ROLE OF THE IRE/XBP-1 PATHWAY IN CIGARETTE SMOKE AFFECTED MACROPHAGE POLARIZATION *IN VITRO*

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Declaration of Academic Achievement

With the assistance of James Murphy I was able to conduct experiments for Figure 6-17, where I planned and analyzed the data. For Tables 2 and 3 along with Figures 15- 17, Anna Dvorkin-Gheva assisted in nCounter and R statistical analysis, where I interpreted data and designed experiments.

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Lay Abstract

Cigarette smoke exposure damages the lungs and over time places the user at risk for increased infections, progressive decreases in lung function and cancer.

A specific cell of the immune system and found in the lungs, macrophages or "Big Eater" cells, responds first by picking up debris and responding to harmful foreign substances by releasing proteins signaling the immune system to become activated.

Within all animal cells, an organelle called the Endoplasmic Reticulum (ER) manufactures a third of proteins produced allowing the cell to adapt to foreign substances, including cigarette smoke. Cigarette smoke could cause the ER, a plastic organelle, to change in size and function at a heightened level due to activation of a sensing protein integrated in the ER, Inositol Requiring Enzyme-1 (IRE-1).

Both activation of the ER and cigarette smoke causes macrophages to behave as "tissue-healing" or M2 subsets that release factors promoting reconstruction of the lungs; alternatively, M1 macrophages fight diseases and promote further inflammation. Using genetic analysis of macrophages exposed to cigarette smoke in culture dishes and analyzing the proteins secreted, we determined cigarette smoke inhibits M1 macrophages and the "tissue-healing" subset, while increasing adhesion molecule expression.

Overall, cigarette smoke affected the polarization of M1 and M2 phenotype, analyzed through proteins and genes expression. We observed an increase in sXBP-1, indicative of IRE-1/XBP-1 pathway activation, from cigarette smoke extract exposure in macrophages. However, the use of IRE-1 inhibitors increased ER stress markers while affecting M1 and M2 markers. This suggests ER compensation from the use of inhibiting one arm of the ER stress response.

Abstract

Cigarette smoke contributes to 90% of lung cancer cases and 80% of COPD cases [1-4]. These concerns loom large as lung cancer represents 13% of all cancer deaths and estimates report by 2020 COPD will be the third leading cause of death in the world [5, 6]. The master regulator of the ER stress response, IRE-1, in the context of cigarette smoke exposure lacks study. Interestingly, its downstream pathways are activated [7, 8]. In fact, the 2014 Surgeon General's report on the health consequences of smoking highlighted the endoplasmic reticulum (ER) stress response as a potential mechanism leading to the development of lung cancer and Chronic Obstructive Pulmonary Disorder (COPD) [1].

Following acute cigarette smoke exposure, mouse lung homogenates exhibited increased levels of XBP-1 along with downstream mediators of IRE-1 activation— GRP-78 and CHOP. Specifically observing macrophages, an important immune cell and source of acute inflammation, cigarette smoke induced activation of IRE-1/XBP-1 pathway through splicing of XBP-1 mRNA. However, upon assaying for pro-inflammatory cytokines we were unable to determine that cigarette smoke directly caused inflammation *in vitro*. Furthermore, cigarette smoke inhibited the activation of M2 macrophages, an anti-inflammatory and tissue healing subset seen through CCL18 inhibition.

A majority of M2 and M1 macrophage markers were decreased from IRE-1/XBP-1 inhibition. This suggests a different phenotype than classical M1 or M2 polarization being induced by cigarette smoke. In addition, it suggests the IRE-1/XBP-1 pathway having a robust role in controlling gene expression and balance of cellular proteomics.

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Chapter 1 – Introduction

Endoplasmic Reticulum

History

The endoplasmic reticulum (ER) was discovered in 1902 in eukaryotes— well after discoveries of other cellular organelle such as the mitochondria, nucleus and golgi apparatus [9]. Discovered by Emilio Veratti, who observed a subcellular organelle with differential staining compared to the golgi, it initially did not take hold of the scientific community's interest [9]. Later in 1945, with the advancements of electron microscopy, Porter, Claude and Fullam—using ground up cellular lysates—visualized a "lace" like structure within the cell; hence, naming it the endoplasmic reticulum, translating to "within the cytoplasm" and "little net" [9-11]. The cellular organelle consisting of a smooth and rough component, where the latter is associated with bound ribosomes, became better defined as the field of electron microscopy grew [12, 13]. Up until the 1960s the best descriptions of the endoplasmic reticulum came from three cell types:

1) Plasma cells, differentiated B-cells with a high secretory capacity, producing thousands of antibodies per second by holding expansive smooth and rough ERs [10, 14].

2) Acinar cells of the pancreas, with robust rough ER, capable of storing granules and releasing mucin [10].

3) Neurons with Nissl bodies, which are groups of ER with rosettes of ribosomes that associate with the soma and dendrites, releasing neurotransmitters and integrating intracellular lipids and protein [10].

At that point in time the idea of the ER acting as an "intracellular skeleton", that provided cellular structure and a site for protein synthesis, was generally accepted [15]. In the 1970s, work by Palade outlined greater sophistication of the organelle machinery

stating the ER initiated secretion of proteins and postulated that a signal must be required for secretion, from his observations in Acinar cells [16]. Later, Porter determined the specific signal required for the integration of nascent protein into the ER [13, 17]. However, since these findings, numerous more ER processes have been discovered including calcium storage, intracellular protein trafficking, lipid production, signal transduction and gene regulation [15].

Cells that undergo differentiation require endoplasmic reticulum expansion to be capable of meeting heightened metabolic needs [7, 15]. The functions of the ER are mainly to maintain proteomic balance within the cell, making it an essential organelle for eukaryotic cells to function [18]. Commonly known as the "warehouse" of the cell, it facilitates over 30% of the cells protein maturation and is a major lipid biosynthesis component [18]. However, the ER maintains connections with different parts of the cell, remaining proximally close to the nucleus [10]. The connection with vesicles, mitochondria and golgi systems highlight the ER as a central site of the protein and lipid trafficking system [15]. Given these points, cellular adaptation machinery responding to environmental and genetic changes occurs through the ER stress response [18].

The Unfolded Protein Response (UPR)

The cell constantly surveys its environment, adapting to changes [18]. These changes can be caused by, but not limited to, activation of oncogenes or loss of tumour suppressor gene, heat shock, viral infections, wound healing, inflammation, extracellular and intracellular nutrient and ion imbalances [19-21]. The endoplasmic reticulum is a dynamic organelle allowing for these changes to manifest and a focal point of cellular activity [15, 22]. Changes to the ER occur in seconds, based on cisternae and tubule

formation, demonstrating the plasticity of the ER [15]. The increased ER function allows a greater accommodation of protein and lipids; thus, increasing production and cellular activity [19-21, 23]. Common changes associated with the endoplasmic stress response are increases in chaperone availability, ER capacity and recycling of misfolded protein [18, 21, 23]. Upon endoplasmic reticulum stress response activation, GRP-78 preferentially binds to misfolded protein and disassociates from the three transmembrane domains: Activating Transcription Factor 6 (ATF6), Protein kinase R-like Endoplasmic Reticulum Kinase (PERK) and Inositol requiring enzyme 1 (IRE-1) [24, 25]. A representation can be seen in Figure 1 below:

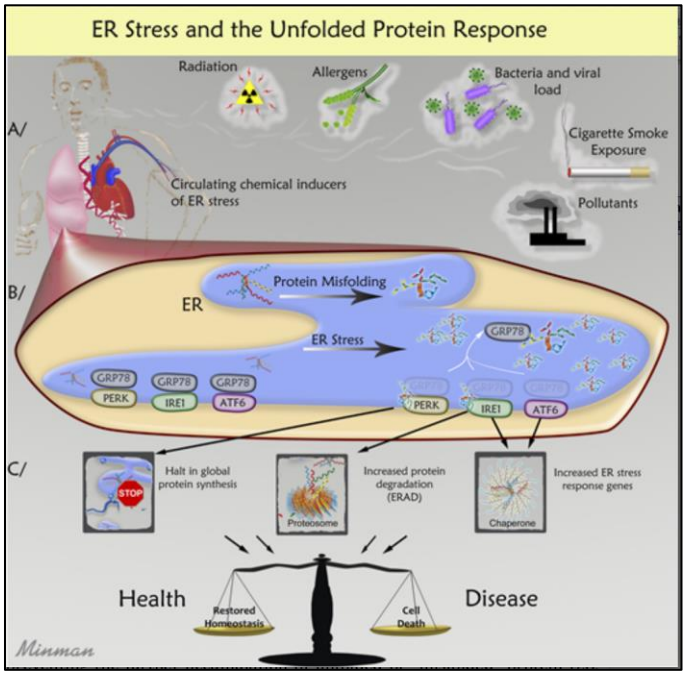

Figure 1: A representation of ER stress and the UPR in the lungs, identifying that (A) exposure to airborne xenobiotics causes (B) unfolded protein responses that leads to (C) cellular adaptions by GRP78 and the three axes of the ER stress response: PERK activation, IRE-1 phosphorylation and ATF-6 golgi translocation [26]. These processes help maintain cellular homeostasis controlling balance between health and disease or life and death.

