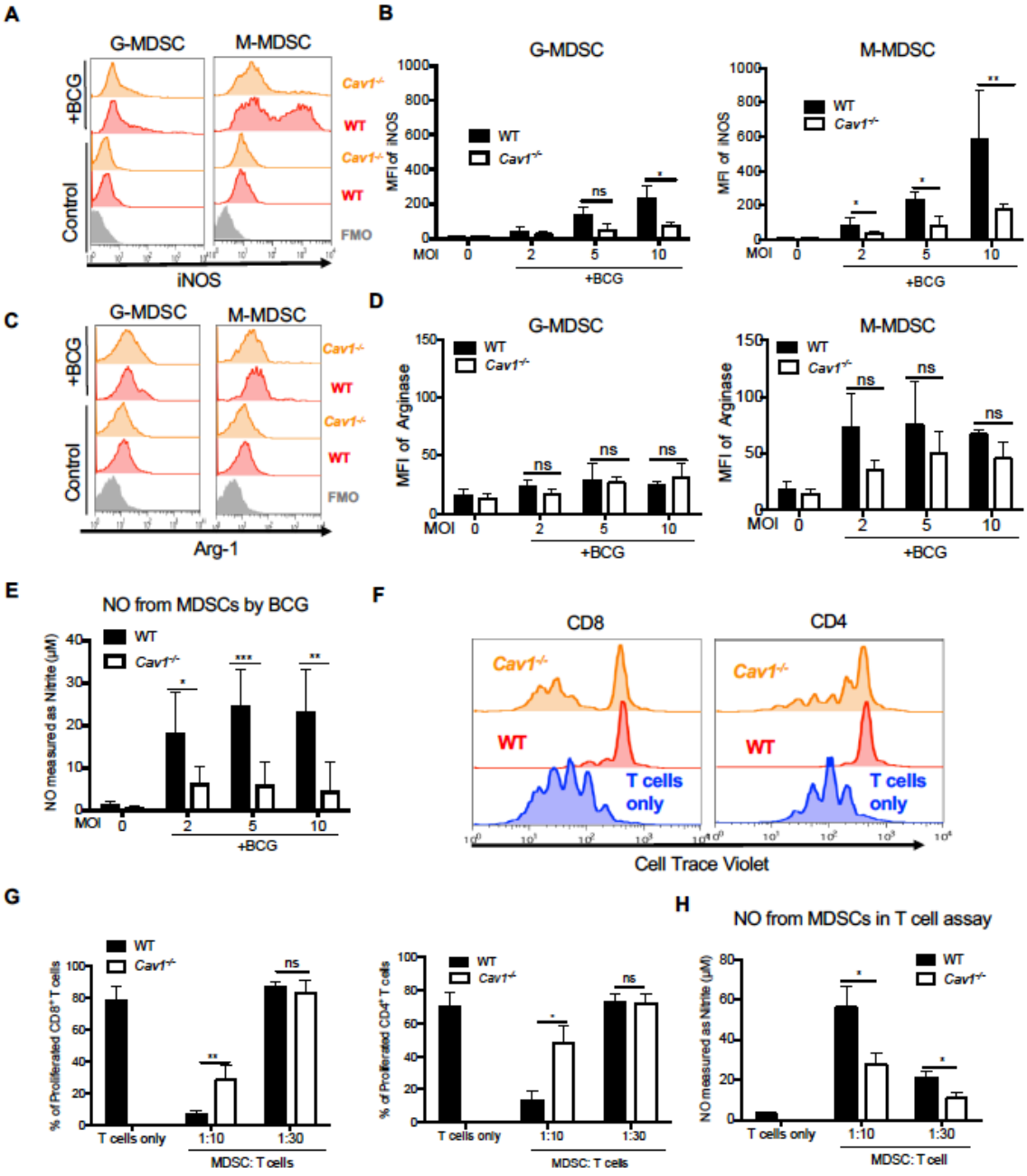

Figure 22: Cav-1 is essential for inducible nitric oxide synthase expression required for suppressive function in BCG-activated MDSCs. BM-MDSCs were stimulated with BCG for 16h at 2, 5, or 10 MOI. Cells were then harvested and G-MDSCs and M-MDSCs were separately analyzed by flow cytometry for intracellular iNOS (A, B quantified) or Arg1 (C, D quantified). Cell supernatants from WT and *Cav-1*$^{-/-}$ BM-MDSCs were stimulated with BCG-GFP at 2, 5, or 10 MOI for 16 hours. Then NO was measured as nitrite by Griess reaction (E). T cell suppressor assay. Syngeneic lymph node and spleen cells as a source of T cells were labelled with the proliferation dye Cell Trace Violet and then stimulated with anti-CD3 and anti-CD28. Then 1h BCG pre-activated BM-MDSCs from WT and *Cav-1*$^{-/-}$ mice were added or, as a control, T cells remained without MDSCs. Co-cultures were analyzed after 3 days. Cells were harvested and stained for CD4 and CD8 and analyzed by FACS to detect T cell proliferation (F). Quantification of proliferated $CD8^+$ and $CD4^+$ T cells (G). Cell supernatants from the suppressor assay were measured for NO production by Griess assay (H). Data shown are from n=3-7 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.2.10 Cav-1 is required for p38 MAPK and NF-ĸB signaling in BCG-activated MDSCs

Next, we addressed the signaling pathway leading to Cav-1 mediated reduction of NO production and thereby decreased T-cell suppression. Recognition of mycobacterial ligand by TLR2 can activate MAPK (p38 and ERK1/2) or AKT which merge to further enhance NF-ĸB signaling, required for NO secretion. We stimulated BM-MDSCs from WT and *Cav1*$^{-/-}$ mice with BCG at indicated time points and analyzed by western blot for the native and phosphorylated forms of p38, AKT, ERK1/2, and NF-ĸB-p65.

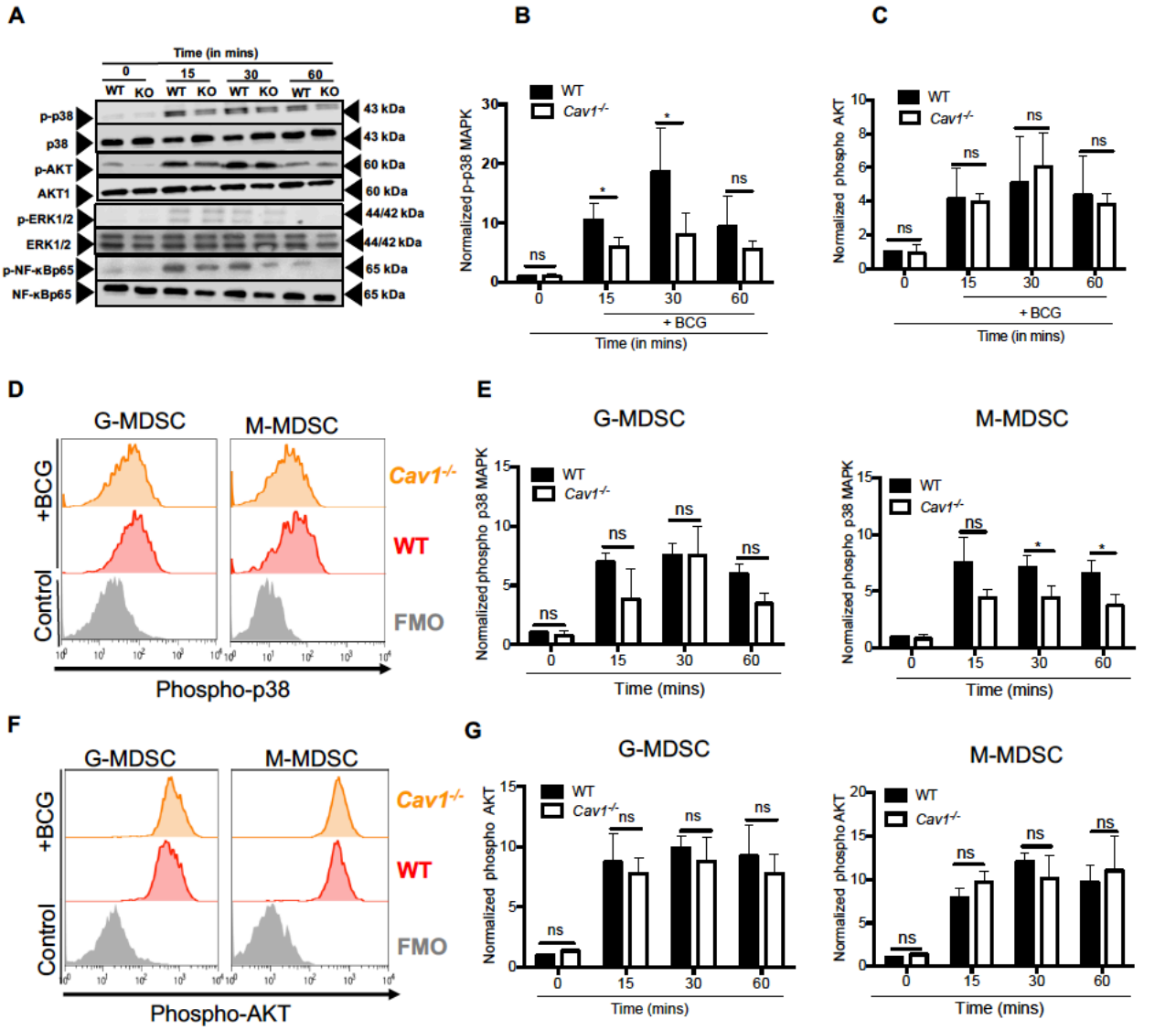

Figure 23: Cav-1 is required for p38-MAPK and NF-ĸB signaling in MDSCs upon BCG infection. (A) BM-MDSCs from WT or *Cav1* $^{-/-}$ mice were stimulated at 5 MOI BCG for the indicated time periods. Cell lysates were prepared, and Western blot analysis was used to examine the total and phosphorylated forms of p38, ERK1/2, AKT1 and NF-ĸBp65. Fold changes in phosphorylated proteins were normalized to band densities of total protein and total revert stain for phospho-p38 MAP Kinase, phospho-AKT **(C)** Experimental setting as in A but G-MDSCs and M-MDSCs were analyzed separately for phospho-p38 (**D, E** quantified) or phospho-AKT (**F, G** quantified) by flow cytometry. Data shown are from n=3-5 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

As expected, WT BM-MDSCs had increased phosphorylated p38 MAPK, ERK1/2 and NF-ΚB-p65 (Figure.23A-C). *Cav-1*$^{-/-}$ BM-MDSCs generated less phosphorylated forms of p38 MAPK and NF-ΚBp65 compared to WT MDSCs (Figure. 23 A-C). However, there was no significant difference observed in p-AKT of WT and Cav-1$^{-/-}$ MDSCs (Figure.23A, C). We also tested sorted MDSCs for their phosphorylated levels of p-38, p-AKT and p-ERK1/2. We observed that after sorting, G-MDSCs and M-MDSCs were already activated in unstimulated conditions (data not shown).

We also analyzed p-AKT and p-p38 MAPK in both the subsets by flow cytometry. Similar to western blot results, BCG stimulation resulted in increased p-AKT and p-p38 MAPK in both G-MDSCs and M-MDSCs compared to unstimulated control (Figure.23 D-G). Interestingly, the subset of M-MDSCs specifically had a reduced p38 MAPK phosphorylation in the absence of Cav-1 as compared to WT M-MDSCs (Figure. 23D, E). No significant difference was observed in G-MDSCs. Also, there was no significant difference between BCG-infected WT and *Cav1*$^{-/-}$ G-MDSCs or M-MDSCs in the phosphorylated form of AKT (Figure. 23F, G). Together, our findings implicate that Cav-1 is required for TLR2-mediated activation of p-38 MAPK and NF-ΚBp65 in BCG-infected M-MDSCs to secrete cytokines and NO production to suppress T cell proliferation.

4.3 Role of Caveolin-1 in DCs and macrophages during mycobacterial infections

4.3.1 Cav-1 is dispensable for mycobacterial uptake in DCs but required for cytokine and NO secretion upon BCG infection.

Next, we also analyzed the Cav-1 deficient dendritic cells (DCs) for the uptake of BCG and secretion of cytokines and NO production upon BCG infection. We wanted to investigate whether the results obtained from Cav$^{-/-}$ MDSCs are specific to MDSCs. For this purpose, we generated DCs from murine BM. DCs were then stimulated with BCG at different MOIs and analyzed the uptake, cytokine secretion and NO production in response to BCG infection. DCs were gated on CD11c^{hi} and MHC II^{Low} for detecting the uptake of BCG-GFP^{+} cells by flow cytometry. No significant difference was observed in the uptake of BCG by WT and *Cav1*$^{-/-}$ DCs *(*Figure.24 A, B). However, similar to MDSCs, BCG-stimulated DCs in the absence of Cav-1 had also defect in IL-6 secretion (Fig. 24 C) and NO production (Figure. 24 D). These results suggest that Cav-1 deficient DCs also had impaired NO production and cytokine signaling in response to mycobacterial infection.

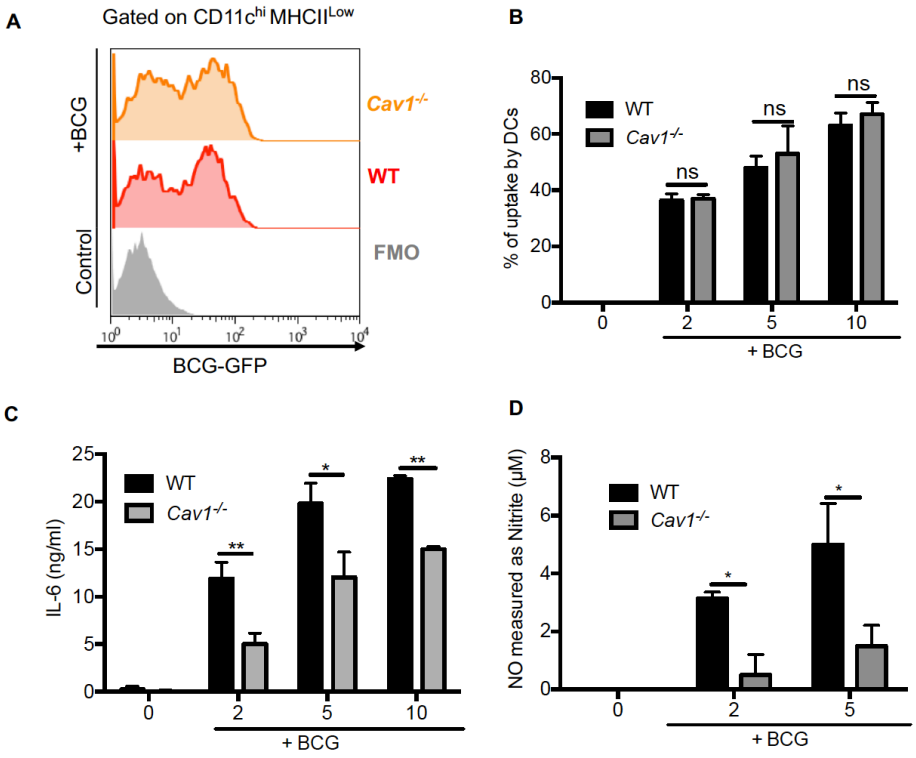

Figure 24: Cav-1 is dispensable for mycobacterial uptake in DCs but required for cytokine and nitric oxide secretion upon BCG infection. D8-DCs from WT or *Cav1* $^{-/-}$ mice were stimulated with BCG-GFP at MOI of 2, 5 or 10 for 6h. After gating out live cells and doublet exclusion. CD11c^{hi} and MHC II^{Low} were then analyzed by flow cytometry for BCG uptake by GFP detection as exemplified **(A)** and quantified **(B)**. Cell supernatants were analyzed from same experimental settings were analyzed for IL-6(C) by ELISA or NO (D) by Griess assay. Data shown are from n=2 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.3.2 Cav-1 is not required for BCG uptake in macrophages but essential for nitric oxide secretion upon BCG infection

Macrophages serve as one of the major cells in TB pathogenesis (Guirado, Schlesinger and Kaplan, 2013). Therefore, in the next study, we evaluated if Cav-1 deficient macrophages had also similar defect like MDSCs and DCs. To evaluate this, we stimulated macrophages from murine BM. Macrophages were then stimulated with BCG at different MOI and analyzed for the uptake of BCG and iNOS expression by flow cytometry. Macrophages were gated on CD11b^{hi} and F4/80^{hi} cells (Figure.25A). *Cav1*$^{-/-}$ macrophages showed no significant difference in the internalization of BCG in macrophages similar to DCs and MDSCs (Figure.25 B, D). Furthermore, lack of Cav-1 impaired iNOS expression upon BCG infection (Figure.25 C, E). Together, these results suggest that *Cav1*$^{-/-}$ DCs and macrophages have impaired nitric oxide signaling in response to BCG but no mycobacterial uptake.

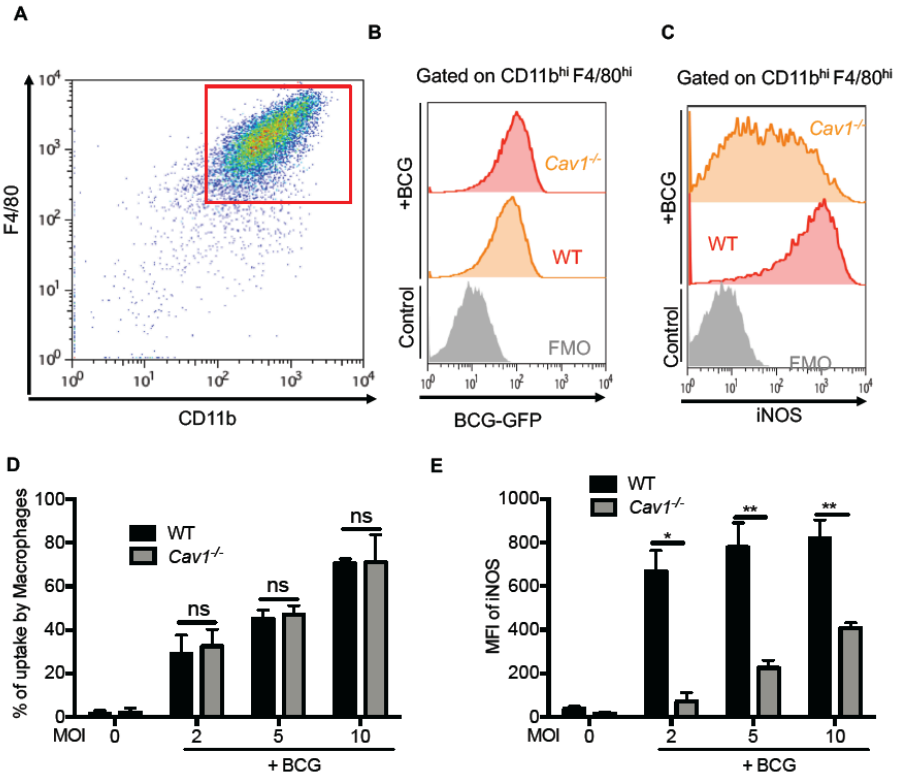

Figure 25: Cav-1 is not required for BCG uptake in macrophages but essential for NO secretion upon BCG infection. (A) D8-macrophages from WT or *Cav1* $^{-/-}$ mice were stimulated with BCG-GFP at MOI of 2, 5 or 10 for 6h. After gating out live cells and doublet exclusion. Macrophages were identified as CD11b^{hi} and F4/80^{hi} cells. **(B)** CD11b^{hi} and F4/80^{hi} were then analyzed by flow cytometry for BCG uptake by GFP detection as exemplified **(B)** and quantified **(D)**. Cells were also analyzed for iNOS expression **(C, E** quantified) by macrophages from the same experimental set up. Data shown are from n=2 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.4 Role of Acid Sphingomyelinase in BCG-infected MDSCs

4.4.1 Blocking acid sphingomyelinase (ASM) by pharmacological inhibitors do not interfere with phagocytosis of BCG into MDSCs

Acid sphingomyelinase (ASM) plays a major role in internalization of *Neisseria gonorrhoeae* (C R Hauck *et al.*, 2000). In this study, we wanted to investigate if ASM has any role in the phagocytosis of mycobacteria. ASM can be blocked by functional inhibitors or anti-depressants such as amitryptiline and desipramine (Beckmann *et al.*, 2014). Administration of these drugs results in the accumulation of anti-depressants in the lysosome which further interferes with the binding of ASM to the plasma membrane and leads to subsequent inactivation of the enzyme by proteolytic degradation (Kölzer, Werth and Sandhoff, 2004). Here, MDSCs were treated with amitryptiline or desipramine at increasing concentrations prior to BCG-GFP infection and then analyzed for the endocytosis of BCG after 6 hours by flow cytometry. Pharmacological inhibition by amitryptiline or desipramine did not block BCG uptake into G-MDSC or M-MDSC (Figure. 26 A, B). Inhibition with positive control

cytochalasin-D (Cyt-D) which blocks actin polymerization reduced the uptake significantly in both G-MDSC and M-MDSC subsets. Therefore, these results show that ASM is dispensable for mycobacterial entry by both G- MDSCs and M-MDSCs.

