# Effects of chronic LPS stimulation on the response of macrophages to subsequent stimuli

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**Dedication**

***To the greatest support in my life, My husband***

***Waleed***

**Acknowledgement**

**I acknowledge Dr. Kara Spiller for all the support that she provided to facilitate my success.**

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**Abstract**

**Effects of chronic LPS stimulation on the response of macrophages to subsequent stimuli**

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Wound healing constitutes a brief inflammatory phase, followed by proliferative phase and ends with a longer period of tissue remodeling. Post tissue injury, macrophages initiate an inflammatory cascade to propagate wound healing. Macrophages initially adapt a pro-inflammatory "M1" activation state, followed an anti-inflammatory "M2" macrophages that are associated with the resolution of initial inflammation. The M1-to-M2 transition has been shown to be crucial to facilitate healing. Impairment of this phenotypic switch is associated with chronic inflammation. Chronic inflammation is characterized by a sustained M1. Noteworthy, patients suffering from chronic inflammatory conditions have a systemically higher that normal LPS levels, which is thought to trigger chronic and systemic pro-inflammatory activation of macrophages. Surprisingly, while normal wounds posses an initial and robust inflammatory reaction, recent studies have highlighted that chronic wounds suffer lower than normal initial inflammatory state in response to otherwise potent pro-inflammatory stimulation. The goal of this work is to study the impact of chronic LPS stimulation on macrophages pro-inflammatory reaction to a fresh LPS treatment and its subsequent capacity to respond to IL4/IL13, "M2" promoting cytokines. This work highlighted that chronic LPS stimulation rendered macrophages hypo-responsive to fresh LPS stimulation. However, it did not impact their "M2" switching capacity. IFNg, a pro-inflammatory cytokine, treatment of chronic "M1" macrophages did not improve their responsiveness to LPS.

**Effects of chronic LPS stimulation on the response of macrophages to subsequent stimuli**

## 1. Introduction

Macrophages are indispensable innate immune cells, which orchestrate a multitude of functions in our bodies ranging from fighting infections and clearing debris to promoting tissue healing and angiogenesis[1]. Their significant role in tissue regeneration and angiogenesis has been increasingly recognized [2-5]. While the mechanism in which they promote healing is not well understood, evidence indicates that macrophage depletion has detrimental effects on healing in various tissue types including the skin[5], heart[6], cornea[7] and liver[8].

In response to tissue injury, tissue resident macrophages initiate an inflammatory cascade that mediates the recruitment of neutrophils and then monocytes into the injury site[9]. Upon the arrival of monocytes to the tissue, they mature into macrophages[10,11]. Macrophages initially adapt a pro-inflammatory "M1" activation state [12,13] , which can be mimicked *in vitro* using Th1 cytokines like interferon gamma (IFNg) or bacterial lipopolysaccharides (LPS)[14]. M1 macrophages are characterized by their secretion of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-a), interleukin beta (IL1-B), and the potent angiogenesis stimulator vascular endothelial growth factor (VEGF)[15].

Acute inflammation, initiated by M1 macrophages, facilitates the proper propagation of wound healing[16]. Besides the established role of inflammation in clearing debris and bacteria, it has been shown to play a role in tissue vascularization [15,17]. However, a prolonged inflammatory state is problematic and impairs healing [18]. Therefore, in healthy wound healing, an acute period of inflammation resolves over time and is accompanied with a shift in the macrophage population from pro-inflammatory "M1" to anti-inflammatory "M2" [13,19]. M2 macrophages are

associated with resolution of inflammation, cellular proliferation, and blood vessel maturation [15,20] . M2 macrophages can be generated *in vitro* using Th2 cytokines such as interleukin 4 and interleukin 13 (IL4/13)[14], and they secrete various chemokines like CCL18, CCL22, and growth factors like platelet derived growth factor- beta (PDGF-BB)[14].

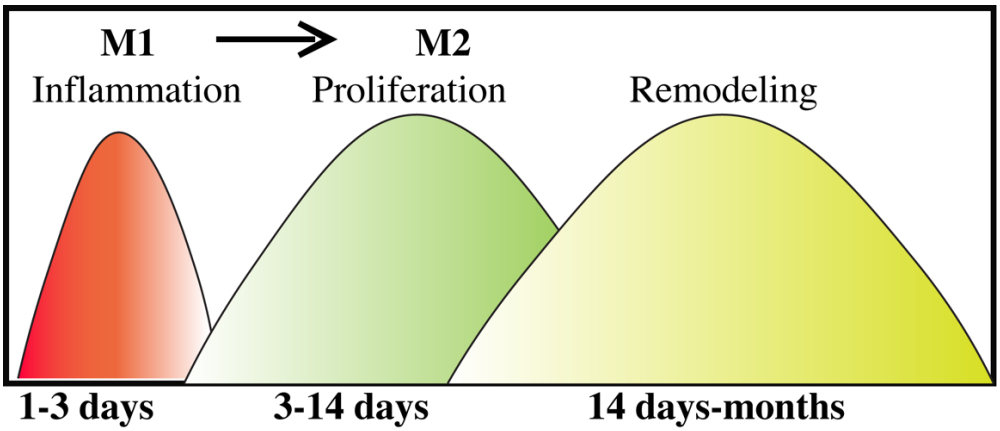


Figure 1. Wound healing phases modulated by macrophages M1-to-M2 transition[12,13]

The M1-to-M2 shift is temporally controlled to facilitate the proper progression of wound healing stages, starting with a brief phase of inflammation followed by cellular proliferation and ending with a longer period of remodeling (Figure 1)[12,13]. The mechanism of macrophage transition from M1-to- M2 macrophage in an injury site remains controversial; while some evidence suggest that recruitment of a new wave of M2 macrophages plays a major role[21,22], others suggest that macrophage repolarization via local signals is the important mechanism contributing to the M1-to- M2 transition [21,23,24].

### *1.1.* M1-to-M2 transition *in vivo*

M1 macrophages are the first subtype of macrophages that reacts to tissue injury and initiate the inflammatory response[13]. While their fate over time is not well understood, *in vivo* studies have suggests that they can repolarize to M2 phenotype overtime. Arnold et al.[13] showed the infiltration of M1 macrophages to a skeletal muscle injury was followed by dynamic switch to the M2 phenotype. Their results suggested that both recruitment and local conditioning were essential for the M1-to-M2 transition[13]. Additionally, Del Secco et al.[23] used sophisticated fluorescent labeling techniques to image macrophage phenotypes in a sterile liver wound murine model over time[23]. Their data indicated that M1 macrophages dominate the wound site initially [23]. As healing progressed, some macrophages switched their surface markers from M1 to M2 associated markers, while others expressed a hybrid of both M1 and M2 surface markers[23], suggesting an M1-to-M2 transition of individual cells. Interestingly, blocking IL10 or IL14 cytokines delayed the shift from M1 phenotype to a hybrid and M2

phenotypes indicating the importance of local cytokines in facilitating the M1-to-M2 switch[23]. Interestingly, they did not observe any M2 macrophages infiltrating the site over the duration of this experiment suggesting the importance of local cytokines in the generation of M2 macrophages[23].

### 1.2. Macrophage exhaustion in chronic inflammatory diseases

Chronic inflammatory diseases are characterized by an impaired M1-to-M2 transition[18,25,26]. Mechanisms leading to this impairment remains elusive. A common theme across many chronic inflammatory conditions including inflammatory bowl diseases[27], diabetes[28] and hyperlipidemia[29,30] , is the systemic increase in LPS levels. The systemic increase in LPS is thought to originate from gut derived commensal bacteria that is leaked to the circulatory system due to intestinal permeability which is triggered by the increased consumption of high fat diet and environmental toxins prevalent in the modern life style [28,31,32].

Macrophages in people with higher systemic LPS levels are characterized by M1 associated surface markers, including Tol-like receptor [33-40]. For example, it has been indicated that newly diagnosed patients with diabetes posses a higher level of systemic Tol-Like receptor 2 and 4 (TLR2/4), which are major LPS receptors, and correlated with increased LPS levels and pro-inflammatory cytokines [41]. However, although higher LPS levels has been associated with increased systemic pro-inflammatory markers initially, its chronic effects on macrophages remain elusive. Some evidence suggests that although LPS levels are high in patients susceptible to developing diabetic complications [42], those patients exhibit a lower level of systemic pro-inflammatory cytokines relative to other diabetic patients without complications

[40]. Since the risk of developing complications increases with poor disease management for prolonged duration [42], such observation suggests that the pro-inflammatory reaction to LPS decreases with time. The observed reduction in macrophages pro-inflammatory cytokine secretion in patients in response to prolonged LPS stimulation [43-45], could be possibly explained by macrophage exhaustion. Immune exhaustion has been defined by the inability of immune cells to mount a proper immune reaction to a pro-inflammatory signal due to its chronic activation[46] by factors like chronic diseases[47] and aging [46].

Of the immune cells, most studies focused on T cell exhaustion [48]. For example, In chronic viral infections, CD8 T-cells loss their capacity to perform their cytotoxic functions or proliferate[48]. Additionally, they are resistant to apoptosis and accumulate in high numbers[48]. Indeed, the accumulation of exhausted CD8 T-cells in chronic infections predicts the severity of the disease[48]. CD8 T-cells in chronic diseases are exhausted due to the programmed cell death-1 receptor (PD-1) and PD-L1 ligand interactions that activate immune inhibitory signaling [49]. Blockade of PD-1/PD-L1 interactions has been shown successful in restoring exhausted CD8 T-cells functions [50].

Unlike, CD8 T-cell exhaustion, macrophage exhaustion remains less understood [43]. Recently, a similar increase in PD-1 expression has been described in macrophages treated with LPS for 24 hours[43]. However, the effect of chronic LPS stimulation was not directly tested. Additionally, the increased PD-1 expression was shown to be associated with an increase in IL10 production [51]. A recent study showed the importance of IL-10 signaling in the maintenance of the exhausted macrophage phenotype and the development of pathological angiogenesis in chronic eye disease model [52]. It was also shown that exhausted macrophages posses hyper-

responsiveness to IL10 signaling[52]. Interestingly, it was shown that inhibiting IL-10/STAT3 signaling may have the potential to rescue exhausted macrophages[52].

Similar to macrophage exhaustion, a hypo-responsive macrophage response has been described in cases of tolerance. However, macrophage tolerance is a more understood macrophage behavior. It is a state of macrophage hypo responsiveness to pro-inflammatory stimulations post a high and acute and high TNFa or LPS stimulation[53,54]. Short-term exposure to LPS (1 hr -to- 8 hr) was shown to be sufficient to induce some macrophage hypo-responsiveness to secondary LPS stimulation[55]. The tolerant macrophage memory can be maintained up to ~5 days post treatment [55]. The tolerant state is thought to be mediated by IL-10 and transforming growth factor TGF-B production by macrophages during their primary LPS stimulation [56]. However, a clear distinction between exhaustion and tolerance is, while tolerant macrophage state is known to be a temporary state that abolish with time[57], immune-exhaustion is thought to cause a prolonged state of immune hypo-responsiveness[58].

