HHS Public Access

Author manuscript

*Alcohol Clin Exp Res*. Author manuscript; available in PMC 2023 December 01.

Published in final edited form as:

*Alcohol Clin Exp Res*. 2022 December ; 46(12): 2214–2224. doi:10.1111/acer.14960.

# Alcohol impairs recognition and uptake of *Mycobacterium tuberculosis* by suppressing toll-like receptor 2 expression

**Gregory W Wigger, MD**[1], **Darya Khani**[1], **Mariam Ahmed**[1], **Layal Sayegh, MD**[2], **Sara C. Auld, MD MSc**[1,3], **Xian Fan, MD**[1], **David M Guidot, MD**[1], **Bashar S Staitieh, MD**[1]

[1]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[2]Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[3]Rollins School of Public Health, Emory University, Atlanta, GA, USA

## Abstract

**Background:** Alcohol impairs pulmonary innate immune function and is associated with an increased risk for tuberculosis (TB). Toll-like receptor 2 (TLR2) is a pattern recognition receptor on alveolar macrophages that recognizes *Mycobacterium tuberculosis* (*Mtb*). Its expression depends, in part, on granulocyte macrophage-colony stimulating factor (GM-CSF) signaling. Given our prior work demonstrating suppression of GM-CSF signaling in chronic alcohol ingestion, we hypothesized that alcohol impairs TLR2 expression via suppression of GM-CSF and thereby reduces the ability of the macrophage to recognize and phagocytose *Mtb*.

**Methods:** Primary alveolar macrophages were isolated from control-fed and alcohol-fed rats. Prior to cell isolation, some alcohol-fed rats were treated with intranasal GM-CSF and then endotracheally inoculated with an attenuated strain of *Mtb*. Primary macrophages were then isolated and immunofluorescence (IF) was used to determine phagocytic efficiency and TLR2 expression in the presence and absence of GM-CSF treatment as well as phagocytic efficiency in the presence and absence of TLR2 neutralization.

**Results:** TLR2 expression and phagocytosis of *Mtb* were significantly decreased in the alveolar macrophages of alcohol-fed rats compared to control-fed rats. In parallel, blocking TLR2 signaling recapitulated this decreased phagocytosis of *Mtb*. In contrast, intranasal GM-CSF treatment restored TLR2 expression and *Mtb* phagocytosis in the alveolar macrophages of alcohol-fed rats to levels comparable to control-fed rats.

**Conclusions:** Chronic alcohol ingestion reduces TLR2 protein expression and phagocytosis of *Mtb*, likely as a result of impaired GM-CSF signaling. GM-CSF restores membrane-bound TLR2 expression and phagocytic function.

### Keywords

tuberculosis; alcohol use disorder; alveolar macrophage; GM-CSF; TLR2

**Corresponding Author Information**: Gregory Wigger, 615 Michael St. NE Ste. 205 Atlanta, GA 30322, Phone: 404-712-8286, Fax: 404-712-2974, gwigger@emory.edu.

***Conflict of Interest***: The authors have no conflict of interest to declare

## INTRODUCTION

Alcohol use disorders (AUD) are common worldwide and result in an estimated 3 million deaths each year (WHO, 2018). Unfortunately, the COVID-19 pandemic and its associated lockdowns has led to an increase in reported alcohol consumption as well as hospitalizations for alcohol-related end-organ damage (Kim et al., 2020, Rutledge et al., 2021). In addition to the well-known complications in the liver, AUD cause pulmonary complications as well, such as an increased risk for acute respiratory distress syndrome (ARDS) and bacterial pneumonia (Moss et al., 1996, Moss et al., 2003, Simou et al., 2018). Since the 1800s, an increased occurrence of pneumonia, including tuberculosis (TB), has been noted among patients who consume alcohol (Osler, 1898). More recent studies have confirmed the increased risk of TB among persons with AUD, and found that the latter is an independent risk factor for TB even after controlling for confounders such as comorbidities, lifestyle, or social determinants of health (Lonnroth et al., 2008, Rehm et al., 2009, Imtiaz et al., 2017). Further, those with AUD and TB are more infectious and have more clinically severe TB, including more cavitary disease and larger areas of affected lung, compared to those without AUD (Diel et al., 2004, Classen et al., 1999, Fiske et al., 2009, Volkmann et al., 2015, Wang, 2012). Even when treated, AUD are associated with poor outcomes, including increased risk of treatment failure and death (Ragan et al., 2020, Volkmann et al., 2015, Thomas et al., 2019).

Alcohol negatively impacts pulmonary innate immunity in many ways that favor *Mtb*, including impaired mucociliary function, increased alveolar oxidative stress, and a more permeable alveolar epithelium (Wigger et al., 2022). Further, alcohol impairs alveolar macrophage function, interfering with macrophage maturation, antioxidant defense, and phagocytosis (Moss et al., 2000, Jensen et al., 2013, Shibata et al., 2001, Joshi et al., 2005, Lloberas et al., 1999). The alveolar macrophage is a key component of the pulmonary immune system with a plethora of functions including pathogen recognition, phagocytosis, and initiation and recruitment of the innate and adaptive immune responses.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is essential for the maturation and function of the alveolar macrophage (Shibata et al., 2001). GM-CSF is also vital to the innate immune response to *Mycobacterium tuberculosis* (*Mtb*), the causative organism of TB, restricting bacillary growth and promoting mycobacterial clearance (Bryson et al., 2019, Mishra et al., 2020, Benmerzoug et al., 2018). We previously demonstrated that chronic alcohol exposure interferes with GM-CSF signaling by decreasing expression of the β-subunit of the receptor for granulocyte/macrophage-colony stimulating factor (GMCSF-Rβ) on the alveolar macrophage surface (Joshi et al., 2005). This results in diminished activity of PU.1, a GM-CSF-dependent regulatory transcription factor required for normal alveolar macrophage differentiation, phagocytosis, pathogen killing, and cytokine production (Brown et al., 2009, Shibata et al., 2001, Joshi et al., 2006, Joshi et al., 2005).

Toll-like receptor 2 (TLR2) is an important pattern recognition receptor (PRR) expressed on macrophages that recognizes lipoproteins and lipomannans specific to mycobacteria, including *Mtb*, and subsequently initiates expression of various effector molecules

(Oliveira-Nascimento et al., 2012, Kirschning et al., 1998, Aliprantis et al., 1999). TLR2 deficiencies have been associated with an increased susceptibility to TB as well as impaired immunologic response and mycobacterial killing by the macrophage (Yim et al., 2006, Ogus et al., 2004, Ben-Ali et al., 2004, Drennan et al., 2004, Texereau et al., 2005, Reiling et al., 2002, Kramarska et al., 2021, Palucci et al., 2016, Thoma-Uszynski et al., 2001). Alcohol has been shown to inhibit TLR2 function (Goral and Kovacs, 2005, Pruett et al., 2004). Further, GM-CSF and PU.1, both downregulated with chronic alcohol exposure, are promoters of TLR2 expression and function (Shibata et al., 2001, Haehnel et al., 2002, Liu et al., 2017, Kurt-Jones et al., 2002). This link between TLR2 and GM-CSF, as well as the importance of TLR2 to the alveolar macrophage's response to *Mtb*, led us to hypothesize that alcohol-induced impairment of alveolar macrophage GM-CSF signaling results in impaired TLR2 expression and thereby impairs recognition and uptake of *Mtb*.

## MATERIALS & METHODS

### Alcohol feeding and primary alveolar macrophage isolation

Adult male Sprague-Dawley littermate rats were fed either an alcohol or a control diet as previously described (Fan et al., 2011). Briefly, animals were fed the standard Lieber-DeCarli liquid diet containing either alcohol (ethanol, 36% of total calories) or an isocaloric carbohydrate substitution *ad libitum* for a total of 6 weeks. All animal work was performed with the approval of the Institutional Use and Care of Animals Committee at Emory University.

Primary alveolar macrophages were obtained by bronchoalveolar lavage (BAL) from alcohol-fed and control-fed rats after sacrifice as previously described (Staitieh et al., 2018). Alveolar macrophages were washed before plating in F12-K with gentamycin (50ug/mL), amphotericin B (2.5 ng/mL), and 10% fetal bovine serum. Each independent experiment made use of cells from 3–4 different animals per feeding group that were plated at a density of 80,000 cells/well on 16-well slide for immunofluorescence (IF). Two hours after plating, non-adherent cells were removed to obtain an enriched population of alveolar macrophages.

### Airway treatments *in vivo*

In selected experiments, rats were anesthetized with vaporized 3% isoflurane and then treated intranasally with either recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF) (PeproTech, Cranbury, NJ/USA), 500 ug in 100 μL of phosphate-buffered saline (PBS), or 100 μL of PBS alone, by gently instilling the volume into one nostril with a 200 μL pipette. Rats sniff repetitively by a characteristic reflex that delivers a significant volume of the instilled liquid into the lower airways as we have published previously (Pelaez et al., 2004). Rats were given 3 separate rGM-CSF or PBS treatments over 3–6 days prior to *Mtb* endotracheal inoculation.