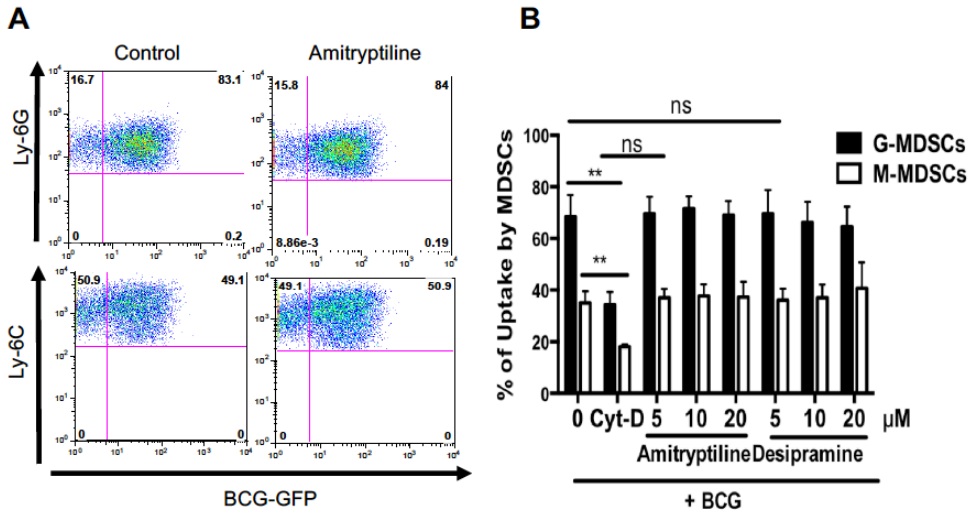

Figure 26: Blocking acid sphingomyelinase (ASM) by pharmacological inhibitors do not interefere with phagocytosis of BCG into MDSCs. BM-MDSCs were incubated with Cytochalasin-D or desipramine or amitryptiline for 1h and then stimulated with BCG-GFP at MOI of 5 for 6h. Cells were then analyzed by flow cytometry for BCG uptake by GFP detection in G-MDSC and M-MDSC subsets as exemplified **(A)** and quantified **(B)**. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.4.2 Blocking ASM by pharmacological inhibitors alters cytokine and NO production upon BCG infection in MDSCs

Bacterial infection causes activation of acid sphingomyelinase activity which results in the hydrolysis of sphingomyelin (SM) to form ceramide (Simonis *et al.*, 2014). ASM inhibitors suppress LPS-mediated secretion of cytokines (Sakata *et al.*, 2007b). Therefore, we asked if ASM inhibitors can suppress BCG-induced cytokine and NO production by MDSCs. To evaluate this, we stimulated MDSCs with increasing concentration of amitryptiline and desipramine before infecting with BCG and then analyzed for the cytokine production and NO production after 16 hours. Blocking ASM resulted in significantly decreased secretion of IL-6 and IL-10 by MDSCs in response to BCG in dose-dependent fashion (Figure.27A, B). However, there was also significantly reduced IL-1β and IL-12p40 secretion after blocking ASM on BCG-stimulated MDSCs but not in a dose-dependent manner (Figure.27C, D). Also, blocking ASM impaired the NO production by MDSCs in response BCG infection (Figure.27E). Together, these data suggest that blocking ASM affects the secretion of cytokines and NO in BCG-infected MDSCs but not mycobacterial uptake.

4.4.3 Inhibition of ASM alters AKT signaling upon BCG infection in MDSCs

Mycobacteria induces phosphorylation of AKT, ERK1/2 and p38 MAPK on DCs or macrophages upon activation (Jo *et al.*, 2007) . Activation of PI3K/AKT is important for cytokine secretion from peritoneal macrophages (Tapia-Abellán *et al.*, 2013) . Since we observed a reduced cytokine secretion in BCG-activated MDSCs, we analyzed for phosphorylation of AKT by flow-cytometry in MDSCs treated with ASM inhibitor amitryptiline. As expected, we noted increased AKT phosphorylation in both G-MDSCs and M-MDSCs upon BCG infection (Figure.28 A, B). However, blocking of ASM resulted in significantly reduced phosphorylated form of AKT in BCG-infected M-MDSCs. G-MDSCs also showed a trend of reduced AKT phosphorylation but it was not statistically significant. Hence, from these results we conclude that blocking of ASM by pharmacological drugs inhibited AKT signaling in BCG-stimulated M-MDSCs.

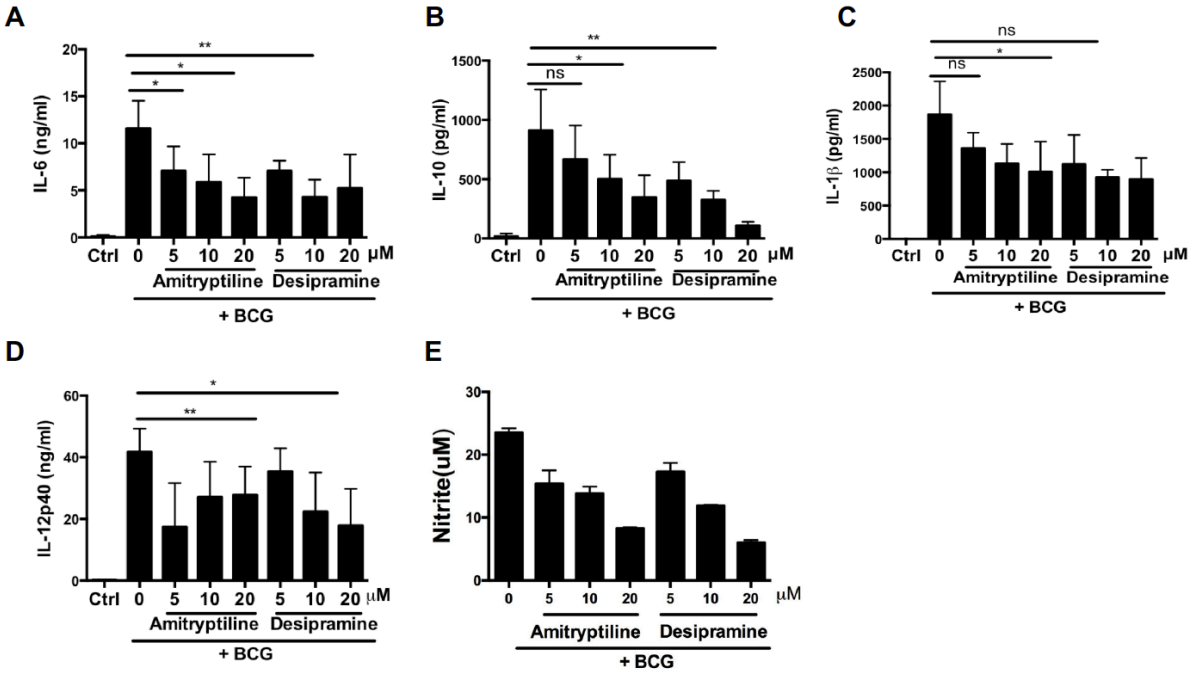

Figure 27: Blocking acid sphingomyelinase (ASM) by pharmacological inhibitors impairs cytokine and NO production upon BCG infection in MDSCs. BM-MDSCs were incubated with amitryptiline and desipramine at increasing concentrations for 1 hour and then stimulated with BCG-GFP at 5MOI. Cell supernatants were measured for IL-6**(A)**, IL-10 **(B)**, IL-1 β **(C)**and IL-12P40**(D)**, by ELISA and NO**(E)** by griess assay after 16 hrs. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

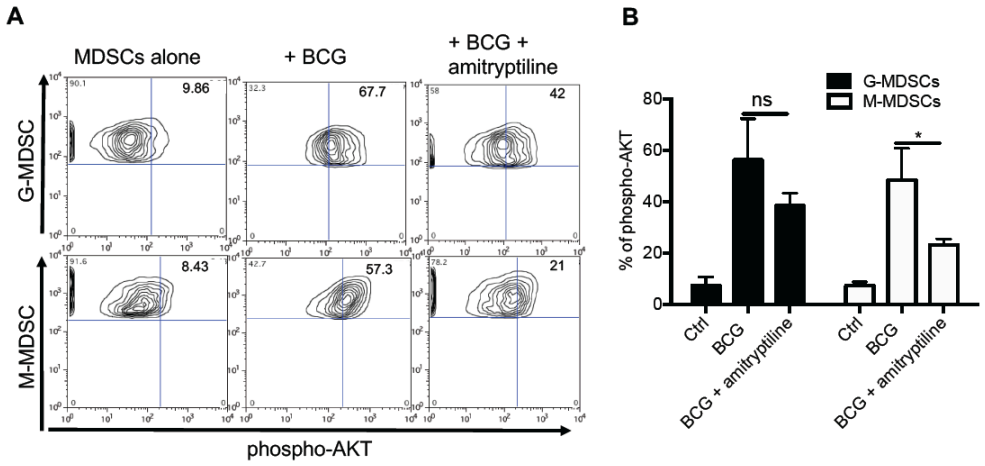

Figure 28: Blocking ASM alters AKT signaling upon BCG infection in MDSCs. A&B) D3-MDSCs were incubated with amitryptiline for 1 hour and then stimulated with BCG-GFP at 5MOI. Cells were harvested and stained for the surface marker Ly-6C and Ly-6G and intracellularly for p-AKT and analyzed by flow cytometry. Data shown are from n=2 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.4.4 ASM deficient MDSCs take up BCG similarly to wildtype MDSCs

We did not find any difference in the uptake of BCG in MDSCs by using pharmacological drugs to block ASM. Thus, we wanted to confirm these findings by using MDSCs from ASM knockout mice. Similar to blocking studies, gene deficient MDSCs also did not show any difference in the uptake of BCG between WT and ASM$^{-/-}$ G-MDSCs or M-MDSCs. (Figure.29 A-E). Taken together, these results implicate that blocking ASM or its genetic deficiency has no effect on the mycobacterial uptake into G-MDSCs or M-MDSCs

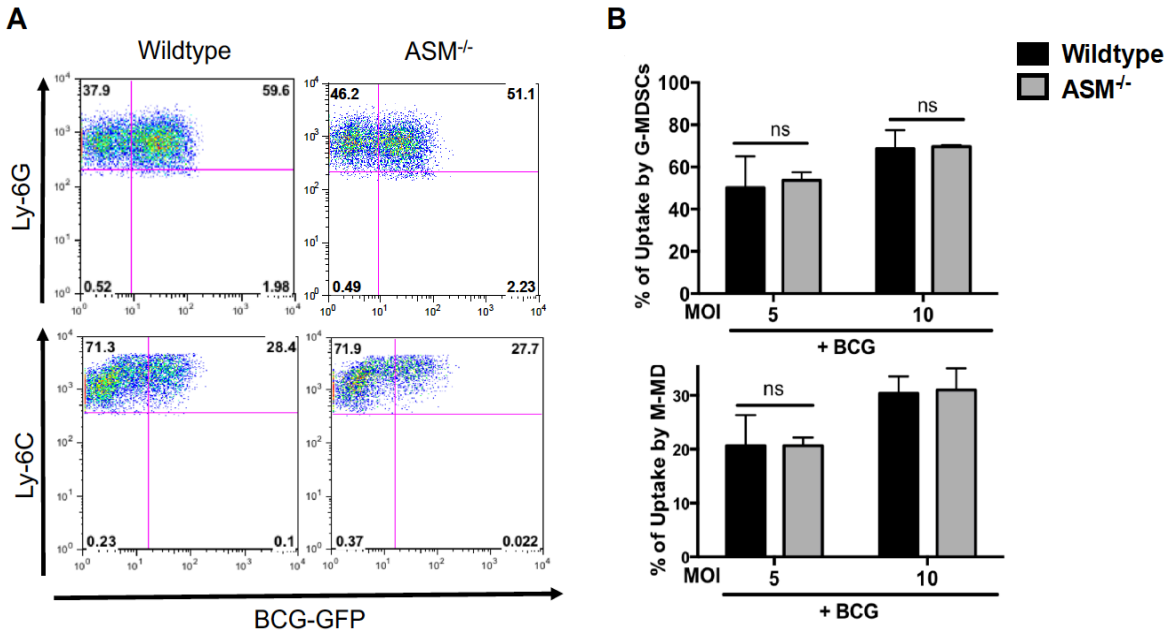

Figure 29: ASM deficient MDSCs uptake BCG similar to Wildtype MDSCs. BM-MDSCs were generated from Wildtype or ASM$^{-/-}$ mice and stimulated with BCG-GFP at 5,10 (MOI) and tested for BCG uptake of G-MDSCs and M-MDSCs. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.4.5 ASM deficiency impairs cytokine and NO production in BCG-infected MDSCs

Next, we also examined for cytokine production and NO production in BCG-stimulated wildtype and ASM$^{-/-}$ MDSCs to confirm the results obtained by blocking ASM with drugs. For this purpose, wildtype and ASM deficient MDSCs were treated with BCG at different MOI and analyzed for their cytokine levels by ELISA and NO levels by Griess assay. Interestingly, we observed reduced IL-6, IL-1β and TNF in ASM deficient BCG-infected MDSCs as compared to wildtype MDSCs in dose dependent fashion (Figure.30 A, B, C). Also, NO production by ASM$^{-/-}$ MDSCs was impaired in response to BCG infection (Figure.3E). Together, results from blocking studies and genetically deficient ASM MDSCs suggest that ASM is an essential component for cytokine and NO production in mycobacterial infections in MDSCs but not required for mycobacterial uptake into MDSCs.

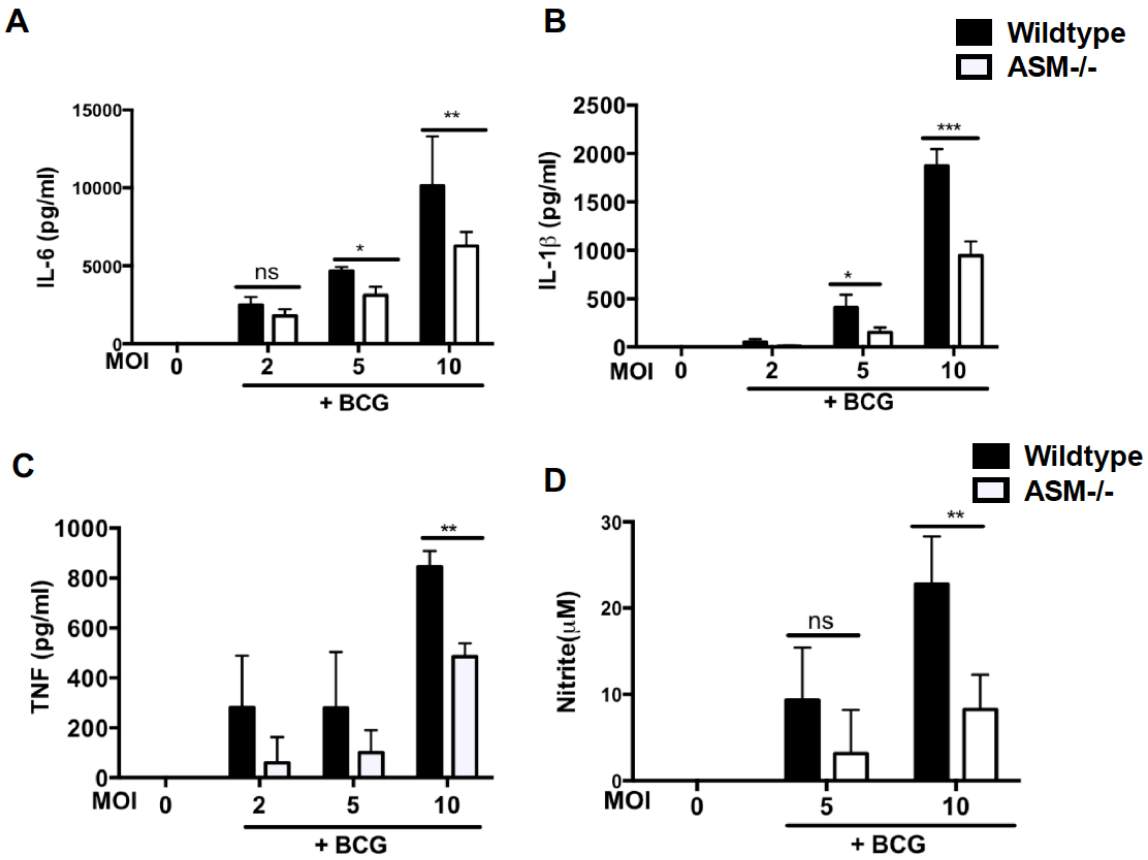

Figure 30: ASM deficiency impairs cytokine production and NO production in BCG-infected MDSCs. BM-MDSCs were generated from Wildtype or ASM$^{-/-}$ mice and stimulated with BCG-GFP at 5,10(MOI). Cell supernatants were measured for IL-6**(A)**, IL-1 β **(B)**and TNF**(C)**, by ELISA and NO**(D)** by Griess assay after 16 hrs. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.5 Asialo-GM1 in BCG-stimulated MDSCs

4.5.1 Asialo-GM1 is expressed on both subsets of MDSCs and further upregulated specifically upon mycobacterial stimulation

Asialo GM1 is a ganglioside which has been shown to be expressed on MDSC cell surface (Rößner *et al.*, 2005b). In this study, we wanted to investigate the significance of asialo-GM1 on mycobacteria-activated G-MDSCs and M-MDSCs. To address this, we infected MDSCs with BCG-live, BCG-killed, Mtb killed, Listeria killed and also with TLR2 agonist (Pam_3CSK_4) and TLR4 agonist (LPS) and analyzed for the surface expression of asialo-GM1 on both subsets of MDSCs by FACS. Interestingly, BCG Live, BCG killed and Mtb killed specifically upregulated asialo-GM1 expression on both G-MDSCs and M-MDSCs (Figure. 31A, B). However, no significant difference was observed upon LPS, Pam_3CSK_4 or Listeria killed stimulation on both subsets of MDSCs. We also acquired confocal microscopy images of BM-MDSCs and stimulated them with BCG. Unstimulated MDSCs had less asialo-GM1 staining as compared to BCG-stimulated MDSCs (Figure.31C). Together, these results indicate that both the subsets of MDSCs express asialo-GM1 and is further upregulated specifically upon mycobacterial stimulation.

4.5.2 Asialo-GM1 binds to BCG in dose-dependent manner

Previous reports showed that *Pseudomonas aeruginosa* binds to ganglioside asialo-GM1 (Gupta *et al.*, 1994). Since we observed upregulation of asialo-GM1 on MDSCs, we hypothesized that mycobacteria might bind to asialo-GM1 and result in immune activation. To test if asialo-GM1 binds to BCG, we performed a binding assay. BCG-GFP was incubated with soluble form of asialo-GM1 at increasing concentrations overnight and binding was accessed using asialo-GM1 antibody by flow cytometry (Figure. 32 A). There was increase in BCG-GFP and asialo-GM1 double positive cells in a dose dependent manner (Figure. 32 B, C). BCG alone and BCG without soluble form of asialo-GM1 were used as controls. Hence, these results indicate that BCG bind to asialo-GM1.