Attempts to rescue tolerance includes using IFNg , a pro-inflammatory cytokine treatment. It has been indicated that interferon gamma partially inhibits the negative feedback loop induced by LPS tolerant macrophages[59,60]. Additionally, IFNg treatment, partially restored energy metabolism and cytokine production capability in tolerant macrophgaes[61]. Interestingly, IFNg treatment has been also shown to rescue macrophages capacity to phagocytose dead cells in chronic granulomatous[62]. Additionally, IFNg has been previously shown to inhibit IL10 secretion from macrophages[63]. Since more research is available on macrophage tolerance, and owing to the similarities between tolerance and exhaustion of macrophages[58,61], delivering IFNg may have the potential to rescue macrophage exhaustion and promote the proper M1

response that is impaired in chronic inflammation.

### 1.3. Impaired macrophage inflammatory functions in chronic inflammation

It is thought that macrophage exhaustion due to aging or chronic diseases is the cause for impaired macrophage functions [44]. For example, injecting allogenic macrophages from young donors, not old ones, to patients with chronic wounds showed improved healing outcomes, suggesting that macrophages from older patient are reaching an exhausted phase, impairing their wound healing functions[64]. However, to our knowledge, the direct impact of chronic pro-inflammatory stimulation of macrophages functions is poorly understood. Recently, increased chronic LPS levels have been directly linked to impaired tissue healing[65]. For example, Yuan et al[65] injected mice with LPS or phosphate buffered saline (PBS) for 10 days, then induced a full thickness cutaneous injury[65]. Vascularization and collagen deposition were significantly reduced in mice chronically stimulated with LPS[65]. Vascularization and collagen deposition are both associated with M1 and M2 functions in normal wound healing[15,20], suggesting that macrophage impairment is linked to the impaired healing response[65].

Nassiri et al[25] compared the temporal expression of pro-inflammatory and anti-inflammatory markers in diabetic human ulcers. Interestingly, they showed that non-healing ulcers had an initially lower pro-inflammatory gene expression signatures relative to healing ulcers [25]. However, non-healing ulcers increased their pro-inflammatory gene expression signature overtime, unlike healing ulcers[25]. This study suggests that patients that develops chronic wounds complications, have lower capacity to mount a rapid and robust inflammatory response in response to injury. Similarly, Marks et al.[66] showed that patients with inflammatory

bowl disease express defective acute inflammatory response[66]. Their peripheral blood monocytes were isolated, cultured *in vitro* and stimulated with TNFa or with wound fluid gathered from the early inflammatory phase from healthy individuals[66]. Patients' macrophages expressed significantly less pro-inflammatory cytokine in response to either stimulation relative to healthy macrophages[66] . Similarly, Malaponte et al.[67] showed that continuous inflammatory stimulation by dialysis treatment caused impaired response of macrophages to inflammatory stimulation, which was directly proportional to the number of months they have been undergoing dialysis. They quantified pro-inflammatory cytokines released into plasma of healthy patients and patients that go through dialysis treatments before and after treatment[67]. The patients were categorized into patients that have been going through dialysis treatments for short, medium and long time[67]. The results showed that ability of macrophages to secrete pro-inflammatory cytokines *in vivo* and *in vitro* in response to LPS was inversely proportional to the time of dialysis treatment[67]. These results suggest that chronic pro-inflammatory stimulation exhausts inflammatory capacity of macrophages in response to fresh pro-inflammatory stimulation. Collectively, these studies suggest the impaired M1 response in chronic inflammatory cases consequently impairs the propagation of wound healing[68].

Since the the proper wound healing response has been associated with a temporally controlled M1-to-M2 switch in macrophage phenotypes, many researchers investigated possible ways to facilitate this process by using local cytokines delivery in order to override impaired macrophages behavior in disease[15].

### *1.4.* M1-to-M2 switching *in vitro*

The impaired M1-to-M2 macrophages shift in chronic inflammatory conditions motivated further investigations to highlight the plasticity of macrophages, defined by their capacity to respond to different and sequential environmental stimulations by changing their phenotype.

*In vitro* evidence suggest that pro-inflammatory and un-activated macrophages have the capacity to make anti-inflammatory "M2" macrophages upon stimulation with IL4/IL13 cytokines[15]. Macrophages gene expression, surface marker expression, and protein secretion have been shown to reflect their response to the most recent stimuli[15]. Moreover, Porcheray et al[69] questioned the if the M2 macrophage population originated from previously resting macrophage, or if they arise from an initially pro-inflammatory M1 macrophages population. They suggested that the heterogeneity of M2 macrophages allude to the concept that the M1 to M2 switching is an important contributor to the complexity of the M2 population. They treated macrophages with TNFa to promote M1 followed by transforming growth factor beta (TGF-B), interleukin 10 or 4 (IL10, IL4) treatments to promote the sequential M1-to-M2 transition[69]. They observed changes in macrophage surface markers and morphologies in response to different treatments to reflect their M2 phenotype[69]. Their data showed that macrophages were fully and actively reversing from M1 phenotype to M2 phenotypes[69]. These significant findings suggest that M1-to-M2 phenotype switching could be a mechanism to resolve inflammation[69].

Additionally, Davis et al. [70] showed that macrophage repolarization in response to cryptococcal pneumonia is part of the pathology of this infection. They noted that lung macrophages changed their phenotype over the duration of the infection in response to the

invading pathogen in a way that affected healing outcomes[70]. Macrophages dynamically changed their gene expression, morphology, and protein secretion to reflect their sequential response to IFNg then IL4 stimulation[70]. Their study highlighted the potential importance of developing therapies for controlling macrophages dynamic polarization in diseases to control healing outcomes [70]. Similarly, Stout et al[71] treated macrophages with a variety of sequential cytokines before being exposed to LPS to investigate macrophages functional adabtability and capacity to sequentially change their phenotype[71]. They stimulated macrophages with different cytokines including IL10 , IFNg or IL4. The differences in their response to the later LPS was determined to be due to their memory from the previous type of cytokine treatment[71]. For example, treating macrophages with IL4 prior to being exposed to LPS significantly enhanced their secretion of inflammatory cytokines including TNFa and reduced their anti-inflammatory cytokines secretion including IL10[71]. Their data suggested the capacity of macrophages to functionally adapt to changing microenvironment by altering their protein secretion profile, highlighting the effect of macrophages memory in modifying their response to subsequent stimulations in some cases[71].

### 1.5. Significance

Post tissue damage, monocytes must traffic to the tissue and subsequently differentiate to macrophages in order for wound healing to occur[13]. Macrophages play a major role in tissue regeneration and wound healing and their dysfunctional behavior have been observed in chronic inflammatory situations and impaired healing. Since LPS levels are systemically higher in many chronic inflammatory diseases, we suspect that it may directly cause macrophage exhausted response to fresh pro-inflammatory and anti-inflammatory environmental

stimulation. Understanding the effect of chronic LPS stimulation on functional M1 activation and their subsequent M2 transition is important because it could elucidate a potential reason for the impaired M1-to-M2 switch in chronic inflammation. In addition, since sequential cytokines delivery is a potential therapy to facilitate the M1-to-M2 transition [15,72], it is imperative to understand the impact of chronic LPS stimulation observed in patients, on their macrophage's ability to respond to fresh cytokines treatments which may inform the development of more effective therapeutic strategies.

## 2. Summary of Aims

Macrophages, are central players in modulating the inflammatory and wound healing response [13]. Their behavior was shown to be dysregulated by various chronic pathologies, including diabetes [68]. In normal wound healing, macrophages behavior is temporally controlled to possess a pro-inflammatory "M1" phenotype at early stages post injury and an anti-inflammatory "M2" phenotype at later stages [13]. The impaired M1-to-M2 shift has been shown to be linked to chronic, non-healing injuries [25]. However, factors causing this impairment remain poorly understood. **Since chronic pro-inflammatory stimulation of macrophage is linked to impaired healing[65], we propose that macrophages exhaustion (hypo-responsiveness to environmental stimulation) mediates the delay in the initial inflammatory response, and subsequently impair the propagation of the healing phases facilitated by the M1-to-M2 switch.** We seek to delineate the effects of chronic macrophages exposure to LPS (M1 promoting stimuli) on their ability to respond to fresh LPS and subsequently to IL13/4(M2 promoting stimuli) to examine their M1-to-M2 switching capacity. **The first aim is to examine the effect of chronic versus acute LPS stimulation on human monocytes and macrophages**

**subsequent response to fresh LPS.** We hypothesize that the chronic LPS stimulation of monocytes and macrophages will decrease their response over time because they may develop exhaustion leading to hypo-responsiveness to environmental stimulation. **The second aim is to test the impact of chronic LPS stimulation on macrophages M1-to-M2 transition capacity**. We expect that LPS chronic exposure will impair macrophages response to IL4/IL13 by reducing their PDGBB and CCL18 protein secretion, which are common M2 markers [14], relative to acutely stimulated and naïve macrophages that are exposed to IL13/4**. The third aim of this work, is to study the impact of a pro-inflammatory cytokine treatment (IFNg) to restore the responsiveness of exhausted** macrophages to LPS and to IL13/IL4 cytokines. We hypothesize that similar to IFNg's ability to partially restore macrophages functions in tolerant macrophages [59], IFNg may restore the responsiveness of exhausted macrophages, since both exhaustion and tolerance are mediated by an inflammatory negative feedback mechanism.

## 3. Aim 1. Determine the effect of chronic LPS stimulation on TNFa secretion of human macrophages.

### 3.1. Background

A common theme noted across patients with chronic inflammatory diseases is the systemic and chronic increase in LPS levels [73,74]. Macrophages response to acute LPS stimulation is well characterized. Their response is governed by a negative feedback loop which is initiated a few hours following their first exposure to LPS, causing their pro-inflammatory cytokines secretion to subside overtime, and their anti-inflammatory markers including CC-chemokines CCL22, CCL2 and CCL17 to increase [73,75]. The activation of the negative feedback loop causes

macrophage hyporesponsiveness to secondary pro-inflammatory stimulation, also known as tolerance[56,59,61].

Although the acute impact of LPS on macrophages' response to secondary LPS stimulation has been extensively studied [57,76,77], its chronic effect on macrophages remains poorly explored. The purpose of this aim is to directly investigate the ability of macrophages to respond to a fresh dose of LPS following their prolonged LPS exposure. We hypothesize that the chronic LPS stimulation of macrophages will decrease their response over time because they may develop exhaustion leading to their hypo responsiveness to environmental stimulation. We will investigate macrophages responsiveness by measuring TNFa protein secretion, a pro-inflammatory cytokine [78,79]. Protein secretion was selected as a measurement to understand cellular response on a functional level.

## 3.2. Materials and methods

### 3.2.1. Experimental design

Monocytes were cultured under different treatment conditions to make the different experimental groups that represent chronically and acutely stimulated macrophages under two different doses of LPS to investigate if the impact of chronic stimulation is dose dependent (**Table 1**; Figure 1). Based on our standardized protocol, monocytes take five days to differentiate macrophages, mostly marked by cellular adherence to the culture dish[14]. Early chronic priming-Hi and Early chronic priming-Lo groups are chronically stimulated starting at day zero of the culture. **They are aimed to model the systemic chronic inflammatory stimulation of monocytes to investigate the effect of monocytes pro-inflammatory**

**conditioning on their later response to stimulation as macrophages (day six).** Groups 3 and 4 are controls aimed to model the acute response of mature macrophages. Moreover, **to investigate the effect of chronic LPS stimulation on mature macrophages on their response to fresh LPS stimulation,** groups 5 and 6, which are late chronic primed-Hi and late chronic primed-Lo were created **(Table 1**; Figure 1**).** Unactivated macrophages (M0) exposed only to media containing MCSF were used as a control throughout the experiment. Media and stimuli were refreshed every three days.