Specifically, PERK leads to EIF-2alpha activation and upregulation of Endoplasmic Reticulum Stress Elements (ERSE) in DNA [27]. These downstream factors initiate cell death factor CCAAT/Enhancer-binding Protein Homologous Protein (CHOP) and translational attenuation, preventing the further accumulation of unfolded or "misfolded" protein [27, 28]. ATF-6, a transcription factor, undergoes proteolysis in the golgi further activating the ERSE, transcribing chaperones for protein folding [28]. IRE-1, an evolutionary conserved eukaryotic protein found integrated in the ER membrane has kinase and endonuclease activity [24, 29-33].

IRE-1/XBP-1 pathway activation: Role in ER stress and cellular adaptation

IRE-1 activation specifically acts as a master regulator of the ER stress response [7, 28, 34-38]. The kinase activity interacts with downstream pathways JNK and TRAF2 which can control cell survival, homeostasis of calcium signaling coupled with the mitochondria and inflammation [24, 30].

Conventionally, splicing of mRNA occurs exclusively in the nucleus with use of a spliceosome, a complex of protein and RNA [29, 30, 33, 39-43]. However, upon disassociation with GRP-78, due to higher affinity for unfolded protein, freed IRE-1 can undergo autophosphorylation, homodimerize and confer specific cytoplasmic ribonuclease activity [24, 29, 44, 45]. X-ray crystallography of activated IRE-1 demonstrates protein autophosphorylation at ser724 in the amino acid chain [29, 30, 36]. In addition, dynamic clustering, through the G547 amino acid, is necessary for the RNase activity but not sufficient— phosphorylation significantly increases RNase activity by stabilizing the resultant dimer [46].

Activation of ribonuclease activity in the cytosol splices XBP-1 RNA [47-50]. The spliced variant (sXBP-1) produced from IRE-1 RNase activity is then ligated through catalysis from RtcB RNA ligase— independent of the spliceosome [51-53]. The production of sXBP-1 both requires IRE-1 activation and tRNA ligase catalyzed by RtcB [29, 30, 33, 46, 50]. Deletion of RtcB halts differentiation of B-cells into plasma cells by preventing sXBP-1 production [51, 52]. The two-step cytoplasmic splicing event results in removal of a 26nt intron and subsequent ligation of the RNA— translating to a basic leucine zipper transcription factor 54kda in size [49, 51, 54-56]. Previous reports outlined the unspliced variant acting as negative feedback for the spliced variant by allosterically inhibiting the nuclear localization signal [57]. Normally, the unspliced XBP-1 protein is rapidly degraded; however, scientists utilizing unsplicable XBP-1 demonstrated similar activity to sXBP-1 with decreased effectiveness [57-59].

The product of xbp-1 splicing, a 54 kda basic leucine zipper transcription factor, specifically activates promoter regions such as UPR element (UPRE) and ER stress-response elements I and II (ERSE-I and ERSE-II) [31, 32, 60]. These factors are involved in cellular survival along with increased chaperone production for increased protein folding [61, 62]. Furthermore, sXBP-1 alleviates ER stress through degradation of misfolded protein, the expansion of the endoplasmic reticulum through lipid production, membrane formation and increased ER function through production of chaperones for protein folding [7, 37, 38, 42, 63-65]. A list of numerous genes regulated by sXBP-1 transcription factor is seen in the table below relevant to our investigation:

Table 1: Select downstream genes affected by sXBP-1 transcription factor activity		
Genes	**Function**	**Reference**
EDEM	Protein degradation	[66-68]
$p58^{IPK}$	Negative regulator of UPR	[66, 69]
ERdj4	Protein degradation	[66]
PDI-P5	Chaperone	[66, 70, 71]
Ramp4	Facilitates protein glycosylation and interacts with nascent protein entering ER	[66]
HSP40	Chaperone	[66]
mgat2	Golgi enzyme converting oligomannose to n-glycans	[66]
XBP-1	Transcription factor	[66]
MGP	Binds calcium ions	[66, 72]
Splicing factor, arginine/serine-rich 1	Control splicing in nucleus, involved in proliferation and cell death	[66, 73]
Solute carrier family 2, member 1	Glucose transporter for blood-brain barrier	[66, 74]
Glucosamine-phosphate N-acetyltransferase 1	Acetyltransferase	[66]
GRP78	Chaperone	[66]
nfκb	Survival signal, mediator of inflammation	[58]
TXNIP	Activates NLRP3 inflammasome	[75]
CHOP	Pro-cell death signal and caspase 11 activator	[76, 77]
defender against cell death-1	Negative regulator of cell death	[7]
SRP and SRP54	Mediates protein translocation into ER	[7]
Calnexin	Chaperone, where ATP and calcium act as cofactors	[78]
Calreticulin	Binds calcium and misfolded protein	[78]
MHC-II	Maturation of antigen presenting cells, presenting antigen to T-cells for adaptive immune response	[79]
FKBP-2, 7, 10 and 14	Family of ER chaperones with prolyl isomerase activity and act as chaperones, binds immunosuppressive drug FK506 with high affinity	[7]
Mannose Binding Lectin 2	Component of innate immune system that can activate the Lectin pathway of the complement system causing inflammation	[7]

IRE-1 activation is involved in RNA degradation, through the process denoted Regulated IRE-1 Dependent Decay (RIDD), aiding cell survival and further alleviating protein load through controlling genetic expression at the pre-translational stage promoting homeostasis [43, 80]. However, the broad range destruction of ER-resident pre-micro, micro and messenger RNA (pre-miRNA, miRNa and mRNA, respectively) leads to lowered antibody secretion in B-cells and NLRP3 inflammasome and caspase-2 activation promoting cell death; thus, the reduction or absence of XBP-1 promotes the RIDD pathway [43, 80]. However, a study by Sun et al. (2016) observed pre-miRNA's degraded by RIDD promoting apoptosis independent of XBP-1 status [81].

Pharmaceutical manipulators of IRE-1 activation have been discovered and tested *in vitro* and *in vivo* [62, 82]. An imine based compound, STF-083010, was found to irreversibly inhibit splicing of XBP-1 by selectively binding to the active site of the ribonuclease, through K907— inducing selective cell death in multiple myeloma cells [41, 82]. Another ribonuclease inhibitor, 4μ8C— a coumarin based molecule, reversibly binds to the K907 by Schiff base formation with its aldehyde moiety [41]. In addition, it inhibits the K599 amino acid, competing with ADP, to inhibit the kinase activity [41]. Other inhibitors, APY29 and suntinib, inhibits phosphorylation preventing kinase activity but causes XBP-1 splicing— while Compound 3 halts IRE-1 phosphorylation and subsequent kinase and endonuclease activity [34, 37, 83].

There are two isoforms of IRE-1, the alpha form found ubiquitously within cells and the beta form which is limited to the airway and intestinal epithelial cells [84, 85]. Thus, there is applicability to multiple cell types and processes. These include the differentiation of macrophages, plasma cells, eosinophils, osteoblasts and osteoclasts, adipocyte

development and myelin production [86-92]. Interestingly, the anti-tumour responses from CD8+ dendritic cells requires sXBP-1, however, constitutive expression of sXBP-1 leads to impaired anti-tumor responses caused by accumulation of lipids [47, 55, 93]. Thus, there must be balance. Other than protein and lipid production, the ER also acts as a calcium store in non-muscular cells [15]. The extracellular release of calcium is paramount to the initiation of the ER stress response [15, 94, 95].