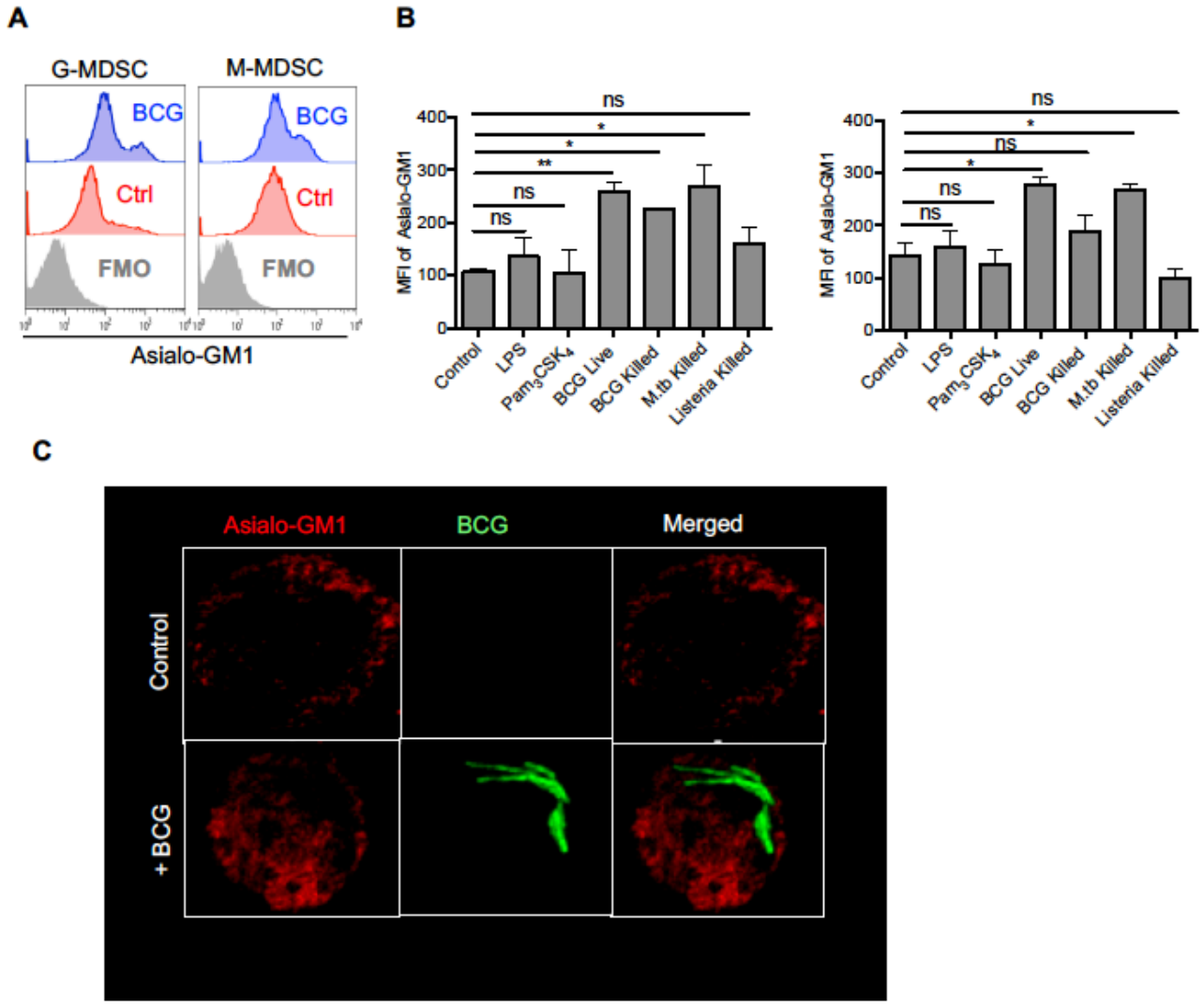

Figure 31: Asialo-GM1 is expressed on both subsets of MDSCs and further upregulated specifically upon mycobacterial stimulation. BM-MDSCs were stimulated with BCG Live, BCG Killed, Mtb Killed and after 16h analyzed for Asialo-GM1 expression on G-MDSCs and M-MDSCs as **(A)** exemplified and **(B)** quantified. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

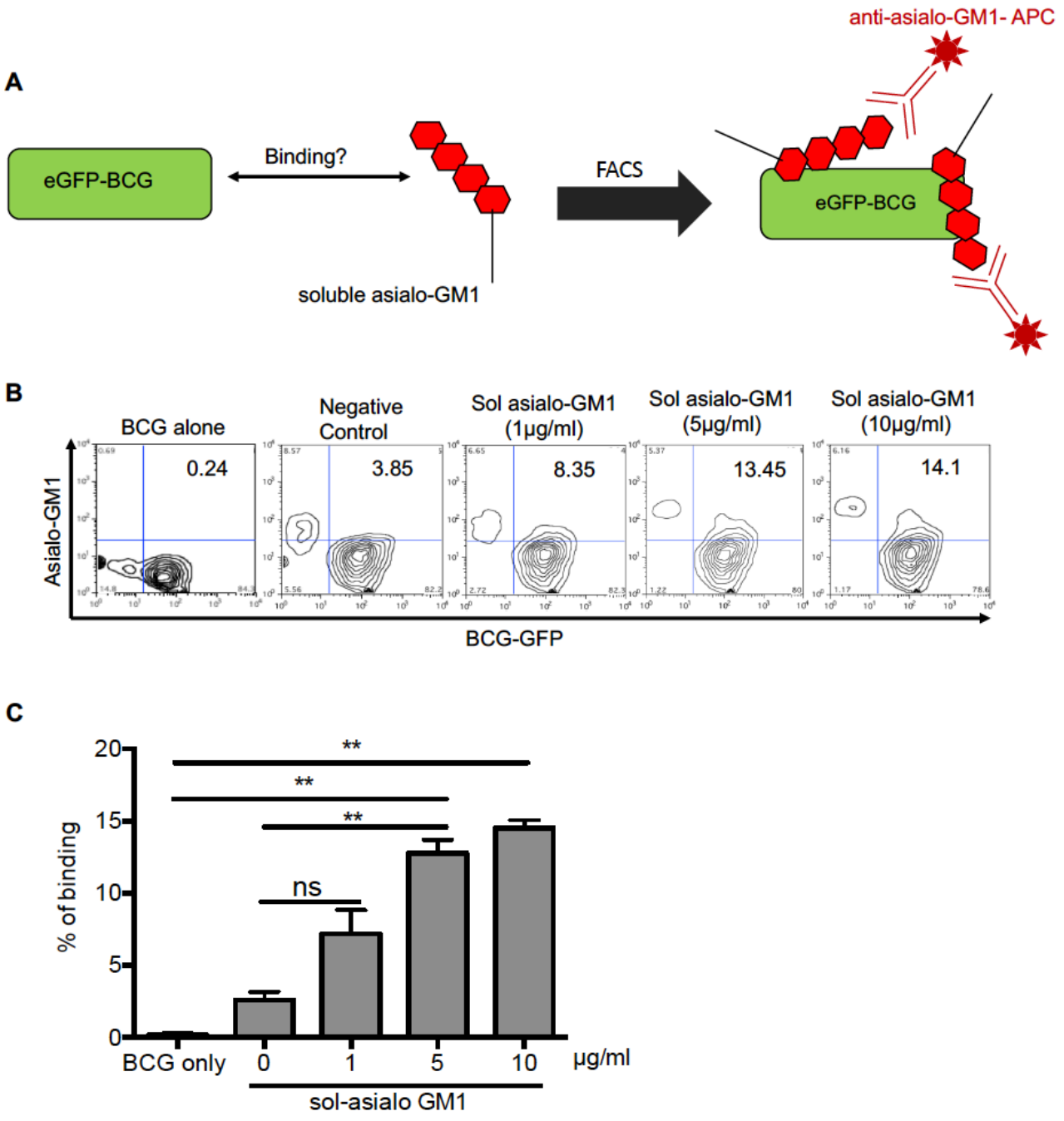

Figure 32: Asialo-GM1 binds to BCG in a dose dependent manner. **(A)** Binding assay: GFP-BCG was incubated with soluble asialo-GM1 at increasing concentrations (1,5,10 µg/ml) overnight. Binding was detected using APC conjugated asialo-GM1 antibody. **(B&C)** Binding was analyzed by % of double positive BCG and asialo-GM1.BCG alone was used as a control. Negative control was also used where no soluble form of asialo-GM1 was added. **(C)** quantified data. Data shown are from n=2 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5; (ns) P>0.5 unpaired, two-tailed, student's t-test.

4.5.3 Asialo-GM1 is dispensable for mycobacterial uptake into G-MDSCs and M-MDSCs

Mtb steal lipids from the host environment for their intracellular survival inside the cell (Stehr, A. and Singh, 2013). There are several routes of entry for this deadly pathogen which can induce immune activation, signal transduction and bacterial persistence (van Crevel, Ottenhoff and van der Meer, 2002). Gangliosides have been implicated in providing a portal for entry of intracellular bacteria in murine macrophages (Naroeni and Porte, 2002). Since Mtb

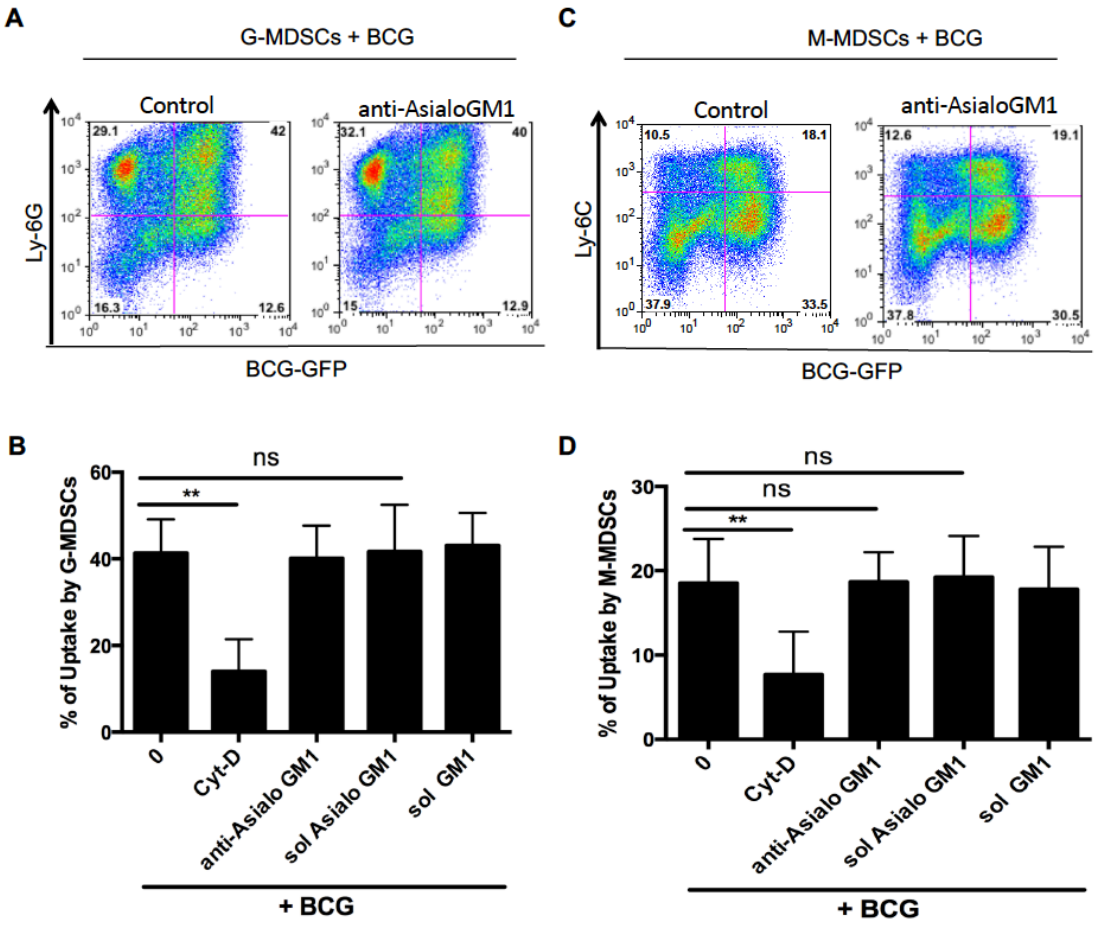

Figure 33: Asialo-GM1 is dispensable for mycobacterial uptake into G-MDSCs and M-MDSCs. BM-MDSCs were incubated with Cyt-D or anti-asialo GM1 or soluble asialo GM1 or soluble GM1 for 1h and then stimulated with BCG-GFP at MOI of 5 for 6h. Cells were then analyzed by flow cytometry for BCG uptake by GFP detection in G-MDSC and M-MDSC subsets as exemplified **(A & C)** and quantified **(B & D)**. Data shown are from n=3 independent experiments. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test.

cell wall contains mainly lipid contents, we hypothesized that mycobacteria might use asialo-GM1 for gaining entry in MDSCs. We blocked asialo-GM1 on MDSCs either with asialo GM1 antibody, soluble form of asialo-GM1 or soluble form of GM1 and then stimulated with BCG and analyzed for the uptake by flow cytometry. We also used cytochalasin-D (Cyt-D) as a positive control to block actin polymerization (Schliwa, 1982). Blocking asialo-GM1 with antibody or with soluble form of asialo-

GM1 or GM1 did not affect BCG endocytosis into G-MDSCs and M-MDSCs (Figure.33A-D). However, treating MDSCs with cytochalsin-D reduced the uptake of BCG as expected. These findings implicate that asialo-GM1 is upregulated upon mycobacterial stimulation but is dispensable for their entry into MDSCs.

4.5.4 Blocking asialo-GM1 has no effect on cytokine and NO production by MDSCs upon BCG infection

Apart from phagocytosis, asialo-GM1 plays a pivotal role in amyloid β-induced cytokine secretion in monocytic cell line,THP-1 (Ariga and Yu, 1999). Therefore, we wanted to examine whether asialo-GM1 has any role in cytokine and NO production. We used asialo-GM1 antibody to block asialo-GM1. Asialo-GM1 antibody was a polyclonal rabbit antibody and therefore, we also used rabbit serum as a control. Galectin-1 was also used as a control to block GM1 since it is a major receptor for ganglioside GM1 (Kopitz *et al.*, 1998). MDSCs were treated with these blocking agents and then infected with BCG and analyzed for cytokine and NO production after 16 hours. Surprisingly, treatment of anti-asialo GM1 or Galectin-1 had no effect on cytokine release or nitric oxide production by MDSCs upon BCG infection (Fig.34 A-E). Also, rabbit serum did not alter cytokine or NO release confirming the specificity of asialo-GM1antibody. These findings indicate that asialo-GM1 is not required for cytokine or nitric oxide secretion by BCG-infected MDSCs.

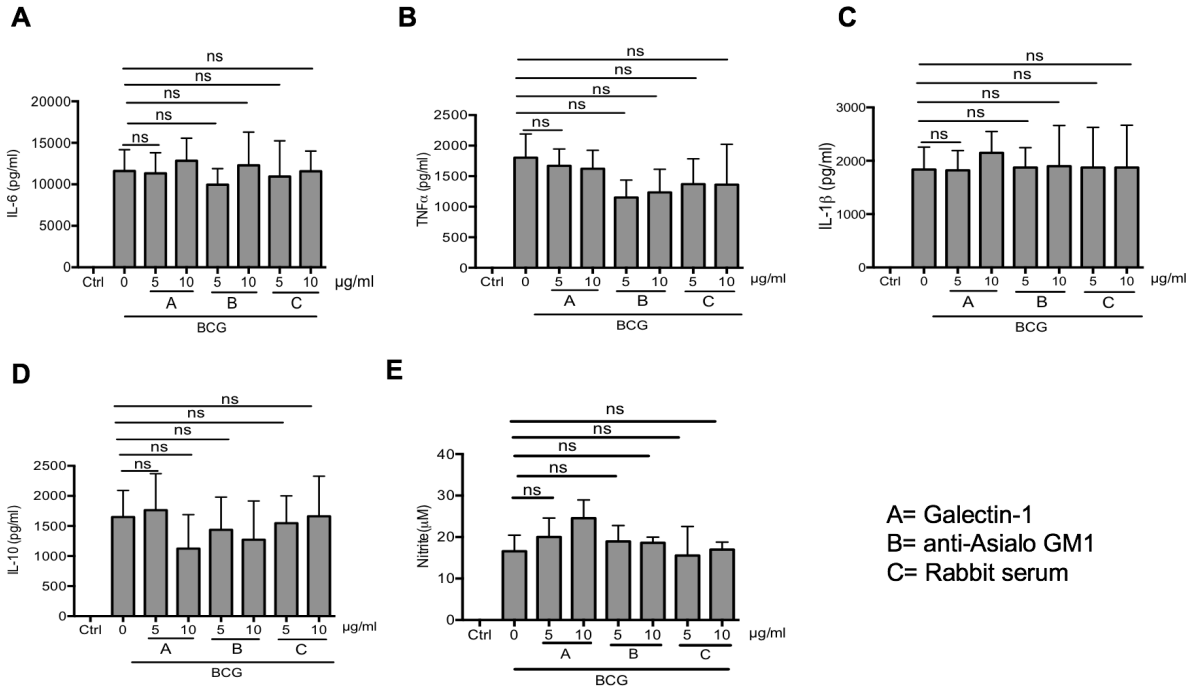

Figure 34: Blocking Asialo-GM1 has no effect on cytokine and NO production upon BCG infection. BM-MDSCs were incubated with anti-asialo-GM1 or Galectin-1 or rabbit serum at increasing concentrations for 1 hour and then stimulated with BCG-GFP at 5MOI. Cell supernatants were measured for IL-6**(A)**, TNF-α**(B)**, IL-1 β (C)and IL-10(D), by ELISA and NO**(E)** by Griess assay after 16 hrs. Data shown are from n=6 independent experiments. *P<0.5 (ns) P>0.5 unpaired, two-tailed, student's t-test

4.5.5 Blocking asialo-GM1 do not impair BCG induced AKT signaling in G-MDSC or M-MDSC

Mycobacterial virulence factors induce phosphorylation of AKT and subsequent signaling for the cell survival (Maiti, Bhattacharyya and Basu, 2001). Gangliosides have also been shown to be important for inducing PI3K/AKT/mTOR signaling in human neuroblastoma cell lines (DURBAS *et al.*, 2015). Hence, we wanted to examine the role of asialo-GM1 in BCG induced AKT signaling. For this purpose, we incubated MDSCs with anti-asialoGM1 for one hour and infected them with BCG for 4 hours and then analyzed them for AKT phosphorylation by G-MDSCs and M-MDSCs by flow cytometry. BCG infection induced AKT phosphorylation on both G-MDSCs and M-MDSCs (Figure.35 A, B). However, we did not observe any significant differences between BCG or blocking with anti-asialoGM1 on MDSCs. Together, these findings imply that blocking asialo-GM1 did not affect p-AKT signaling in G-MDSCs or M-MDSCs

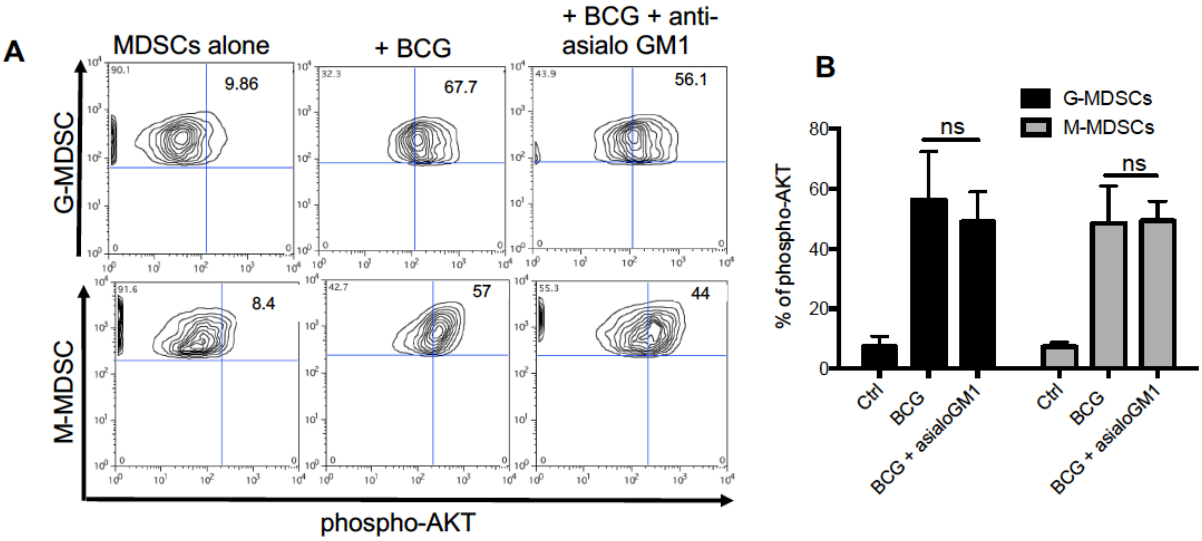

Figure 35: Blocking asialo-GM1 do not impair BCG induced AKT signaling in G-MDSCs or M-MDSCs. A&B) BM-MDSCs were incubated with anti-asialo-GM1 for 1 hour and then stimulated with BCG-GFP at 5 MOI. Cells were harvested and stained for the surface marker Ly-6C and Ly-6G and intracellularly for p-AKT and analyzed by flow cytometry. Data shown are from n=2 independent experiments. (ns) P>0.5 unpaired, two-tailed, student's t-tesT

5.Discussion

MDSCs are the major myeloid regulatory cells massively induced during TB infection in both human and mice (du Plessis *et al.*, 2018). The data provided in this thesis provides insights into the functional role of lipid-rich areas, Cav-1, ASM and asialo-GM1 on mycobacteria-infected MDSCs (Rößner *et al.*, 2005a). These lipid-rich components are not involved in phagocytosis of mycobacteria in MDSCs as reported for other cell types but affect signaling pathways in MDSCs upon mycobacterial activation. Cav-1 influences MDSC activation through TLR2 signaling required for T-cell suppression by NO production. ASM is an essential component for cytokine secretion, NO production and activation of AKT signaling in BCG activated MDSCs. Asialo-GM1 is upregulated specifically upon mycobacterial stimulation on MDSCs and binds to BCG but are not required for cytokine and NO production or AKT signaling.

5.1 Role of MDSCs in mycobacterial infections

In this study, we observed that both G-MDSCs and M-MDSCs subsets readily phagocytose BCG and produce NO and cytokines in response to BCG. Moreover, functional activity of BCG-activated MDSCs in suppressing $CD4^+$ and $CD8^+$ T-cell proliferation was NO-dependent. These results confirmed that *in vitro* generated MDSCs behave similarly to human MDSCs in TB infection.
We also compared BCG-live, BCG-heat-killed and Mtb-heat-killed for their ability to activate MDSCs. Although MDSCs phagocytosed BCG-live and BCG-killed in a similar manner, the killed form of BCG could not secrete cytokines and NO as efficient as BCG-live. Previous reports also showed that heat-killed *Mycobacterium indicus pranii* activated macrophages secrete significantly reduced cytokines and NO as compared to its live form (Kumar *et al.*, 2014). Heat treatment denatures immune-active molecules such as TLR ligands (e.g. LPS) and results in diminished TNF-α inducing activity (Gao, Wang and Tsan, 2006). The possible reason for the lower level of cytokine and NO production in MDSCs activated by BCG-killed compared to BCG-live might be due to denaturation of immune-active molecules during the heating process.

DCs and macrophages have been a topic of extensive research in TB infection but there are only a few studies exploring the role of MDSCs in Mtb infection. Previous findings suggested that MDSCs have weak phagocytic activity compared to DCs and macrophages but MDSCs secrete increased nitrogen intermediates (Veglia, Perego and Gabrilovich, 2018c). In this study, we also found that macrophages had an increased phagocytic capacity to take up BCG as compared to MDSCs. Moreover, DCs secreted lower amount of NO upon BCG infection than MDSCs. These immunogenic (DCs and macrophages) and immunosuppressive (MDSCs) perform different functions during TB infection. In the TB granuloma, DCs are involved in stimulating antigen-specific T-cells whereas,

MDSCs have a role in T-cell suppression by secreting suppressive molecules such as NO.Thus, MDSCs may impair the activity of protective anti-TB immune responses. Reduced MDSC population after successful treatment of TB (Knaul *et al.*, 2014) indicate that it might be detrimental to the host.