**Table 1. Experimental design groups.**

| Experimental Group | Treatment |
|---|---|
| 1) Early chronic priming$^{Hi}$ | Starting with monocytes at day zero, chronically stimulated macrophages were cultured for 7 days in complete media with a high dose of LPS (100 ng/ml). |
| 2) Early chronic priming$^{Lo}$ | Starting with monocytes at day zero, chronically stimulated macrophages were cultured for 7 days in complete media with a low dose of LPS (1 ng/ml). |
| 3) Acute priming$^{Hi}$ | Starting at day 6, mature macrophages were exposed to (100 ng/ml) LPS for 24 hours. |
| 4) Acute priming$^{Lo}$ | Starting at day 6, mature macrophages were exposed to (1 ng/ml) LPS for 24 hours. |
| 5) Late chronic priming$^{Hi}$ | Starting at day six, mature macrophages were stimulated with (100 ng/ml) LPS for 7 days, until day 13. |
| 6) Late chronic primed$^{Lo}$ | Starting at day six, mature macrophages were stimulated with (1 ng/ml) LPS for 7 days, until day 13. |

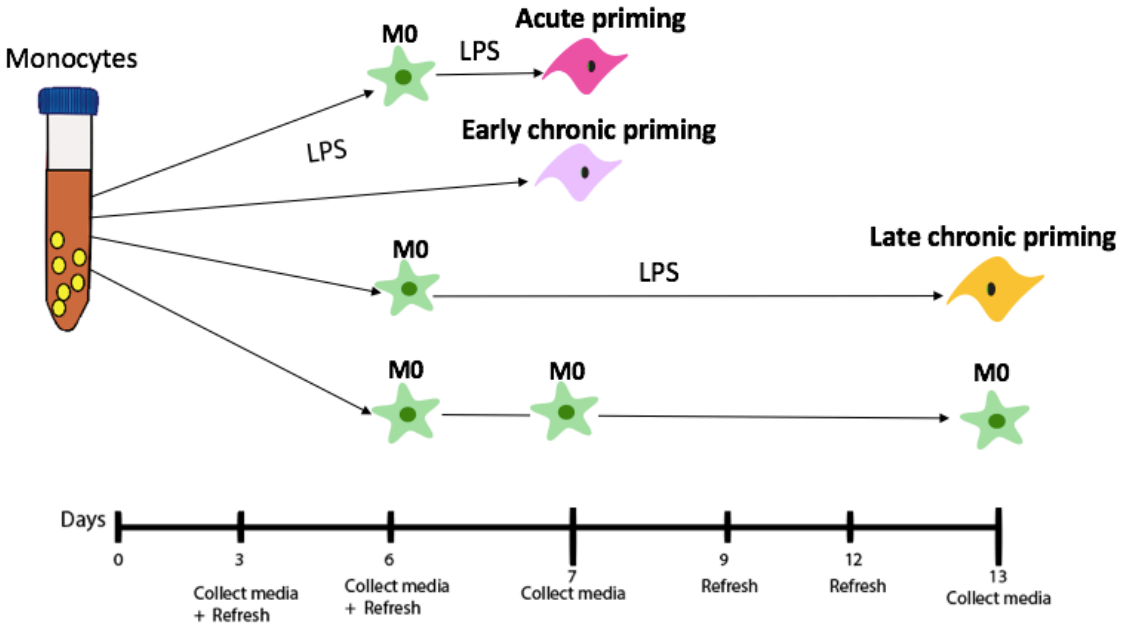


Figure 2. Experimental design timeline for aim one. The experiment was repeated with high and low LPS dose.

**3.2.2. Cell culture-** Primary human monocytes (purchased from University of Pennsylvania Human Immunology Core, Philadelphia, PA). Cells were cultured in at 37 C and 5% CO2 in ultralow 24 wells culture plates (Corning) in complete media (RPMI (Thermofisher), %10 heat-inactivated human serum ( Sigma Aldrich), 1% penicillin streptomycin (Thermofisher) and 20 ng/ml and macrophage colony stimulating factor MCSF (Peprotech) ). The seeding density of cells was approximately 800 thousand cells/ml. Lipopolysaccharides (LPS) was purchased from Sigma Aldrich and used to stimulate pro-inflammatory “M1” macrophages.

**3.2.3. Enzyme linked immunoassay** - At day 3,6,7 and 13, media was collected, centrifuged and the supernatants were stored at -80 until later Enzyme linked immunoassay for cellular secretion of TNFa (Peprotech). To count the total cell number in the well, adhered cells were scraped and combined with the centrifuged cell pullet and resuspended in 1 ml media. Cells were counted using countess image analysis processer and live cells number was recorded.

**3.2.4. Statistical analysis -** Our hypothesis was tested on six biological replicates from one human donor using One-way or two-way analysis of variance, with Tukey's post-hoc multiple comparison. $P < 0.05$ was considered significant for all analyses. Error bars in all figures represents standard error of mean.

## 3.2. Results

To confirm that macrophages respond to acute LPS stimulation by increasing their TNFa secretion, macrophages were differentiated from monocytes by culturing for six days in complete media with MCSF, according to our standard protocol. At day six, they were stimulated with a high or low doses of LPS for 24 hours and their culture media were analyzed. Although TNFa secretion was not different between groups treated with high or low LPS, they were both higher than M0 macrophages (Figure 3). **These results confirm that secretion of**

**TNFa is a good indicator of the response of macrophages to LPS stimulation**.

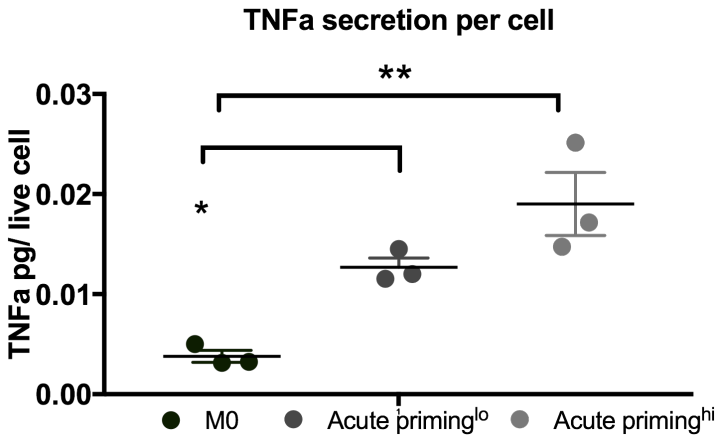


Figure 3. TNFa secretion from macrophages either un-activated, stimulated with low dose of LPS (1ng/ml) or with high dose of LPS(100ng/ml). Data collected from n=3 experimental replicates, one biological donor. Error bars are mean +/- SEM. Two way ANOVA with Tukey's post hoc.

To investigate the effect of chronic LPS stimulation on macrophages' TNFa secretion, macrophages were stimulated with high or low LPS doses for six days starting with monocytes at day zero, with the media being refreshed at day three. **TNFa secretion was significantly reduced over time following the prolonged exposure to either high or low doses of LPS treatment (Figure 4).** Interestingly, by day six, chronically stimulated macrophages by high or low LPS doses produced significantly less TNFa relative to M0.

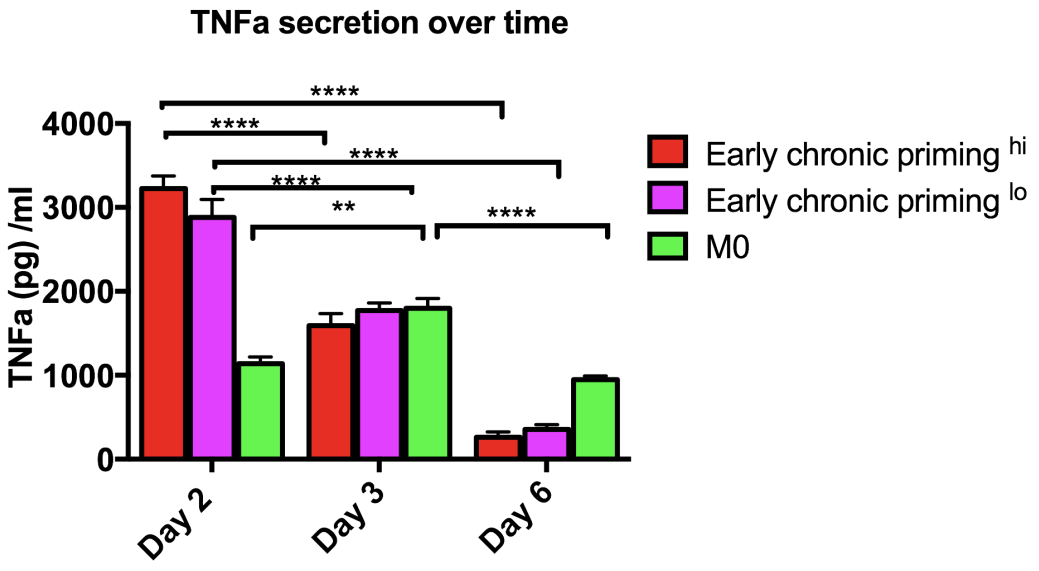


Figure 4. TNFa secretion overtime in response to chronic stimulation with 1 ng/ml LPS or 100 ng/ml LPS. Data collected from n=6 experimental replicates, one biological donor. Error bars are mean +/- SEM. Two way ANOVA with Tukey's post hoc.

Furthermore, to compare the response of macrophages that are acutely stimulated by LPS relative to ones that has been already exposed to LPS for prolonged period, we simultaneously cultured M0 and chronically stimulated macrophages using high or low LPS doses for six days, with the media being refreshed at day three. By day six, chronically stimulated macrophages and M0 macrophages were stimulated with a fresh high or low LPS dose**.** The collected media from both groups at day seven were analyzed for their TNFa secretion. A higher TNFa secretion was observed in the acutely stimulated group in both high and low doses of LPS (Figure 5; Figure 6). **These data suggest that chronic stimulation of monocytes with high LPS dose exhausts their response to fresh LPS stimulation.**

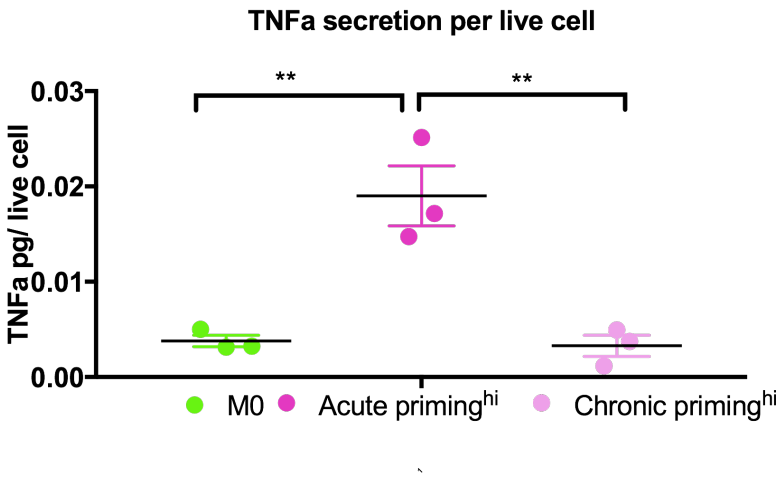


Figure 5. Macrophage population stimulated with with high (100ng/ml) dose of LPS for chronic versus acute duration.