IRE-1 activation & Mitochondria cross talk

The mitochondria and ER maintain an intimate link of communication through forming a Mitochondrial Associated Membrane (MAM), mediated through calcium signaling [24, 96-99]. The MAM uses the IRE-1/XBP-1 regulated Insp3R calcium channel and upregulated chaperones for communication [48, 100]. The cross talk between ER and mitochondria occurs through oxidative stress governed by XBP-1— the calcium efflux leads to activation of mitochondria to produce ROS [48].

Increased ROS production in XBP-1 KO mouse embryonic fibroblasts (MEFs), administered hydrogen peroxide and arsenic trioxide, demonstrate increased susceptibility to mitochondrial membrane potential damage and oxidative stress induced cell death [101]. A similar loss in redox homeostasis was observed in XBP-1 knock down MEFs; however, oxidative stress resolved from XBP-1 upregulation [101].

ER-Golgi network: Potential control by IRE-1/XBP-1 pathway

Another organelle intimately tied with ER cellular function is the golgi, where together they act synchronously to secrete and integrate protein through an intricate trafficking system [15]. Computational analysis suggests that ER signal transduction affects the functions of the golgi [102]. There are secretion deficiencies affecting growth and

development in XBP-1 deficient mice, leading to death at birth [103]. Understanding the ER-golgi network could provide insight to the mechanism of disorder development.

After synthesis of mRNA transcripts and its subsequent exit from the nucleus, free and bound ribosomes translate RNA transcripts into protein [12, 13]. Afterwards, only nascent protein with the signal recognition protein (SRP) signal enter into the ER [12]. Glycoproteins undergo n-linked glycosylation and are further processed for ER-golgi secretion or integration into membranes [104]. Anterograde transport from the ER to the golgi requires soluble NSF attachment protein receptors (SNAREs) to load vesicles for transport [105]. These vesicles mediate their retrograde (golgi to ER) and anterograde (ER to golgi) transport using actin and microtubule filaments with kinesin and dynein motors, respectively [106, 107]. The overall process once membrane fusion has occurred is irreversible and thus tightly regulated as these processes are involved in secretion, phagocytosis and integrating proteins into the membrane [108-110].

The ER-golgi network assists in phagosome production and antibacterial killing. Specifically, the IRE-1/XBP-1 pathway is necessary for ROS mediated bactericidal killing of bacteria but not sufficient, it requires the use of SNARE protein sec22p [111]. The ER-golgi system mediates ROS accumulation into the phagosome allowing for effecting killing of Methicillin Resistant Staphylococcus Aureus (MRSA) [111]. Furthermore, yeast knockdowns of SNARE protein, sec24, exhibited secretory deficits with phenotype reversal with overexpression of HAC-1, the XBP-1 homolog in yeast [105]. Macrophage mediated *Brucella* killing has also been identified as a role of IRE-1 activation through interacting with YIP1a [112]. Thus, IRE-1/XBP-1 pathway increases sec24 and other accessory

proteins in coat protein complex II (COPII) mediated anterograde transport during a stress response [105, 112, 113].

The importance of the COPII mediated transport is essential for ER produced proteins for integration and secretion [104]. COPII mediated transport allows for integration of membranes, cytokine secretion as described in macrophages and as previously mentioned, plasma cells for the secretion of antibodies [56, 104]. Thus, the ER-golgi network allows for destruction of material phagocytized by macrophages, suggesting an involvement of the IRE-1/XBP-1 pathway in breakdown of oxidized lipids and tar from cigarette smoke has not been studied.

IRE-1/XBP-1 pathway implications in survival and disease pathology

Studies of XBP-1 polymorphisms demonstrate positive correlations between activation of IRE-1/XBP-1 pathway and disease phenotypes, such as multiple myeloma, Alzheimer's, schizophrenia, personality disorders, ischemic stroke and hyperhomocysteinemia [114-121]. The wide spectrum of diseases may account for the multiple roles IRE-1/XBP-1 pathway has in cellular homeostasis. For example, IRE-1 helps control the balance between life and cell death [49, 55]. Specifically, activation of IRE-1 upregulates the NFκB response, a survival signal [58, 122]. However, other downstream responses from the UPR and the IRE-1/XBP-1 pathway cause cellular adaptations: alleviating the stress of misfolded protein, aiding in metabolic processes in the mitochondria and bolstering cell membranes via the ER-golgi axis [54].

In terms of proliferation, cancer cells can utilize the UPR for their advantage, specifically the IRE-1/XBP-1 pathway [58, 123]. Activation of the IRE-1/XBP-1 pathway was attributed to loss of p53 expression, a tumour suppressor, through accumulation of

misfolded protein [124]. Furthermore, it was determined p53 degraded IRE-1 through proteasome activation and subsequent tumour growth significantly reduced in p53 knockout tumours treated with STF-083010 *in vivo* [124].

Other forms of cancer cell death are seen in plasma cell related cancers, that require sXBP-1 for antibody secretion; thus, multiple myeloma and chronic lymphatic leukemia have been the main target of IRE-1/XBP-1 inhibitors inducing cell death [82]. In addition, targeting dopaminergic neurons prevented Parkinson's disease related neurotoxins that are produced by surviving neurons, suggesting applications to the brain as well [125]. The well-studied XBP-1 -116(G/C) polymorphism, homozygous for guanine, has been implicated in obesity and stroke, outlining a need to understand the mechanisms of ER stress in disease pathology [120].

Another pathway important in regulating cell cycle, mTOR has overlap with the UPR [126]. An inhibitor of mTORC1, an mTOR pathway kinase, prevented splicing of XBP-1 in the liver and prevented lipogenesis, where sXBP-1 induces the differentiation of adipose (fat) cells [90, 127, 128]. However, Benhamron et al. (2015) postulated that there were important cell energetic profiles that were induced independent of XBP-1 and under normal conditions would not be activated, including plasma cell differentiation [126]. Another report shows mTOR overexpression compensated for the loss of XBP-1 splicing allowing for plasma cell maturation, addressing the metabolic changes caused by IRE-1/XBP-1 deletion [126].

Wnt signaling, involved in proliferation, affects IRE-1/XBP-1 activation [129]. ER stress has been implicated in the activation of Wnt signaling and specifically negative

regulation via sXBP-1 [129, 130]. Thus, understanding the direct effects of sXBP-1 could help understand the processes that overlap with its regulatory functions in the cell.

Along with unfolded protein, downstream inflammatory pathways are activated from the IRE-1/XBP-1 pathway. Activation of the IRE-1/XBP-1 pathway acts to produce IL-6 and TNF-alpha and activate the NLRP3 inflammasome cleaving precursor cytokines to their mature form (IL-1beta, IL-18 and IL-33) [62, 131-134]. In terms of viral infection, IRE-1 activation occurs in the context of influenza virus infection, the virus uses the machinery of the UPR to produce virions [135]. In addition to the processing of cytokines for secretion, XBP-1 also binds to IRF3 involved in type I IFN production for viral defense [136-138].

The IRE-1/XBP-1 pathway also activates assembly of the NLRP3 inflammasome [133]. The inflammasome is responsible for the cleavage of caspase 1 and caspase 11 leading to the production of activated forms of IL-1beta, IL-18 and IL-33 [139]. In addition, activation of the inflammasome led to pyroptosis, a caspase independent form of cell death in which factors such as HMGB1 are released, causing further inflammation [139]. Inhibiting the NLRP3 inflammasome prevented secretion of these mature cytokines for secretion [62].

The Macrophage or “Big Eater”

Macrophage development and function

Macrophages, arising from bone marrow and myeloid precursors, are a diverse resident and infiltrating cell type of the innate immune system [140]. The robust cell type is found in virtually every tissue ranging from connective tissue to the liver to the lungs. They offer important innate roles in maintaining homeostasis from anti-microbial functions to

wound healing [141]. These roles, depending on the stimulus, are mediated through secretion of inflammatory cytokines or through phagocytosis. [104, 141]. Macrophages upon infection act as first responders, clearing pathogens upon arrival; ultimately, preventing the need for a further response. If needed, macrophages produce a chemotactic gradient allowing for a more robust innate and adaptive response [141].

In addition, to dispel the damaging inflammatory response, macrophages clear debris and promote an end to the cycle using anti-inflammatory cytokines and producing tissue healing factors [140-142]. The study outlined a positive trend towards atherosclerosis—also with macrophage polarization playing a role in pathogenesis [120]. Thus, to further our understanding of different macrophage polarization subsets and their role in disease pathogenesis future treatments are able to be discovered.