For endotracheal inoculation of *Mtb*, an attenuated *Mtb* strain, H37Rv ($mc^2$ 6206) (Jain et al., 2014), was obtained from the laboratory of Dr. William R. Jacobs Jr., PhD at Albert Einstein of College Medicine (New York, NY/USA) and was grown in Middlebrook's H9 broth supplemented with Middlebrook's OADC enrichment using the methods described

by Nazarova et al (Nazarova and Russell, 2017). *Mtb* was labeled with fluorescein-5-Isothiocyanate (FITC) (Invitrogen, Carlsbad, CA/USA) using the methods described by Astarie-Dequeker et al (Astarie-Dequeker et al., 1999). Briefly, $2\times10^8$ mycobacteria were added to 1 mL of 0.01% FITC in 0.2M $Na_2CO_3$–$NaHCO_3$ buffer containing 150 mM NaCl (pH 9.2) for 30 minutes. Mycobacteria were then washed twice in PBS and subsequently resuspended in the enriched Middlebrook broth at a concentration of $1\times10^8$/mL. Animals were endotracheally intubated as described by Kastl et al (Kastl et al., 2004) and inoculated with *Mtb* as described by Leong et al (Leong et al., 2010). Briefly, animals were anesthetized with intraperitoneal injections of ketamine and xylazine and subsequently endotracheally intubated with a 14-gauge intravenous catheter. Using a 1 mL syringe, $10^7$ of FITC-labeled *Mtb* in 100 μL was injected through the endotracheal catheter followed by an immediate 900 μL of air and an additional 1 mL of air, to facilitate dispersion of *Mtb* into the lower airways. Twenty-four hours after inoculation, the animals were sacrificed.

### GM-CSF treatment, TLR2 neutralization and Mtb infection *ex vivo*

Primary alveolar macrophages were obtained from wild-type rats by BAL and plated as described above. Cells were incubated with 50 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) for 48 hours. Cells were treated with a TLR2 neutralizing antibody (Anti-mTLR2-IgG, ID: C9A12, InvivoGen, San Diego, CA/USA) at 5 ug/mL. After 30 minutes, cells were infected with FITC-labeled *Mtb*, H37Rv ($mc^2$ 6206), at a multiplicity of infection (MOI) of 10:1 for 24 hours.

### Phagocytic Efficiency

Phagocytosis was assessed via immunofluorescence. Images were obtained with a fluorescence microscope and cellSens software (Olympus Life Science, Waltham, MA/USA). The point maxima function of ImageJ (Fiji, Madison, WI/USA) was used to measure the number of fluorescent, intracellular FITC-labeled *Mtb*. Multiple random fields from multiple replicates were assessed for a total of 250–350 cells per condition. *Mtb* phagocytosis was expressed as the phagocytic efficiency, which was calculated as the total number of FITC-fluorescent puncta within the cellular border per total number of cells. The phagocytic efficiencies are presented % relative to control.

### Immunofluorescence

Immunofluorescence staining was performed using primary antibodies to TLR2 (1:250 dilution) (ID: MA5–32787, Invitrogen, Carlsbad, CA/USA), Alexa Fluor-labeled secondary antibodies, and 4′,6-diamidino-2-phenylindole (DAPI, 1:5000 dilution). Prior cell fixation and image acquisition, wells were treated with 1:10 diluted trypan blue solution for approximately 2 minutes to quench background fluorescence and enhance image quality. Images were obtained with a fluorescence microscope and cellSens software. Corrected total cell fluorescence was calculated with ImageJ. Multiple random fields from multiple replicates for a total of 200–300 cells per condition were analyzed to determine the mean fluorescence for each condition. Area and integrated density were calculated, and the fluorescence of each cell was then determined by dividing the integrated density by the area. The levels of fluorescence are presented % relative to control.

### Statistical Analyses

Student's t-test was used for single comparisons and analysis of variance (ANOVA) was used for multiple comparisons using the Analysis ToolPak in Microsoft Excel (Redmond, WA/USA). Data are presented as mean ± standard error of the mean. Significance was accepted at $p < 0.05$.

## RESULTS

### Chronic alcohol ingestion impairs phagocytosis of attenuated Mtb in a rat model and reduces TLR2 expression in rat alveolar macrophage

As chronic alcohol exposure decreases alveolar macrophage bacterial phagocytosis (Brown et al., 2009), we first assessed phagocytosis of *Mtb* from alcohol-fed rats and their control-fed littermates. As shown in Figure 1, the relative phagocytic efficiency was significantly lower in the primary alveolar macrophages isolated from alcohol-fed rats compared to macrophages from control-fed rats (40 ± 2% vs. 100 ± 17%, $p<0.05$), a 60% decrease in phagocytosis compared to control. Given the importance of TLR2 for *Mtb* recognition, as well as previous work showing alcohol's detrimental effects on TLR2 in splenic, peritoneal, and peripheral white blood cells in murine models (Thoma-Uszynski et al., 2001, Goral and Kovacs, 2005, Pruett et al., 2004, Hu and Spaink, 2022), the effect of chronic alcohol ingestion on TLR2 expression from the primary alveolar macrophage was assessed in our particular animal model. As shown in Figure 2, the relative TLR2 expression was significantly reduced in the alveolar macrophages of alcohol-fed rats (81 ± 1% vs. 100 ± 1%, $p<0.05$) indicative of nearly a 20% decrease in TLR2 expression compared to control.

### Blocking TLR2 signaling impairs phagocytosis of attenuated *Mtb* in a rat model

To determine the functional consequences of decreased TLR2 expression, alveolar macrophages were cultured with TLR2 neutralizing antibody prior to adding *Mtb* in the culture medium. As shown in Figure 3, blocking TLR2 on the cell membrane by neutralizing antibody significantly decreases the relative phagocytic efficiency of alveolar macrophages compared to control (72 ± 6% vs. 100 ± 7, $p<0.05$). We found that this significant decrease in the relative phagocytic efficiency persisted even if the alveolar macrophages were incubated with GM-CSF for 48 hours prior to exposure to the TLR2 neutralizing antibody (68 ± 6% vs 100 ± 7%, $p<0.05$), Phagocytic efficiency of the alveolar macrophages was not significantly affected by GM-CSF treatment alone (96 ± 4% vs. 100 ± 7%, $p>0.05$) (Figure 3).

### Intranasal GM-CSF treatment restores alveolar macrophage TLR2 expression and phagocytosis of attenuated *Mtb* in alcohol-fed rat alveolar macrophages

Given the role of GM-CSF and PU.1 in the regulation of TLR2 (Shibata et al., 2001, Haehnel et al., 2002), we next assessed whether GM-CSF treatment could restore TLR2 expression in primary alveolar macrophages from alcohol-fed rats. Alcohol-fed rats and their littermate controls were treated with recombinant rat GM-CSF with 3 days by nasal instillation. We found that GM-CSF improves relative TLR2 expression in the alveolar macrophages of alcohol-fed rats (Figure 4) and restores it to the level of control-fed rats

(100 ± 1% vs. 100 ± 1%, p>0.05). There is no change in the TLR2 expression in the control animals treated with or without GM-CSF (97 ± 2% vs. 100 ± 1%, p>0.05) (Figure 4).

Since chronic alcohol exposure reduced GMCSF-Rβ expression on the alveolar macrophage surface and consequently impaired macrophage function (Brown et al., 2009, Trapnell and Whitsett, 2002), alveolar macrophage phagocytosis of *Mtb* was used to analysis of the macrophage function. We found that there is an increase in the relative phagocytic efficiency of alveolar macrophages from alcohol-fed rats treated with GM-CSF to the level of their control-fed littermates (100 ± 10% vs 100 ± 9%, p>0.05) without significantly impacting the phagocytic efficiency of the alveolar macrophages in control animals with or without GM-CSF treatment (116 ± 7% vs. 100 ± 9%, p>0.05) (Figure 5).

## DISCUSSION

This study provides evidence that GM-CSF-dependent TLR2 suppression is a novel mechanism by which alcohol impairs the alveolar macrophage response to *Mtb*. It builds on our prior work on the relationship between GM-CSF and innate immunity in alcohol-induced lung disease and illustrates a previously unrecognized pathway by which alcohol renders the lung more susceptible to *Mtb*, suggesting novel research pathways into host-directed immune therapeutics.

We utilized a rat model for chronic alcohol ingestion that has previously been established as a robust model for alcohol-associated lung disease in human subjects. Both this animal model and human subjects with AUD has previously been shown to have depleted alveolar levels of glutathione, an important antioxidant needed for oxidative stress defense, as well as increased alveolar epithelial permeability that puts the lung at risk for edematous injury (Holguin et al., 1998, Moss et al., 2000, Guidot et al., 2000, Burnham et al., 2009). Further, this rat model has previously been shown to recapitulate alveolar macrophage dysfunction seen in human subjects with AUD, specifically including impaired development, bacterial phagocytosis, and expression of GM-CSF receptor β-subunit expression (Joshi et al., 2005, Mehta et al., 2013). To our knowledge, this is the first study to use this rodent model for AUD in the setting of TB, in particular investigating the response of the alveolar macrophage. The attenuated *Mtb* strain used in these experiments, while having an improved biosafety and limited virulence, has been shown to result in similar levels of macrophage uptake and induce a comparable cytokine release when compared to more pathogenic strains of *Mtb* – making it a suitable model for investigations of the initial innate immune response (Mouton et al., 2019).