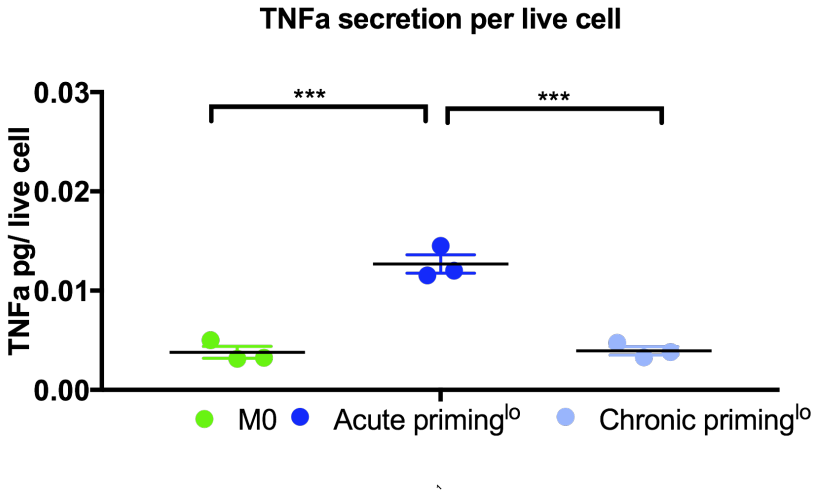


Figure 6. Macrophage population stimulated with with low(1ng/ml) dose of LPS for chronic versus acute duration.

**Macrophage population stimulated for chronic versus acute duration with LPS:** Macrophage population stimulated with with high(100ng/ml) or low (1ng/ml) dose of LPS for chronic versus acute duration. TNFa secretion from macrophage population stimulated with high or low LPS for seven days versus macrophage population stimulated high or low LPS for one day. The media was refreshed 24 hours prior to collection in all groups. Data collected from n=3 experimental replicates, one biological donor. Error bars are mean +/- SEM. One way ANOVA with Tukey's post hoc.

Moreover, to understand the effect of chronic LPS stimulation on fully differentiated macrophages, we repeated the above experiment but we started the chronic stimulation of macrophages at day six of the culture when the cells were mature macrophages (M0), instead of day zero (monocytes). The chronic LPS stimulation was continued for seven days. Interestingly, chronic stimulation of starting at the mature macrophage (M0) stage and using high or low LPS doses, lead to significantly less TNFa secretion relative to acutely primed group (Figure 7; Figure 8). **These data suggests that chronically stimulating mature macrophages with high or low LPS doses, exhausts their response to fresh LPS.**

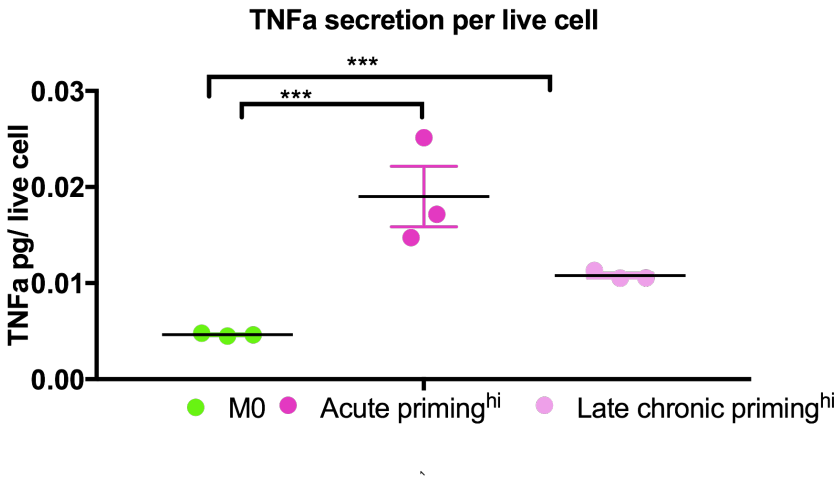


Figure 7. Effects of chronic high LPS stimulation on TNFa secretion from starting at the mature macrophage stage.

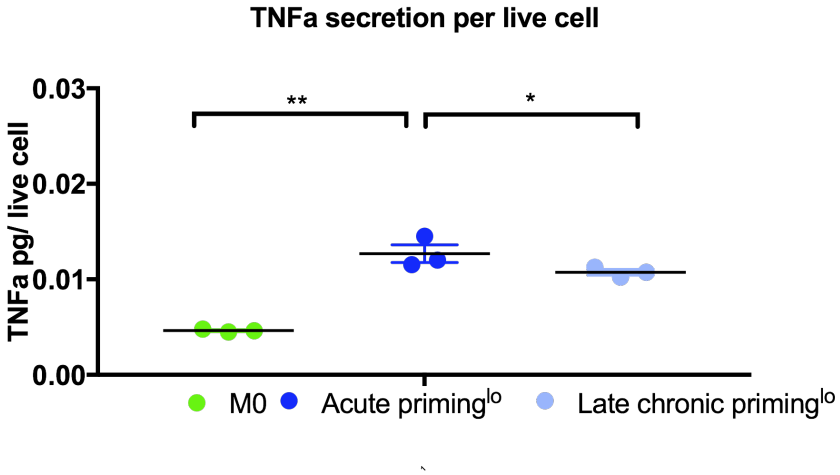


Figure 8. Effects of chronic low LPS stimulation on TNFa secretion from starting at the mature macrophage stage.

**Effects of chronic high LPS stimulation on TNFa secretion from starting at the mature macrophage stage:** Macrophages treated with high or low LPS for seven days starting at day six from the start of the culture, until day 13. M0 represents an un-activated macrophage group that remained in the culture for 13 days. Data collected from n=3 experimental replicates from one biological donor. Error bars are mean +/- SEM. One-way ANOVA with Tukey's post hoc was used.

### 3.3. Discussion

Acute inflammation has a plethora of beneficial effects on tissue regeneration. Beside its known role in clearing debris and fighting infections, it is an important initiator of angiogenesis[15] and cellular proliferation[16]. For example, it has been shown *in vivo* that early and high expression of LPS receptor called toll like receptor 2 (TLR2), expressed on M1 macrophages, is associated with improved healing outcomes due to enhancing cellular proliferation and migration[80]. Similarly, it has been suggested that acute LPS stimulation of wounds facilitate faster healing by accelerating inflammatory resolution, increasing macrophage infiltration further enhancing angiogenesis and cellular proliferation by upregulating TGFB and VEGF [81]. Macrophages are the major players in the inflammatory response[4]. Therefore, understanding the impact of different environmental conditions on their behavior is essential to understand potential reasons for the changes in their behavior in pathological conditions, ultimately to inform new treatments.

Patients with chronic inflammatory diseases have been shown to also possess a slightly elevated levels of LPS that are also linked to high cholesterol and sugar diet, chronic smoking or drinking , which collectively are associated with increasing the risk of developing complications [29,42,74,82-84]. Therefore, it is important to understand how chronic exposure to LPS impact macrophages, which are essential players in tissue regeneration[5].

It is established that high and acute dosage of LPS stimulation cause macrophages to mount an acute, resolving inflammation and tolerant behavior to subsequent stimulation which recover with increasing their resting phase[74,85]. While the impact of primary and acute LPS stimulation on macrophage tolerance against a secondary LPS stimulation has been extensively

studied, the direct effect of chronic and continuous exposure of macrophage to LPS in the context of wound healing remains poorly understood. It has been highlighted that aging causes a general macrophages exhaustion due to continuous immune stimulation[46]. However, how macrophages exhaustion caused by chronic stimulation impact macrophages wound healing functions, remains poorly understood. We hypothesized that chronic LPS stimulation of macrophages will exhaust macrophages pro-inflammatory activation. Our findings suggested that monocytes and macrophages chronically stimulated with LPS exhausted their TNFa secretion in response to further LPS stimulation (Figure 5- 8). Our findings may have a major physiological relevance as it was recently shown that mature tissue macrophages in addition to circulating monocytes are essential initial responders in the wound healing response[86]. Future studies are needed to further understand the mechanisms of macrophage exhaustion.

We speculate that macrophage hyporesponsiveness to LPS may render cells unable to mount a proper acute inflammatory response to injury, leading to impaired/delayed wound healing. We think that chronic LPS stimulation may activate negative regulatory pathways making the cells hyporesponsive to other pro-inflammatory stimulation. It has been previously shown that NFKB signaling is tightly regulated by a negative feedback loop that prevents excessive inflammation [87]. Classical inflammatory activation of NFKB is mediated via p65 and p50 heterodimer[87]. P50 homodimer has been highlighted as an important repressor of the inflammatory response [87]. NFKB is repressed when p50/p50 homodimer binds to the DNA because p50, unlike p65 lacks a transtrascriptional domain[87]. Thus far, little is known about the regulation of p50/p50 binding [87]. P50/P50 homodimer binding has been suggested to be a mechanism through which IL-10 promotes its anti-inflammatory activities. In acute macrophage

tolerance, p50/p50 homodimer repressive binding has been shown to play a role in macrophage hypo-responsiveness to LPS [88]. Future study needs to confirm if p50/p50 homodimer repressive binding was mediating macrophage chronic hyporesponsiveness and if this mechanism was IL10 or IL10 dependent.

## 4. Aim 2. The impact of chronic LPS stimulation on macrophages M1-to-M2 switching

### 4.2. Background

The M1-to-M2 shift in macrophages population is essential for normal wound healing and it has been shown to be impaired in chronic wounds [18,25]. Evidence suggests that macrophages in type 2 diabetes are hyporesponsive to anti-inflammatory stimulation. For example, Barry et al. [89] showed that macrophages cultured in hyperglycemic conditions were hyporesponsive to interleukin 10 (IL10), an anti-inflammatory stimulation, which was not explained by a change in IL10 receptor[89].

Additionally, O'Connor et al.[90] showed impaired IL4 signaling in in macrophages isolated from a diabetic mouse model. The impaired signaling was mediated by increased expression of suppressor of cytokine signaling 3 (SOSC3) [90]. Since we previously mentioned that LPS levels are increased in chronic inflammatory diseases, and the prolonged LPS exposure also causes SOSC3 activation in macrophages [91], we expect that LPS chronic exposure will impair macrophages response to IL4/IL13.

Therefore, the purpose of this aim is to examine the impact of chronic LPS stimulation on macrophages M1-to-M2 switching, by investigating their PDGBB, CCL18 (M2 markers)[14] and TNFa protein secretion (M1 marker) , relative to M0-to-M2 switching and acute M1-to-M2

switching controls.