Alveolar macrophages are commonly differentiated during fetal development and among that pool of cells they regenerate and replicate naturally [141]. They are different than infiltrating macrophages that arrive during lung insult as monocytes and differentiate once entering tissue [141, 142]. Alveolar macrophages are long-lived and express different surface receptors that allow them to interact with type II epithelial cells [143]. Given their role in waste removal, these macrophages are associated with an M2 phenotype at steady-state, with abilities to stimulate tissue regeneration [142, 144, 145]. The association of latent TGF-beta with the cells means it becomes activated during tissue regeneration [143]. These cells compose of 90% of airway macrophages and mainly are involved in dead cell recycling otherwise known as efferocytosis [144]. The surfactants produced by type II epithelial cells are used for bacteriocidal killing and if the functions of these macrophages are reduced then bacterial colonization may occur more readily [146]. These causes are a

main inducer of acute exacerbations of COPD which lead to the heavy burden of long-term hospital visits on patients and health providers and mortality in patients [147]. Alveolar macrophages in particular are very phagocytic in their sentinel cell ability; thus, their oxidative burst to dispose of pathogens and debris is also heightened compared to infiltrating monocytes [144, 148-150]. These phagolysosomes which destroy debris and pathogens require the ER to traffic to lysosomes [148].

Macrophage Polarization: A role for IRE-1/XBP-1

Currently, macrophages are defined as classically activated (M1) or alternatively activated (M2) macrophages, respectively [141]. More recently, other classifications of macrophage polarization have been suggested within disease pathology [151-153]. ER stress has been identified to specifically activate an M2 macrophage polarization [154, 155]. Based on the IRE-1/XBP-1 pathway able to control the ER stress pathway, the connection between the splicing of XBP-1 and macrophage M2 polarization would be plausible. In fact, the study completed by Oh et al. (2011) pointed towards JNK-PPARγ pathway, where IRE-1 activation promotes the JNK pathway through its kinase activity, however, the role of the ribonuclease activity remains unstudied [154, 156]. Furthermore, M2 macrophage activation was downregulated by PBA a chemical inhibitor of ER stress through its chaperone ability.

A summary of the differences between the M1 and M2 macrophages are demonstrated in the image below:

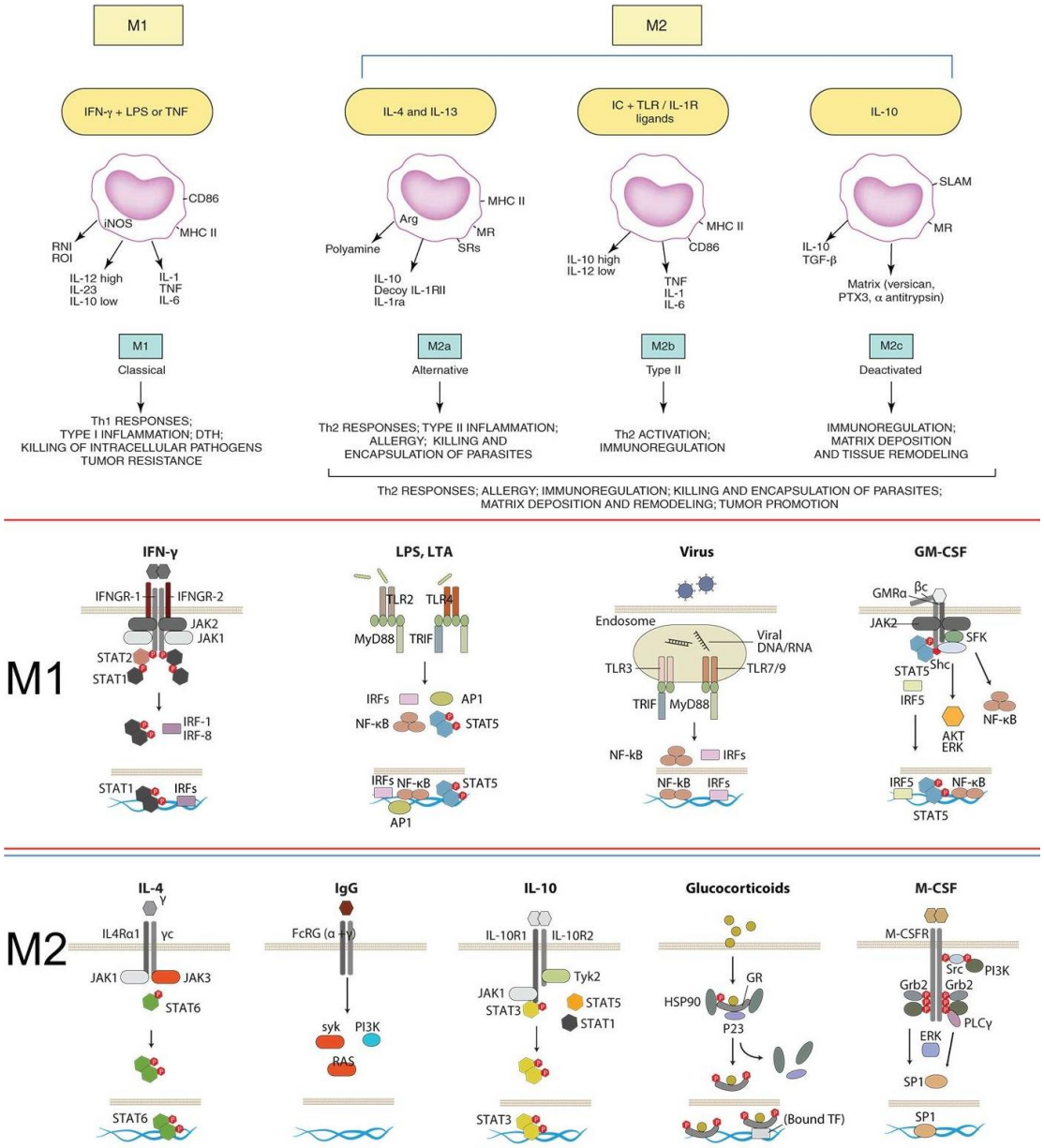

Figure 2: An outline of the in vitro models of M1/M2 macrophages and markers, from: [157]. An MØ macrophage may be polarized when administered different polarizing mixtures can become an M1 macrophage and different subsets of M2 macrophages, M2a (alternatively activated macrophages), M2b (Type II macrophages) and M2c (Deactivated macrophages). Different polarized states are associated with different behaviour to *in vitro* stimuli with phenotypes observed in vivo systems as well.

The Faculty of 1000 called for reassessment of the M1/M2 polarization definitions based on the vast number of conditions that give rise to various polarized M1 and M2 macrophages [157]. A selected list of M1 and M2 markers can be observed in Table 2 including secreted protein and surface markers utilized within this study. As previously noted, M1 (classic) macrophages use ROS mediated killing of bacteria, antibody dependent cell cytotoxicity (ADCC), inflammatory cytokine production and initiation of the adaptive immune system [111, 157, 158].

The release of cytokines, IL-1, IL-2, IL-6, IL-8, IL-15, IL-18 promote inflammation and allow for a transition to an adaptive immune response characterized by a T-helper 1 response – activing CD8+ Killer T-cells [141]. IL-2 and IL-15 are involved in the maturation of lymphocytes and activating them towards a T-helper 1 response for intracellular pathogens, including viruses and bacteria [159]. IL-6 produces acute phase proteins involved in complement activation leading to increased chemotaxis of innate immune cells [159]. IL-1 further promotes inflammation and involves increased chemotaxis of macrophages to the site of inflammation. IL-8 has chemotactic properties that allows for the infiltration of neutrophils via higher integrin expression leading to extravasation into lung interstitium and alveoli [159]. Additional markers for the M1 macrophage include production of Nitric Oxide (NO) (from the iNOS enzyme) and Reactive Oxygen Species (ROS) (Figure 2) that act to destroy intracellular pathogens within vesicles [112].