Phagocytic impairment is crucial as the alveolar macrophage is the resident innate immune effector in the lung and is responsible for recognizing, phagocytosing, and clearing pathogens, including *Mtb*. *Mtb* recognition and subsequent phagocytosis by the alveolar macrophage is the first step in the intracellular killing process, but also classically activates macrophages and leads to production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), which are responsible for recruiting additional macrophages, polymorphonuclear leukocytes (PMNs), and antigen-presenting cells to the site of infection (Jayachandran et al., 2013). This cytokine release,

as well as *Mtb* antigen presentation by various cells, mobilizes the adaptive immune response resulting in the T-cell activation crucial for control of *Mtb* infection (Jasenosky et al., 2015). Thus, an impairment to *Mtb* recognition and phagocytosis not only dampens initial mycobacterial control and intracellular killing but could later blunt and/or delay comprehensive innate and adaptive immune response to *Mtb* infection. We found that *Mtb* phagocytosis and TLR2 expression is decreased in the alveolar macrophages of alcohol-fed rats compared to control-fed rats after endotracheal inoculation of *Mtb*. Endotracheal inoculation of *Mtb* strengthens the results and is the first to utilize this type of exposure to investigate phagocytosis of *Mtb* in this rat model. We used immunofluorescence to assess the phagocytic efficiency, as opposed to measurements of colony-forming units (CFU) or polymerase chain reaction (PCR) after cell lysis, as it allowed for direct visualization of the fluorescently labeled *Mtb* as well as a quantification per cell. Notably, this method has limitations as it is 2-dimensional and the lacks quantification of the mycobacteria in each intracellular, fluorescent puncta. In addition, although we were able to assess uptake, this method does not allow us to comment on phagolysosomal fusion or pathogen killing. Our use of immunofluorescence for TLR2 assessment was undertaken without membrane permeabilization and is complemented by DAPI staining to assist with visualization of the surface protein staining and was further enhanced by the use of trypan blue exposure to quench background fluorescence.

Utilizing a TLR2 neutralizing antibody, we assessed the functional consequences of decreased TLR2 expression in primary rat alveolar macrophages. We found that blocking of TLR2 impaired phagocytosis of *Mtb*. Further, this impairment in phagocytosis persisted even when macrophages were supplemented with GM-CSF prior to exposure to the TLR2 neutralizing antibody. This result directly links TLR2 to the macrophage's phagocytosis of *Mtb*, a relationship previously suggested, and confirms the importance of TLR2 to GM-CSF-dependent phagocytosis (Palucci et al., 2016). TLR2 is an important PRR that specifically recognizes mycobacteria and subsequently induces intracellular signaling via the transcription factor nuclear factor kB (NF-kB) leading to production of reactive oxygen species (ROS), type 1 interferons, and effector molecules essential for intracellular killing, recruitment of additional PMNs, and adaptive immune activation including interleukin (IL)-1, IL-6, and IL-12 (Oliveira-Nascimento et al., 2012, Kirschning et al., 1998, Aliprantis et al., 1999). TLR2 is vital for restricting intracellular growth of *Mtb* and containing *Mtb* infection. TLR2 has previously been shown to enhance macrophage proinflammatory cytokine release, antimicrobial effector pathways, and macrophage apoptosis in response to *Mtb* (Kramarska et al., 2021, Palucci et al., 2016, Thoma-Uszynski et al., 2001, Bocchino et al., 2005, Lopez et al., 2003). Further, TLR2-deficient murine models exhibit significantly decreased macrophage activation, proinflammatory cytokine release, and early death after exposure to *Mtb* compared to controls (Drennan et al., 2004, Reiling et al., 2002). To our knowledge, we are the first to link impaired GM-CSF signaling to a reduction in alveolar macrophage phagocytosis of *Mtb* as a result of diminished TLR2 expression.

In the rat model used in these experiments, alcohol has been previously shown to significantly reduce PU.1 expression and its nuclear binding (Joshi et al., 2005, Brown et al., 2009). Alcohol's inhibition of PU.1 expression is multifactorial, however alcohol is known to downregulate GMCSF-Rβ expression on the alveolar macrophage surface, thus

impairing GM-CSF signaling and diminishing PU.1 expression (Shibata et al., 2001, Joshi et al., 2006, Joshi et al., 2005, Lloberas et al., 1999, Brown et al., 2009). Nonetheless, treatment with GM-CSF seems to override any proximal suppression. Intranasal treatment with rat rGM-CSF prior to endotracheal *Mtb* infection restored both TLR2 expression and phagocytic function in alveolar macrophages of alcohol-fed rats. Our data further solidifies the relationship of functional GM-CSF and PU.1 signaling to adequate TLR2 expression and function (Shibata et al., 2001, Haehnel et al., 2002, Liu et al., 2017, Kurt-Jones et al., 2002). To our knowledge, this is the first experiment to use intranasal rGM-CSF treatments to induce a functional response to *Mtb* that mitigates the effects of chronic alcohol ingestion.

In summary, we determined that chronic alcohol ingestion impairs alveolar macrophage TLR2 expression and subsequent phagocytosis of *Mtb*. The increasing recognition of TB associated with AUD underscores the need to further investigate pathways mediating alcohol-induced macrophage dysfunction. In this study, we build on known impairments in phagocytosis and GM-CSF signaling in AUD by linking the macrophages' dysfunctional response to *Mtb* to impairments in TLR2 expression. In the animal model, these deleterious effects appear to be due, in part, to the downregulation of GM-CSFRβ and PU.1 that we previously identified (Joshi et al., 2005), and the subsequent reduction in TLR2 expression identified in this study. Treatment with GM-CSF restores both membrane-bound TLR2 levels and macrophage phagocytic function, indicating that the proper function of TLR2 is dependent on functional GM-CSF signaling. Future studies will ultimately need to confirm our results in human alveolar macrophages. As a potential translation to the clinical setting, this study suggests that enhancing GM-CSF signaling in TB associated with AUD will increase PU.1 activity and TLR2 expression, and may, after appropriate confirmation and clinical testing, be considered as an adjunctive therapy in people with AUD at high risk for pulmonary TB.

## Sources of Support:

GWW: T32 HL116271

SCA: K23 AI134182

DMG: R01 AA025857

BSS: K08 AA024512

## REFERENCES

ALIPRANTIS AO, YANG RB, MARK MR, SUGGETT S, DEVAUX B, RADOLF JD, KLIMPEL GR, GODOWSKI P & ZYCHLINSKY A 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science, 285, 736–9. [PubMed: 10426996]

ASTARIE-DEQUEKER C, N'DIAYE EN, LE CABEC V, RITTIG MG, PRANDI J & MARIDONNEAU-PARINI I 1999. The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. Infect Immun, 67, 469–77. [PubMed: 9916047]

BEN-ALI M, BARBOUCHE MR, BOUSNINA S, CHABBOU A & DELLAGI K 2004. Toll-like receptor 2 Arg677Trp polymorphism is associated with susceptibility to tuberculosis in Tunisian patients. Clin Diagn Lab Immunol, 11, 625–6. [PubMed: 15138193]

BENMERZOUG S, MARINHO FV, ROSE S, MACKOWIAK C, GOSSET D, SEDDA D, POISSON E, UYTTENHOVE C, VAN SNICK J, JACOBS M, GARCIA I, RYFFEL B & QUESNIAUX VFJ 2018. GM-CSF targeted immunomodulation affects host response to M. tuberculosis infection. Sci Rep, 8, 8652. [PubMed: 29872095]

BOCCHINO M, GALATI D, SANDUZZI A, COLIZZI V, BRUNETTI E & MANCINO G 2005. Role of mycobacteria-induced monocyte/macrophage apoptosis in the pathogenesis of human tuberculosis. Int J Tuberc Lung Dis, 9, 375–83. [PubMed: 15830742]

BROWN SD, GAUTHIER TW & BROWN LA 2009. Impaired terminal differentiation of pulmonary macrophages in a Guinea pig model of chronic ethanol ingestion. Alcohol Clin Exp Res, 33, 1782–93. [PubMed: 19645732]

BRYSON BD, ROSEBROCK TR, TAFESSE FG, ITOH CY, NIBASUMBA A, BABUNOVIC GH, CORLEIS B, MARTIN C, KEEGAN C, ANDRADE P, REALEGENO S, KWON D, MODLIN RL & FORTUNE SM 2019. Heterogeneous GM-CSF signaling in macrophages is associated with control of Mycobacterium tuberculosis. Nat Commun, 10, 2329. [PubMed: 31133636]

BURNHAM EL, HALKAR R, BURKS M & MOSS M 2009. The effects of alcohol abuse on pulmonary alveolar-capillary barrier function in humans. Alcohol Alcohol, 44, 8–12. [PubMed: 18567916]

CLASSEN CN, WARREN R, RICHARDSON M, HAUMAN JH, GIE RP, ELLIS JH, VAN HELDEN PD & BEYERS N 1999. Impact of social interactions in the community on the transmission of tuberculosis in a high incidence area. Thorax, 54, 136–40. [PubMed: 10325918]

DIEL R, MEYWALD-WALTER K, GOTTSCHALK R, RUSCH-GERDES S & NIEMANN S 2004. Ongoing outbreak of tuberculosis in a low-incidence community: a molecular-epidemiological evaluation. Int J Tuberc Lung Dis, 8, 855–61. [PubMed: 15260277]

DRENNAN MB, NICOLLE D, QUESNIAUX VJ, JACOBS M, ALLIE N, MPAGI J, FREMOND C, WAGNER H, KIRSCHNING C & RYFFEL B 2004. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. Am J Pathol, 164, 49–57. [PubMed: 14695318]

FAN X, JOSHI PC, KOVAL M & GUIDOT DM 2011. Chronic alcohol ingestion exacerbates lung epithelial barrier dysfunction in HIV-1 transgenic rats. Alcohol Clin Exp Res, 35, 1866–75. [PubMed: 21569054]

FISKE CT, HAMILTON CD & STOUT JE 2009. Alcohol use and clinical manifestations of tuberculosis. J Infect, 58, 395–401. [PubMed: 19418621]

GORAL J & KOVACS EJ 2005. In vivo ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inflammatory response by limiting p38 and ERK1/2 activation. J Immunol, 174, 456–63. [PubMed: 15611271]

GUIDOT DM, MODELSKA K, LOIS M, JAIN L, MOSS IM, PITTET JF & BROWN LA 2000. Ethanol ingestion via glutathione depletion impairs alveolar epithelial barrier function in rats. Am J Physiol Lung Cell Mol Physiol, 279, L127–35. [PubMed: 10893211]

HAEHNEL V, SCHWARZFISCHER L, FENTON MJ & REHLI M 2002. Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. J Immunol, 168, 5629–37. [PubMed: 12023360]

HOLGUIN F, MOSS I, BROWN LA & GUIDOT DM 1998. Chronic ethanol ingestion impairs alveolar type II cell glutathione homeostasis and function and predisposes to endotoxin-mediated acute edematous lung injury in rats. J Clin Invest, 101, 761–8. [PubMed: 9466970]

HU W & SPAINK HP 2022. The Role of TLR2 in Infectious Diseases Caused by Mycobacteria: From Cell Biology to Therapeutic Target. Biology (Basel), 11.