## 4.3. Materials and methods

4.3.3. **Experimental design-** Groups 1,2,3, and 4 from Aim 1 **(Table 1)** were used to examine the impact of LPS chronic stimulation starting at the monocyte stage (modeling the systemic LPS effect) on the capacity of macrophages to respond to IL4/IL13 by secreting M2-associated proteins, especially PDGFBB and CCL18. At day 7 of the culture, mature macrophages were switched to complete media containing 40 ng/ml IL4 and 20 ng/ml IL13 cytokines for 24 hours. IL13/IL4 cytokines and fresh media volume were decided based on cell number at day seven to ensure a ratio of 1 million cells per ml. Unactivated macrophages (M0) exposed to media containing MCSF and directly stimulated by IL4/IL13 at day seven were used as controls. For all groups, the media was refreshed every three days and at day seven.

**4.2.2. Cell culture-** Primary human monocytes (purchased from University of Pennsylvania Human Immunology Core, Philadelphia, PA). Cells were cultured in at 37 C and 5% CO2 in ultralow 24 wells culture plates (Corning) in complete media (RPMI (Thermofisher), %10 heat-inactivated human serum ( Sigma Aldrich), 1% penicillin streptomycin (Thermofisher) and 20 ng/ml and macrophage colony stimulating factor MCSF (Peprotech) ). The seeding density of cells was approximately 800 thousand cells/ml. Lipopolysaccharides (LPS) was purchased from Sigma Aldrich and used to stimulate pro-inflammatory “M1” macrophages. Interleukin 4 and interleukin 13 (IL4

/IL13) cytokines were purchased from Peprotech and used to stimulate the anti-inflammatory “M2” response.

**4.2.3. Enzyme linked immunoassay-** ***At day 7 (M1) and 8 (M2),*** media was collected, centrifuged and the supernatants were stored at -80 until later Enzyme linked immunoassay for cellular secretion of TNFa, PDGFBB (Peprotech) and CCL18 (PARC, R&D systems). Additionally, at day 7 and 8, cells were scraped gently to count their number using a countess automated cell counter (Invitrogen). To count the total cell number in the well, adhered cells were scraped and combined with the centrifuged cell pullet and resuspended in 1 ml media. Cells were counted using countess image analysis processer and live cells number was recorded.

**4.2.4. Statistical analysis**- please refer to section 3.2.4.

## 4.3. Results

To investigate M2 repolarization capacity of acutely stimulated macrophages using high or low LPS doses, different macrophage groups were stimulated with IL13/IL4 cytokines for 24 hours. Their M1-to-M2 switching capacity was measured by their increased secretion of CCL18, PDGFBB (M2 markers) and decreased secretion of TNFa (M1 marker). To test the impact of chronic stimulation of LPS on macrophages M1-to-M2 switching. Macrophages were chronically activated using high or low LPS doses, ***until day 7***. All groups were then switched to (IL13/IL4) for 24 hours, ***until day 8.***

On a population level, chronic stimulation using high LPS did not impact macrophages increase in CCL18 secretion post IL13/IL4 treatment (Figure 9 A). However, normalizing to live cell number revealed that CCL18 secretion only significantly increased post IL13/IL4 treatment in chronically primed groups (Figure 9 C). Fold change increase in CCL18 secretion was the highest in chronically primed group (Figure 9 D). Acute stimulation with high LPS reduced macrophages response to IL13/IL4 as shown by their lower CCL18 secretion relative to M0-to-M2 control (Figure 9 D).

PDGFBB protein increased secretion in response to IL13/IL4 was impaired on a population level in chronically stimulated macrophages (Figure 10 A). However, they increased their PDGFBB secretion on a single cell level (Figure 10 C). Fold change increase in PDGFBB secretion was the highest in chronically primed group (Figure 10 D). Acute stimulation with high LPS reduced macrophages response to IL13/IL4 as shown by their lower PDGFBB secretion relative to M0-to-M2 control (Figure 10 D).

TNFa secretion was not impacted post IL13/4 stimulation on a populations and single cell level in chronically stimulated groups and reduced in acutely stimulated groups (Figure 11).

Interestingly, at day 7 (before IL4/IL13 stimulation), chronically stimulated macrophages with high LPS had a higher CCL18 secretion relative to acutely stimulated group ( Figure 13 C). **These results suggest that chronic high LPS stimulation rendered cells more responsive to IL13/IL4 as it was measured by their higher PDGFBB and CCL18 secretion relative to M0-to-M2 control.**

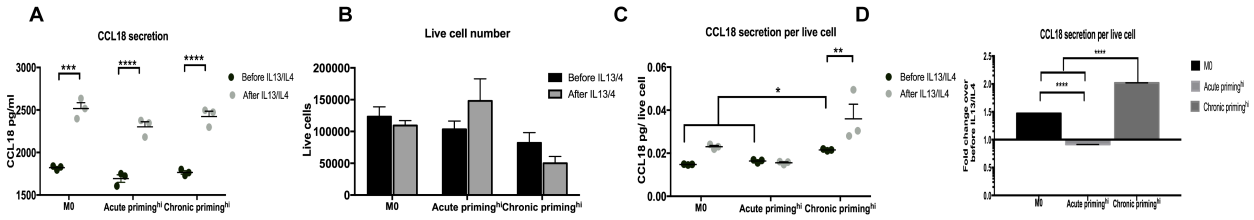


Figure 9. CCL18 secretion: The impact of chronic high LPS stimulation on macrophages M1-to-M2 switch. Interaction is not significant (p=0.2974)

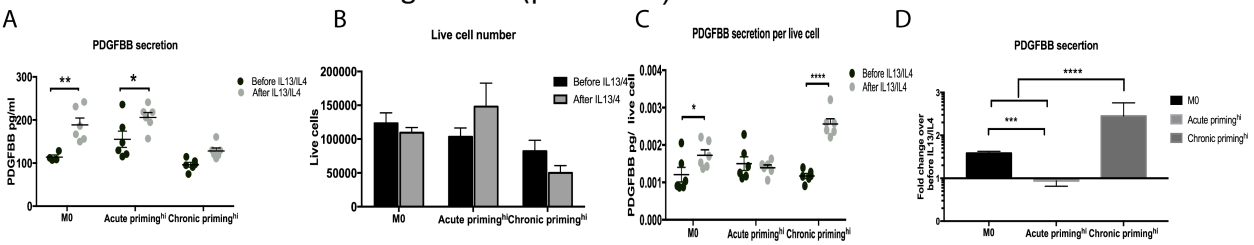


Figure 10. PDGFBB secretion: The impact of chronic high LPS stimulation on macrophages M1-to-M2 switch. Interaction was significant (p<0.0001)

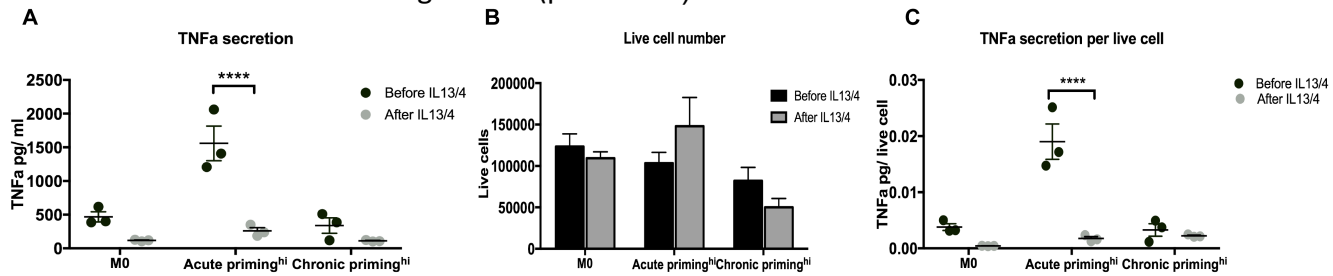


Figure11. TNFa secretion: The impact of chronic high LPS stimulation on macrophages M1-to-M2 switch. Interaction was significant (p=0.0015)

**M1-to-M2 switch from chronically stimulated macrophages with high LPS** :Macrophages activated by 100 ng/ml LPS for seven days versus for one day, were switched to IL13/IL4 containing media for 24 hours, until day 8. M0 control switched to IL13/4 was used as a control. A) Protein was measured before (day 7) and after IL4/IL13 stimulation (day 8). B) Live cell number in different treatment groups using trypan blue exclusion assay before and after IL4/IL13 stimulation. C) Protein secretion normalized to the average of live cell number. D) Fold change of protein secretion. The media was refreshed 24 hours prior to collection in all groups. PDGFBB Data collected from n=6 experimental replicates. CCL18 and TNFa data were collacted from n=3 experimental replicates. All data came from one biological replicates. Error bars are mean +/- SEM. Two way and one way ANOVA with Tukey's post hoc.

Chronic stimulation using low LPS did not impact macrophages increase in CCL18 secretion (Figure 12). Fold change over "before IL13/IL4 treatment" revealed that chronic stimulation made macrophages secrete the highest CCL18 in response to IL13/IL4 (Figure 12 D). PDGFBB secretion did not increase in chronically primed group post IL13/IL4 treatment (figure 13). Also, all groups decreased their TNFa secretion post IL13/4 stimulation (Figure 14 ). Interestingly, at day 7 (before IL4/IL13 stimulation), chronically stimulated macrophages with low LPS had a higher M2 associated proteins secretion relative to acutely stimulated group. **These results suggest that chronic low LPS stimulation rendered cells more responsive to IL13/IL4 in terms of their CCL18 secretion, not PDGFBB secretion, relative to M0-to-M2 control. However, acute stimulation with low LPS did not impact macrophages response to IL13/IL4 as shown by their similar CCL18 and PDGFBB secretion relative to M0-to-M2 control.**

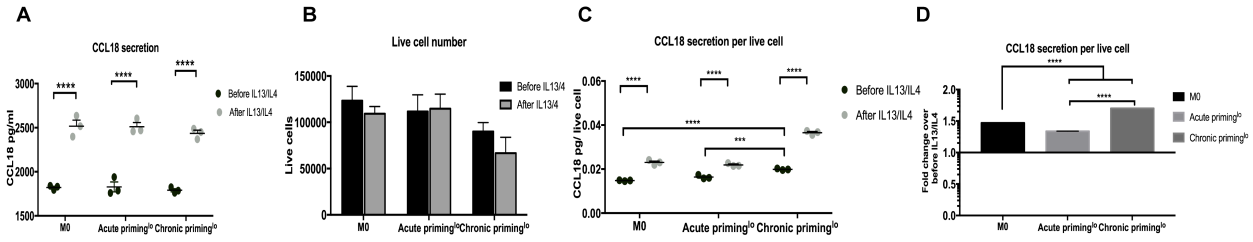


Figure12. CCL18 secretion: The impact of chronic low LPS stimulation on macrophages M1-to-M2 switch. Interaction was significant ($p<0.0001$)