Alternatively, M2 (healer) macrophages mainly clear debris through phagocytosis and promote tissue healing with expression of Mannose Receptor (MR) and Scavenger Receptors (SR) [157]. These cytokines released include TGF-beta, CCL18, IL-10 along with autocrine production of IL-4 and IL-13 [157, 160, 161]. IL-10 and TGF-beta suppress the

inflammatory response denoted by the cytokines outlined above. The CCL18 chemokine released by M2 macrophage acts a chemotaxis agent for macrophages and T-cells [162]. The subsets involved in M2 macrophage polarization may involve increased activity based on the cytokine milieu promoted *in vitro*, such as IL-4 and IL-10 with IL-6 that produces a high amount of arginase [152]. Other macrophage subsets differentiated with Immune Complexes (ICs) or IL-10, M2b and c respectively, demonstrate different secretions [157]. M2b are involved mainly with immune regulation causing the immune response to become dampened while M2c release matrix remodeling proteins such as MMP9, IL-10 and TGF-beta (Figure 3).

Protein or Gene Abbreviation	Protein Name	Type of Marker	Reference
EGF	Epidermal Growth Factor	M2	[163]
FGF-2	Basic Fibroblast growth factor	M2	[163, 164]
TGF-a	Transforming growth factor alpha	M2	[165]
G-CSF	Granulocyte colony-stimulating factor	M1/M2	[166, 167]
GM-CSF	Granulocyte macrophage colony-stimulating factor	M1/M2	[157, 167]
CX3CL1 (Fractalkine)	Fractalkine or chemokine C-X3-C motif ligand 1	M1	[168]
IFNa2	Interferon alpha-2	Mixed	[169]
IFNy	Interferon gamma	M1	[170]
CXCL1or GRO alpha	Growth-related oncogene-alpha or chemokine C-X-C motif ligand 1	M1	[171]
CCL2 (MCP-1)	Chemokine (C-C motif) ligand 2 or Monocyte Chemoattractant protein-1	M1	[172-174]
CCL3 (MIP-1a)	Macrophage inflammatory protein-1a	M1	[175]
CCL4 (MIP-1B)	Chemokine C-C motif ligand 4 or Macrophage inflammatory protein beta (MIP-1B)	M1	[176]
CCL5 (RANTES)	Chemokine C-C motif ligand 5 or Regulated on activation, normal T-Cell expressed and secreted (RANTES)	M1	[164]
CCL7 (MCP-3)	Chemokine C-C motif ligand 7 or Monocyte-chemotactic protein 3	M1	[171]
CCL11 (Eotaxin-1)	Chemokine C-C motif 11 or Eotaxin-1	M2	[177]
CCL18	Chemokine (C-C motif) ligand 18	M2	[173, 178]
CCL22 or MDC	Chemokine C-C motif ligand 22 or Macrophage Derived Chemokine	M2	[179]
PDGF-BB	Platelet-derived growth factor BB	M2	[180]
sCD40L	Soluble cluster of differentiation 40 ligand	M1	[181]
IL-1RA	Interleukin-1 receptor antagonist	M1/M2	[162]
IL-1a	Interleukin-1 alpha	M1	[182]

IL-1b	Interleukin-1 beta	M1	[172]
IL-2	Interleukin-2	M1	[183]
IL-3	Interleukin-3	M2	[184]
IL-4	Interleukin-4	M2	[171]
IL-5	Interleukin-5	M2	[185]
IL-6	Interleukin-6	M1	[171]
IL-7	Interleukin-7	M1	[178]
IL-8	Interleukin-8	M1	[157]
IL-9	Interleukin-9	M2	[186]
IL-10	Interleukin-10	M2	[172] [187]
IL-12P40	Interleukin-12 subunit 40	M1	[188]
IL-12P70	Interleukin-12 subunit 70	M1	[188]
IL-13	Interleukin-13	M2	[171]
IL-15	Interleukin-15	M1	[178]
IL-17A	Interleukin-17A	M1	[189]
IL-18	Interleukin-18	M1	[190]
TNFa	Tumour necrosis factor alpha	M1	[164, 188]
TNFB	Tumour necrosis factor beta	M1	[169, 191]
CD14	Cluster of Differentiation 14	M2	[174, 192]
CD163	Cluster of Differentiation 163	M2	[181]
CD68	Cluster of Differentiation 68	---	[174]
CLEC10A	C-Type Lectin Domain Family 10 Member A	M2	[193]
COL1A1	Collagen Type I Alpha 1 Chain	M2	[173]
COL3A1	Collagen Type III Alpha 1 Chain	M2	[173]
CXCL10 (IP-10)	C-X-C motif chemokine 10 or Interferon gamma-induced protein- 10	M1	[178, 179]
FN1	Fibronectin 1	M2	[172]
IL1RL1	Interleukin 1 Receptor-Like 1	M1	
IL33	Interleukin-33	M2	[194]
IL4R	Interleukin-4 Receptor	M2	
ITGAM (CD11b)	Intergrin Subunit Alpha M or Cluster Differentiation 11b	M2	[190]
ITGAX (CD11c)	Integrin, alpha X (complement component 3 receptor 4 subunit) or Cluster Differentiation 11c	M1	[195]
MRC1	Mannose Receptor C 1	M2	[172]
MSR1	Macrophage Scavanger Receptor 1	M2	[172]
NOS2 (iNOS)	Nitric Oxide Synthase 2 or inducible nitric oxide synthase	M1	[162]
OSM	Oncostatin-M	M1	[196]
OSMR	Oncostatin-M Receptor	M1	[196]
TGFB1	Transforming growth factor beta 1	M2	[188]
VEGFA	Vascular endothelial growth factor	M1/M2	[169]

Table 2: Summary of M1 and M2 macrophage markers at the protein and surface receptor expression level with referenced findings.

Thus, focusing primarily on the functions of these macrophages along with differences in cellular physiology (Figure 2) can better define these macrophages. The IRE-1/XBP-1 pathway could be involved in many of these functions, as previously stated, such

as phagocytosis, ROS mediated killing and secretion of cytokines based on cellular physiology.

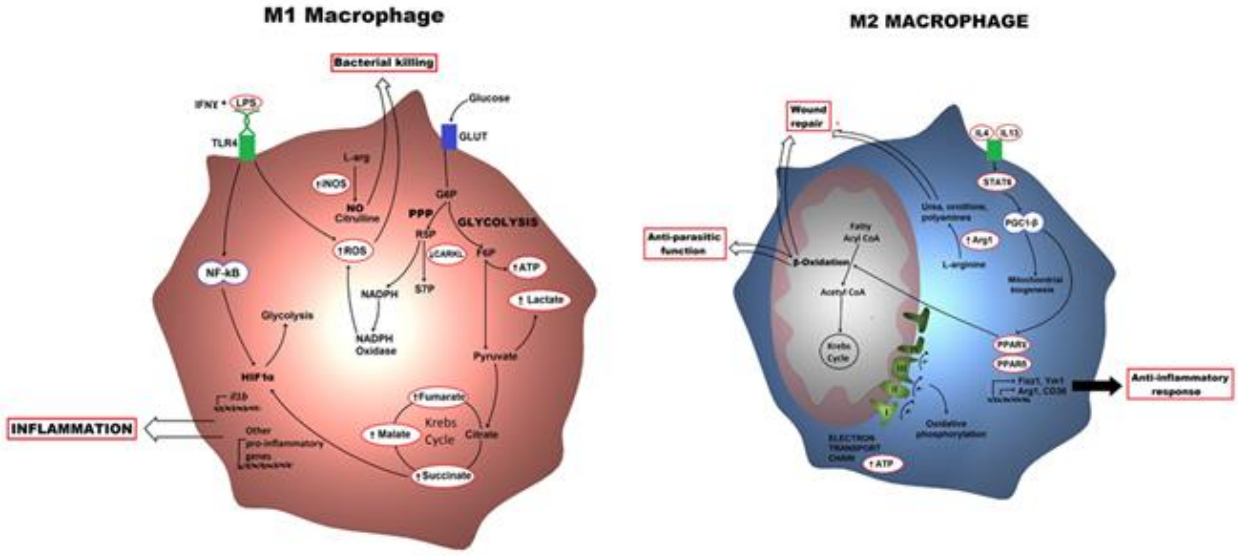

Figure 3: Biochemical differences between M1 and M2 macrophages, from: [197]. The differences between macrophage polarization states can be separated with a glycolytic metabolic pathway for M1 and oxidative phosphorylation for M2.