IMTIAZ S, SHIELD KD, ROERECKE M, SAMOKHVALOV AV, LONNROTH K & REHM J 2017. Alcohol consumption as a risk factor for tuberculosis: meta-analyses and burden of disease. Eur Respir J, 50.

JAIN P, HSU T, ARAI M, BIERMANN K, THALER DS, NGUYEN A, GONZALEZ PA, TUFARIELLO JM, KRIAKOV J, CHEN B, LARSEN MH & JACOBS WR JR. 2014. Specialized transduction designed for precise high-throughput unmarked deletions in Mycobacterium tuberculosis. mBio, 5, e01245–14. [PubMed: 24895308]

JASENOSKY LD, SCRIBA TJ, HANEKOM WA & GOLDFELD AE 2015. T cells and adaptive immunity to Mycobacterium tuberculosis in humans. Immunol Rev, 264, 74–87. [PubMed: 25703553]

JAYACHANDRAN R, BOSEDASGUPTA S & PIETERS J 2013. Surviving the Macrophage: Tools and Tricks Employed by Mycobacterium tuberculosis. In: PIETERS J & MCKINNEY JD (eds.) Pathogenesis of Mycobacterium tuberculosis and its Interaction with the Host Organism. Berlin, Heidelberg: Springer Berlin Heidelberg.

JENSEN JS, FAN X & GUIDOT DM 2013. Alcohol causes alveolar epithelial oxidative stress by inhibiting the nuclear factor (erythroid-derived 2)-like 2-antioxidant response element signaling pathway. Am J Respir Cell Mol Biol, 48, 511–7. [PubMed: 23306837]

JOSHI PC, APPLEWHITE L, MITCHELL PO, FERNAINY K, ROMAN J, EATON DC & GUIDOT DM 2006. GM-CSF receptor expression and signaling is decreased in lungs of ethanol-fed rats. Am J Physiol Lung Cell Mol Physiol, 291, L1150–8. [PubMed: 16877635]

JOSHI PC, APPLEWHITE L, RITZENTHALER JD, ROMAN J, FERNANDEZ AL, EATON DC, BROWN LA & GUIDOT DM 2005. Chronic ethanol ingestion in rats decreases granulocyte-macrophage colony-stimulating factor receptor expression and downstream signaling in the alveolar macrophage. J Immunol, 175, 6837–45. [PubMed: 16272341]

KASTL S, KOTSCHENREUTHER U, HILLE B, SCHMIDT J, GEPP H & HOHENBERGER W 2004. Simplification of rat intubation on inclined metal plate. Adv Physiol Educ, 28, 29–32. [PubMed: 14973009]

KIM JU, MAJID A, JUDGE R, CROOK P, NATHWANI R, SELVAPATT N, LOVENDOSKI J, MANOUSOU P, THURSZ M, DHAR A, LEWIS H, VERGIS N & LEMOINE M 2020. Effect of COVID-19 lockdown on alcohol consumption in patients with pre-existing alcohol use disorder. Lancet Gastroenterol Hepatol, 5, 886–887. [PubMed: 32763197]

KIRSCHNING CJ, WESCHE H, MERRILL AYRES T & ROTHE M 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med, 188, 2091–7. [PubMed: 9841923]

KRAMARSKA E, SQUEGLIA F, DE MAIO F, DELOGU G & BERISIO R 2021. PE_PGRS33, an Important Virulence Factor of Mycobacterium tuberculosis and Potential Target of Host Humoral Immune Response. Cells, 10.

KURT-JONES EA, MANDELL L, WHITNEY C, PADGETT A, GOSSELIN K, NEWBURGER PE & FINBERG RW 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, 1860–8. [PubMed: 12176910]

LEONG F, DARTOIS V & DICK T 2010. A Color Atlas of Comparative Pathology of Pulmonary Tuberculosis, Boca Raton, CRC Press.

LIU C, WANG M, SUN W, CAI F, GENG S, SU X & SHI Y 2017. PU.1 serves a critical role in the innate defense against Aspergillus fumigatus via dendritic cell-associated C-type lectin receptor-1 and toll-like receptors-2 and 4 in THP-1-derived macrophages. Mol Med Rep, 15, 4084–4092. [PubMed: 28440496]

LLOBERAS J, SOLER C & CELADA A 1999. The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. Immunol Today, 20, 184–9. [PubMed: 10203717]

LONNROTH K, WILLIAMS BG, STADLIN S, JARAMILLO E & DYE C 2008. Alcohol use as a risk factor for tuberculosis - a systematic review. BMC Public Health, 8, 289. [PubMed: 18702821]

LOPEZ M, SLY LM, LUU Y, YOUNG D, COOPER H & REINER NE 2003. The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through Toll-like receptor-2. J Immunol, 170, 2409–16. [PubMed: 12594264]

MEHTA AJ, YELIGAR SM, ELON L, BROWN LA & GUIDOT DM 2013. Alcoholism causes alveolar macrophage zinc deficiency and immune dysfunction. Am J Respir Crit Care Med, 188, 716–23. [PubMed: 23805851]

MISHRA A, SINGH VK, ACTOR JK, HUNTER RL, JAGANNATH C, SUBBIAN S & KHAN A 2020. GM-CSF Dependent Differential Control of Mycobacterium tuberculosis Infection in Human and Mouse Macrophages: Is Macrophage Source of GM-CSF Critical to Tuberculosis Immunity? Front Immunol, 11, 1599. [PubMed: 32793233]

MOSS M, BUCHER B, MOORE FA, MOORE EE & PARSONS PE 1996. The role of chronic alcohol abuse in the development of acute respiratory distress syndrome in adults. JAMA, 275, 50–4. [PubMed: 8531287]

MOSS M, GUIDOT DM, WONG-LAMBERTINA M, TEN HOOR T, PEREZ RL & BROWN LA 2000. The effects of chronic alcohol abuse on pulmonary glutathione homeostasis. Am J Respir Crit Care Med, 161, 414–9. [PubMed: 10673179]

MOSS M, PARSONS PE, STEINBERG KP, HUDSON LD, GUIDOT DM, BURNHAM EL, EATON S & COTSONIS GA 2003. Chronic alcohol abuse is associated with an increased incidence of acute respiratory distress syndrome and severity of multiple organ dysfunction in patients with septic shock. Crit Care Med, 31, 869–77. [PubMed: 12626999]

MOUTON JM, HEUNIS T, DIPPENAAR A, GALLANT JL, KLEYNHANS L & SAMPSON SL 2019. Comprehensive Characterization of the Attenuated Double Auxotroph Mycobacterium tuberculosisDeltaleuDDeltapanCD as an Alternative to H37Rv. Front Microbiol, 10, 1922. [PubMed: 31481950]

NAZAROVA EV & RUSSELL DG 2017. Growing and Handling of Mycobacterium tuberculosis for Macrophage Infection Assays. Methods Mol Biol, 1519, 325–331. [PubMed: 27815890]

OGUS AC, YOLDAS B, OZDEMIR T, UGUZ A, OLCEN S, KESER I, COSKUN M, CILLI A & YEGIN O 2004. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J, 23, 219–23. [PubMed: 14979495]

OLIVEIRA-NASCIMENTO L, MASSARI P & WETZLER LM 2012. The Role of TLR2 in Infection and Immunity. Front Immunol, 3, 79. [PubMed: 22566960]

OSLER W 1898. The principles and practice of medicine, designed for the use of practitioners and students of medicine, New York,, D. Appleton and company.