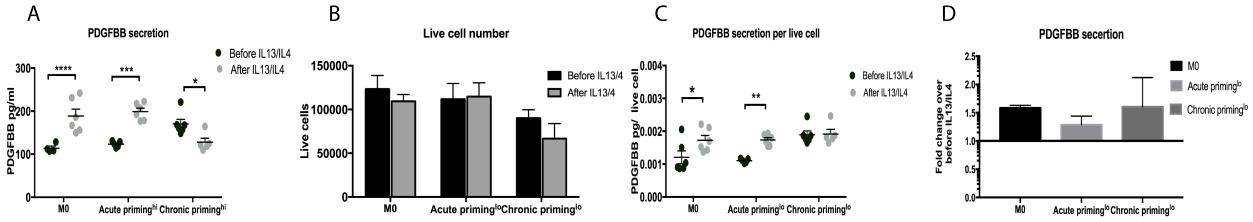

Figure13. PDGFBB secretion: The impact of chronic low LPS stimulation on macrophages M1-to-M2 switch. Interaction was not significant (p=0.0712)

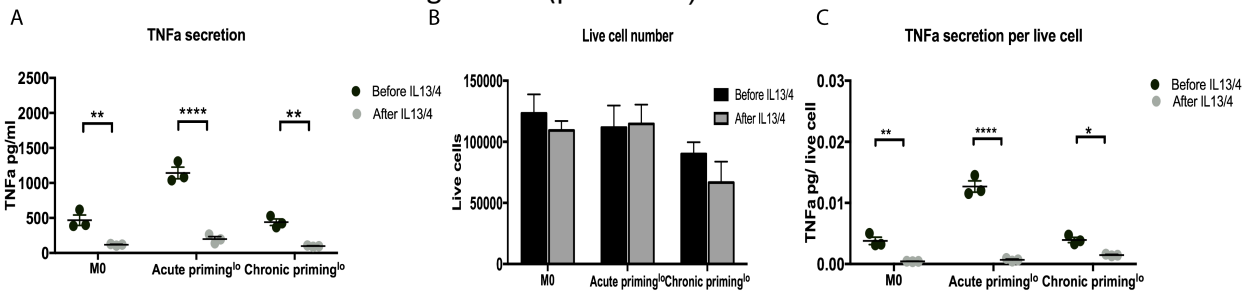


Figure14. TNFa secretion: The impact of chronic low LPS stimulation on macrophages M1-to-M2 switch. Interaction was significant (p<0.0001)

**M1-to-M2 switch from chronically stimulated macrophages with high LPS:** The impact of chronic low LPS stimulation on macrophages M1-to-M2 switch. Macrophages activated by 1 ng/ml LPS for seven days versus for one day, were switched to IL13/IL4 containing media for 24 hours, until day 8. M0 control switched to IL13/4 was used as a control. A) Protein was measured before (day 7) and after IL4/IL13 stimulation (day 8). B) Live cell number in different treatment groups using trypan blue exclusion assay before and after IL4/IL13 stimulation. C) Protein secretion normalized to cell number. The media was refreshed 24 hours prior to collection in all groups. PDGFBB Data collected from n=3 experimental replicates. CCL18 and TNFa data were collected from n=3 experimental replicates. All data came from one biological replicates. Error bars are mean +/- SEM. Two way or one way ANOVA with Tukey's post hoc was used to compare the change in each group over time.

**Table 2. Summary of results from aim 2 reported as it compares to before IL13/IL4 treatment**

| Condition | Population | Single cell |
|---|---|---|
| Chronic high LPS | CCL18_ *increased*<br>PDGFBB_ *not impacted* | CCL18_ *increased*<br>PDGFBB_ *increased* |
| Chronic low LPS | CCL18_ *increased*<br>PDGFBB_ *reduced* | CCL18_ *increased*<br>PDGFBB_ *not impacted* |

## 4.4. Discussion

Although acute inflammation is critical for successful wound healing, chronic inflammation has detrimental effects[25].Many research focus of promoting the M2 phenotype to promote healing of chronic inflammatory conditions[92]. Due to the important functions of an acute M1 activation in wound healing, unlike chronic M1, we investigated if an acute versus chronic M1 activation impact their M2 activation.

Here we showed that acute LPS stimulation with low (1 ng/ml) LPS dose, not high dose, facilitated an efficient cellular repolarization that is not different from direct polarization from M0 to M2 macrophage (Figure 10; Figure 11; Figure 12), suggesting that acute inflammatory phase does not reduce macrophages M1-to-M2 switching capacity. Indeed, Ostuni et al.[93] showed LPS impact macrophages epigenetic memory to facilitate modifications in their responsive capacity. Here, we found that high (100ng/ml) LPS stimulation impaired the efficiency of macrophages to secrete M2 associated proteins upon stimulation with IL13/IL4 (Figure 10; Figure 11).

Interestingly, chronic high LPS stimulation rendered cells more responsive to IL13/IL4 as it was measured by their higher PDGFBB and CCL18 secretion relative to M0-to-M2 control. However, acute stimulation with high LPS rendered macrophages less responsive to IL13/IL4 as shown by their lower CCL18 and PDGFBB secretion relative to M0-to-M2 control. On the other hand, chronic low LPS stimulation rendered cells more responsive to IL13/IL4 in terms of their CCL18 secretion, not PDGFBB secretion, relative to M0-to-M2 control. However, acute

stimulation with low LPS did not impact macrophages response to IL13/IL4 as shown by their similar CCL18 and PDGFBB secretion relative to M0-to-M2 control.

In diabetic ulcers, non-healing patients, expressed a lower initial pro-inflammatory markers relative to patients that healed [25]. Some evidence suggest that we need high level for initially acute inflammation to enable a better inflammatory resolution [25,94,95] . Many studies that investigated macrophage tolerance suggests that stimulating macrophages with two high doses of LPS separated by a resting phase to induce the tolerance response promote their capacity to activate their M2 associated response [45,96,97] by capitalizing on the activation of the opposing inflammatory and anti-inflammatory roles of NFKB [45,75]. Interestingly, we observed that chronically stimulated macrophages lead to a slightly higher M2 associated proteins' secretion relative to acutely stimulated groups. Thus, we speculate that the impaired pro-inflammatory activation in the initial phases of chronic wounds may be due to the impairment of the rapid M1 associated response to injury(shown in aim 1 data), along with skewing their activation to M2 like phenotype by their hyper-response to M2 stimulations, not M1 stimulation. Collectively, this disrupted behavior may lead macrophages to mount a dysfunctional initial inflammatory response and slow the overall propagation of the healing response due to the lack of temporal control over their ordered phenotypic activation and M1-to-M2 switch.

In conclusion, our findings suggest that although chronic LPS stimulation impaired macrophages response to further LPS stimulation, it did enhance their response to IL13/4 possibly because their exhaustion was limited to their pro-inflammatory pathway but not in other pathways. However, we think that the observed hyper-response of chronically stimulated

groups to IL13/IL14 could be partially mediated by their lack of ability to proliferate in response to IL13/IL4, dedicating more energy to synthesizing and secreting M2 proteins, unlike acute M1. Acute M1 was probably more or equally responsive to IL13/IL4 but by means of cellular proliferation and protein secretion, thus, secreting less proteins per cell because they are also investing their energy in proliferation, unlike chronic M1.

Future studies needs to further investigate and support this proposed hypothesis for impaired healing. Additionally, understanding the different proliferation capacities of chronic versus acute M1s in response to IL13/IL4 cytokines could highlight the need to rescue the proliferative capacity of macrophages in chronic wound prior to delivering M2 promoting cytokine therapy.

## 5. Aim 3. The impact of using interferon gamma treatment to restore the responsiveness of exhausted macrophages

### 5.3. Background

Recent evidence suggests that the initial inflammatory phase of chronic inflammatory conditions is impaired[25,94]. We suggested that the chronic LPS increase in patients with chronic diseases cause macrophages exhaustion, a phenomenon that reduces macrophages responsiveness to environmental stimuli, like fresh LPS signals. Some studies suggest that rescuing the initial inflammatory phase may jump start the wound healing process[94]. For example, in a diabetic mouse model of impaired wound healing, using IL1B inflammatory stimuli to rescue the defective macrophage activation improved wound healing[98].

It has been recently showed that IL10 signaling is essential for the maintenance of

exhausted macrophage phenotype which was imperative for the development of pathological angiogenesis in chronic eye disease[52]. IFNg's ability to inhibit IL10 and partially restore macrophages functions in cases of tolerance[63], may enable it from being a promising candidate to restore the responsiveness of exhausted macrophages to LPS since macrophage exhausted response is similar to its tolerant response.

Additionally, macrophage tolerance due to acute high LPS stimulation has been shown to cause metabolic paralysis characterized by decreased oxygen consumption, inability to increase glycolysis and less transport of fatty acid[61]. Collectively, leading macrophages to reduce their cytokine producing capacity[61]. Since macrophage tolerance is mediated by a negative feedback loop, it has been indicated that interferon gamma partially inhibits the negative feedback loop and rescue macrophages[59,60]. IFNg treatment, partially restored energy metabolism and cytokine production capability in tolerant macrophgaes[61]. Additionally, IFNg treatment has been shown to rescue macrophages capacity to phagocytose dead cells in chronic granulomatous[62].

We hypothesized that treating exhausted macrophages with IFNg may restore its responsiveness, similar to its effect of tolerant macrophages. The purpose of this aim is to investigate the capacity of IFNg cytokine to restore the response of exhausted macrophages to LPS, and to investigate how will IFNg pretreatment impact macrophages subsequent response to IL13/IL4 cytokines by analyzing their secretion of TNFa, PDGFBB and CCL18 proteins.

### 5.4. Materials and method

### 5.4.1. Experimental design

Groups 1,2,3,and 4 from aim 1 **(Table 1)** were used to examine the impact of adding 100 ng/ml IFNg treatment at day six of the culture to the chronically stimulated groups on their capacity to respond to LPS. To examine if IFNg pre-treatment impact its later response to IL13/4, at day 7, macrophages were switched to complete media containing 40 ng/ml IL4 and 20 ng/ml IL13 cytokines for 24 hours (Figure 12). IL13/IL4 cytokines and fresh media volume were decided based on cell number at day seven to ensure a ratio of 1 million cells per ml. M0 exposed to media containing MCSF only, M0 directly stimulated by IL4/IL13 and M0 stimulated by IFNg only for 24 hours prior to their Il13/IL4 treatment were used as controls. For all groups, the media was refreshed every three days and at day seven.