Cigarette smoke induced ER stress and inflammation

Cigarette smoking is the highest risk factor of developing chronic obstructive pulmonary disease (COPD) and lung cancer [150, 198-201]. COPD alone will be considered the 3rd leading cause of death in 2020, while lung cancer remains an untreatable disease. For cigarette smokers, they consist of 80% of COPD patients and 90% of lung cancer patients. The threatening nature of cigarette smoke begins with its composition, it contains over 4,500 chemicals, including 10^{15} free radicals per puff, many which have immune activating and suppressive abilities [202, 203]. Nicotine and ceramides specifically act as immunosuppressive agents, while many factors in the cigarette smoke can cause lipid oxidation and oxidative stress leading to activation of ER stress responses and

inflammation from accumulating misfolded protein [202, 204, 205]. The pro-inflammatory response causes dendritic cells to activate CD4+ and CD8+ T-cells further destroying lung parenchyma [202]. This suggests the immune response to cigarette smoke is mainly inflammatory; however, there are conflicting reports.

Specifically in macrophages, cigarette smoke causes release of inflammatory cytokines, promoting neutrophil influx and expression of adhesion molecules, promoting cellular infiltration [206, 207]. As previously mentioned, macrophages collected from cigarette smoker's sputum samples have increased pro-inflammatory profiles [208]. However, human macrophages and bronchial epithelial cells show inhibition of pro-inflammatory factor, nfκb, upon *in vitro* cigarette smoke exposure, a transcription factor encoding for pro-inflammatory cytokines [209, 210]. An article in 2015 demonstrated that inflammatory cell death was driving inflammation through cigarette smoke induced cell death, via CHOP, in epithelial cells and macrophages [211]. Interestingly, in chronic smokers the identification of auto antibodies have also been found, perhaps induced by inflammatory cell death [212]. Thus, the source of inflammation from cigarette smoke requires further elucidation.

In terms of the inflammation observed in cigarette smoke *in vitro* and *in vivo* an initial inflammatory response from epithelial cells and macrophages allow for the infiltration of macrophages, neutrophils and lymphocytes to the lungs in humans and mice [1]. The release of G-CSF and IL-8 creates a gradient for extravasation of neutrophils into the airways affected by cigarette smoke indicating an M1 polarization [213]. Additionally, macrophage derived chemokine (MDC), an M2 chemokine, allows for infiltration of monocytes and innate to adaptive response transition [179]. The adaptive response from

T-cells involves the polarization of T helper 17 cells that produce IL-17 that further propagates inflammation [214]. With M1 and M2 factors playing a role in cigarette smoke pathology, the polarization of macrophages affected by cigarette smoke during infection or clearing extracellular debris requires phenotyping.

The cellular effects of cigarette smoke *in vitro* are subject to conflicting reports. Macrophages isolated from the sputum of long-term smokers have increased inflammation when stimulated with LPS [215]. In addition, within human macrophages IL-8 and TLR-4 secretion via nfkb occurs following treatment of cigarette smoke extract [206, 207]. However, their function to destroy pathogens and even respond to pathogen associated molecular patterns (PAMPs) is altered [202]. Murine macrophages stimulated *in vitro* with cigarette smoke extract exhibit reduced phagocytosis to common respiratory pathogens like *P. aeruginosa* or *S. Pneumococcus* and increased markers of M2 polarization [216, 217]. These markers of M2 polarization observed by Yuan et al. (2014) included TGF-beta, IL-10, IL-6 and CD-163 after stimulation of macrophages with 2% CSE over the course of 6 days [216]. Additionally, in THP-1 cells demonstrated reduced phagocytic capabilities from cigarette smoke in internalizing Dextran-FITC in a dose dependent manner [218]. Fu et al (2015) demonstrated the downregulation of M1 markers such as IL-12 and increase in TGF-beta, CD163 IL-10 indicative of M2 macrophage polarization [218]. These findings outline reduced M1 capabilities and point towards an M2-like phenotype.

Similar findings in COPD patients confirm the lack of phagocytic capacity of alveolar macrophages. Alveolar epithelial cells expressing “eat-me-signals”, such as phosphotidyl serine due to apoptosis, are not effectively engulfed by COPD patient lung macrophages when compared to healthy controls [144]. Seminal works completed Johnson et al. (1970),

outlined the differences in alveolar macrophage engulfment— with cigarette smoker's alveolar macrophages demonstrating a reduced capacity for phagocytosing bacteria. Additionally, they outlined the increased endoplasmic reticulum expansion and golgi vesicles, using electron microscopy [148].

Stämpfli & Anderson (2009) suggest that perhaps there is an immune profile skewing occurring from cigarette smoke exposure in macrophages that prevents clearance of apoptotic cells, furthering the tissue remodeling occurring *in vivo* [202]. A study of cigarette smoking vs. various degrees of COPD demonstrated that polarization of M1 and M2 macrophages increased as COPD severity increased compared to cigarette smoking patients [219]. Thus, the phenotype suggested would be of the deactivated M2 characteristics [160, 220]. Deactivated macrophages show impairment in their ability to produce inflammatory cytokines, reduced ability to phagocytize bacteria, indicative of classical M1 macrophage polarization and a reduced phagocytic of cellular debris [160, 180, 220, 221].

Macrophages from cigarette smoke exposed mice and COPD patients show impaired vesicle formation possibly from altered autophagy machinery [222, 223]. The direct effects of cigarette smoke may not cause classical M1 or M2 polarization and may provide a modulated macrophage polarization state that may have M1 and M2 characteristics as mentioned by Stämpfli and Hogg (2009) or may be deactivated from cigarette smoke.

The 2014 Surgeon General's report reflecting the past 20 years fighting cigarette smoke induced disease specifically outlined the important role of the ER stress response in cigarette smoke induced pathology [1]. The report cited a link between the immune system and ER stress requiring study [1]. *In vitro*, XBP-1 has been activated in epithelial cells

treated with cigarette smoke along with activation of GRP-78 and ER chaperones [224, 225]. It was shown that CHOP, downstream of XBP-1, was highly upregulated [224, 226, 227]. These suggest a change of cell physiology due to cigarette smoke potentially altering the polarization state.

Central Aim and Hypotheses

The heightened ER stress response garners interest in its cigarette smoke related effects and it could be postulated an overactive UPR could be detrimental due to cigarette smoke exposure [26, 228, 229]. **However, the activation of macrophages from sXBP-1 by cigarette smoke has not been studied.** It would be plausible that macrophages accumulating tar and lipids, induced by IRE-1/XBP-1 pathway, would be prevented from undergoing bactericidal activity because of the added cellular cargo [222, 230]. In addition, inhibiting the IRE-1/XBP-1 pathway prior to cigarette smoke exposure could prevent these changes from occurring and restore normal macrophage functions promoting misfolded protein and ER stress to reach homeostasis.

Thus, observing the involvement of the IRE-1/XBP-1 pathway in cigarette smoke induced macrophage polarization remains an elusive avenue for future treatments of cigarette smoke induced pathology leading to lung cancer and COPD, where macrophage behavior remains unclear [162, 197, 220, 231]. Thus, understanding the physiological implications of the polarization could better define the effects cigarette smoke has on macrophages [1, 202, 223].

Thus, my overall hypothesis is that macrophages exposed to cigarette smoke leads to ER stress and activation of the unfolded protein response, affecting macrophage polarization and immune phenotype. I further hypothesize that UPR

inhibition will modulate the cigarette smoke induced effects on macrophage polarization.

To test my hypothesis, I have designed three specific aims as follows:

AIM I: To establish that cigarette smoke activates the IRE-1/XBP-1 pathway

a. Is there evidence of XBP-1 splicing from cigarette smoke exposure in macrophages?

AIM II: To determine if cigarette smoke exposure affects M1 or M2 macrophage polarization

a. Does cigarette smoke modulate M1 polarization and secretion of M1-related cytokines and chemokines?
b. Does cigarette smoke modulate M2 polarization and secretion of M2-related cytokines and chemokines?

AIM III: To examine if attenuation of IRE-1/XBP-1 endonuclease activity alters cigarette smoke-mediated macrophage polarization

a. Does use of IRE-1/XBP-1 inhibitor modulate cigarette smoke effects on M1 macrophage polarization?
b. Does use of IRE-1/XBP-1 inhibitor modulate cigarette smoke effects on M2 macrophage polarization?