PALUCCI I, CAMASSA S, CASCIOFERRO A, SALI M, ANOOSHEH S, ZUMBO A, MINERVA M, IANTOMASI R, DE MAIO F, DI SANTE G, RIA F, SANGUINETTI M, PALU G, BRENNAN MJ, MANGANELLI R & DELOGU G 2016. PE_PGRS33 Contributes to Mycobacterium tuberculosis Entry in Macrophages through Interaction with TLR2. PLoS One, 11, e0150800. [PubMed: 26978522]

PELAEZ A, BECHARA RI, JOSHI PC, BROWN LA & GUIDOT DM 2004. Granulocyte/macrophage colony-stimulating factor treatment improves alveolar epithelial barrier function in alcoholic rat lung. Am J Physiol Lung Cell Mol Physiol, 286, L106–11. [PubMed: 14504066]

PRUETT SB, ZHENG Q, FAN R, MATTHEWS K & SCHWAB C 2004. Ethanol suppresses cytokine responses induced through Toll-like receptors as well as innate resistance to Escherichia coli in a mouse model for binge drinking. Alcohol, 33, 147–55. [PubMed: 15528012]

RAGAN EJ, KLEINMAN MB, SWEIGART B, GNATIENKO N, PARRY CD, HORSBURGH CR, LAVALLEY MP, MYERS B & JACOBSON KR 2020. The impact of alcohol use on tuberculosis treatment outcomes: a systematic review and meta-analysis. Int J Tuberc Lung Dis, 24, 73–82. [PubMed: 32005309]

REHM J, SAMOKHVALOV AV, NEUMAN MG, ROOM R, PARRY C, LONNROTH K, PATRA J, POZNYAK V & POPOVA S 2009. The association between alcohol use, alcohol use disorders and tuberculosis (TB). A systematic review. BMC Public Health, 9, 450. [PubMed: 19961618]

REILING N, HOLSCHER C, FEHRENBACH A, KROGER S, KIRSCHNING CJ, GOYERT S & EHLERS S 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol, 169, 3480–4. [PubMed: 12244136]

RUTLEDGE SM, SCHIANO TD, FLORMAN S & IM GY 2021. COVID-19 Aftershocks on Alcohol-Associated Liver Disease: An Early Cross-Sectional Report From the U.S. Epicenter. Hepatol Commun, 5, 1151–1155. [PubMed: 34533000]

SHIBATA Y, BERCLAZ PY, CHRONEOS ZC, YOSHIDA M, WHITSETT JA & TRAPNELL BC 2001. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. Immunity, 15, 557–67. [PubMed: 11672538]

SIMOU E, BRITTON J & LEONARDI-BEE J 2018. Alcohol and the risk of pneumonia: a systematic review and meta-analysis. BMJ Open, 8, e022344.

STAITIEH BS, EGEA EE, FAN X, AMAH A & GUIDOT DM 2018. Chronic Alcohol Ingestion Impairs Rat Alveolar Macrophage Phagocytosis via Disruption of RAGE Signaling. Am J Med Sci, 355, 497–505. [PubMed: 29753380]

TEXEREAU J, CHICHE JD, TAYLOR W, CHOUKROUN G, COMBA B & MIRA JP 2005. The importance of Toll-like receptor 2 polymorphisms in severe infections. Clin Infect Dis, 41 Suppl 7, S408–15. [PubMed: 16237639]

THOMA-USZYNSKI S, STENGER S, TAKEUCHI O, OCHOA MT, ENGELE M, SIELING PA, BARNES PF, ROLLINGHOFF M, BOLCSKEI PL, WAGNER M, AKIRA S, NORGARD MV, BELISLE JT, GODOWSKI PJ, BLOOM BR & MODLIN RL 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science, 291, 1544–7. [PubMed: 11222859]

THOMAS BE, THIRUVENGADAM K, S R, KADAM D, OVUNG S, SIVAKUMAR S, BALA YOGENDRA SHIVAKUMAR SV, PARADKAR M, GUPTE N, SURYAVANSHI N, DOLLA CK, GUPTE AN, KOHLI R, PRADHAN N, SIVARAMAKRISHNAN GN, GAIKWAD S, KAGAL A, DHANASEKARAN K, DELUCA A, GOLUB JE, MAVE V, CHANDRASEKARAN P, GUPTA A & STUDY CT-RI 2019. Smoking, alcohol use disorder and tuberculosis treatment outcomes: A dual co-morbidity burden that cannot be ignored. PLoS One, 14, e0220507. [PubMed: 31365583]

TRAPNELL BC & WHITSETT JA 2002. Gm-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. Annu Rev Physiol, 64, 775–802. [PubMed: 11826288]

VOLKMANN T, MOONAN PK, MIRAMONTES R & OELTMANN JE 2015. Tuberculosis and excess alcohol use in the United States, 1997–2012. Int J Tuberc Lung Dis, 19, 111–9. [PubMed: 25519800]

WANG HH, J; LAUZARDO M 2012. The Effect of Alcohol Use on the Clinical Presentation of Tuberculosis. J Mycobac Dis, 2.

WHO. 2018. Alcohol: Fact Sheet [Online]. Available: https://www.who.int/en/news-room/fact-sheets/detail/alcohol [Accessed December 1 2021].

WIGGER GW, BOUTON TC, JACOBSON KR, AULD SC, YELIGAR SM & STAITIEH BS 2022. The Impact of Alcohol Use Disorder on Tuberculosis: A Review of the Epidemiology and Potential Immunologic Mechanisms. Frontiers in Immunology, 13.

YIM JJ, LEE HW, LEE HS, KIM YW, HAN SK, SHIM YS & HOLLAND SM 2006. The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. Genes Immun, 7, 150–5. [PubMed: 16437124]

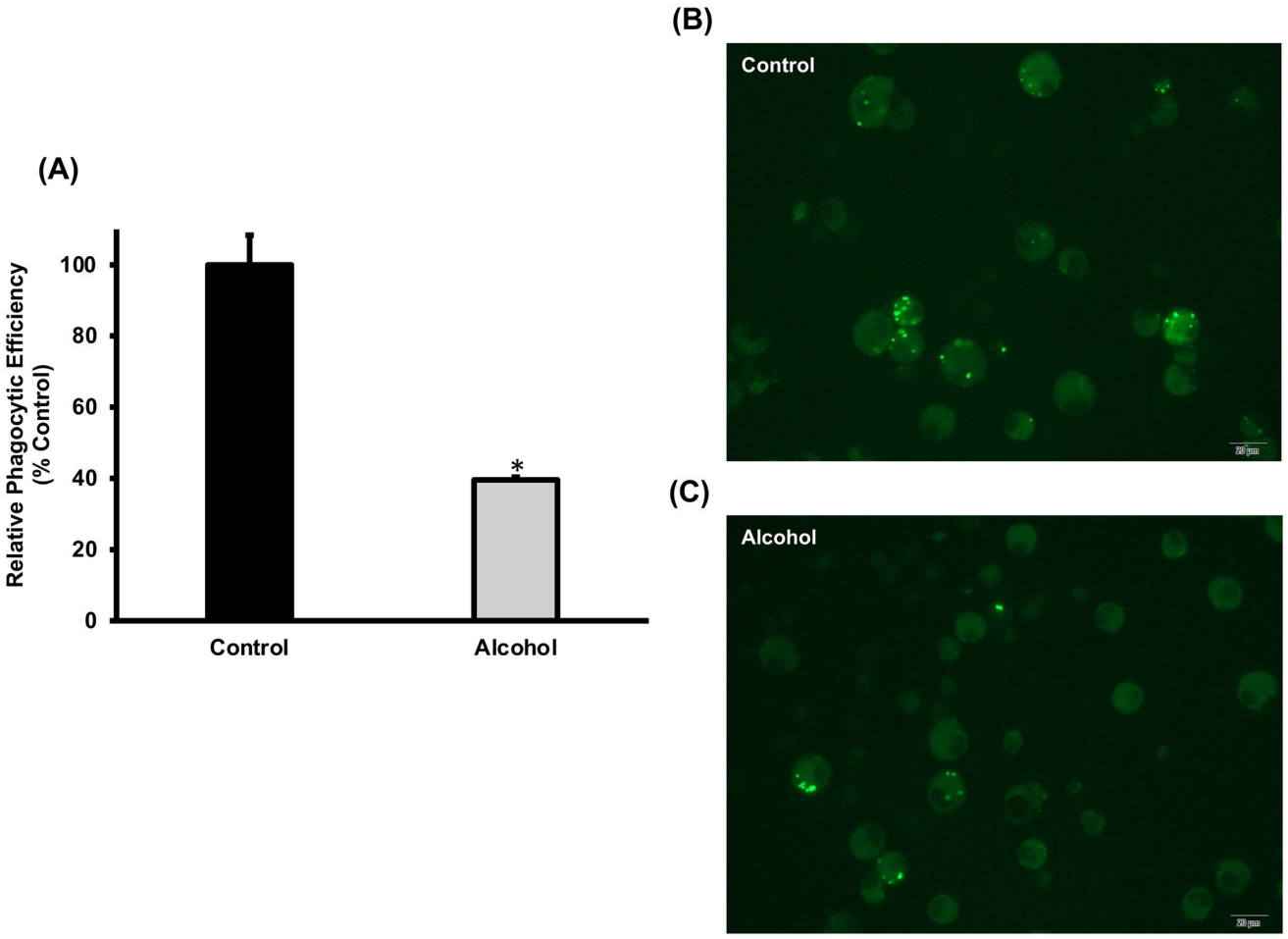



**Figure 1:**

Chronic alcohol ingestion impairs alveolar macrophage phagocytosis of attenuated *Mycobacterium tuberculosis* (*Mtb*). (A) Alveolar macrophages from alcohol-fed rats (Alcohol) have significantly decreased phagocytic efficiency of *Mtb* compared to control-fed rats (Control). Rats were endotracheally inoculated with $10^7$ of fluorescein-5-Isothiocyanate (FITC)-labeled *Mtb*. Primary alveolar macrophages were isolated 24 hours after inoculation by bronchoalveolar lavage. Phagocytic efficiency was quantified by immunofluorescence. Representative images are shown from control-fed (B) and alcohol-fed (C) rats. Data presented as mean ± SEM. *$p \leq 0.05$ compared to control. n = 250 or more cells per group from at least 3 animals/condition.