5.2 Cav-1 expression on BCG-infected MDSCs

In this study, we found that Cav-1 is expressed and further upregulated with BCG infection on the surface of both G-MDSCs and M-MDSCs. However, basal expression of Cav-1 was observed to be higher in M-MDSCs as compared to G-MDSCs. Our data add on previous findings showing Cav-1 upregulation in HIV infected macrophages (Mergia, 2017). Cav-1 expression is also increased on DCs upon maturation by LPS (Oyarce *et al.*, 2017). In contrast, there is also a report showing Cav-1 down-regulation in human colon and ovarian cancer (Wiechen *et al.*, 2001) (Bender *et al.*, 2000). Previous findings implicate that Cav-1 on the cell membrane might have different functions depending on the cell type or pathogen the cell encounters. In DCs, increased expression of Cav-1 plays a role in the migration of DCs to lymph nodes through Rac1 signaling to elicit effective $CD8^+$ T cell response (Oyarce *et al.*, 2017). In HIV infected macrophages, upregulation of Cav-1 was important for virus pathogenesis and persistence (Mergia, 2017). Therefore, Cav-1 upregulation in MDSCs indicates its functional role of MDSCs in cell signaling and mycobacterial persistence

5.3 Expression of PRRs on MDSCs in the absence of Cav-1

Multiple PRRs for recognition of mycobacterial ligands have been characterized in DCs and macrophages (Philips and Ernst, 2012b). TLR2 and TLR4 are the most prominent PRRs for mycobacterial recognition. However, there are no studies reported on PRRs of MDSCs for Mtb recognition. We found that G-MDSCs had upregulated expression of surface TLR2 and TLR4 upon BCG infection. M-MDSCs had upregulated expression of surface TLR2 but not TLR4 in response to BCG infection. However, we observed increased expression of TLR4 intracellularly in BCG-infected M-MDSCs. Previous findings have shown that surface TLR2 is upregulated upon bacterial stimulus on human neutrophils (Kurt-Jones *et al.*, 2002). Monocytes from HIV-infected patients have increased surface expression of TLR2 but not TLR4 (Heggelund *et al.*, 2004). These results together indicate that innate immune cells upregulate TLR2 or TLR4 depending on the cell type and infection to initiate the signaling upon pathogen exposure.

Lipid-rich surfaces can act synergistically with TLRs in enhancing their signaling intensity. Cav-1 is also associated with these lipid rafts and linked with TLR4 (Tsai *et al.*, 2011a)(X.M. Wang *et al.*, 2009). Cav-1 has been shown to be required for the TLR4 expression and signaling in peritoneal macrophages (Tsai *et al.*, 2011a). In contrast to this, we could not find a differential surface or intracellular expression and up-regulation of TLR4 in the absence of Cav-1 after BCG infection on MDSCs.Cav-1 has been also associated with TLR2 function. In a murine chronic asthma model, inhibition of airway inflammation occurred via Cav-1 through TLR2 mediated activation of MyD88 and NF-KB (Fang *et al.*, 2016). We found that TLR2 showed no differences for its surface expression

but showed lower amounts of TLR2 in the cytoplasm of *Cav1*$^{-/-}$ M-MDSCs before BCG infection and a failure to increase TLR2 after BCG infection. Previous reports suggested that TLR2 localized around yeast containing phagosomes in macrophages (David M.Underhill *et al.*, 1999). It is possible that TLR2 expression on the cell surface may not require Cav-1 but other lipid raft components such as GM1. TLR2/4 activation results in the recruitment of GM1 ganglioside (Lonez, 2015).Together with previous data, it indicate that TLR2 binds to mycobacterial ligands on the M-MDSC cell surface and localize around phagosome containing mycobacteria. TLR2 recruitment on the phagosome might need Cav-1 and therefore, in the absence of Cav-1, intracellular TLR2 is diminished. We also found that deficiency of Cav-1 did not change the intracellular level of TLR2 in G-MDSCs. The reason for this might be due to that G-MDSCs had lower levels of basal Cav-1 expression and less upregulation upon BCG infection as compared to M-MDSCs.

Among other PRRs, we also evaluated the expression of other mycobacterial receptors such as Mincle and DC-SIGN on WT and *Cav1*$^{-/-}$ MDSCs upon BCG infection. Mincle and DC-SIGN have been reported to act as receptors on macrophages and DCs respectively., for the mycobacterial ligand (Matsunaga and Moody, 2009) (Tailleux *et al.*, 2003). We noted that Mincle and DC-SIGN expression was not increased upon BCG infection. Moreover, we also did not find any difference between WT and *Cav1*$^{-/-}$ MDSCs expression of Mincle and DC-SIGN. Mincle expression is shown to be upregulated on peritoneal macrophages upon BCG infection (Kerscher *et al.*, 2016). Mincle binds the glycolipids TDM and activates macrophages which are required for immune response to mycobacteria (Schoenen *et al.*, 2010). These data could suggest that Mincle expression is not regulated by mycobacteria-infected MDSCs in contrast to macrophages. Mincle may not be involved in MDSC activation and immune response to mycobacteria.

Human DC-SIGN binds to Man-LAM on human DCs and downregulates costimulatory molecules and immune responses by IL-10 production (Geijtenbeek *et al.*, 2003). Mouse SIGN3 is considered the best candidate as ortholog for human DC-SIGN because of its binding ability to mannose content (Lang, 2013). DC-SIGN expression is increased in phagocytic cells upon Mtb infection and contributes to early defense against Mtb (Tanne *et al.*, 2009). Thus, no change in the expression of DC-SIGN upon BCG infection in MDSCs may be due to (1) difference in the homolog between mouse and human, (2) may be its a specific feature of DCs and not MDSCs.

5.4 Surface markers and cytokine profile in Cav-1 deficient BCG-infected MDSCs

PD-L1 is an inhibitory ligand expressed by MDSCs that binds to PD-1 to suppress T-cell activation. PD-L1 is upregulated on MDSCs in the tumor microenvironment (Lu *et al.*, 2016) and are involved in exhaustion of T-cell immunity by the cell to cell contact (Blank and Mackensen, 2007). In this study, we found that PD-L1 was upregulated upon BCG infection on the surface of both G-MDSCs and M-MDSCs. Our findings were in agreement with the previous reports which have shown increased PD-L1 expression on neutrophils from the blood of TB patients (McNab *et al.*, 2011). M-

MDSCs were also shown to upregulate PD-L1 in TB granuloma of human *in vitro* model (Agrawal *et al.*, 2018). G-MDSCs isolated from the HIV-infected patients suppress T cell response via PD-L1/PD-1 interaction (Bowers *et al.*, 2014). These results indicate a possible role of PD-L1 in immune suppression by MDSCs in tumor microenvironment or during infection. In contrast to these findings, MDSC suppression was shown to be dependent on NO and not on PD-L1 dependent mechanism (Rößner *et al.*, 2005a). This might be due to the difference in the models used for different studies. MDSCs were shown to be suppressing T-cells via PD-L1 molecule in the immune cells of the tumor, HIV or TB patients *in vivo* while the study showing PD-L1 independent suppression used *in vitro* generated MDSCs.

CD40 is a costimulatory molecule of the TNF family expressed mainly by antigen presenting cells (Gerlach *et al.*, 2012). CD40 has been shown to be important for inducing protective immunity against Mtb aerosol infection (Khan *et al.*, 2016). Several reports have shown that expression of CD40 on MDSCs is required for MDSC-mediated immune suppression (Huang *et al.*, 2012) (Shen *et al.*, 2014) (Pan *et al.*, 2010). Previous findings suggested that Mtb down-regulates CD40 expression on DCs and inhibit Th1 and Th17 responses (Sia *et al.*, 2017). We found that CD40 surface marker was upregulated upon BCG infection on both G-MDSCs and M-MDSCs cell surfaces. These results together with our data indicate that CD40 can act as both costimulatory or inhibitory molecule depending on the cell type. Thus, in MDSCs, it acts as an inhibitory molecule while in DCs or other immune stimulatory cells CD40 has a costimulatory function. Although Mtb is reported to downregulate CD40 expression in DCs, in this study we found upregulated expression of CD40 in BCG infected MDSCs. This contradictory finding of CD40 expression might be because authors used Mtb in their study which has virulence factor ESAT-6 might be involved in the downregulation of CD40 whereas, we used BCG in this study which lacks this virulence factor.

In this study, we observed that BCG-stimulated Cav-1$^{-/-}$ M-MDSCs had diminished PD-L1, CD40 and CD69 compared to WT MDSCs. Down-regulated activation marker CD69 expression on *Cav-1*$^{-/-}$ M-MDSC indicate that M-MDSCs are not activated efficiently in the absence of Cav-1. The previous study showed that deficiency of Cav-1 in DCs did not affect the co-stimulatory markers CD14, CD86, CD38 and CD40 (Oyarce *et al.*, 2017). Cav-1 deficient macrophages had impaired CD36 and CD14 expression upon LPS exposure. These results suggest that CD40 expression is not affected upon LPS stimulations in DCs and macrophages but only in MDSCs in response to BCG infection.

Next, we found that *Cav1*$^{-/-}$ BCG-activated MDSCs had impaired IL-6, IL-10, IL-12p40 and TNF-α secretion as compared to WT. However, there was no significant difference in IL-1β secretion by WT or *Cav1*$^{-/-}$ BCG-stimulated MDSCs. Furthermore, we also observed that mainly M-MDSCs secreted cytokines upon BCG infection. G-MDSCs did not secrete IL-6 after BCG stimulation. Therefore, reduced cytokine production was mainly in M-MDSCs and not G-MDSCs. Silencing of Cav-1 by siRNA in murine alveolar and peritoneal macrophages has been shown to result in increased LPS-induced TNF-α and IL-6 and decreased IL-10 production (Wang *et al.*, 2006).

Infection with *Pseudomonas aeruginosa* infection in *Cav-1*$^{-/-}$ mice results in increased cytokines such as IL-1β, IFN-γ, IL-6, TNF-α in the lung. Previously, it has been shown that functional caveolae are essential for CD40-mediated signaling, MAPK activation and IL-8 secretion by epithelial cells (Li and Nord, 2004). Our findings add to the MDSC field that PD-L1 and CD40 expression and cytokine production depend on Cav-1 to mediate TLR2 intracellular expression after BCG infection in M-MDSCs. Together with previous findings, we conclude that Cav-1 has a multi-faced role and might have different function during intracellular mycobacterial infection and extracellular *Pseudomonas* infection. The possible reason of opposite outcome might be also that authors have shown increased cytokines in the bronchoalveolar lavage fluid of *Pseudomonas aeruginosa* infected *Cav-1*$^{-/-}$ mice while in this study we used *in vitro* generated MDSCs.

5.5 Cav-1 mediated entry of mycobacteria in MDSCs

Cav-1 mediated endocytosis in DCs, macrophages, neutrophils and kidney fibroblast cells has been implicated for several pathogens such as, respiratory syncia virus, *Leishmania chagasi*, *Pseudomonas aeruginosa, E.coli,* SV40 (simian virus) (Werling *et al.*, 1999)(Pelkmans, Kartenbeck and Helenius, 2001) (Rodriguez, Gaur and Wilson, 2006). MDSCs have been shown to harbor mycobacteria in infected mice (Knaul *et al.*, 2014). Therefore, we examined whether Cav-1 is required to take up BCG by MDSCs. Surprisingly, we did not find any differences between WT or *Cav1*$^{-/-}$ in the phagocytosis of BCG into G-MDSC or M-MDSCs. Previous reports showed a role of Cav-1 for pathogen entry by using pharmacological inhibitors to block caveolae (Rodriguez, Gaur and Wilson, 2006)(Muñoz, Rivas-Santiago and Enciso, 2009). Mtb is internalized in mast cells by lipid raft domains (Muñoz, Rivas-Santiago and Enciso, 2009). Others have shown the role of Cav-1 in phagocytosis by using *Cav1*$^{-/-}$ mice for pathogens such as *Pseudomonas aeruginosa and E.coli* (Gadjeva *et al.*, 2010)(Tsai *et al.*, 2011a). In our experimental set up, both pharmacological inhibitors and genetic deficiency of Cav-1 did not show any influence on BCG phagocytosis into MDSCs. Mycobacteria are intracellular pathogens known to persist inside phagocytic cells and use the nutrients from the host to survive. Therefore, they can use a variety of mechanisms for entering the phagocytic cell. These results indicate that in the absence of Cav-1 in MDSCs, mycobacteria can switch to other phagocytic receptors such as mannose receptors, scavenger receptors, complement receptors to gain entry in the cell.

After mycobacterial uptake by a macrophage cell line, the accumulation of Mtb in caveosomes has been reported previously (Shin and Abraham, 2001). Caveosome formation by Cav-1 to form phagosomes has been reported to serve as an intracellular niche for pathogen survival (Nichols, 2003). However, there are also reports questioning the existence of caveosomes (Hayer *et al.*, 2010). Since coronin-1 inhibits the fusion of cytoplasmic vesicles with lysosomes for bacterial degradation (Jayachandran *et al.*, 2008b), this may reflect a mechanism of immune evasion. In this study, we did not find any evidence for a co-localization of Cav-1 with genetically GFP-labelled BCG within MDSCs. Together, these results indicate that different subsets of immunogenic (macrophages and DCs) and

suppressive immune cells (MDSCs) mediate mycobacterial uptake via partially different mechanisms and there is also no evidence of caveosome formation.

5.6 Role of Cav-1 in iNOS secretion and T cell suppression by BCG-activated MDSCs

MDSCs suppressive activity is associated with L-arginine metabolism. L-arginine is a substrate of two enzymes namely, iNOS and Arg1 (Talmadge and Gabrilovich, 2013). MDSCs express a high amount of both Arg1 and iNOS which are involved in suppressing T-cell function (Gabrilovich and Nagaraj, 2009). NO suppress T-cell function by inducing apoptosis or blocking JAK3 and STAT5 function in T cells (Gabrilovich and Nagaraj, 2009). In this study, we observed that M-MDSCs had increased secretion of iNOS upon BCG infection compared to G-MDSCs. This was corresponding to previous data shown by others that M-MDSCs suppress by secreting iNOS whereas G-MDSCs suppress T cells by secreting ROS (Veglia, Perego and Gabrilovich, 2018a). We also found that *Cav1*$^{-/-}$ G-MDSCs and M-MDSCs displayed a defect in the iNOS expression after BCG activation. Our data is in accordance with the previous findings which showed that Cav-1 KO macrophages had hindered expression of iNOS upon LPS exposure (Tsai *et al.*, 2011b). NO production and apoptotic cell death have been shown to be regulated by Cav-1 in human neuroblastoma cells (Shen *et al.*, 2008). In endothelium, eNOS is hyperactive in the absence of Cav-1. Our results together with previous data indicate that Cav-1 has a major role in iNOS expression in MDSCs and macrophages upon mycobacterial or LPS exposure respectively, while in endothelial cells it has opposite outcome.

Decreased CD40, PD-L1, iNOS expression as well as a reduced cytokine and NO secretion from *Cav1*$^{-/-}$ MDSCs in response to BCG infection gave us a hint that Cav-1 might be involved in T-cell suppression. We found that MDSCs deficient in Cav-1 exhibited reduced $CD4^+$ and $CD8^+$ T cell suppression in NO-dependent manner. Since NO production represents the major mechanism of BM-MDSCs in vitro (Rößner *et al.*, 2005a), these findings indicate that MDSCs lose their functional property to suppress T cells in the absence of Cav-1. However, there are no reports linking Cav-1 to T-cell suppression. We conclude here that due to the reduced ability of Cav-1 deficient MDSCs to secrete NO, it results in impaired T-cell suppression.

5.7 MDSC signaling pathways in the absence of Cav-1 in BCG-infected MDSCs

We found that *Cav1*$^{-/-}$ M-MDSCs failed to up-regulate TLR2 and displayed an impaired downstream p38 MAPK and NF-κB activation after BCG infection. However, G-MDSCs exhibited no defect in phosphorylation of p38 MAPK pathway in the absence of Cav-1. From our results, it appears that ERK1/2 signaling is also not affected by Cav-1. In MDSCs, the induction of iNOS and NO secretion relies on NF-κB signaling induced by TLR stimulation, which needs further support by mobilization of the IRF-1 transcription factor (Ribechini *et al.*, 2017). Previous reports suggested that NF-κB is also involved in MDSC expansion and immune suppressive function (Trikha, Carson and

III, 2014). Previous studies demonstrated that Cav-1 expression is required for p38 MAPK pathway mediated anti-proliferative effect of CO in fibroblasts (Kim *et al.*, 2005). Cav-1 knockdown resulted in enhanced phosphorylation of AKT but did not modify phosphorylation of ERK1/2 in VEGF induced endothelial cells (Gonzalez *et al.*, 2004). In contrast to this finding, Cav-1 deficiency has also been shown to downregulate PI3K/AKT signaling in response to insulin-like growth factor in mouse embryonic fibroblast cells (Matthews, Taggart and Westwood, 2008).

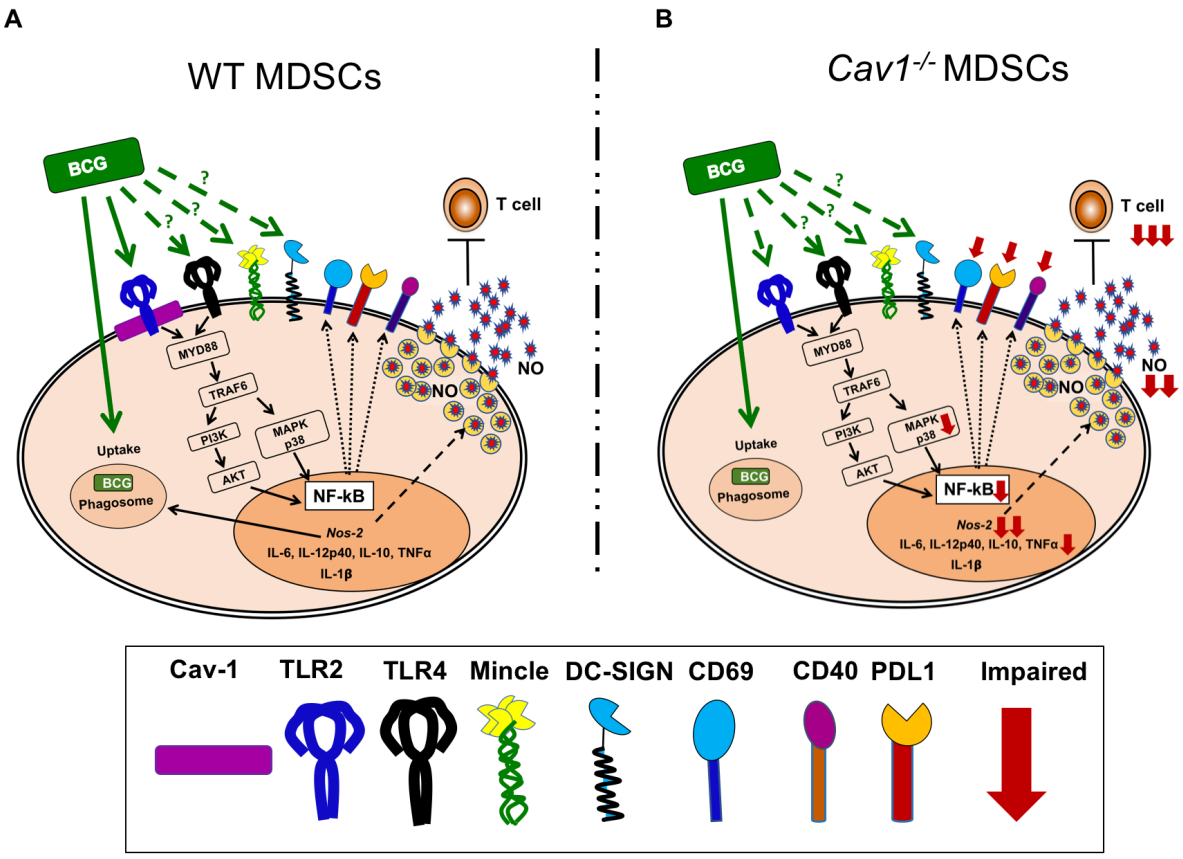

Figure 36: Schematic representation of WT and *Cav1*$^{-/-}$ BCG-activated MDSCs. (A) WT M-MDSCs take up BCG. BCG can be recognized by TLR2 and TLR4 to further activate AKT, p38 MAPK and NF-KB to secrete cytokines and NO. MDSCs release NO which is required for T cell suppression. MDSCs also express CD40, PD-L1 after activation by BCG. **(B)** *Cav1*$^{-/-}$ MDSCs take up BCG, similar to WT MDSCs. Deficiency of Cav-1 results in impaired p38 MAPK and NF-KB and reduced selected cytokine secretion in response to BCG. *Cav1*$^{-/-}$ MDSCs have diminished iNOS and NO production thereby, reduced T-cell suppression. Lack of Cav-1 also affects surface markers such as PD-L1, CD40 and CD69.

In endothelial cells, Cav-1 interacts with TLR4 upon LPS exposure and activates NF-KB to secrete cytokines (Jiao *et al.*, 2013). Deficiency of Cav-1 results in hyperactive eNOS inhibits NF-KB activation and diminished expression of pro-inflammatory cytokines (Garrean *et al.*, 2006a). Others

have shown that *S. aureus* binds both asialo-GM1 and TLR2 in lipid rafts leading to synergistic signals in airway epithelial cells (Soong *et al.*, 2004). The asialo-GM1 mediated co-signals have been identified for flagellin binding to TLR5 to enhance NF-κB signals via the ERK pathway (McNamara *et al.*, 2006). These data indicate that different co-receptors or membrane lipid-rich area components may cooperate with specific TLRs to shape specific immune responses. Here, in this study, we found cooperation of TLR2 with Cav-1 for mycobacterial recognition. In the absence of Cav-1, the intracellular expression level of TLR2, but not TLR4, and its upregulation after BCG infection is impaired. Our results showed a defect of p38 and NF-κB signals affecting several subsequent activation processes such as surface markers, cytokines, iNOS and NO release, finally impairing M-MDSC suppressor function (Figure.36). In conclusion, our data together with previous results indicate that Cav-1 is required for TLR2 signaling in M-MDSCs and TLR4 signaling in endothelial cells and thereby, regulates NF-κB signaling. We did not find any difference in p-AKT in the absence of Cav-1 in MDSCs upon BCG infection, however, others found enhanced and reduced AKT phosphorylation in endothelial cells and mouse embryonic fibroblast, respectively. This might be possibly due to different cell types used in different studies and phosphorylation of AKT depends on several other factors.