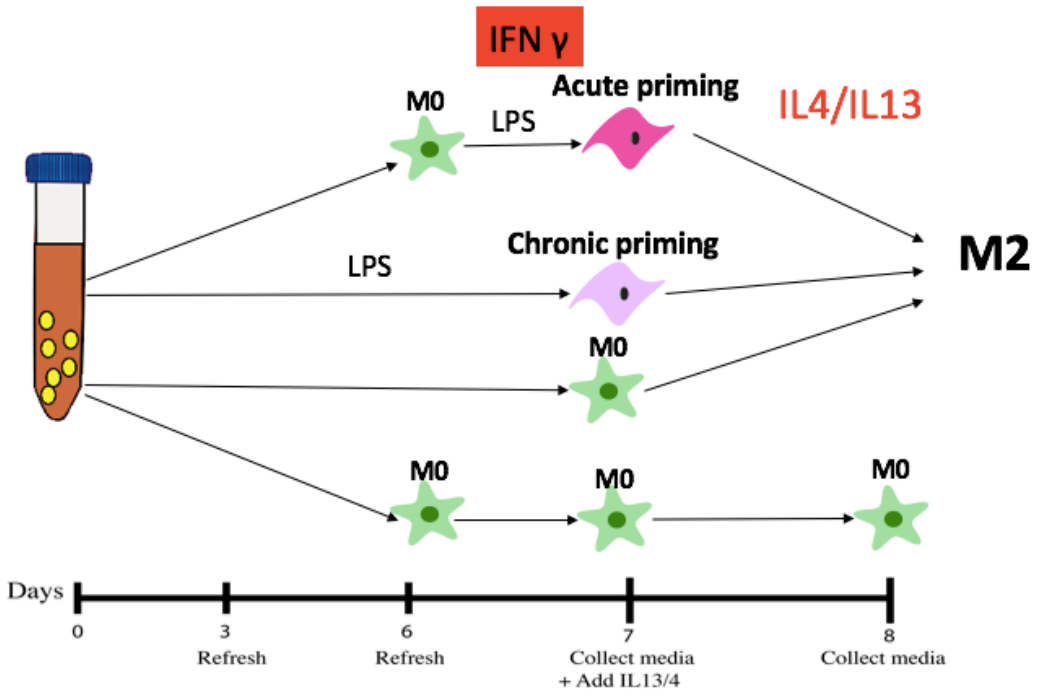

Figure 15. Aim three experimental design timeline. The study was repeated using high and low LPS dose.

**5.4.2. Cell culture -** Primary human monocytes (purchased from University of Pennsylvania Human Immunology Core, Philadelphia, PA). Cells were cultured in at 37 C and 5% CO2 in ultralow 24 wells culture plates (Corning) in complete media (RPMI (Thermofisher), %10 heat-inactivated human serum ( Sigma Aldrich), 1% penicillin streptomycin (Thermofisher) and 20 ng/ml and macrophage colony stimulating factor MCSF (Peprotech) ). The seeding density of cells was approximately 800 thousand cells/ml. Lipopolysaccharides (LPS) was purchased from Sigma Aldrich. Interleukin 4 and interleukin 13 (IL4 /IL13) and IFNg cytokines were purchased from Peprotech.

**5.4.3. Enzyme linked immunoassay** - ***At day 7(M1) and 8(M2),*** media was collected, centrifuged and the supernatants were stored at -80 until later Enzyme linked immunoassay for cellular secretion of TNFa, PDGFBB and CCL18. Additionally, cells were scraped gently to count their number using a countess automated cell counter (Invitrogen). To count the total cell number in the well, adhered cells were scraped and combined with the centrifuged cell pullet and resuspended in 1 ml media. Cells were counted using countess image analysis processer and live cells number was recorded.

5.4.4. **Statistical analysis** – refer to section 3.2.4.

## 5.5. Results

Adding an additional dose of IFNg with fresh LPS treatment did not increase TNFa secretion in any of the chronically or acutely LPS stimulated macrophage groups (Figure 16;

Figure 17). **These results indicate that IFNg dis not rescue the hyporesponsiveness of chronically stimulated macrophages to subsequent stimulation with LPS.**

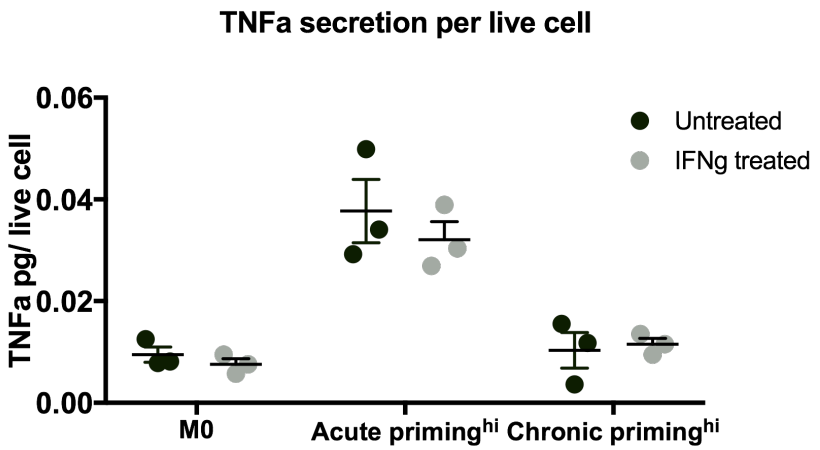


Figure 16. TNFa secretion from chronically treated macrophage group with high (100ng/ml) LPS alone for seven days or also receiving IFNg treatment at day six. Interaction was not significant (p= 0.613).

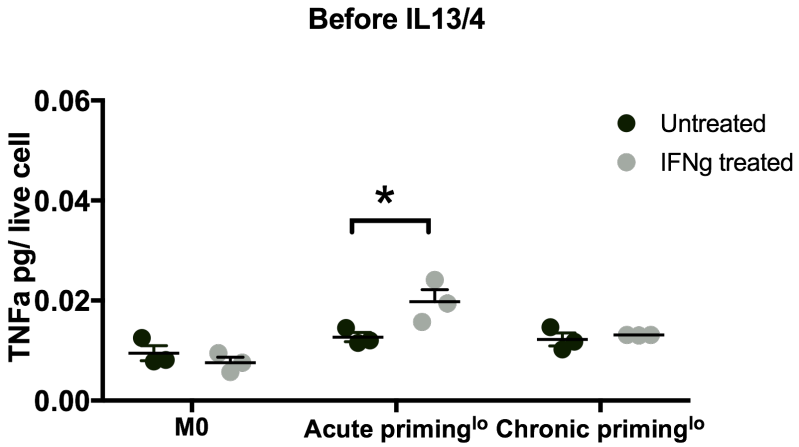


Figure 17. TNFa secretion from chronically treated macrophage group with high (1ng/ml) LPS alone for seven days or also receiving IFNg treatment at day six. Interaction was significant (p=0.0225).

**IFNg treatment of chronically stimulated macrophages**: TNFa secretion from chronically stimulated macrophages treated with LPS alone for seven days OR were given the same treatment but with an additional IFNg treatment at day six, for 24 hours prior to collection. A) TNFa protein secretion. The media was refreshed 24 hours prior to collection in all groups. Data

collected from n=3 experimental replicates and one biological replicates. Error bars are mean +/- SEM. Two way ANOVA with Tukey's post hoc .

To investigate if IFNg pre-treatment impacted macrophages response to IL13/IL4, CCL18, PDGFBB and TNFa protein secretion were analyzed at day seven of the (IFNg/LPS) treated groups and at day eight of the same group which subsequently were exposed to IL13/IL4 treatment. IFNg pre- treatment did not impact the decrease in TNFa secretion post IL13/4 treatment in groups primed using low LPS doses (Figure 18; Figure 21). However, TNFa was significantly higher in pretreated groups that were primed with high LPS dose (Figure 18). Interestingly, IFNg pre-treatment significantly enhanced CCL18 fold change secretion in response to IL13/4 relative to untreated groups in acutely primed and M0 groups, and impaired chronically primed group (Figure 19 D).

PDGFBB secretion was impaired in all groups relative to untreated groups (Figure 20). **These results suggest that IFNg pretreatment of chronically and acutely (high) primed macrophages, enhanced CCL18 secretion, impaired PDGFBB secretion, did not impact TNFa. Chronically primed group impaired their CCL18 and PDGFBB secretion in IFNg treated groups relative to untreated groups (Figure 19 D; Figure 20).**

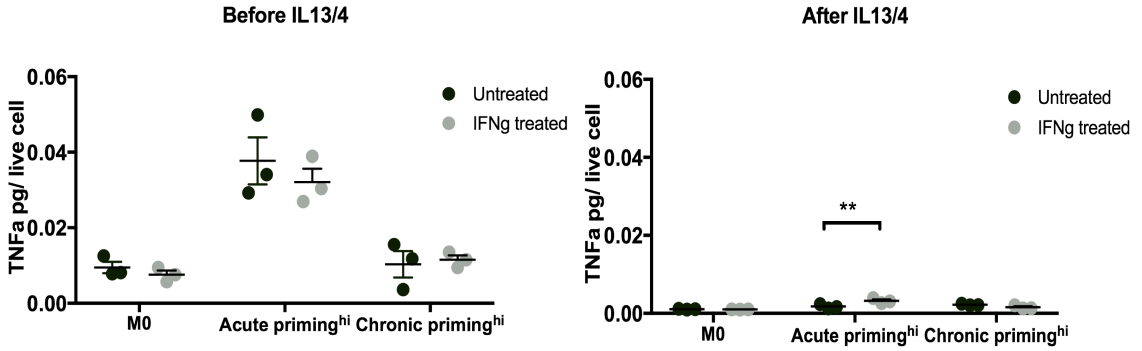


Figure 18. TNFa secretion: the effect of IFNg pre-treatment on the M1-to-M2 switching of acutely stimulated macrophages

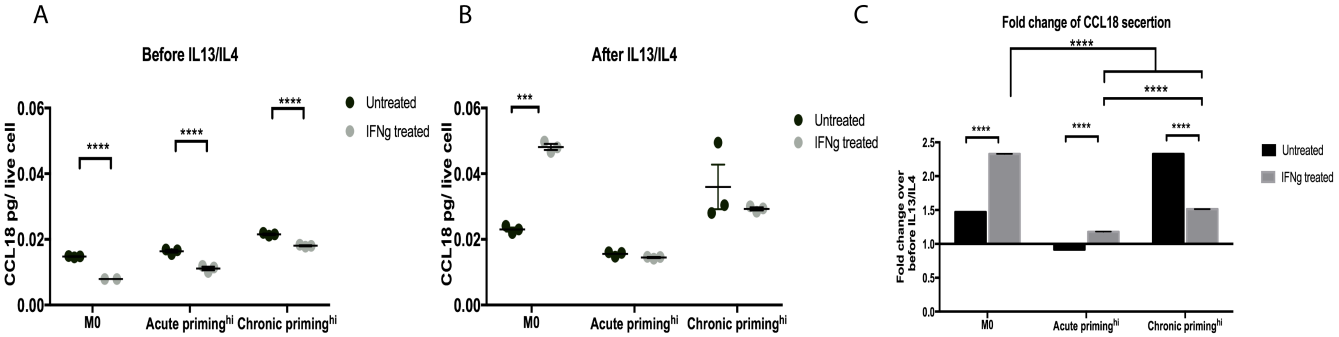


Figure 19. CCL18 secretion: the effect of IFNg pre-treatment on the M1-to-M2 switching of chronically stimulated macrophages with high LPS.

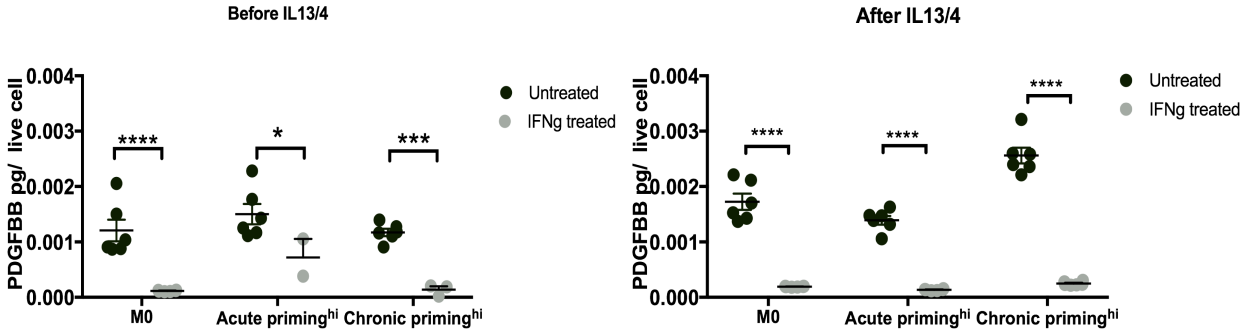


Figure 20. PDGFBB secretion: the effect of IFNg pre-treatment on the M1-to-M2 switching of chronically stimulated macrophages with high LPS.