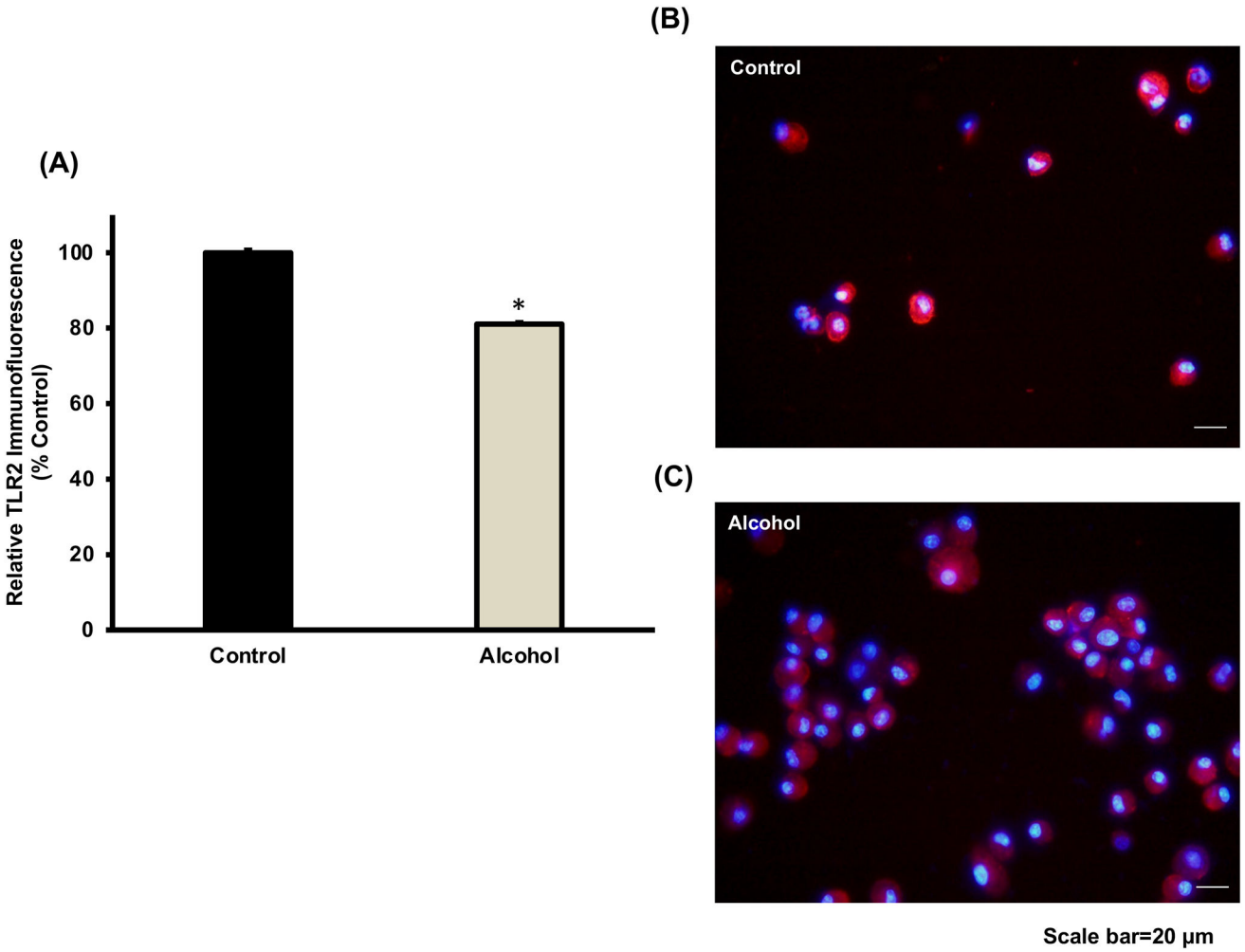



**Figure 2:**

Chronic alcohol ingestion significantly reduces alveolar macrophage toll-like receptor 2 (TLR2) expression. (A) Alveolar macrophages from alcohol-fed rats have significantly decreased TLR2 expression compared to control-fed rats. The effect of chronic alcohol ingestion was examined in primary alveolar macrophages of rats fed a control vs. alcohol diet for > 6 weeks. TLR2 expression was quantified using immunofluorescence via primary antibodies to TLR2 and Alexa Fluor-labeled secondary antibodies. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Representative images are shown from control-fed (B) and alcohol-fed (C) rats. Data presented as mean ± SEM. *$p \leq 0.05$ compared to control. n = 250 or more cells per group from at least 3 animals/condition.

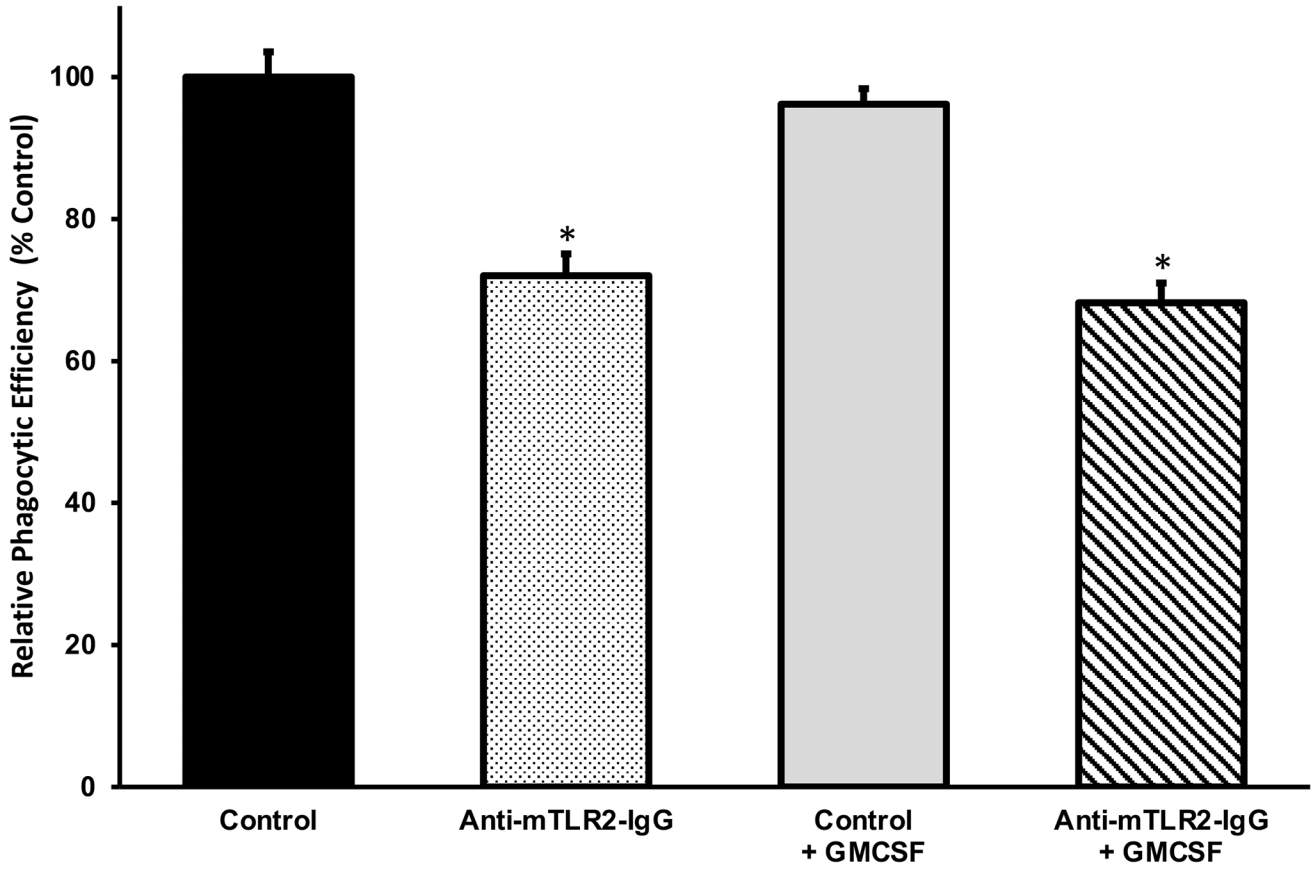
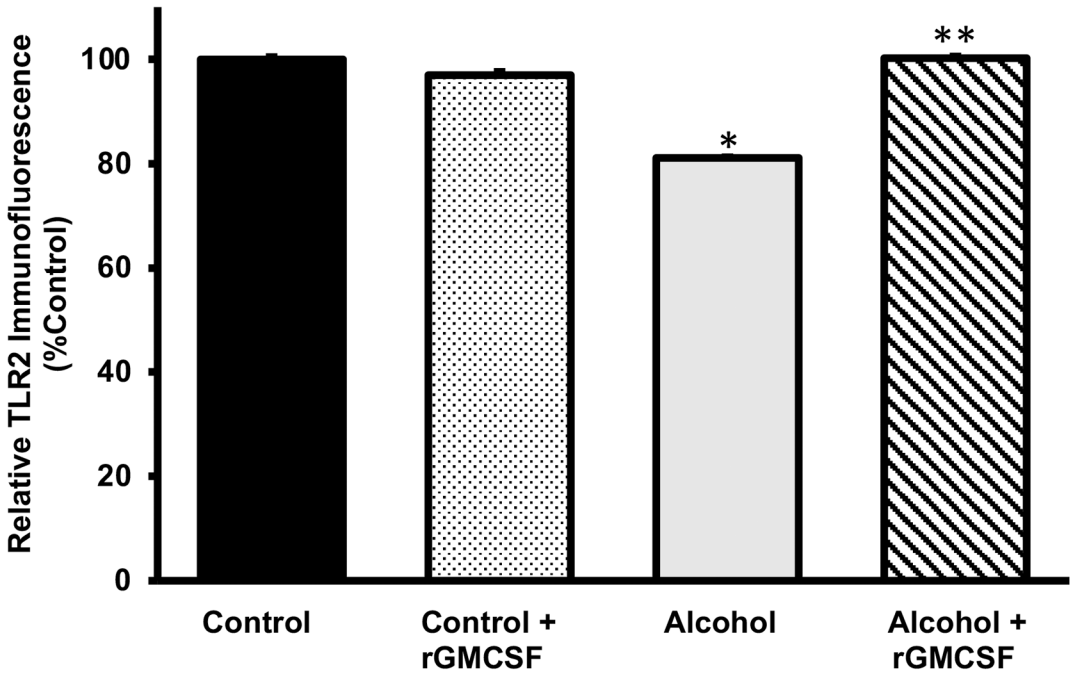