5.8 Acid Sphingomyelinase in BCG-activated MDSCs

ASM has been shown to reorganize the cell surface and activate signaling proteins within microdomains (Gulbins and Kolesnick, 2004). Our studies revealed that MDSCs phagocytosed BCG independent of inhibiting ASM using pharmacological inhibitors (amitryptiline and desipramine) or MDSCs from $ASM^{-/-}$ mice comparably internalized BCG. Previously, ASM has been shown to be involved in the internalization of *Neisseria gonorrhoeae* and modulates the subsequent immune response in neutrophils (C.R. Hauck *et al.*, 2000). These results suggest that ASM is dispensable for mycobacterial uptake in MDSCs but required for *Neisseria gonorrhoeae* internalization in neutrophils, fibroblast and epithelial cells. This is possible because different cell types use different mechanisms of endocytosis. Although we did not find any difference in the endocytosis, suppression of ASM or using $ASM^{-/-}$ MDSCs resulted in reduced NO production and decreased IL-6, IL-10, TNF-α and IL-1ß cytokine secretion by MDSCs in response to BCG. The previous report has shown suppression of LPS-induced cytokine release and activation of NF-κB by ASM inhibited THP-1 macrophage cell line (Sakata *et al.*, 2007a). Palmitic acid (PA) together with LPS have been shown to synergistically increase hydrolysis of sphingomyelin by stimulating ASM activity which is involved in increased ceramide production and IL-6 secretion in RAW macrophages (Jin *et al.*, 2013). Another sphingomyelinase known as neutral sphingomyelinase (NSM) has been investigated during systemic infection of mice and murine macrophages with *Mycobacterium bovis* BCG. Using genetic knockdown studies on the RAW macrophage cell line, they demonstrated that NSM blocking result in the prevention of superoxide production in response to BCG infection (Li *et al.*, 2016). In RAW

macrophage cell line, LPS and fatty acid-mediated activation of ASM activity was dependent on TLR4 but not TLR2 signaling (Jin *et al.*, 2013). LPS-activated TLR4 clustering and activation were shown to be dependent on ceramide, generated by ASM in THP-1 macrophage cell line (Cuschieri *et al.*, 2007). These results together indicate that ASM is essential for NO production in BCG infected MDSCs whereas NSM is required for superoxide production in BCG infected macrophages. Moreover, LPS and mycobacteria activate ASM activity and affect the cytokine response in both macrophages and MDSCs. We also observed decreased phosphorylation of AKT signaling after blocking ASM with amitryptiline. ASM activation and ceramide production results in the activation of PI3K/AKT signaling in hepatocytes and regulates apoptosis (Osawa *et al.*, 2005). Together, these results suggested that inhibition of ASM results in the impaired phosphorylation of AKT signaling in MDSCs and hepatocytes. ASM inhibition might also regulate apoptosis in MDSCs as it does in hepatocytes.In conclusion, these results indicate that ceramide generated by ASM might be required for clustering and activation of TLRs upon mycobacterial stimulus in MDSCs. In the absence of ASM, clustering and activation of TLR is inefficient in BCG-activated MDSCs and thereby, affecting the NF-KB signaling to secrete cytokines (Figure.37).

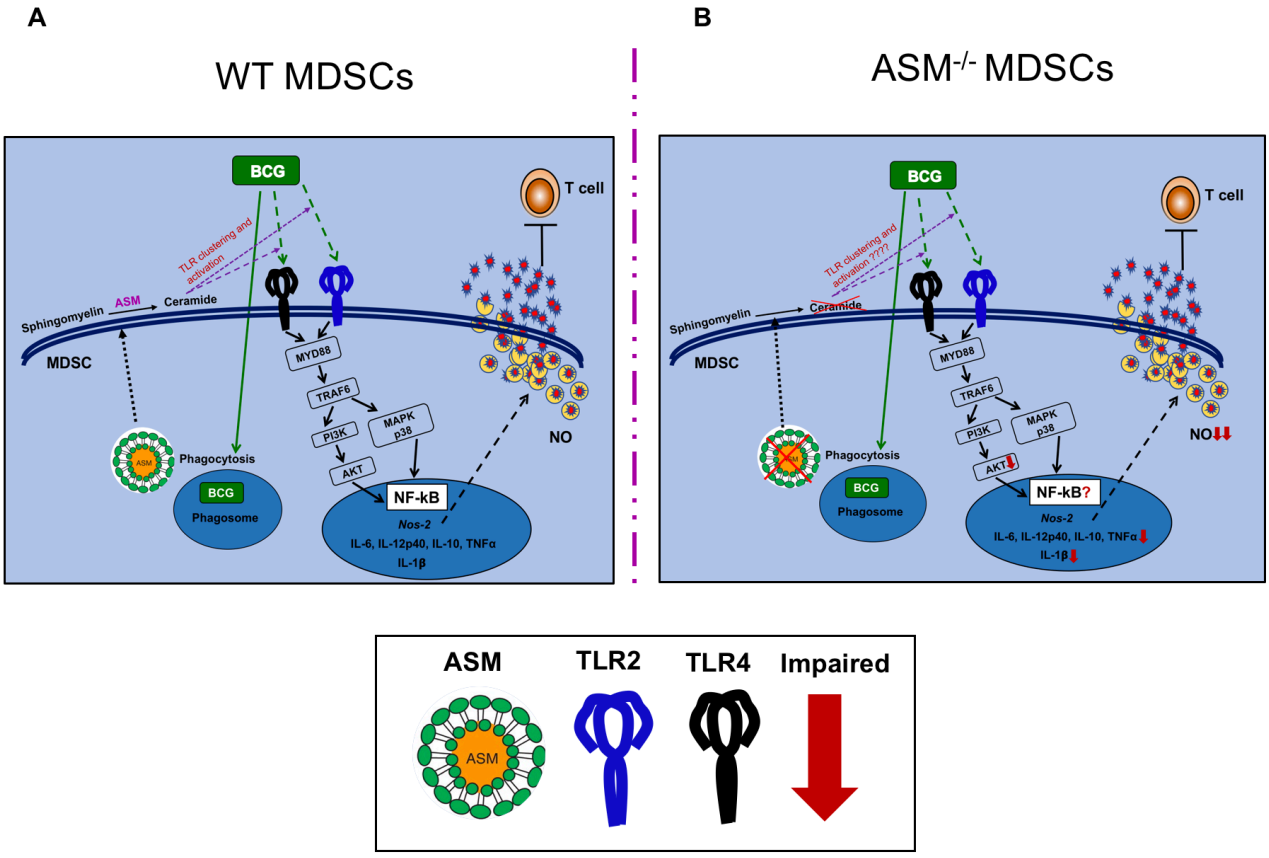

Figure 37: Schematic representation of ASM deficient BCG-activated MDSCs. (A) ASM is an enzyme located in the lysosome which migrates to the plasma membrane to hydrolyze sphingomyelin to ceramide which might be important for clustering and activation of TLRs. WT-MDSCs take up BCG. BCG can be recognized by TLR2 and TLR4 to further activate AKT, p38 MAPK and NF-KB to secrete cytokines and NO. **(B)** ASM was blocked by using pharmacological inhibitors such as amitryptiline or desipramine or ASM$^{-/-}$ MDSCs were used in this study. ASM is dispensable

for BCG phagocytosis by MDSCs. Blocking of ASM results in the impaired AKT signaling and reduced NO and cytokine secretion.

5.9 Asialo-GM1 in BCG-stimulated MDSCs

Asialo-GM1 has been shown to be present on NK cells, macrophages, blood monocytes, T cells and on the MDSC cell surface (Riser, Laybourn and Varani, 1988)(Moore *et al.*, 2008) (Rößner *et al.*, 2005a). In this study, we found that asialo-GM1 was upregulated on the cell surface of both the subsets of MDSCs after mycobacterial infections. Interestingly, only mycobacterial preparations such as BCG-live, BCG-killed and Mtb-killed upregulated asialo-GM1 expression on MDSCs while other stimulations such as LPS, Pam_3CSK_4 or listeria killed did not affect the expression of asialo-GM1. The possible reason for this might be due to remarkable complexity of mycobacterial cell wall compared to TLR2 or TLR4 agonist alone. Our findings correlate with the previous reports that found asialo-GM1 upregulation in T cells upon viral infection (Moore *et al.*, 2008). These results indicate that asialo-GM1 upregulates specifically on MDSCs upon mycobacterial infection and T cells upon RSV infection but not upon TLR2 or TLR4 stimulations. Previously it has been shown several times that *Pseudomonas aeruginosa* binds to the glycolipid asialo-GM1 on epithelial cells and acts as a receptor for *Pseudomonas aeruginosa* pilin (Gupta *et al.*, 1994) (Saiman and Prince, 1993) (de Bentzmann *et al.*, 1996). We observed a dose- dependent increase in the binding of BCG to asialo-GM1. These results together suggested that asialo-GM1 on MDSCs binds to mycobacteria whereas on epithelial cells it binds to *Pseudomonas aeruginosa.* Ganglioside GM1 has been previously implicated in the phagocytosis and survival of *Brucella suisin* in murine macrophages (Williams and Palmer, 2014). We used anti-asialo-GM1 antibody in order to block asialo-GM1 on the cell surface of MDSCs. We observed that blocking asialo-GM1 or GM1 did not affect the mycobacterial internalization in both G-MDSCs and M-MDSCs. These results together suggest that asialo-GM1 and GM1 do not have any role in the internalization of mycobacteria but GM1 might be required for uptake of other pathogens. Previous report demonstrated that the treatment with GM1 in amyloid β protein activated THP-1 monocytic cell line results in reduced cytokine release (Ariga and Yu, 1999). In contrast to this, we did not observe any significant difference in cytokine and NO production by BCG-activated MDSCs after blocking asialo-GM1. Removal of sialic acid results in the diminished LPS-mediated expression of cytokines by monocyte-derived DCs (Stamatos *et al.*, 2010). These data together suggest that GM1 is important for cytokine production in monocytes upon activation but in the absence of sialic acid, asialo-GM1 upon stimulation has no effect on cytokine secretion by MDSCs. Furthermore, we also found phosphorylation of AKT was not affected after blocking asialo-GM1 on BCG-stimulated MDSCs. TLR2 in association with asialo-GM1 has been shown to amplify the signaling at the apical surface of airway cells in response to *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection (Soong *et al.*, 2004). Together, our results indicate that despite BCG binding and induced upregulation, asialo-GM1 remained dispensable for mycobacterial phagocytosis, cytokine, NO production or AKT signaling in BCG-activated MDSCs (Figure. 38).

Asialo-GM1 might bind to mycobacteria and cooperate with TLR2 or TLR4 in BCG activated MDSCs and this interaction may be important to amplify the signaling or in the recycling of TLR2 or TLR4 in the phagosomal compartment of the cell.

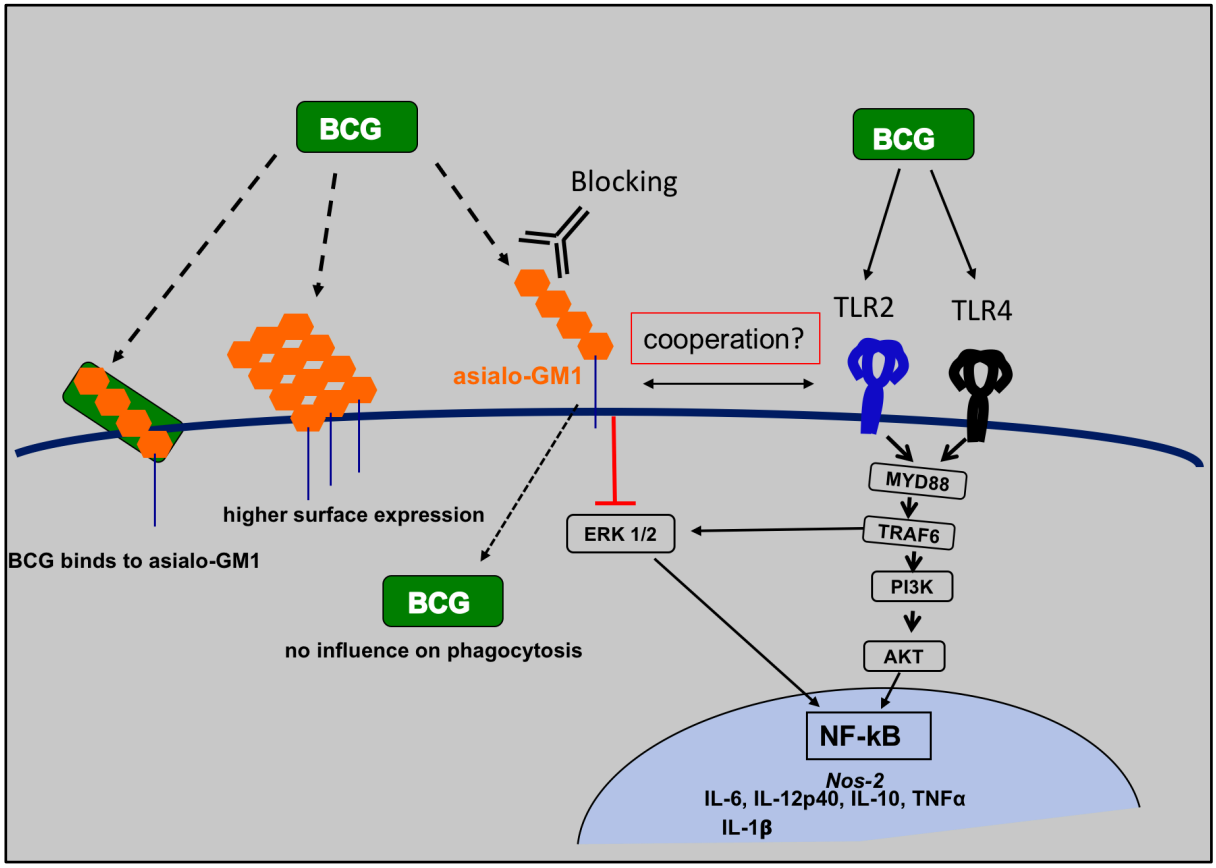

Figure 38: Schematic representation showing role of asialo-GM1 in BCG-activated MDSCs. Asialo-GM1 expression is increased upon BCG stimulation. BCG binds to asialo-GM1. Asialo-GM1 is dispensable for BCG phagocytosis by MDSCs. Blocking of asialo-GM1 has no effect on cytokine or NO production or AKT signaling by MDSCs.

Summary

Myeloid-derived suppressor cells (MDSCs) constitute of monocytic (M-MDSCs) and granulocytic cell subsets (G-MDSCs)and were initially described as suppressors of T-cell function in tumor microenvironments. Recent studies have shown the involvement of MDSCs in a number of infectious diseases including *Mycobacterium tuberculosis* (Mtb) infection. MDSCs are tremendously accumulated in patients with Mtb infection and exert a suppressive effect on T cell responses against mycobacteria. *Mycobacterium bovis* BCG, the only available vaccine against Mtb fails to protect against the adult pulmonary tuberculosis (TB). Understanding the mechanisms of MDSC suppression for immunity against mycobacterial infection will provide a rational basis to improve anti-TB vaccination and host-directed therapies against TB. In this study, we investigated the role of three lipid-rich components of the plasma membrane, Caveolin-1(Cav-1), Acid Sphingomyelinase (ASM) and asialo-GM1 on BCG-activated MDSCs.

Cav-1 is one of the vital components of caveolae (plasma membrane invaginations) which regulates apoptosis and lipid metabolism. In this work, we found that MDSCs upregulated Cav-1, TLR4 and TLR2 expression after BCG infection on the cell surface. However, Cav-1 deficiency resulted in a selective defect in the intracellular TLR2 accumulation in the M-MDSC, but not G-MDSC subset. Further analysis indicated no difference in the phagocytosis of BCG by M-MDSCs from WT and *Cav1*$^{-/-}$ mice but a reduced capacity to up-regulate surface markers, to secrete various cytokines, induce iNOS and NO production. These defects correlated with deficits of *Cav1*$^{-/-}$ MDSCs in the suppression of T cell proliferation. Among the signaling pathways that were affected by Cav-1 deficiency, we found lower phosphorylation of NF-κB and p38 mitogen-activated protein kinase (MAPK) in BCG - activated MDSCs.

ASM is an enzyme present in lysosomes and is translocated to the cell surface where it hydrolyzes sphingomyelin into ceramide. Flow cytometric studies revealed that MDSCs phagocytosed BCG independent of inhibiting ASMase using pharmacological inhibitors (amitryptiline or desipramine) or MDSCs from WT and ASM$^{-/-}$. Suppression of ASMase or using ASM$^{-/-}$ MDSCs resulted in reduced NO production and decreased cytokine secretion by MDSCs in response to BCG. Furthermore, MDSCs inhibited by amitryptiline had impaired AKT phosphorylation upon BCG infection.

Asialo-GM1 is a ganglioside expressed on the cell surface of MDSCs reported to cooperate with TLR2 for activating ERK signaling. Here, in this study, we found that asialo-GM1 expression was upregulated specifically upon mycobacterial infection and not upon any other stimulus. We noted that the soluble form of asialo-GM1 bound to BCG. Flow cytometric studies revealed that blocking

asialo-GM1 did not affect the phagocytosis of BCG into MDSCs. Furthermore, blocking of asialo-GM1 had no effect on the cytokine and NO secretion or AKT signaling.

Collectively, the data presented in this work implicated that Cav-1, ASM, asialo-GM1 are dispensable for the internalization of BCG. Rather, Cav-1 and ASM are required for the functional activation of MDSCs. Although asialo-GM1 binds to BCG, we did not find any difference in the functional activation of MDSCs after blocking asialo-GM1. This study provides insights into the role of lipid raft components of the MDSC cell membrane during mycobacterial infection.

Zusammenfassung

Myeloide Suppressorzellen (engl, *myeloid-derived suppressor cells* MDSCs) bestehen aus monozytischen (M-MDSCs) und granulozytären Subtypen (G-MDSCs) und wurden anfangs als Suppressoren der T-Zellfunktion in Tumormikroumgebungen beschrieben. Kürzlich durchgeführte Studien haben gezeigt, dass MDSCs an einer Reihe von Infektionskrankheiten beteiligt sind, einschließlich einer Infektion mit *Mycobacterium tuberculosis* (Mtb). MDSCs sind bei der Patienten Mtb-Infektion enorm akkumuliert und üben eine supprimierende Wirkung auf die T-Zell-Antworten gegen Mykobakterien aus. *Mycobacterium bovis* BCG, der einzige verfügbare Impfstoff gegen Mtb, schützt nicht gegen die Lungentuberkulose bei Erwachsenen (TB). Das Verständnis der Mechanismen über welche MDSCs eine der Immunsuppression bei mykobakteriellen Infektionen vermitteln, bilden eine rationale Grundlage für die Verbesserung der Anti-TB-Impfung und Therapien gegen TB. In dieser Studie wurden die Rolle der drei lipidreichen Komponenten der Plasmamembran, Caveolin-1 (Cav-1), Saure Sphingomyelinase (ASM) und Asialo-GM1 bei BCG-aktivierten MDSCs untersucht.

Cav-1 ist eine der Komponenten von Caveolae (Plasmamembran-Invagination), die die Apoptose und den Fettstoffwechsel regulieren. Diese Arbeit zeigte, dass MDSCs die Expression von Cav-1, TLR4 und TLR2 nach BCG-Infektion auf der Zelloberfläche hochregulierten. Eine Cav-1 Defizienz führte jedoch zu einem selektiven Defekt in der intrazellulären TLR2-Akkumulation bei M-MDSCs, jedoch nicht bei G-MDSCs. Weitere Analysen zeigten keinen Unterschied in der Phagozytose von BCG durch M-MDSCs von WT- und Cav1$^{-/-}$ Mäusen, jedoch eine verringerte Fähigkeit, Oberflächenmarker hoch zu regulieren, verschiedene Zytokine zu sekretieren und die Produktion von iNOS und NO zu induzieren. Diese Defekte korrelierten mit Defiziten von Cav1$^{-/-}$ MDSCs bei der Unterdrückung der T-Zell-Proliferation. Unter den von Cav-1-Mangel betroffenen Signalwegen fanden wir eine geringere Phosphorylierung der NF-ĸB- und p38- Mitogen-aktivierten Proteinkinase (MAPK) in BCG-aktivierten MDSCs.

ASM ist ein in Lysosomen vorhandenes Enzym, das an die Zelloberfläche transloziert wird, wo es Sphingomyelin zu Ceramid hydrolysiert. Durchflusszytometrische Studien ergaben, dass MDSCs BCG unabhängig von der Hemmung von ASMase mit pharmakologischen Inhibitoren (Amitryptilin oder Desipramin) oder MDSCs von ASM$^{-/-}$ Mäusen BCG phagozytierten. Die Suppression von ASMase oder die Verwendung von ASM$^{-/-}$ MDSCs führte zu einer verringerten NO Produktion und einer verringerten Zytokinsekretion durch MDSCs als Antwort auf BCG. Darüber hinaus hatten MDSCs, die durch Amitryptilin inhibiert wurden, die AKT-Phosphorylierung bei einer BCG-Infektion beeinträchtigt.