Chapter 2 – Material & Methods

Bone Marrow Isolation and differentiation of monocytes to macrophages

Bone marrow-derived macrophages were isolated and cultured as previously described [232]. In brief, 6-week female naïve C57BL6/J mice were euthanized and bone marrow progenitor cells from femur and tibia extracted using sterile PBS. Cells were centrifuged for 10 minutes at 1500rpm using a Beckman Coulter centrifuge (Allegra 6R rotor). The cellular pellet was re-suspended in DMEM and cultured for 7 days in 20ng/mL M-CSF media in a 15 mm petri dish. Following 7 days of culture, cells were gently lifted from the respective culture dish and re-seeded at a density of $1x10^5$ in 96 well plates.

Human THP-1 macrophage culture and differentiation

Human THP-1 monocytic cells (ATCC) were grown in HEPES buffered 1640 RPMI (containing 10% FBS, 1% PenStrep and 1% L-Glut) and differentiated using phorbol-12-myristate-13-acetate (PMA) previously described [233]. At passage 9-16, cells were centrifuged for 10 minutes at 1200rpm using a Beckman Coulter centrifuge (Allegra 6R rotor). The resultant cell pellet was re-suspended in the above media containing 100nM of PMA and seeded at a density of $1x10^5$ per well in a flat bottom 96 well plate in 100uL (for cytokine analysis) or $2x10^4$ in 96 well plates (for RNA extraction protocol). Cells were incubated at 37C at 5.0% CO2 concentration and after 48 hours these cells were denoted as "macrophage-like cells".

Polarization of Macrophages and CSE treatment

Polarization of MØ was completed using cells seeded in triplicate. M1 macrophages were given LPS with 20ng/mL IFN-γ and M2 macrophages were exposed to recombinant

IL-4 (20 ng/mL) and IL-13 (50 ng/mL) (PeproTech Canada) for 72 hours to differentiate to M2 polarized macrophages in serum-free Dulbecco's Modified Eagle's Medium (DMEM).

Cigarette Smoke Extract System

Cigarette smoke extract (CSE) was produced using a previously described method [8]. In brief, two research cigarettes (IRF4, Kentucky Tobacco Research) were smoked, through a negative pressure system, into 8mL of serum free RPMI with 1% PenStrep and 1% L-Glutamine: which was considered complete or 100% cigarette smoke extract. Room air extract was collected through running the system for 15 minutes with an unlit cigarette. Cigarette smoke was diluted in the same aforementioned media and used within 45 minutes of production.

Cytokine Assay

A multiplex assay assaying for 42 proteins (Eve Technologies) was utilized to determine the secretion in supernatants tested from human macrophages stimulated with cigarette smoke and aforementioned polarization mixtures (Appendix, Table 4).

Arginase Assay

Arginase was determined as previously described [234]. In brief, BDMS were lysed and lysates collected using Triton X-100 with protease inhibitors. After lysate extraction, the ability to convert L-arginine into urea was determined after 30 minutes. Negative control was treated with serum free media and positive control, represented by black bar, with M2 mixture with IL-6.

RNA extraction

RNA was extracted as previously described. RNA from cellular lysates was extracted and purified using Nucleospin RNA extraction kit (Macherey-Nagel). RNA concentrations

were determined using a NanoDrop spectrophotometer (General Electric). Reverse transcription was performed using SuperScript II reverse transcriptase protocol (Invitrogen).

qPCR analysis

qPCR was demonstrated as previously described. In brief, semi-quantitative gene expression analysis (ΔΔCT method) was performed using Applied Biosystems 7500 fast Real-Time PCR System using SYBR Green (Invitrogen) to evaluate the gene expression level of spliced XBP-1 normalized to 18S expression.

Nanostring analysis

Gene expression profiling using Nanostring technologies was utilized as per manufacturer's directions and previously described (NanoString Technologies) [235]. The array utilized 32 human genes and normalized for overall counts (See Appendix I, Table 4).

Statistical Analysis

Statistical significance was established using GraphPad, Prism 5.0 with one way ANOVA using Bonferroni's Multiple Comparison Test with SEM to assess the difference between groups using P value < 0.05 (represented as *) and P values <0.01 (represented as **) P value < 0.001 (represented as ***) and P value <0.0001 (represented as ****). For nanostring statistics, limma package which uses differential expression analysis via linear models. In brief, data was entered within a design and contrast matrix to be modelled using linear model for comparisons [236].

Chapter 3— Results

The overall purpose of this thesis was to examine the direct impact of cigarette smoke on macrophage immune phenotype, focusing on ER stress and the activation of the UPR.

Previously, we had examined lungs of mice exposed to cigarette smoke for 2 weeks and determined an approximate 10-fold increase of GRP78 and 3-fold increase of spliced XBP-1, indicative of a typical UPR response (Appendix, Figure 12). Although this response was observed in total lung homogenates of mice exposed to cigarette smoke for a two-week period, we hypothesized here that isolated macrophages exposed to cigarette smoke extract (CSE) *in vitro* also would lead to UPR activation.

To determine if CSE directly activates the UPR in macrophages, we exposed PMA-activated THP-1 macrophages to various concentrations of CSE. After 6 hours of exposure, the spliced form of XBP1 (sXBP1) was examined by qPCR. Using 18s as a housekeeping gene, we concluded that 10% CSE exposure induced significantly sXBP1 expression and to the same extent as tunicamycin, a well-known ER stress inducer, used here as a positive control (Figure 4). This data confirms that CSE may directly induce an activation of the UPR in macrophages.

Previously, CSE exposure alone had been shown to promote the polarization of human macrophages (PMA-activated THP-1 cells) toward the M2 phenotype, as assessed by the increase in the M2 marker CD163 after 72 hours of 2% CSE exposure [216]. This prompted us to determine if CSE exposure enhanced the conventional IL-4/IL-13 mediated M2 polarization process. Here we used various concentrations of CSE (0.5, 1, 2.5 and 5%) and examined the secretion of CCL-18 after utilizing PMA-activated THP-1 cells with the

known M2 polarizing cytokine IL-4 and IL-13 and increasing concentrations of CSE (0.5, 1, 2.5 and 5%) for 72 hours.

To confirm M2 polarization, we assessed the level of the established M2 cytokine CCL18 [187] in the supernatants after 72 hours of incubation. Consistent with our hypothesis, the M2-polarizing cocktail including IL-4 and IL-13 induced CCL18 secretion from ~200 pg/mL to ~2200 pg/mL (an 11-fold increase). To test the effects of cigarette smoke on M2 macrophage polarization increased concentrations of CSE (from 1 to 5%) were used and a lowered CCL18 secretion was observed in a dose-dependent fashion. As it was previously shown, that the addition of the gp130 cytokine IL-6 to the conventional M2 polarizing cocktail IL-4 and IL-13 resulted in a hyperpolarization, we assessed the effect of CSE in this system. Here, we confirmed that the addition of IL-6 resulted in an ~1.7-fold increase in CCL18 secretion compared to IL-4/IL13 stimulation alone observed in Figure 5, and that CSE exposure resulted in a similar dose-dependent inhibition of CCL18 secretion. To verify if cigarette smoke lowering M2 macrophage polarization was a species-dependent observation, we repeated the experiment on primary bone marrow derived macrophages (BMDM) derived from female C57BL6 mice. M2 hyperpolarization was assessed by examining arginase activity in cell lysates after 30 hours of concomitant exposure with IL-4/IL-13/IL-6. CSE concentrations were examined at 0.5, 2.5, 5 and 10% as preliminary experiments did not show toxicity at these concentrations. As shown in Figure 7, the effect of CSE on the M2 polarizing process was similar as observed in the human THP-1 cell line.

Given these results, we attempted to study the secretory activity of macrophages after 72 hours of stimulation with M1 and M2 polarizing mixtures with or without the

presence of CSE. Here, as above, THP-1 macrophages were polarized with either LPS/INF-γ (M1) or IL-4/IL-13 (M2) cocktails in addition to various concentrations of CSE (2.5, 5 and 10%) for 72 hours. Supernatants were submitted to Ive technologies and assessed for a total of 42 cytokines. Please see Figure 8 for a selection of cytokines assessed. A comprehensive list and graphs of the proteins analyzed can be seen in the Appendix (Table 5).