**Figure 3:**

Blocking of TLR2 impairs alveolar macrophage phagocytosis of attenuated *Mtb*. Alveolar macrophages from wild-type rats exposed to a neutralizing TLR2 antibody (Anti-mTLR2-IgG) have significantly decreased phagocytic efficiency of *Mtb* compared to unexposed alveolar macrophages from wild-type rats (Control). A significant decrease in phagocytic efficiency persisted even when alveolar macrophages were incubated with granulocyte macrophage colony-stimulating factor (GMCSF) for 48 hours prior to neutralizing antibody exposure (Anti-mTLR2-IgG+GMCSF). Phagocytic efficiency was quantified using immunofluorescence via FITC fluorescence. Data presented as mean ± SEM. *$p \leq 0.05$ compared to control. n = 250 or more cells per group from at least 3 animals/condition.

**(A)**



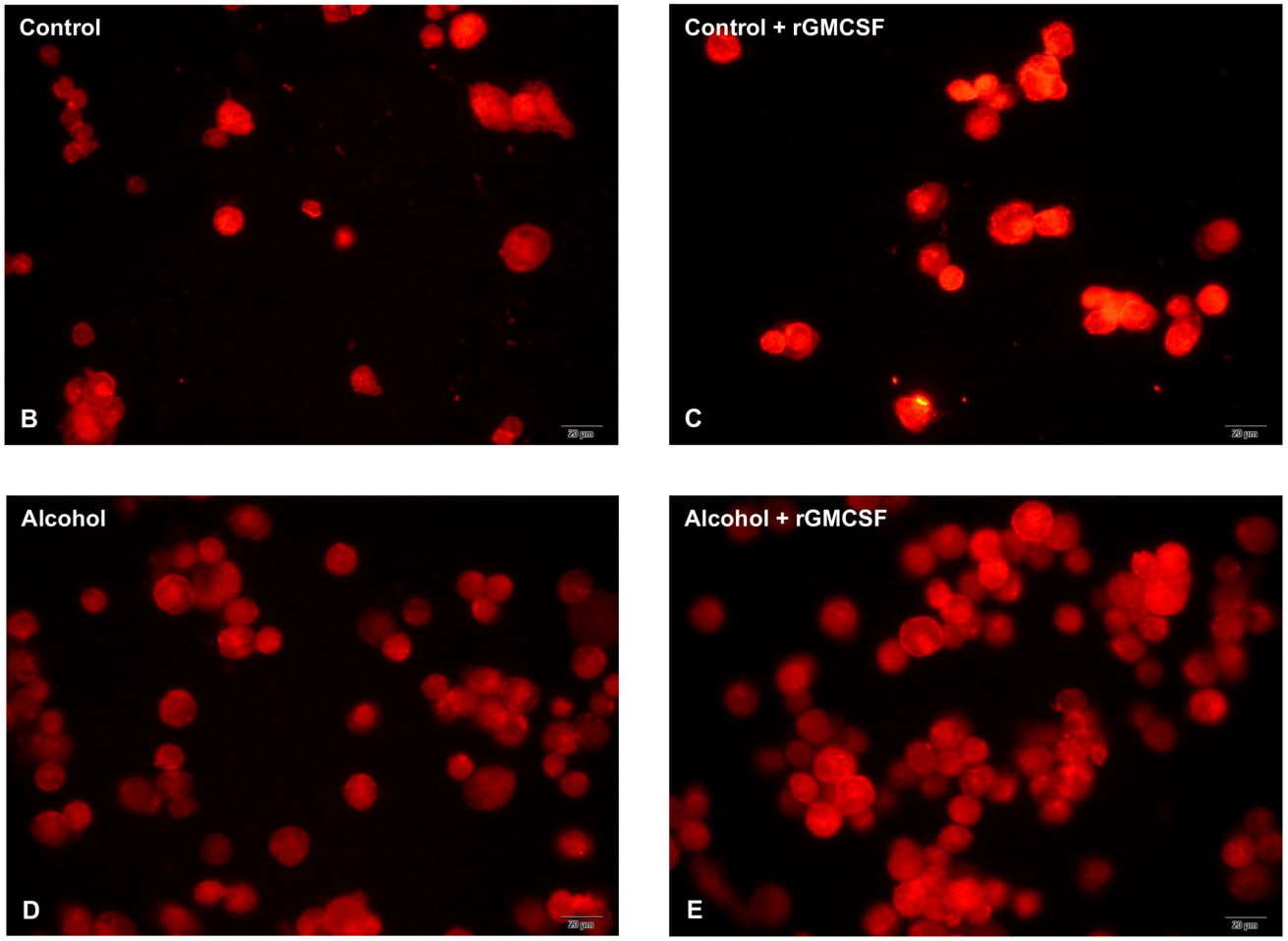
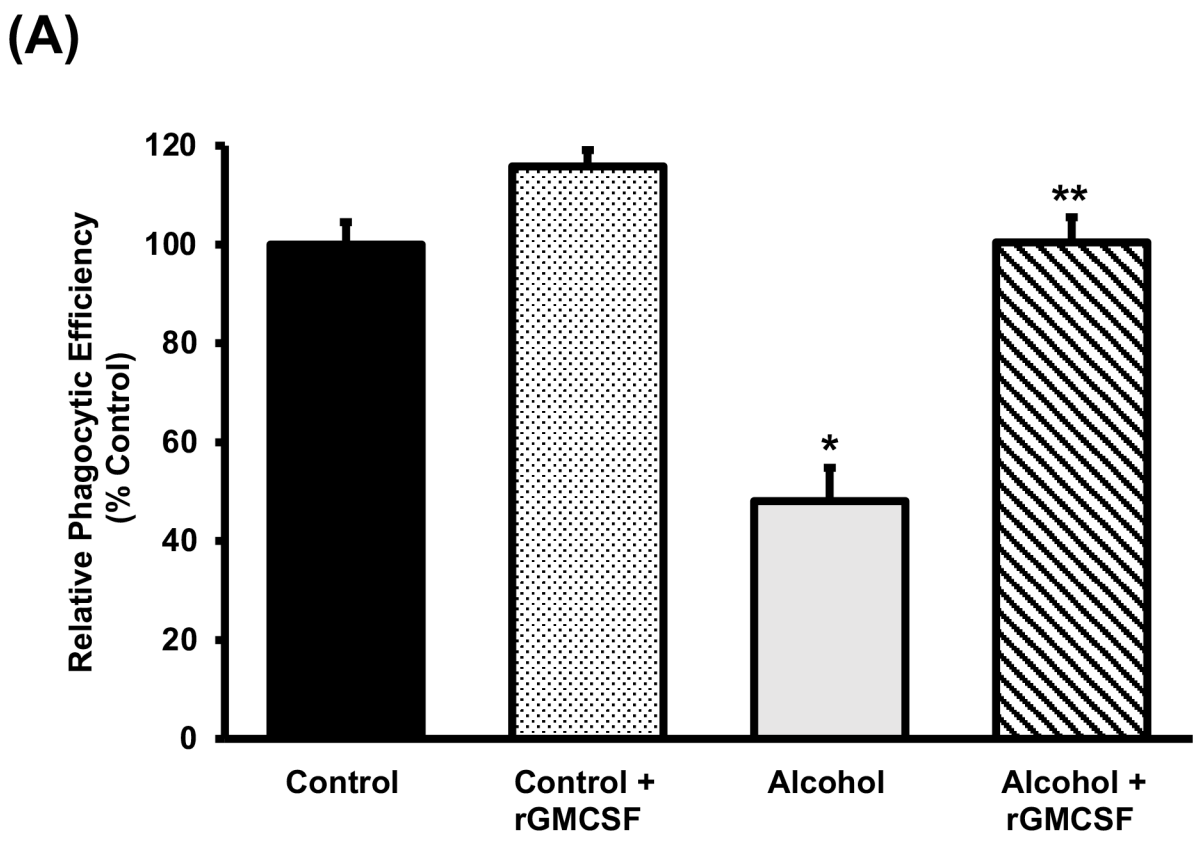



**Figure 4:**

Intranasal recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) treatment rescues alcohol-induced reduction in TLR2 expression. (A) Alveolar macrophages from alcohol-fed rats treated with intranasal recombinant GM-CSF (Alcohol + rGMCSF) have similar levels of TLR2 expression compared to control-fed rats and significantly greater TLR2 expression compared alcohol-fed rats without GM-CSF treatment. TLR2 expression was quantified using immunofluorescence via primary antibodies to TLR2 and Alexa Fluor-labeled secondary antibodies. Representative images are shown from control-fed (B), control-fed with intranasal GM-CSF (C), and alcohol-fed (D), and alcohol-fed with intranasal GM-CSF (E) rats. Data presented as mean ± SEM. *$p \leq 0.05$ compared to control, **$p \leq 0.05$ compared to alcohol. n = 250 or more cells per group from at least 3 animals/condition.