Asialo-GM1 ist ein Gangliosid, das auf der Zelloberfläche von MDSCs exprimiert wird, von dem berichtet wurde, dass es mit TLR2 kooperiert, um ERK-Signale zu aktivieren. Hier in dieser Studie haben wir festgestellt, dass die Expression von Asialo-GM1 spezifisch bei mycobakterieller Infektion und nicht bei einem anderen Stimulus hochreguliert wurde. Wir haben festgestellt, dass die lösliche Form von Asialo-GM1 an BCG binden kann. Durchflusszytometrische Studien ergaben, dass die Blockade von Asialo-GM1 die Phagozytose von BCG in MDSCs nicht beeinflusst. Darüber hinaus hatte die Blockierung von Asialo-GM1 keinen Einfluss auf die Zytokin- und NO-Sekretion oder das AKT-Signal.

Zusammenfassend ergaben die in dieser Arbeit präsentierten Daten, dass Cav-1, ASM, asialo-GM1 für die Internalisierung von BCG entbehrlich sind. Dagegen sind Cav-1 und ASM für die funktionale Aktivierung von MDSCs erforderlich. Obwohl Asialo-GM1 an BCG bindet, konnten wir nach der Blockierung von Asialo-GM1 keinen Unterschied in der funktionellen Aktivierung von MDSCs feststellen. Diese Studie liefert Einblicke in die Rolle einiger Komponenten der lipid-reicher Areale der MDSC-Zellmembran bei mykobakteriellen Infektionen.

Bibliography

Abraham, S. N. *et al.* (1997) 'Survival of FimH-expressing enterobacteria in macrophages relies on glycolipid traffic.', *Nature*, 389(6651), pp. 636–639. doi: 10.1038/39376.

Agrawal, N. *et al.* (2018) 'Human Monocytic Suppressive Cells Promote Replication of Mycobacterium tuberculosis and Alter Stability of in vitro Generated Granulomas', *Frontiers in Immunology*, 9, p. 2417. doi: 10.3389/fimmu.2018.02417.

Albouz, S. *et al.* (1986) 'Modifications of sphingomyelin and phosphatidylcholine metabolism by tricyclic antidepressants and phenothiazines.', *Life sciences*, 38(4), pp. 357–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3945166 (Accessed: 22 December 2018).

Aman, A. T. *et al.* (2001) 'A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(15), pp. 8536–41. doi: 10.1073/pnas.161273098.

Andersen, P. and Doherty, T. M. (2005) 'The success and failure of BCG — implications for a novel tuberculosis vaccine', *Nature Reviews Microbiology*, 3(8), pp. 656–662. doi: 10.1038/nrmicro1211.

Ariga, T. and Yu, R. K. (1999) 'GM1 Inhibits Amyloid β-Protein-Induced Cytokine Release', *Neurochemical Research*. Kluwer Academic Publishers-Plenum Publishers, 24(2), pp. 219–226. doi: 10.1023/A:1022557920150.

Beckmann, N. *et al.* (2014) 'Inhibition of acid sphingomyelinase by tricyclic antidepressants and analogons.', *Frontiers in physiology*. Frontiers Media SA, 5, p. 331. doi: 10.3389/fphys.2014.00331.

Behler, F. *et al.* (2012) 'Role of Mincle in Alveolar Macrophage-Dependent Innate Immunity against Mycobacterial Infections in Mice', *The Journal of Immunology*, 189(6), pp. 3121–3129. doi: 10.4049/jimmunol.1201399.

Bender, F. C. *et al.* (2000) 'Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity.', *Cancer research*, 60(20), pp. 5870–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11059785 (Accessed: 9 January 2019).

Benlimame, N., Le, P. U. and Nabi, I. R. (1998) 'Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum.', *Molecular biology of the cell*, 9(7), pp. 1773–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9658170 (Accessed: 13 September 2018).

Bennett, J. A., Rao, V. S. and Mitchell, M. S. (1978) 'Systemic bacillus Calmette-Guérin (BCG)

activates natural suppressor cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 75(10), pp. 5142–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/283421 (Accessed: 11 September 2018).

de Bentzmann, S. *et al.* (1996) 'Asialo GM1 is a receptor for Pseudomonas aeruginosa adherence to regenerating respiratory epithelial cells.', *Infection and immunity*. American Society for Microbiology (ASM), 64(5), pp. 1582–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8613364 (Accessed: 22 December 2018).

Blank, C. and Mackensen, A. (2007) 'Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion', *Cancer Immunology, Immunotherapy*, 56(5), pp. 739–745. doi: 10.1007/s00262-006-0272-1.

Boscher, C. and Nabi, I. R. (2012) 'CAVEOLIN-1: Role in Cell Signaling', in *Advances in experimental medicine and biology*, pp. 29–50. doi: 10.1007/978-1-4614-1222-9_3.

Van den Bossche, J. *et al.* (2012) 'Regulation and function of the E-cadherin/catenin complex in cells of the monocyte-macrophage lineage and DCs.', *Blood*. American Society of Hematology, 119(7), pp. 1623–33. doi: 10.1182/blood-2011-10-384289.

Bowers, N. L. *et al.* (2014) 'Immune Suppression by Neutrophils in HIV-1 Infection: Role of PD-L1/PD-1 Pathway', *PLoS Pathogens*. Edited by D. C. Douek. Public Library of Science, 10(3), p. e1003993. doi: 10.1371/journal.ppat.1003993.

Brewer, T. F. and Colditz, G. A. (1995) 'Relationship between bacille Calmette-Guérin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 20(1), pp. 126–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7727638 (Accessed: 10 September 2018).

Bruns, R. R. and Palade, G. E. (1968) 'Studies on blood capillaries. I. General organization of blood capillaries in muscle.', *The Journal of cell biology*. Rockefeller University Press, 37(2), pp. 244–76. doi: 10.1083/JCB.37.2.244.

Bunt, S. K. *et al.* (2009) 'Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4', *Journal of Leukocyte Biology*. John Wiley & Sons, Ltd, 85(6), pp. 996–1004. doi: 10.1189/jlb.0708446.

Cambi, A. and Figdor, C. G. (2003) 'Dual function of C-type lectin-like receptors in the immune system.', *Current opinion in cell biology*, 15(5), pp. 539–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14519388 (Accessed: 9 December 2018).

Cambi, A., Koopman, M. and Figdor, C. G. (2005) 'How C-type lectins detect pathogens', *Cellular Microbiology*, 7(4), pp. 481–488. doi: 10.1111/j.1462-5822.2005.00506.x.

Cella, M. *et al.* (1996) 'Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation.', *The Journal of experimental medicine*, 184(2), pp. 747–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8760829 (Accessed: 7 December 2018).

Chen, X. *et al.* (2007) 'CD4+CD25+FoxP3+ regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease', *Clinical Immunology*, 123(1), pp. 50–59. doi: 10.1016/j.clim.2006.11.009.

Chidlow, J. H. and Sessa, W. C. (2010) 'Caveolae, caveolins, and cavins: complex control of cellular signalling and inflammation', *Cardiovascular Research*, 86(2), pp. 219–225. doi: 10.1093/cvr/cvq075.

Chieppa, M. *et al.* (2003) 'Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program.', *Journal of immunology (Baltimore, Md.: 1950)*, 171(9), pp. 4552–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14568928 (Accessed: 9 December 2018).

Choudhury, A. *et al.* (2006) 'Regulation of caveolar endocytosis by syntaxin 6-dependent delivery of membrane components to the cell surface.', *Nature cell biology*, 8(4), pp. 317–28. doi: 10.1038/ncb1380.

Cohen, A. W. *et al.* (2003) 'Caveolin-1 null mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts', *American Journal of Physiology-Cell Physiology*, 284(2), pp. C457–C474. doi: 10.1152/ajpcell.00380.2002.

Condamine, T. *et al.* (2016) 'Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients.', *Science immunology*. NIH Public Access, 1(2). doi: 10.1126/sciimmunol.aaf8943.

Copin, R. *et al.* (2014) 'Impact of in vitro evolution on antigenic diversity of Mycobacterium bovis bacillus Calmette-Guerin (BCG)', *Vaccine*, 32(45), pp. 5998–6004. doi: 10.1016/j.vaccine.2014.07.113.

Corzo, C. A. *et al.* (2009) 'Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells.', *Journal of immunology (Baltimore, Md.: 1950)*. NIH Public Access, 182(9), pp. 5693–701. doi: 10.4049/jimmunol.0900092.

van Crevel, R., Ottenhoff, T. H. M. and van der Meer, J. W. M. (2002) 'Innate immunity to Mycobacterium tuberculosis.', *Clinical microbiology reviews*. American Society for Microbiology Journals, 15(2), pp. 294–309. doi: 10.1128/CMR.15.2.294-309.2002.

Cuschieri, J. *et al.* (2007) 'Acid Sphingomyelinase Is Required for Lipid Raft TLR4 Complex Formation', *Surgical Infections*. Mary Ann Liebert, Inc. 2 Madison Avenue Larchmont, NY 10538 USA , 8(1), pp. 91–106. doi: 10.1089/sur.2006.050.

d'Azzo, A., Tessitore, A. and Sano, R. (2006) 'Gangliosides as apoptotic signals in ER stress response', *Cell Death and Differentiation*. Nature Publishing Group, 13(3), pp. 404–414. doi: 10.1038/sj.cdd.4401834.

El Daker, S. *et al.* (2015) 'Granulocytic Myeloid Derived Suppressor Cells Expansion during Active Pulmonary Tuberculosis Is Associated with High Nitric Oxide Plasma Level', *PLOS ONE*. Edited by Y. Hoshino. Public Library of Science, 10(4), p. e0123772. doi: 10.1371/journal.pone.0123772.

Darrah, P. A. *et al.* (2014) 'Aerosol Vaccination with AERAS-402 Elicits Robust Cellular Immune Responses in the Lungs of Rhesus Macaques but Fails To Protect against High-Dose *Mycobacterium tuberculosis* Challenge', *The Journal of Immunology*, 193(4), pp. 1799–1811. doi: 10.4049/jimmunol.1400676.

Das, R. *et al.* (2013) 'Macrophage migration inhibitory factor (MIF) is a critical mediator of the innate immune response to Mycobacterium tuberculosis', *Proceedings of the National Academy of Sciences*, 110(32), pp. E2997–E3006. doi: 10.1073/pnas.1301128110.

Davenne, T. and McShane, H. (2016) 'Why don't we have an effective tuberculosis vaccine yet?', *Expert Review of Vaccines*. Taylor & Francis, 15(8), pp. 1009–1013. doi: 10.1586/14760584.2016.1170599.

David S. Park, ‡,§ *et al.* (2003) 'Caveolin-1 Null (−/−) Mice Show Dramatic Reductions in Life Span†'. American Chemical Society . doi: 10.1021/BI0356348.

Delano, M. J. *et al.* (2007) 'MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis.', *The Journal of experimental medicine*. Rockefeller University Press, 204(6), pp. 1463–74. doi: 10.1084/jem.20062602.

Divangahi, M. *et al.* (2008) 'NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity.', *Journal of immunology (Baltimore, Md.: 1950)*, 181(10), pp. 7157–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18981137 (Accessed: 20 December 2018).

Doz, E. *et al.* (2007) 'Acylation Determines the Toll-like receptor (TLR)-dependent Positive *Versus* TLR2-, Mannose Receptor-, and SIGNR1-independent Negative Regulation of Pro-inflammatory Cytokines by Mycobacterial Lipomannan', *Journal of Biological Chemistry*, 282(36), pp. 26014–26025. doi: 10.1074/jbc.M702690200.

Drab, M. *et al.* (2001) 'Loss of Caveolae, Vascular Dysfunction, and Pulmonary Defects in Caveolin-1 Gene-Disrupted Mice', *Science*, 293(5539), pp. 2449–2452. doi: 10.1126/science.1062688.

DURBAS, M. *et al.* (2015) 'GD2 ganglioside specific antibody treatment downregulates PI3K/Akt/mTOR signaling network in human neuroblastoma cell lines', *International Journal of Oncology*. Spandidos Publications, 47(3), pp. 1143–1159. doi: 10.3892/ijo.2015.3070.

Engelman, J. A. *et al.* (1998) 'Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo: A role for the caveolin-scaffolding domain', *FEBS Letters*. No longer published by Elsevier, 428(3), pp. 205–211. doi: 10.1016/S0014-5793(98)00470-0.

Enioutina, E. Y., Bareyan, D. and Daynes, R. A. (2011) 'A Role for Immature Myeloid Cells in Immune Senescence', *The Journal of Immunology*, 186(2), pp. 697–707. doi: 10.4049/jimmunol.1002987.

Ernst, J. D. (2012) 'The immunological life cycle of tuberculosis', *Nature Reviews Immunology*. Nature Publishing Group, 12(8), pp. 581–591. doi: 10.1038/nri3259.

Ernst, J. D. (2018) 'Mechanisms of M. tuberculosis Immune Evasion as Challenges to TB Vaccine Design', *Cell Host & Microbe*, 24(1), pp. 34–42. doi: 10.1016/j.chom.2018.06.004.

Esen, M. *et al.* (2001) 'Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells.', *Apoptosis : an international journal on programmed cell death*, 6(6), pp. 431–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11595832 (Accessed: 22 December 2018).

Fang, P. *et al.* (2016) 'Targeted inhibition of GATA-6 attenuates airway inflammation and remodeling by regulating caveolin-1 through TLR2/MyD88/NF-κB in murine model of asthma', *Molecular Immunology*. Pergamon, 75, pp. 144–150. doi: 10.1016/J.MOLIMM.2016.05.017.

Feng, H. *et al.* (2013) 'Role of caveolin-1 and caveolae signaling in endotoxemia and sepsis.', *Life sciences*. NIH Public Access, 93(1), pp. 1–6. doi: 10.1016/j.lfs.2013.05.016.

Gabrilovich, D. I. and Nagaraj, S. (2009) 'Myeloid-derived suppressor cells as regulators of the immune system', *Nature Reviews Immunology*. Nature Publishing Group, 9(3), pp. 162–174. doi: 10.1038/nri2506.

Gadjeva, M. *et al.* (2010) 'Caveolin-1 Modifies the Immunity to *Pseudomonas aeruginosa*', *The Journal of Immunology*, 184(1), pp. 296–302. doi: 10.4049/jimmunol.0900604.

Gandotra, S. *et al.* (2007) 'Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with Mycobacterium tuberculosis.', *Infection and immunity*. American Society for Microbiology Journals, 75(11), pp. 5127–34. doi: 10.1128/IAI.00458-07.

Gao, B., Wang, Y. and Tsan, M. (2006) 'The heat sensitivity of cytokine-inducing effect of lipopolysaccharide', *Journal of Leukocyte Biology*. John Wiley & Sons, Ltd, 80(2), pp. 359–366. doi: 10.1189/jlb.1205738.

Garcia-Vallejo, J. J. and van Kooyk, Y. (2013) 'The physiological role of DC-SIGN: A tale of mice and men', *Trends in Immunology*, 34(10), pp. 482–486. doi: 10.1016/j.it.2013.03.001.

Garrean, S. *et al.* (2006a) 'Caveolin-1 regulates NF-kappaB activation and lung inflammatory response to sepsis induced by lipopolysaccharide.', *Journal of immunology (Baltimore, Md. : 1950)*, 177(7), pp. 4853–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16982927 (Accessed: 18 December 2018).

Garrean, S. *et al.* (2006b) 'Caveolin-1 regulates NF-kappaB activation and lung inflammatory response to sepsis induced by lipopolysaccharide.', *Journal of immunology (Baltimore, Md. : 1950)*, 177(7), pp. 4853–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16982927 (Accessed: 14 November 2018).

Geijtenbeek, T. B. H. *et al.* (2003) 'Mycobacteria target DC-SIGN to suppress dendritic cell function.', *The Journal of experimental medicine*, 197(1), pp. 7–17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12515809 (Accessed: 24 January 2019).

Gerlach, A.-M. *et al.* (2012) 'Role of CD40 ligation in dendritic cell semimaturation.', *BMC immunology*. BioMed Central, 13, p. 22. doi: 10.1186/1471-2172-13-22.

Glenney, J. R. (1989) 'Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation

by Rous sarcoma virus.', *The Journal of biological chemistry*, 264(34), pp. 20163–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2479645 (Accessed: 12 September 2018).

GLOBAL TUBERCULOSIS REPORT 2018 (2018). Available at: http://apps.who.int/bookorders. (Accessed: 28 December 2018).

Goedegebuure, P. *et al.* (2011) 'Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer.', *Current cancer drug targets*. NIH Public Access, 11(6), pp. 734–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21599634 (Accessed: 3 January 2019).

Gonzalez, E. *et al.* (2004) *siRNA-mediated down-regulation of caveolin-1 differentially modulates signaling pathways in endothelial cells Downloaded from*. JBC Papers in Press. Available at: http://www.jbc.org/ (Accessed: 25 January 2019).

Grassmé, H. *et al.* (2001) 'CD95 Signaling via Ceramide-rich Membrane Rafts', *Journal of Biological Chemistry*, 276(23), pp. 20589–20596. doi: 10.1074/jbc.M101207200.

Greifenberg, V. *et al.* (2009) 'Myeloid-derived suppressor cell activation by combined LPS and IFN-γ treatment impairs DC development', *European Journal of Immunology*, 39(10), pp. 2865–2876. doi: 10.1002/eji.200939486.

Gringhuis, S. I. *et al.* (2007) 'C-Type Lectin DC-SIGN Modulates Toll-like Receptor Signaling via Raf-1 Kinase-Dependent Acetylation of Transcription Factor NF-κB', *Immunity*, 26(5), pp. 605–616. doi: 10.1016/j.immuni.2007.03.012.

Guirado, E., Schlesinger, L. S. and Kaplan, G. (2013) 'Macrophages in tuberculosis: friend or foe.', *Seminars in immunopathology*. NIH Public Access, 35(5), pp. 563–83. doi: 10.1007/s00281-013-0388-2.

Gulbins, E. and Kolesnick, R. (2004) 'Acid Sphingomyelinase-derived Ceramide Signaling in Apoptosis', in *Phospholipid Metabolism in Apoptosis*. Boston: Kluwer Academic Publishers, pp. 229–244. doi: 10.1007/0-306-47931-1_12.

Gupta, S. K. *et al.* (1994) 'Pili and lipopolysaccharide of Pseudomonas aeruginosa bind to the glycolipid asialo GM1.', *Infection and immunity*, 62(10), pp. 4572–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7927723 (Accessed: 4 January 2019).

Han, F. *et al.* (2015) 'Caveolin-1 regulates cell apoptosis and invasion ability in paclitaxel-induced multidrug-resistant A549 lung cancer cells.', *International journal of clinical and experimental pathology*, 8(8), pp. 8937–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26464635 (Accessed: 1 January 2019).

Harris, J. *et al.* (2002) 'Caveolae and caveolin in immune cells: distribution and functions', *Trends in Immunology*. Elsevier Current Trends, 23(3), pp. 158–164. doi: 10.1016/S1471-4906(01)02161-5.

Hauck, C. R. *et al.* (2000) 'Acid sphingomyelinase is involved in CEACAM receptor-mediated phagocytosis of Neisseria gonorrhoeae.', *FEBS letters*, 478(3), pp. 260–6. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/10930579 (Accessed: 22 December 2018).

Hauck, C. R. *et al.* (2000) 'Acid sphingomyelinase is involved in CEACAM receptor-mediated phagocytosis of Neisseria gonorrhoeae', *FEBS Letters*. No longer published by Elsevier, 478(3), pp. 260–266. doi: 10.1016/S0014-5793(00)01851-2.

Hayer, A. *et al.* (2010) 'Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation', *The Journal of Cell Biology*, 191(3), pp. 615–629. doi: 10.1083/jcb.201003086.

Heggelund, L. *et al.* (2004) 'Increased Expression of Toll-Like Receptor 2 on Monocytes in HIV Infection: Possible Roles in Inflammation and Viral Replication', *Clinical Infectious Diseases*. Oxford University Press, 39(2), pp. 264–269. doi: 10.1086/421780.

Heitmann, L. *et al.* (2013) 'Mincle is not essential for controlling Mycobacterium tuberculosis infection', *Immunobiology*, 218(4), pp. 506–516. doi: 10.1016/j.imbio.2012.06.005.

Herz, J. *et al.* (2009) 'Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes', *Nature Immunology*, 10(7), pp. 761–768. doi: 10.1038/ni.1757.

Huang, J. *et al.* (2012) 'Elevated serum soluble CD40 ligand in cancer patients may play an immunosuppressive role', *Blood*, 120(15), pp. 3030–3038. doi: 10.1182/blood-2012-05-427799.

Huwiler, A. *et al.* (2000) 'Physiology and pathophysiology of sphingolipid metabolism and signaling.', *Biochimica et biophysica acta*, 1485(2–3), pp. 63–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10832090 (Accessed: 22 December 2018).

Igbavboa, U. *et al.* (2009) 'Amyloid β-protein stimulates trafficking of cholesterol and caveolin-1 from the plasma membrane to the Golgi complex in mouse primary astrocytes', *Neuroscience*. doi: 10.1016/j.neuroscience.2009.04.049.

Ishikawa, E. *et al.* (2009) 'Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle', *The Journal of Experimental Medicine*, 206(13), pp. 2879–2888. doi: 10.1084/jem.20091750.

Janssens, S. and Beyaert, R. (2002) 'A universal role for MyD88 in TLR/IL-1R-mediated signaling.', *Trends in biochemical sciences*, 27(9), pp. 474–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12217523 (Accessed: 19 December 2018).

Jayachandran, R. *et al.* (2008a) 'RNA Interference in J774 Macrophages Reveals a Role for Coronin 1 in Mycobacterial Trafficking but Not in Actin-dependent Processes', *Molecular Biology of the Cell*. Edited by S. Schmid, 19(3), pp. 1241–1251. doi: 10.1091/mbc.e07-07-0640.