**The effect of <u>IFNg pretreatment of chronically stimulated macrophages with high LPS on their subsequent M1-to-M2 switch</u>:** Chronically stimulated macrophages with high (100ng/ml) LPS alone for seven days or with LPS alone for six days and LPS+(IFNg) during the seventh day. Then all groups were exposed to IL13/IL4 treatment for 24 hours until day eight. Each group was compared to non-IFNg treated control groups at day 7 and 8. PDGFBB data collected from n=6 experimental replicates. CCL18 and TNFa data collected from n=3 experimental replicates. All data are collected from one biological replicate. Error bars are mean +/- SEM. Two way ANOVA with Tukey's post hoc was used to test the difference between IFNg and non-IFNg treated groups in each day.Interaction was significant ($p<0.0001$) for CCL18 fold change.

IFNg pre-treatment significantly reduced CCL18 fold change secretion in response to IL13/4 relative to untreated groups in acutely primed (low) macrophages (Figure 22 D). Chronically primed group (low) had the greatest fold CCL18 in response to IL13/IL4 relative to acute and M0 macrophage groups. secretion was impaired in all groups relative to untreated groups (Figure 22).PDGFBB secretion was impaired in all IFNg treated groups relative to untreated (Figure 23). **These results suggest that IFNg pretreatment of chronically and acutely (high) primed macrophages, enhanced CCL18 secretion, impaired PDGFBB secretion, did not impact TNFa. Chronically primed group impaired their CCL18 and PDGFBB secertion in IFNg treated groups relative to untreated groups (Figure 19 Dl Figure 20).**

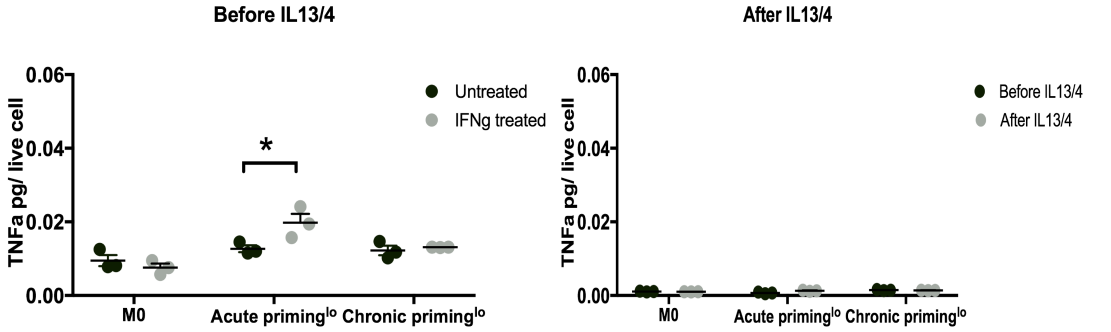


Figure 21. TNFa secretion: The effect of IFNg pre-treatment on the M1-to-M2 switching of chronically stimulated macrophages with low LPS.

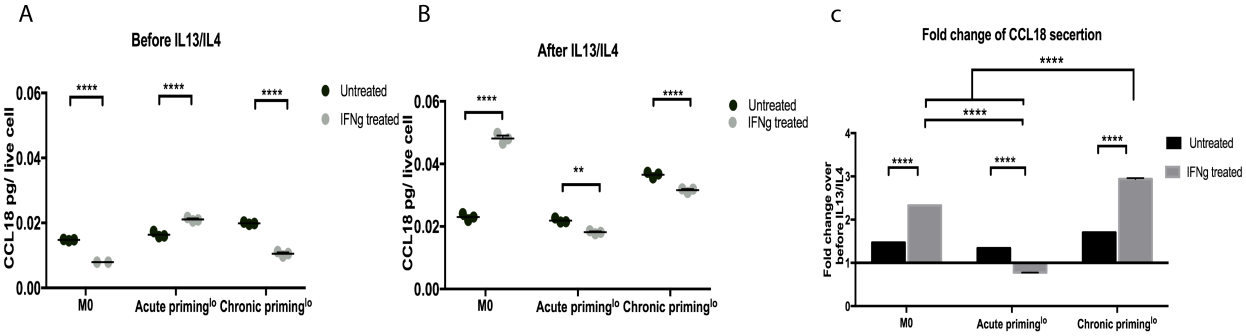


Figure 22. CCL18 secretion: The effect of IFNg pre-treatment on the M1-to-M2 switching of chronically stimulated macrophages with low LPS.Interaction was significant ($p<0.0001$)

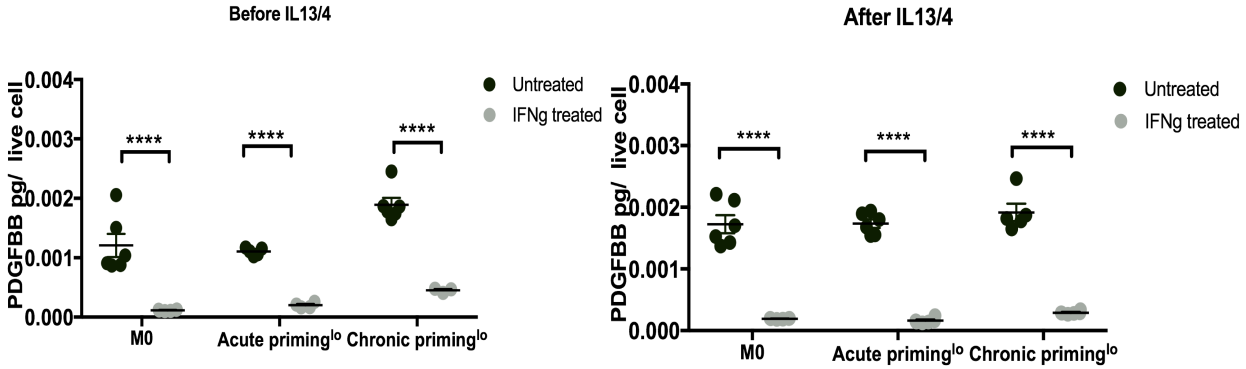


Figure 23. PDGFBB secretion: The effect of IFNg pre-treatment on the M1-to-M2 switching of chronically stimulated macrophages with low LPS

**The effect of IFNg pretreatment of chronically stimulated macrophages with low LPS on their subsequent M1-to-M2 switch:** Chronically stimulated macrophages with low (1ng/ml) LPS alone for seven days or with LPS alone for six days and LPS+(IFNg) during the seventh day. Then all groups were exposed to IL13/IL4 treatment for 24 hours until day eight. Each group was compared to non-IFNg treated control groups at day 7 and 8. A) Protein secretion. B) Live cell number using trypan exclusion assay. C)Protein secretion normalized to the average of live cell number. Data collected from n=6 biological replicates. Error bars are +/- SEM. Two way ANOVA with Tukey's post hoc was used to test the difference between IFNg and non-IFNg treated groups in each day. Significant bars were color coded to match the respective group color.

In summary, these results suggest that regardless of the LPS chronic stimulation dose, upon stimulation with IL4 and IL13, the addition of IFNg impairs primed macrophages secretion of PDGFBB, and does not affect the secretion of TNFa. Furthermore, upon stimulation with IL4/IL13, chronically primed groups with high LPS dose pretreated with IFNg, did not change their CCL18 secretion. In contrast, chronically primed groups with low LPS dose that was pretreated with IFNg, had an impaired CCL18 secretion post IL13/IL4 treatment. Last, IFNg pretreatment always increased M0 CCL18 secretion upon IL13/IL4 treatment.

### 5.6. Discussion

The pretreatment with IFNg have been shown to partially overcome macrophage hyporesponsiveness to a secondary dose of LPS in tolerant macrophages through blocking tolerance mediated suppression of TNF-a and IL-6[59]. Additionally, IFNg was shown to prolong macrophages response to LPS rescue phagocytosis[60]. So, we tested if what was found to rescue tolerance still holds true in the case of exhaustion. We hypothesized that IFNg may rescue macrophages exhausted response to LPS induced by chronic LPS treatment.

Unexpectedly, IFNg was ineffective at rescuing the inflammatory response of chronically stimulated groups characterized by their TNFa secretion. IFNg pre-treatment impaired the response of acutely stimulated macrophages to IL13/4 by decreasing the CCL18 and PDGFBB secretion (Figure 19; Figure 20; Figure 22; Figure 23).

We speculate that IFNg treatment did not rescue the response of exhausted macrophages to LPS because macrophages exhaustion maybe maintained by other negative

suppression mechanisms that is different from the ones maintaining tolerance. However, it impaired the secretion of M2 associated proteins because of its STAT3 suppressive roles[63]. Future studies needs to investigate the mechanisms that mediates macrophage exhaustion and possible treatments to overcome it.

The delivery of allogenic macrophages has been shown to improve chronic wounds' healing[99]. Those allogenic macrophages are obtained from healthy and young people [99]. However, due to the risks associated with cellular therapies, developing methods to rescue endogenous exhausted macrophages will open the door for therapies that harness autologous macrophages via restoring their proper responsiveness.

## 6. Limitations

Some limitations of this current work include the limited panel of proteins screened to understand macrophages behavior. In future studies we aim to use a greater panel of proteins and gene expansion analysis to gain a better understanding of the impact of chronic stimulation on macrophage responsiveness to pro-inflammatory and anti-inflammatory stimuli. Moreover, we hope to test the impact of macrophages chronic exposure to other pro-inflammatory stimuli that are common in some chronic diseases including TNFa and hyperglycemia. Last, we aim to further understand the mechanisms of macrophage exhaustion to enable the development of targeted strategies to rescue their response.

## 7. Conclusions

In conclusion, our work suggests that macrophages chronic exposure to a high or low doses of LPS leads to macrophage exhaustion characterized by a hyporesponsive behavior to subsequent pro-inflammatory stimulation. Importantly, acute and chronic LPS stimulation enhanced their ability to polarize to the M2 phenotype.

In each aim respectively, we showed that:

1) Regardless of the starting time of stimulation, chronic LPS treatment impaired macrophages TNFa secretion in response to fresh dose of LPS.
2) Acute macrophages stimulation using a low dose of LPS does not impact macrophages response to IL13/4. However, stimulating macrophages acutely with high LPS dose impairs macrophages response to IL13/4.Also, chronic macrophage stimulation with LPS enhanced their M2 protein secretion in response to IL13/4.
3) Pre-treating chronically stimulated with IFNg, did not rescue their TNFa secretion. However, it enhanced M0 macrophage CCL18 secretion and inhibited PDGBB secretion of all macrophage groups.

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