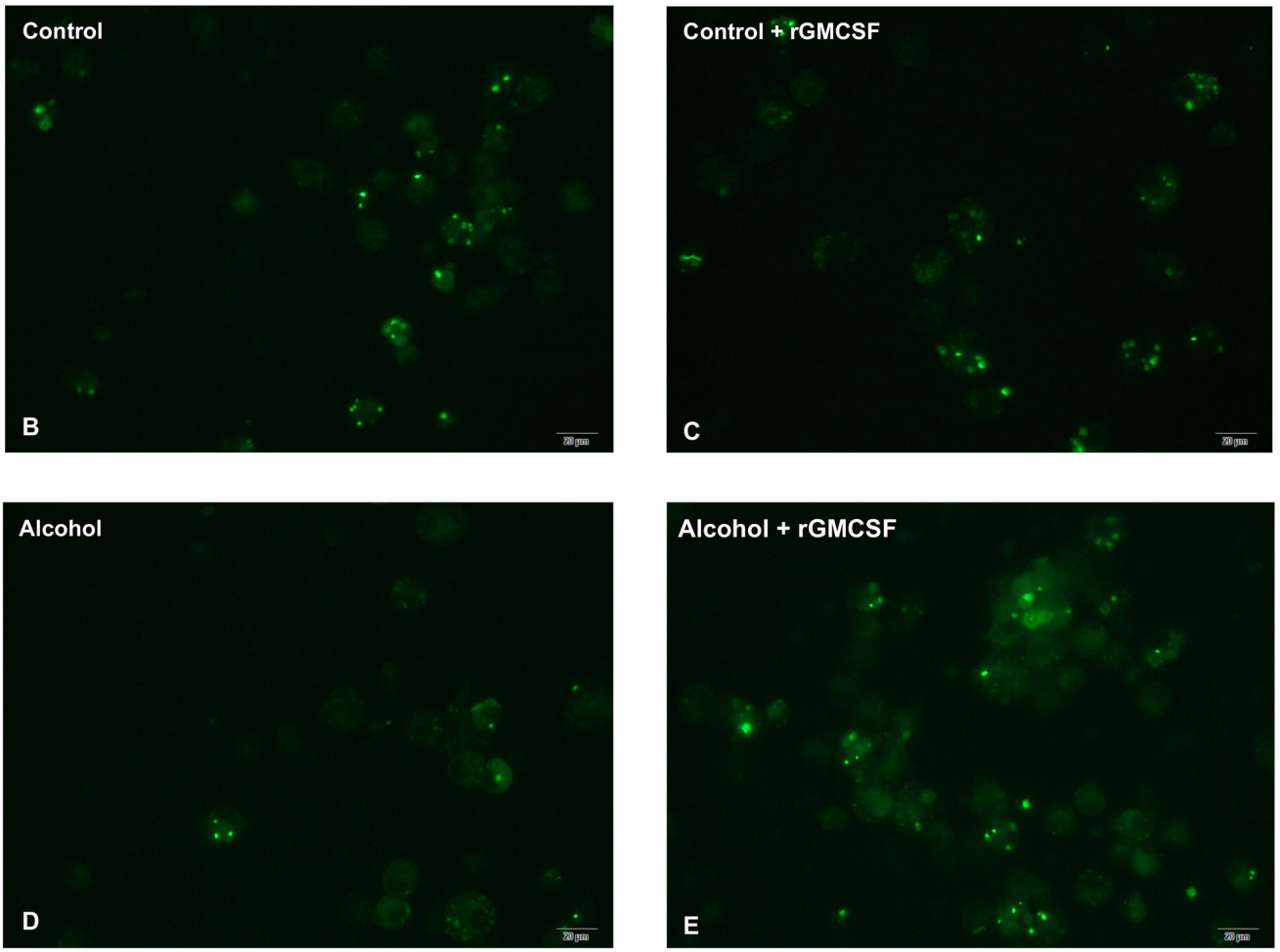



**Figure 5:**

Intranasal recombinant GM-CSF treatment restores the phagocytic efficiency of *Mtb* impaired by chronic alcohol ingestion in alveolar macrophages. (A) Alveolar macrophages from alcohol-fed rats treated with intranasal recombinant GM-CSF have similar phagocytosis of *Mtb* compared to control-fed rats and significantly greater phagocytic efficiency compared in alcohol-fed rats without GM-CSF treatment. Rats were treated with GM-CSF three times prior to endotracheal inoculation with *Mtb*. Primary alveolar macrophages were isolated 24 hours after inoculation. Phagocytic efficiency was quantified using immunofluorescence. Representative images are shown from control-fed (B), control-fed with intranasal GM-CSF (C), and alcohol-fed (D), and alcohol-fed with intranasal GM-CSF (E) rats. Data presented as relative mean ± SEM. *$p \leq 0.05$ compared to control, **$p \leq 0.05$ compared to alcohol. n = 250 or more cells per group from at least 3 animals/condition.

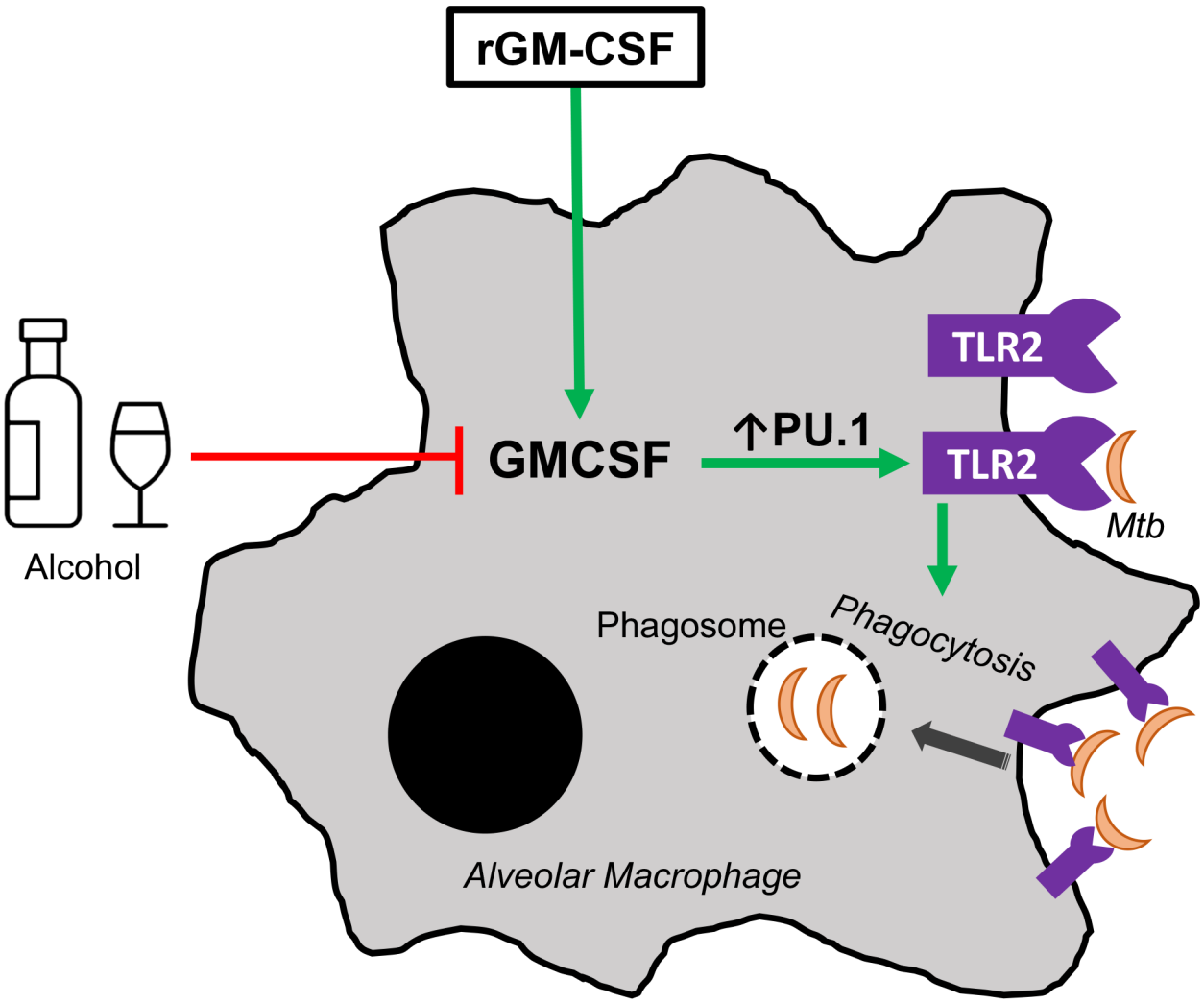



**Figure 6:**

Summary schema of the alveolar macrophage and the impact of alcohol use disorder on the expression of membrane-bound toll-like receptor 2 (TLR2) expression and phagocytosis of *Mycobacterium tuberculosis* (*Mtb*). Chronic alcohol exposure impairs granulocyte macrophage colony-stimulating factor (GM-CSF) signaling, which decreases PU.1 expression, thereby diminishing TLR2 expression. Decreased TLR2 thereby impairs *Mtb* recognition and subsequent phagocytosis. Treatment with recombinant GM-CSF (rGM-CSF) mitigates alcohol-induced impairments to the GM-CSF/PU.1 axis, which allows for recovery of TLR2 expression and *Mtb* phagocytosis.