Jayachandran, R. *et al.* (2008b) 'RNA Interference in J774 Macrophages Reveals a Role for Coronin 1 in Mycobacterial Trafficking but Not in Actin-dependent Processes', *Molecular Biology of the Cell*. Edited by S. Schmid, 19(3), pp. 1241–1251. doi: 10.1091/mbc.e07-07-0640.

Jiao, H. *et al.* (2013) 'Caveolin-1 Tyr14 Phosphorylation Induces Interaction with TLR4 in Endothelial Cells and Mediates MyD88-Dependent Signaling and Sepsis-Induced Lung Inflammation', *The Journal of Immunology*, 191(12), pp. 6191–6199. doi: 10.4049/jimmunol.1300873.

Jin, J. *et al.* (2013) 'Acid sphingomyelinase plays a key role in palmitic acid-amplified inflammatory signaling triggered by lipopolysaccharide at low concentrations in macrophages', *American Journal of Physiology-Endocrinology and Metabolism*, 305(7), pp. E853–E867. doi: 10.1152/ajpendo.00251.2013.

Jo, E.-K. *et al.* (2007) 'Intracellular signalling cascades regulating innate immune responses to Mycobacteria: branching out from Toll-like receptors', *Cellular Microbiology*. John Wiley & Sons, Ltd (10.1111), 9(5), pp. 1087–1098. doi: 10.1111/j.1462-5822.2007.00914.x.

Kachler, K. *et al.* (2017) 'Enhanced Acid Sphingomyelinase Activity Drives Immune Evasion and Tumor Growth in Non-Small Cell Lung Carcinoma.', *Cancer research*. American Association for Cancer Research, 77(21), pp. 5963–5976. doi: 10.1158/0008-5472.CAN-16-3313.

Kaufmann, S. H. E., Hussey, G. and Lambert, P.-H. (2010) 'New vaccines for tuberculosis.', *Lancet (London, England)*. Elsevier, 375(9731), pp. 2110–9. doi: 10.1016/S0140-6736(10)60393-5.

Kerscher, B. *et al.* (2016) 'Mycobacterial receptor, Clec4d (CLECSF8, MCL), is coregulated with Mincle and upregulated on mouse myeloid cells following microbial challenge', *European Journal of Immunology*. John Wiley & Sons, Ltd, 46(2), pp. 381–389. doi: 10.1002/eji.201545858.

Khader, S. A. *et al.* (2007) 'IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge', *Nature Immunology*, 8(4), pp. 369–377. doi: 10.1038/ni1449.

Khan, N. *et al.* (2016) 'Stimulation through CD40 and TLR-4 Is an Effective Host Directed Therapy against Mycobacterium tuberculosis.', *Frontiers in immunology*. Frontiers Media SA, 7, p. 386. doi: 10.3389/fimmu.2016.00386.

Killick, K. E. *et al.* (2013) 'Receptor-mediated recognition of mycobacterial pathogens', *Cellular Microbiology*, 15(9), pp. 1484–1495. doi: 10.1111/cmi.12161.

Kim, H. P. *et al.* (2005) 'Caveolin-1 expression by means of p38beta mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(32), pp. 11319–24. doi: 10.1073/pnas.0501345102.

Kirkegaard, T. *et al.* (2010) 'Hsp70 stabilizes lysosomes and reverts Niemann–Pick disease-associated lysosomal pathology', *Nature*, 463(7280), pp. 549–553. doi: 10.1038/nature08710.

Kiss, A. L. and Botos, E. (2009) 'Endocytosis via caveolae: alternative pathway with distinct cellular compartments to avoid lysosomal degradation?', *Journal of cellular and molecular medicine*. Wiley-Blackwell, 13(7), pp. 1228–37. doi: 10.1111/j.1582-4934.2009.00754.x.

Knaul, J. K. *et al.* (2014) 'Lung-Residing Myeloid-derived Suppressors Display Dual Functionality in Murine Pulmonary Tuberculosis', *American Journal of Respiratory and Critical Care Medicine*, 190(9), pp. 1053–1066. doi: 10.1164/rccm.201405-0828OC.

Ko, J. S. *et al.* (2010) 'Direct and Differential Suppression of Myeloid-Derived Suppressor Cell Subsets by Sunitinib Is Compartmentally Constrained', *Cancer Research*, 70(9), pp. 3526–3536. doi: 10.1158/0008-5472.CAN-09-3278.

Kölzer, M., Werth, N. and Sandhoff, K. (2004) 'Interactions of acid sphingomyelinase and lipid bilayers in the presence of the tricyclic antidepressant desipramine', *FEBS Letters*, 559(1–3), pp. 96–98. doi: 10.1016/S0014-5793(04)00033-X.

Kopitz, J. *et al.* (1998) 'Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture.', *The Journal of biological chemistry*, 273(18), pp. 11205–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9556610 (Accessed: 4 January 2019).

Kornhuber, J. *et al.* (2005) 'High activity of acid sphingomyelinase in major depression', *Journal of Neural Transmission*, 112(11), pp. 1583–1590. doi: 10.1007/s00702-005-0374-5.

Kumar, P. *et al.* (2014) '*Mycobacterium indicus pranii* and *Mycobacterium bovis* BCG lead to differential macrophage activation in Toll-like receptor-dependent manner', *Immunology*, 143(2), pp. 258–268. doi: 10.1111/imm.12306.

Kumar, V., Patel, S., Tcyganov, E. and Gabrilovich, D. I. (2016) 'The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment.', *Trends in immunology*. NIH Public Access, 37(3), pp. 208–220. doi: 10.1016/j.it.2016.01.004.

Kumar, V., Patel, S., Tcyganov, E. and Gabrilovich, D. I. (2016) 'The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment', *Trends in Immunology*. doi: 10.1016/j.it.2016.01.004.

Kurt-Jones, E. A. *et al.* (2002) 'Role of Toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils', *Blood*, 100(5).

Kwan, C. K. and Ernst, J. D. (2011) 'HIV and Tuberculosis: a Deadly Human Syndemic', *Clinical Microbiology Reviews*, 24(2), pp. 351–376. doi: 10.1128/CMR.00042-10.

Lang, R. (2013) 'Recognition of the mycobacterial cord factor by Mincle: relevance for granuloma formation and resistance to tuberculosis.', *Frontiers in immunology*. Frontiers Media SA, 4, p. 5. doi: 10.3389/fimmu.2013.00005.

Latz, E. *et al.* (2002) 'Lipopolysaccharide Rapidly Traffics to and from the Golgi Apparatus with the Toll-like Receptor 4-MD-2-CD14 Complex in a Process That Is Distinct from the Initiation of Signal Transduction', *Journal of Biological Chemistry*, 277(49), pp. 47834–47843. doi: 10.1074/jbc.M207873200.

Li, C. *et al.* (2016) 'Inhibition of neutral sphingomyelinase protects mice against systemic tuberculosis.', *Frontiers in bioscience (Elite edition)*, 8, pp. 311–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26709664 (Accessed: 2 January 2019).

Li, H. and Nord, E. P. (2004) 'Functional caveolae are a prerequisite for CD40 signaling in human renal proximal tubule cells', *American Journal of Physiology-Renal Physiology*. American

Physiological Society, 286(4), pp. F711–F719. doi: 10.1152/ajprenal.00308.2003.

Li, S., Couet, J. and Lisanti, M. P. (1996) 'Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 271(46), pp. 29182–90. doi: 10.1074/JBC.271.46.29182.

Lindau, D. *et al.* (2013) 'The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells.', *Immunology*. Wiley-Blackwell, 138(2), pp. 105–15. doi: 10.1111/imm.12036.

Lonez, C. (2015) 'Role of lipid microdomains in TLR-mediated signalling', *Biochimica et Biophysica Acta (BBA) - Biomembranes*. Elsevier, 1848(9), pp. 1860–1867. doi: 10.1016/J.BBAMEM.2015.03.014.

Lopes Pinheiro, M. A. *et al.* (2016) 'Acid Sphingomyelinase-Derived Ceramide Regulates ICAM-1 Function during T Cell Transmigration across Brain Endothelial Cells.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 196(1), pp. 72–9. doi: 10.4049/jimmunol.1500702.

Lu, C. *et al.* (2016) 'The expression profiles and regulation of PD-L1 in tumor-induced myeloid-derived suppressor cells', *OncoImmunology*, 5(12), p. e1247135. doi: 10.1080/2162402X.2016.1247135.

Lutz, M. B. *et al.* (1999) 'An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow.', *Journal of immunological methods*, 223(1), pp. 77–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10037236 (Accessed: 26 January 2019).

Ma, D. Y. and Clark, E. A. (2009) 'The role of CD40 and CD154/CD40L in dendritic cells.', *Seminars in immunology*. NIH Public Access, 21(5), pp. 265–72. doi: 10.1016/j.smim.2009.05.010.

Maiti, D., Bhattacharyya, A. and Basu, J. (2001) 'Lipoarabinomannan from Mycobacterium tuberculosis promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 276(1), pp. 329–33. doi: 10.1074/jbc.M002650200.

Manzanillo, P. S. *et al.* (2012) 'Mycobacterium Tuberculosis Activates the DNA-Dependent Cytosolic Surveillance Pathway within Macrophages', *Cell Host & Microbe*, 11(5), pp. 469–480. doi: 10.1016/j.chom.2012.03.007.

Martino, A. *et al.* (2010) '*Mycobacterium bovis* Bacillus Calmette-Guérin Vaccination Mobilizes Innate Myeloid-Derived Suppressor Cells Restraining In Vivo T Cell Priming via IL-1R–Dependent Nitric Oxide Production', *The Journal of Immunology*, 184(4), pp. 2038–2047. doi: 10.4049/jimmunol.0903348.

Matsunaga, I. and Moody, D. B. (2009) 'Mincle is a long sought receptor for mycobacterial cord

factor.', *The Journal of experimental medicine*. Rockefeller University Press, 206(13), pp. 2865–8. doi: 10.1084/jem.20092533.

Matthews, L. C., Taggart, M. J. and Westwood, M. (2008) 'Modulation of Caveolin-1 Expression Can Affect Signalling through the Phosphatidylinositol 3-Kinase/Akt Pathway and Cellular Proliferation in Response to Insulin-Like Growth Factor I', *Endocrinology*. Oxford University Press, 149(10), pp. 5199–5208. doi: 10.1210/en.2007-1211.

McNab, F. W. *et al.* (2011) 'Programmed death ligand 1 is over-expressed by neutrophils in the blood of patients with active tuberculosis', *European Journal of Immunology*, 41(7), pp. 1941–1947. doi: 10.1002/eji.201141421.

McNamara, N. *et al.* (2006) 'AsialoGM1 and TLR5 cooperate in flagellin-induced nucleotide signaling to activate Erk1/2.', *American journal of respiratory cell and molecular biology*. American Thoracic Society, 34(6), pp. 653–60. doi: 10.1165/rcmb.2005-0441OC.

Medina, F. A. *et al.* (2006) 'Caveolin-1-deficient mice show defects in innate immunity and inflammatory immune response during Salmonella enterica serovar Typhimurium infection.', *Infection and immunity*. American Society for Microbiology Journals, 74(12), pp. 6665–74. doi: 10.1128/IAI.00949-06.

Mergia, A. (2017) 'The Role of Caveolin 1 in HIV Infection and Pathogenesis', *Viruses*, 9(6), p. 129. doi: 10.3390/v9060129.

Mirza, M. K. *et al.* (2010) 'Caveolin-1 Deficiency Dampens Toll-Like Receptor 4 Signaling through eNOS Activation', *The American Journal of Pathology*, 176(5), pp. 2344–2351. doi: 10.2353/ajpath.2010.091088.

Monteiro, H. P., Arai, R. J. and Travassos, L. R. (2008) 'Protein Tyrosine Phosphorylation and Protein Tyrosine Nitration in Redox Signaling', *Antioxidants & Redox Signaling*, 10(5), pp. 843–890. doi: 10.1089/ars.2007.1853.

Moore, M. L. *et al.* (2008) 'Differential Regulation of GM1 and Asialo-GM1 Expression by T Cells and Natural Killer (NK) Cells in Respiratory Syncytial Virus Infection', *Viral Immunology*, 21(3), pp. 327–339. doi: 10.1089/vim.2008.0003.

Mori, K. *et al.* (2012) 'Formation of GM1 Ganglioside Clusters on the Lipid Membrane Containing Sphingomyeline and Cholesterol', *The Journal of Physical Chemistry B*, 116(17), pp. 5111–5121. doi: 10.1021/jp207881k.

Mundy-Bosse, B. L. *et al.* (2011) 'Myeloid-Derived Suppressor Cell Inhibition of the IFN Response in Tumor-Bearing Mice', *Cancer Research*, 71(15), pp. 5101–5110. doi: 10.1158/0008-5472.CAN-10-2670.

Muñoz, S., Rivas-Santiago, B. and Enciso, J. A. (2009) '*Mycobacterium tuberculosis* Entry into Mast Cells Through Cholesterol-rich Membrane Microdomains', *Scandinavian Journal of Immunology*. Wiley/Blackwell (10.1111), 70(3), pp. 256–263. doi: 10.1111/j.1365-3083.2009.02295.x.

Murata, T. *et al.* (2007) 'Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac,

and pulmonary defects in global caveolin-1 knockout mice', *The Journal of Experimental Medicine*, 204(10), pp. 2373–2382. doi: 10.1084/jem.20062340.

Muzio, M. *et al.* (1997) 'IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling.', *Science (New York, N.Y.)*, 278(5343), pp. 1612–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9374458 (Accessed: 19 December 2018).

Naroeni, A. and Porte, F. (2002) 'Role of cholesterol and the ganglioside GM(1) in entry and short-term survival of Brucella suis in murine macrophages.', *Infection and immunity*. American Society for Microbiology Journals, 70(3), pp. 1640–4. doi: 10.1128/IAI.70.3.1640-1644.2002.

Nichols, B. (2003) 'Caveosomes and endocytosis of lipid rafts.', *Journal of cell science*. The Company of Biologists Ltd, 116(Pt 23), pp. 4707–14. doi: 10.1242/jcs.00840.

Osawa, Y. *et al.* (2005) 'Roles for C_{16} -ceramide and Sphingosine 1-Phosphate in Regulating Hepatocyte Apoptosis in Response to Tumor Necrosis Factor-α', *Journal of Biological Chemistry*, 280(30), pp. 27879–27887. doi: 10.1074/jbc.M503002200.

Oyarce, C. *et al.* (2017) 'Caveolin-1 Expression Increases upon Maturation in Dendritic Cells and Promotes Their Migration to Lymph Nodes Thereby Favoring the Induction of CD8+ T Cell Responses', *Frontiers in Immunology*, 8, p. 1794. doi: 10.3389/fimmu.2017.01794.

Pacher, P., Beckman, J. S. and Liaudet, L. (2007) 'Nitric oxide and peroxynitrite in health and disease.', *Physiological reviews*. NIH Public Access, 87(1), pp. 315–424. doi: 10.1152/physrev.00029.2006.

Pai, M. *et al.* (2016) 'Tuberculosis', *Nature Reviews Disease Primers*. Nature Publishing Group, 2, p. 16076. doi: 10.1038/nrdp.2016.76.

Pan, P.-Y. *et al.* (2010) 'Immune Stimulatory Receptor CD40 Is Required for T-Cell Suppression and T Regulatory Cell Activation Mediated by Myeloid-Derived Suppressor Cells in Cancer', *Cancer Research*, 70(1), pp. 99–108. doi: 10.1158/0008-5472.CAN-09-1882.

Parton, R. G. and Howes, M. T. (2010) 'Revisiting caveolin trafficking: the end of the caveosome.', *The Journal of cell biology*. Rockefeller University Press, 191(3), pp. 439–41. doi: 10.1083/jcb.201009093.

Parton, R. G. and Simons, K. (2007) 'The multiple faces of caveolae', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 8(3), pp. 185–194. doi: 10.1038/nrm2122.

Pelkmans, L., Kartenbeck, J. and Helenius, A. (2001) 'Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER', *Nature Cell Biology*, 3(5), pp. 473–483. doi: 10.1038/35074539.

Pelkmans, L. and Zerial, M. (2005) 'Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae', *Nature*. Nature Publishing Group, 436(7047), pp. 128–133. doi: 10.1038/nature03866.

Peters, P. J. *et al.* (2003) 'Trafficking of prion proteins through a caveolae-mediated endosomal pathway', *The Journal of Cell Biology*, 162(4), pp. 703–717. doi: 10.1083/jcb.200304140.

Peyron, P. *et al.* (2000) 'Nonopsonic phagocytosis of Mycobacterium kansasii by human neutrophils depends on cholesterol and is mediated by CR3 associated with glycosylphosphatidylinositol-anchored proteins.', *Journal of immunology (Baltimore, Md.: 1950)*, 165(9), pp. 5186–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11046051 (Accessed: 3 December 2018).

Philips, J. A. and Ernst, J. D. (2012a) 'Tuberculosis Pathogenesis and Immunity', *Annual Review of Pathology: Mechanisms of Disease*, 7(1), pp. 353–384. doi: 10.1146/annurev-pathol-011811-132458.

Philips, J. A. and Ernst, J. D. (2012b) 'Tuberculosis Pathogenesis and Immunity', *Annual Review of Pathology: Mechanisms of Disease*, 7(1), pp. 353–384. doi: 10.1146/annurev-pathol-011811-132458.

Pietiäinen, V. *et al.* (2004) 'Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events.', *Molecular biology of the cell*. American Society for Cell Biology, 15(11), pp. 4911–25. doi: 10.1091/mbc.e04-01-0070.

du Plessis, N. *et al.* (2013) 'Increased Frequency of Myeloid-derived Suppressor Cells during Active Tuberculosis and after Recent *Mycobacterium tuberculosis* Infection Suppresses T-Cell Function', *American Journal of Respiratory and Critical Care Medicine*, 188(6), pp. 724–732. doi: 10.1164/rccm.201302-0249OC.

du Plessis, N. *et al.* (2018) 'Translational Potential of Therapeutics Targeting Regulatory Myeloid Cells in Tuberculosis', *Frontiers in Cellular and Infection Microbiology*. Frontiers, 8, p. 332. doi: 10.3389/fcimb.2018.00332.

Płóciennikowska, A. *et al.* (2015) 'Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling', *Cellular and Molecular Life Sciences*, 72(3), pp. 557–581. doi: 10.1007/s00018-014-1762-5.

Quesniaux, V. J. *et al.* (2004) 'Toll-like receptor 2 (TLR2)-dependent-positive and TLR2-independent-negative regulation of proinflammatory cytokines by mycobacterial lipomannans.', *Journal of immunology (Baltimore, Md.: 1950)*, 172(7), pp. 4425–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15034058 (Accessed: 8 December 2018).

Razani, B. *et al.* (2001) 'Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities.', *The Journal of biological chemistry*, 276(41), pp. 38121–38. doi: 10.1074/jbc.M105408200.

Razani, B. *et al.* (2002) 'Caveolin-1-deficient Mice Are Lean, Resistant to Diet-induced Obesity, and Show Hypertriglyceridemia with Adipocyte Abnormalities', *Journal of Biological Chemistry*, 277(10), pp. 8635–8647. doi: 10.1074/jbc.M110970200.

Ribechini, E. *et al.* (2017) 'Novel GM-CSF signals via IFN-γR/IRF-1 and AKT/mTOR license monocytes for suppressor function.', *Blood advances*. American Society of Hematology, 1(14), pp. 947–960. doi: 10.1182/bloodadvances.2017006858.

Richterova, Z. *et al.* (2001) 'Caveolae Are Involved in the Trafficking of Mouse Polyomavirus Virions

and Artificial VP1 Pseudocapsids toward Cell Nuclei', *Journal of Virology*, 75(22), pp. 10880–10891. doi: 10.1128/JVI.75.22.10880-10891.2001.

Riser, B. L., Laybourn, K. A. and Varani, J. (1988) 'Treatment of mice with anti-asialo-GM1 antibody or poly-I:C: effects on metastasis dissociable from modulation of macrophage antitumor activity.', *Natural immunity and cell growth regulation*, 7(5–6), pp. 305–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3221908 (Accessed: 28 December 2018).

Rodriguez, N. E., Gaur, U. and Wilson, M. E. (2006) 'Role of caveolae in Leishmania chagasi phagocytosis and intracellular survival in macrophages', *Cellular Microbiology*. John Wiley & Sons, Ltd (10.1111), 8(7), pp. 1106–1120. doi: 10.1111/j.1462-5822.2006.00695.x.

Rodriguez, P. C., Quiceno, D. G. and Ochoa, A. C. (2007) 'L-arginine availability regulates T-lymphocyte cell-cycle progression', *Blood*, 109(4), pp. 1568–1573. doi: 10.1182/blood-2006-06-031856.

Rößner, S. *et al.* (2005a) 'Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide productionin vitro', *European Journal of Immunology*, 35(12), pp. 3533–3544. doi: 10.1002/eji.200526172.

Rößner, S. *et al.* (2005b) 'Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide productionin vitro', *European Journal of Immunology*, 35(12), pp. 3533–3544. doi: 10.1002/eji.200526172.

Rothberg, K. G. *et al.* (1992) 'Caveolin, a protein component of caveolae membrane coats.', *Cell*, 68(4), pp. 673–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1739974 (Accessed: 12 September 2018).

Rothberg, K. G. *et al.* (1992) 'Caveolin, a protein component of caveolae membrane coats', *Cell*. Cell Press, 68(4), pp. 673–682. doi: 10.1016/0092-8674(92)90143-Z.

Saiman, L. and Prince, A. (1993) 'Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 92(4), pp. 1875–80. doi: 10.1172/JCI116779.

Sakai, S. *et al.* (2016) 'CD4 T Cell-Derived IFN-γ Plays a Minimal Role in Control of Pulmonary Mycobacterium tuberculosis Infection and Must Be Actively Repressed by PD-1 to Prevent Lethal Disease', *PLOS Pathogens*. Edited by S. M. Fortune. Public Library of Science, 12(5), p. e1005667. doi: 10.1371/journal.ppat.1005667.

Sakata, A. *et al.* (2007a) 'Acid sphingomyelinase inhibition suppresses lipopolysaccharide-mediated release of inflammatory cytokines from macrophages and protects against disease pathology in dextran sulphate sodium-induced colitis in mice.', *Immunology*. Wiley-Blackwell, 122(1), pp. 54–64. doi: 10.1111/j.1365-2567.2007.02612.x.

Sakata, A. *et al.* (2007b) 'Acid sphingomyelinase inhibition suppresses lipopolysaccharide-mediated release of inflammatory cytokines from macrophages and protects against disease pathology in dextran sulphate sodium-induced colitis in mice.', *Immunology*. Wiley-Blackwell, 122(1), pp. 54–64. doi: 10.1111/j.1365-2567.2007.02612.x.

Sánchez, D. *et al.* (2010) 'Role of TLR2- and TLR4-mediated signaling in Mycobacterium tuberculosis-induced macrophage death', *Cellular Immunology*. Academic Press, 260(2), pp. 128–136. doi: 10.1016/J.CELLIMM.2009.10.007.

Schliwa, M. (1982) 'Action of cytochalasin D on cytoskeletal networks.', *The Journal of cell biology*, 92(1), pp. 79–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7199055 (Accessed: 3 December 2018).

Schoenen, H. *et al.* (2010) 'Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 184(6), pp. 2756–60. doi: 10.4049/jimmunol.0904013.

Schramm, M. *et al.* (2008) 'Acid sphingomyelinase is required for efficient phago-lysosomal fusion', *Cellular Microbiology*, 10(9), pp. 1839–1853. doi: 10.1111/j.1462-5822.2008.01169.x.

Schütze, S. *et al.* (1992) 'TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown.', *Cell*, 71(5), pp. 765–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1330325 (Accessed: 22 December 2018).

Shaw, A. S. (2006) 'Lipid rafts: now you see them, now you don't', *Nature Immunology*. Nature Publishing Group, 7(11), pp. 1139–1142. doi: 10.1038/ni1405.

Shen, J. *et al.* (2008) 'Interaction of caveolin-1, nitric oxide, and nitric oxide synthases in hypoxic human SK-N-MC neuroblastoma cells', *Journal of Neurochemistry*, 107(2), pp. 478–487. doi: 10.1111/j.1471-4159.2008.05630.x.

Shen, J. *et al.* (2014) 'Downregulation of CD40 expression contributes to the accumulation of myeloid-derived suppressor cells in gastric tumors.', *Oncology letters*. Spandidos Publications, 8(2), pp. 775–780. doi: 10.3892/ol.2014.2174.

Shibata, Y. *et al.* (1994) *Macrophage Colony-Stimulating Factor-Induced Bone Marrow Macrophages Do Not Synthesize or Release Prostaglandin E2*. Available at: www.bloodjournal.org (Accessed: 30 January 2019).

Shin, J. S. and Abraham, S. N. (2001) 'Co-option of endocytic functions of cellular caveolae by pathogens.', *Immunology*, 102(1), pp. 2–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11168630 (Accessed: 20 December 2018).

Shin, J. S., Gao, Z. and Abraham, S. N. (2000) 'Involvement of cellular caveolae in bacterial entry into mast cells.', *Science (New York, N.Y.)*, 289(5480), pp. 785–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10926542 (Accessed: 13 September 2018).

Sia, J. K. *et al.* (2017) 'Engaging the CD40-CD40L pathway augments T-helper cell responses and improves control of Mycobacterium tuberculosis infection', *PLOS Pathogens*. Edited by P. Salgame, 13(8), p. e1006530. doi: 10.1371/journal.ppat.1006530.

Simonis, A. *et al.* (2014) 'Differential Activation of Acid Sphingomyelinase and Ceramide Release Determines Invasiveness of Neisseria meningitidis into Brain Endothelial Cells', *PLoS*

Pathogens. Edited by C. Tang. Public Library of Science, 10(6), p. e1004160. doi: 10.1371/journal.ppat.1004160.

Simons, K. and Ikonen, E. (1997) 'Functional rafts in cell membranes', *Nature*. Nature Publishing Group, 387(6633), pp. 569–572. doi: 10.1038/42408.

Sinha, P. *et al.* (2008) 'Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells.', *Journal of immunology (Baltimore, Md. : 1950)*, 181(7), pp. 4666–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18802069 (Accessed: 10 December 2018).

Van Slambrouck, S. *et al.* (2009) 'AsialoGM1 and integrin alpha2beta1 mediate prostate cancer progression.', *International journal of oncology*. NIH Public Access, 35(4), pp. 693–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19724904 (Accessed: 22 December 2018).

Soong, G. *et al.* (2004) 'TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 113(10), pp. 1482–9. doi: 10.1172/JCI20773.

Stamatos, N. M. *et al.* (2010) 'LPS-induced cytokine production in human dendritic cells is regulated by sialidase activity', *Journal of Leukocyte Biology*. John Wiley & Sons, Ltd, 88(6), pp. 1227–1239. doi: 10.1189/jlb.1209776.

Stamm, C. E., Collins, A. C. and Shiloh, M. U. (2015) 'Sensing of Mycobacterium tuberculosis and consequences to both host and bacillus.', *Immunological reviews*. NIH Public Access, 264(1), pp. 204–19. doi: 10.1111/imr.12263.

Stanley, S. A. *et al.* (2007) 'The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis.', *Journal of immunology (Baltimore, Md.: 1950)*, 178(5), pp. 3143–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17312162 (Accessed: 9 December 2018).

Stehr, M., A., A. and Singh, M. (2013) 'Lipid Inclusions in Mycobacterial Infections', in *Tuberculosis - Current Issues in Diagnosis and Management*. InTech. doi: 10.5772/54526.

Sutcliffe, I. C. and Harrington, D. J. (2004) 'Lipoproteins of *Mycobacterium tuberculosis* : an abundant and functionally diverse class of cell envelope components', *FEMS Microbiology Reviews*, 28(5), pp. 645–659. doi: 10.1016/j.femsre.2004.06.002.

Tagawa, A. *et al.* (2005) 'Assembly and trafficking of caveolar domains in the cell', *The Journal of Cell Biology*, 170(5), pp. 769–779. doi: 10.1083/jcb.200506103.

Tailleux, L. *et al.* (2003) 'DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells.', *The Journal of experimental medicine*, 197(1), pp. 121–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12515819 (Accessed: 15 January 2019).

Talmadge, J. E. and Gabrilovich, D. I. (2013) 'History of myeloid-derived suppressor cells', *Nature Reviews Cancer*. Nature Publishing Group, 13(10), pp. 739–752. doi: 10.1038/nrc3581.

Tanne, A. *et al.* (2009) 'A murine DC-SIGN homologue contributes to early host defense against

Mycobacterium tuberculosis', *The Journal of Experimental Medicine*, 206(10), pp. 2205–2220. doi: 10.1084/jem.20090188.

Tapia-Abellán, A. *et al.* (2013) 'Role of MAP Kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from the ascites of cirrhotic patients', *Liver International*, 33(4), pp. 552–560. doi: 10.1111/liv.12072.

THOMAS, S. *et al.* (2004) 'Analysis of lipid rafts in T cells', *Molecular Immunology*, 41(4), pp. 399–409. doi: 10.1016/j.molimm.2004.03.022.

Torres, V. A. *et al.* (2007) 'E-cadherin is required for caveolin-1-mediated down-regulation of the inhibitor of apoptosis protein survivin via reduced beta-catenin-Tcf/Lef-dependent transcription.', *Molecular and cellular biology*. American Society for Microbiology Journals, 27(21), pp. 7703–17. doi: 10.1128/MCB.01991-06.

Trigatti, B. L., Anderson, R. G. W. and Gerber, G. E. (1999) 'Identification of Caveolin-1 as a Fatty Acid Binding Protein', *Biochemical and Biophysical Research Communications*, 255(1), pp. 34–39. doi: 10.1006/bbrc.1998.0123.

Trikha, P., Carson, W. E. and III (2014) 'Signaling pathways involved in MDSC regulation.', *Biochimica et biophysica acta*. NIH Public Access, 1846(1), pp. 55–65. doi: 10.1016/j.bbcan.2014.04.003.

Truman, J.-P. *et al.* (2011) 'Acid sphingomyelinase in macrophage biology.', *Cellular and molecular life sciences : CMLS*. NIH Public Access, 68(20), pp. 3293–305. doi: 10.1007/s00018-011-0686-6.

Tsai, T.-H. *et al.* (2011a) 'Impaired Cd14 and Cd36 Expression, Bacterial Clearance, and Toll-Like Receptor 4-Myd88 Signaling in Caveolin-1-Deleted Macrophages and Mice', *Shock*, 35(1), pp. 92–99. doi: 10.1097/SHK.0b013e3181ea45ca.

Tsai, T.-H. *et al.* (2011b) 'Impaired Cd14 and Cd36 Expression, Bacterial Clearance, and Toll-Like Receptor 4-Myd88 Signaling in Caveolin-1-Deleted Macrophages and Mice', *Shock*, 35(1), pp. 92–99. doi: 10.1097/SHK.0b013e3181ea45ca.

Tsiganov, E. N. *et al.* (2014a) 'Gr-1dimCD11b+ Immature Myeloid-Derived Suppressor Cells but Not Neutrophils Are Markers of Lethal Tuberculosis Infection in Mice', *The Journal of Immunology*, 192(10), pp. 4718–4727. doi: 10.4049/jimmunol.1301365.

Tsiganov, E. N. *et al.* (2014b) 'Gr-1dimCD11b+ Immature Myeloid-Derived Suppressor Cells but Not Neutrophils Are Markers of Lethal Tuberculosis Infection in Mice', *The Journal of Immunology*, 192(10), pp. 4718–4727. doi: 10.4049/jimmunol.1301365.

Tu, S. *et al.* (2008) 'Overexpression of Interleukin-1β Induces Gastric Inflammation and Cancer and Mobilizes Myeloid-Derived Suppressor Cells in Mice', *Cancer Cell*, 14(5), pp. 408–419. doi: 10.1016/j.ccr.2008.10.011.

Underhill, D. M. *et al.* (1999) 'The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens', *Nature*, 401(6755), pp. 811–815. doi: 10.1038/44605.

Vargas, L. *et al.* (2002) 'Functional Interaction of Caveolin-1 with Bruton's Tyrosine Kinase and Bmx',

Journal of Biological Chemistry, 277(11), pp. 9351–9357. doi: 10.1074/jbc.M108537200.

Varshney, P., Yadav, V. and Saini, N. (2016) 'Lipid rafts in immune signalling: current progress and future perspective.', *Immunology*. Wiley-Blackwell, 149(1), pp. 13–24. doi: 10.1111/imm.12617.

Vasquez-Dunddel, D. *et al.* (2013) 'STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients', *Journal of Clinical Investigation*, 123(4), pp. 1580–1589. doi: 10.1172/JCI60083.

Veglia, F., Perego, M. and Gabrilovich, D. (2018a) 'Myeloid-derived suppressor cells coming of age', *Nature Immunology*, 19(2), pp. 108–119. doi: 10.1038/s41590-017-0022-x.

Veglia, F., Perego, M. and Gabrilovich, D. (2018b) 'Myeloid-derived suppressor cells coming of age', *Nature Immunology*, 19(2), pp. 108–119. doi: 10.1038/s41590-017-0022-x.

Veglia, F., Perego, M. and Gabrilovich, D. (2018c) 'Myeloid-derived suppressor cells coming of age', *Nature Immunology*. Nature Publishing Group, 19(2), pp. 108–119. doi: 10.1038/s41590-017-0022-x.

Vendelova, E. *et al.* (2018) 'Tolerogenic Transcriptional Signatures of Steady-State and Pathogen-Induced Dendritic Cells', *Frontiers in Immunology*, 9, p. 333. doi: 10.3389/fimmu.2018.00333.

Volonte, D. *et al.* (2002) 'Expression of Caveolin-1 Induces Premature Cellular Senescence in Primary Cultures of Murine Fibroblasts', *Molecular Biology of the Cell*. Edited by C.-H. Heldin, 13(7), pp. 2502–2517. doi: 10.1091/mbc.01-11-0529.

Walzl, G. *et al.* (2018) 'Tuberculosis: advances and challenges in development of new diagnostics and biomarkers.', *The Lancet. Infectious diseases*. Elsevier, 18(7), pp. e199–e210. doi: 10.1016/S1473-3099(18)30111-7.

Wang, X. M. *et al.* (2006) 'Caveolin-1 Confers Antiinflammatory Effects in Murine Macrophages via the MKK3/p38 MAPK Pathway', *American Journal of Respiratory Cell and Molecular Biology*. American Thoracic Society, 34(4), pp. 434–442. doi: 10.1165/rcmb.2005-0376OC.

Wang, X. M. *et al.* (2009) 'The heme oxygenase-1/carbon monoxide pathway suppresses TLR4 signaling by regulating the interaction of TLR4 with caveolin-1.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 182(6), pp. 3809–18. doi: 10.4049/jimmunol.0712437.

Werling, D. *et al.* (1999) 'Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells.', *Journal of leukocyte biology*, 66(1), pp. 50–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10410989 (Accessed: 13 September 2018).

'WHO | Global tuberculosis report 2017' (2017) *WHO*. World Health Organization. Available at: http://www.who.int/tb/publications/global_report/en/ (Accessed: 14 September 2018).

Wiechen, K. *et al.* (2001) 'Caveolin-1 Is Down-Regulated in Human Ovarian Carcinoma and Acts as a Candidate Tumor Suppressor Gene', *The American Journal of Pathology*, 159(5), pp. 1635–1643. doi: 10.1016/S0002-9440(10)63010-6.

Williams, J. J. L. and Palmer, T. M. (2014) 'Cavin-1: caveolae-dependent signalling and cardiovascular disease: Figure 1', *Biochemical Society Transactions*, 42(2), pp. 284–288. doi: 10.1042/BST20130270.

Wolf, A. J. *et al.* (2008) 'Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs', *The Journal of Experimental Medicine*, 205(1), pp. 105–115. doi: 10.1084/jem.20071367.

Yadav, M. and Schorey, J. S. (2006) 'The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria', *Blood*, 108(9), pp. 3168–3175. doi: 10.1182/blood-2006-05-024406.

YAMADA, E. (1955) 'The fine structure of the gall bladder epithelium of the mouse.', *The Journal of biophysical and biochemical cytology*, 1(5), pp. 445–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13263332 (Accessed: 12 September 2018).

Zal, T., Volkmann, A. and Stockinger, B. (1994) 'Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen.', *The Journal of experimental medicine*, 180(6), pp. 2089–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7964486 (Accessed: 29 January 2019).

Zeller, C. B. and Marchase, R. B. (1992) 'Gangliosides as modulators of cell function.', *The American journal of physiology*, 262(6 Pt 1), pp. C1341-55. doi: 10.1152/ajpcell.1992.262.6.C1341.

List of Figures

Abbreviations

AM	Alveolar macrophages
Arg1	Arginase1
APC	Antigen presenting cell
APS	Ammonium persulfate
ASM	Acid Sphingomyelinase
BCG	Bacillus Calmette-Guérin
BM	Bone marrow
BSA	Bovine serum albumin
Cav-1	Caveolin-1
CD	Cluster of differentiation
CLR	C-type lectin receptor
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EDTA	Ethylenediaminetetraacidic acid
ELISA	Enzyme linked immunoabsorbant assay
ER	Endoplasmic reticulum
FA	Formaldehyde
FACS	Fluorescence activated cell sorting
GM-CSF	Granulocyte-macrophage colony stimulating factor
G-MDSCs	Granulocytic-MDSCs
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFN	Interferon

IL	Interleukin
iNOS	inducible nitric oxide synthase
kDa	kilo Dalton
KO	Knockout
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
MAPK	Mitogen-activated protein kinases
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MFI	Mean Fluorescence intensity
Mtb	*Mycobacterium tuberculosis*
M-MDSCs	Monocytic-MDSCs
MyD88	Myeloid differentiation factor 88
NaCl	sodium chloride
PAMPs	Pattern associated molecular patterns
PDL-1	Programmed death-ligand 1
PBS	Phosphate buffered saline
PI3K	phosphoinositol-3-OH-Kinase
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium-dodecylsulfate
SM	Sphingomyelin
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor

TB	Tuberculosis
Treg	Regulatory T cell
WT	Wildtype

Acknowledgments

This work was conducted at the Institute of Virology and Immunobiology, University of Würzburg, Germany from March 2015 to December 2018 in the lab of Prof. Manfred Lutz. This study was supported by a PhD fellowship from the German Excellence Initiative to the Graduate School of Life Sciences. This work could not have been possible without the help and support of the people mentioned in this acknowledgment and I am extremely thankful to all of them.

My first and foremost words of thankfulness and gratefulness should be in the name of my PhD supervisor, Prof. Manfred Lutz, whose support and guidance always encouraged me during my doctoral training years. Under his supervision, I not only learned to plan and execute the experiments but also learned to be always optimistic. He taught me to be calm and always helped me to work on my weaknesses with a positive attitude. I greatly appreciate his scientific teaching abilities which have been instrumental for carrying out this research work. I consider myself very lucky to be trained under his supervision.

I would like to sincerely thank my secondary supervisors Dr. Martin Fraunholz and Prof. Andreas Beilhack for spending their precious time and efforts during my PhD. Their critical comments and suggestions during the annual meetings were very helpful.

I would like to thank Gabriele Blum Oehler and the entire GSLS team for offering a wonderful PhD study program, for nicely handling the administrative procedures and for their help and support right from my first day and all throughout my stay in Würzburg. I am extremely thankful to GSLS for providing me the funding to pursue my doctoral thesis.

I should also thank all organizers and graduate colleagues of Graduate program ‘‘Immunomodulation’’ for scientific discussions, Jour Fixe every Thursday and informative meetings and for arranging retreats in the network meetings with other Universities in Tübingen and Erlangen. I have learned a lot during all these PhD years and it helped me to improve my skills in research and networking.

I would like to convey my heartiest gratitude to my lab-members of AG Lutz, Diyaaaa, Ina, Marion, Eliana, Szymon, Julia and also former lab members Mili, Ramon, Cinthia, Andrea, Ulrike for helping me in one way or the other throughout my stay in this lab and their moral support. Coffee breaks and late-night dinners in the institute with them was always a stress buster. Lab outings with all of them were always fun and I spent a memorable time with them which I will cherish forever.

I would like to cordially thank, Dr. Sabine Kranz and all the staff of animal facility for always helping me with animal breeding and housing. I want to thank Prof. Dr. Sibylle Schneider-Schaulies

for providing us the ASM$^{-/-}$ mice and for her suggestions for the ASM project. I am also thankful to lot a of people in the Institute who helped me in technical problems and scientific discussions, Dr. Nora Müller, Vishakha, Thomas, Bhaskar, Claudia, Steffi, Sherry, Lisa, Adam, Lara, Elita and many others who helped me unknowingly.

I owe a very special thanks to my first friend in Würzburg, Hemant, for his continuous support and bearing my madness even after knowing that there is no chance of improvement and for always cheering me up. I also want to thank, Khushi for being such a wonderful soul and always standing by my side during my tough times. Her presence has always been a soothing sense of comfort and I consider myself very fortunate to have them my closest friends in Würzburg. I also want to thank my other friends in Würzburg Amod, Vishakha, cute Vreha, Rakesh, Nitish, Janice ,Pratibha, , Sohail, etc., for being a wonderful people and helping me in one way or the other. I also want to thank my guardian angels in Germany, Aaradhita, Shivam, Jass, Vishal and Ritika di.

I do not have adequate strength of vocabulary to express my deepest sincere gratitude to my Mom, Dad, brother and Neethu bhabhi for their unconditional love, motivation and inspiration throughout my PhD years.

I would like to conclude this acknowledgment with the honest confession that I came to Würzburg with the aim to learn. I am very happy that I got plenty of opportunities to learn and all these doctoral training years has made me in believing that ‘Stop doubting yourself, work hard and make it happen’.

Publication List

1. **Vini John**, Leigh A. Kotze, Nelita Du Plessis, Gerhard Walzl, and Manfred B. Lutz (2019) Caveolin-1 controls TLR2-mediated NO production for T cell suppression by monocytic myeloid-derived suppressor cells after mycobacterial infection. Manuscript to be submitted.

2. Pawan Kumar[1], **ViniJohn[1]**, Ananya Gupta, Sangeeta Bhaskar (2018) Enhanced survival of BCG-stimulated dendritic cells: involvement of anti-apoptotic proteins and NF-kB. Biology Open (BiO) (Shared First Author) (7): bio032045

3. Silva-Vilches C, Pletinckx K, Lohnert, Ashour D, **Vini John**, Vendelova E, Kneitz S, Zhou J, Mueller TD, Bodem J, Lutz MB (2017) Low Doses of Cholera Toxin and its Mediator cAMP Induce CTLA-2 Secretion by Dendritic Cells to Enhance Regulatory T Cell Conversion. PLOS ONE 12(7): e0178114

4. Pawan Kumar, **Vini John**, Soumitra Marathe, Gobardhan Das & Sangeeta Bhaskar (2015) Mycobacterium indicus pranii induces dendritic cell activation, survival and Th1/1/Th17 polarization potential in a TLR-dependent manner. Journal of Leukocyte Biology9(7): 511-520.

Curriculum Vitae

Vini John