Overall, the exposure to cigarette smoke resulted in decreased secretion of several M1-induced related proteins, including IL-1alpha (Figure 8k), IL-12p40 (Figure 8h), IL-12p70 (Figure 8i) and MDC (Figure 8f), while no effect was observed with Rantes (Figure 8i) IL-1RA (Figure 8i). For GM-CSF (Figure 8d), IL-18 (Figure 8a), IL1beta (Figure 8b) and VEGF (Figure 8n), we observed a reduced secretion at 10% cigarette smoke exposure, indicating a possible toxic effect of cigarette smoke. However, when the PMA-activated THP-1 macrophages were polarized toward the M2 phenotype, CSE exposure increased the secretion of G-CSF dose-dependently, with the highest secretion at 10% CSE. For most other cytokines assessed, we did not observe a decrease or modulation of the secretion when CSE was added to the polarization cocktail, suggesting that CSE exerted specific effects towards certain cytokines in this model system. To further examine the effect of CSE, we repeated this experiment with the addition of specific IRE1 inhibitors and assessed the transcriptomic changes using a nanoString® CodeSet containing 34 M1 and M2 related genes.

As shown in Table 4, THP-1 cells polarized toward the M1 phenotype resulted in an increase in 24 genes, including CXCL10 (1058-fold increase), as well as CCL2 (35-fold increase) and CCL22 (13-fold increase), indicating successful polarization. Of note, several

UPR-related genes were also upregulated in the M1 direction, including FKBP2, DDIT3 and XBP1 as well as genes coding for ECM proteins Col1A3 and Col1A1. In comparison, when THP-1 cells were polarized in the M2 direction with the conventional polarization cocktail IL-4 and IL-13, only CCL-18, CCL2, FN1 and ITGAM were upregulated. The addition of CSE in the M1 direction led to remarkable few genes modulated compared to M1 alone (Table 3, Figure 3). Only CD14, IL6R and ITGAM were found to be statistically increased, whereas the addition of CSE led to the downregulation of CD163, CLEC10A and FN1. The addition of the IRE1 inhibitor STF 083010 achieved a virtually identical gene expression pattern as CSE alone, with the only differences that CCL2 was reduced and ITGAM was unaffected, this was in stark contrast to the IRE1 inhibitor 4μ8C, where numerous additional differences could be observed, including the downregulation of XBP1.

In Table 4, we have summarized changes of gene expression when THP1 cells were in the M2 direction with the addition of IL-6 to the conventional polarization cocktail IL-4 and IL-13 as well as the effect of CSE exposure toward the M2 direction and the effect of IRE1 inhibitors on this process. Here, the addition of IL-6 significantly increased the expression of CCL-18 mRNA (10-fold compared to M2 alone), CD14, FN1 and IL4R as previously shown [237]. The addition of CSE to the M2 polarization cocktail resulted in a decrease in CCL-18 expression as well as FN-1 expression while CCL2, CD14 and CLEC10A were increased.

Contrary to what was observed when the IRE1 inhibitor STF083010 was added to CSE and M1 polarization, the addition of this inhibitor in the M2 direction resulted in numerous changes, including a strong downregulation of CCL18, and FN1, while numerous upregulations were observed, including the ones observed above (CCL2, CD14 and

CLEC10A), but also DDIT3 (51-fold) FGF2, IL4Ra, ITGAM, PDGFA, TGFB1 and VEGFA. Contrary to what was observed in the M1 direction, the addition of the IRE1 inhibitor 4μ8C resulted in a similar pattern as observed with STF083010, with significant downregulation of CCL18 and CXCL10, while no effect was observed with the UPR related marker DDIT3. As the addition of IL-6 was shown to substantiate the M2 polarization, we examined the effect of CSE and the IRE1 inhibitor STF083010 on these polarized cells. As can be seen in Figure 10 and associated table 3, 10% CSE reduced CCL18 gene expression 5-fold, whereas the addition of STF083010 further reduced the expression 3.6-fold. This was contrary to CCL2 expression which was increased 2.5-fold by adding CSE exposure and further increased 2.6-fold by the addition of STF083010. In fact, the addition of CSE led to the decrease of FN1 and an increase of CD14, MSR1, CD163, CLEC10A, while the further addition of STF083010 led to a 46-fold increase in FGF2 as well as the UPR markers DDIT3 and GRP78, VEGFA and CCL2 already mentioned. In addition to the decrease of CCL18, the expression of CD14 and MSR1 also decreased 2.4 and 2.6-fold, respectively. Overall, this data demonstrates significant alteration of the gene expression profile of macrophages polarized in both the M1 and the M2-direction as well as with the addition of IL-6 to a hyperpolarization of the M2 phenotype. Some of these changes are further altered when IRE1 is blocked, indicating a potential dependency on IRE1 activation in these systems.

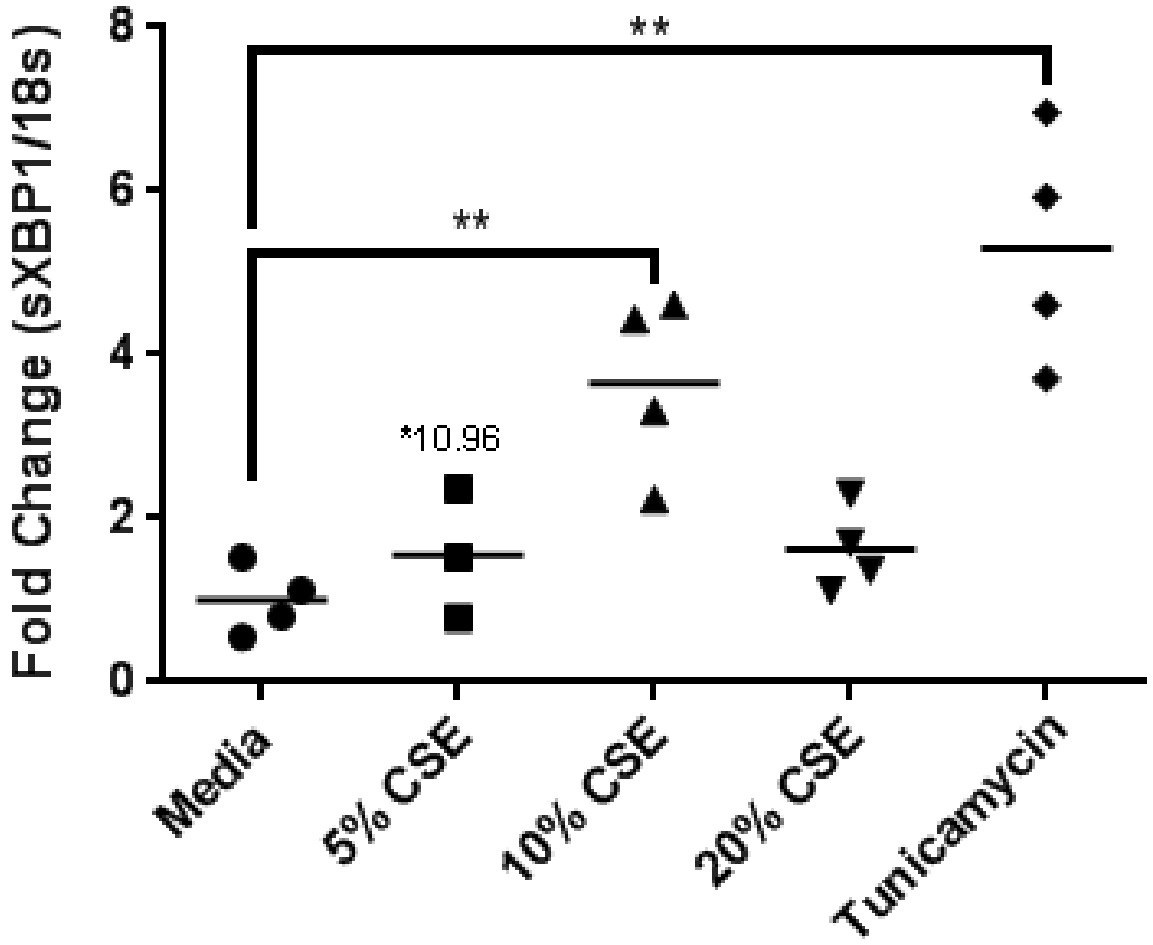

Figure 4: Measurement of sXBP-1 from extracted RNA from THP-1 macrophage-like cells after 6h stimulation with appropriate negative control (media), cigarette smoke and Tunicamycin. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferonni correction. (* $p<0.05$, ** $P<0.01$)

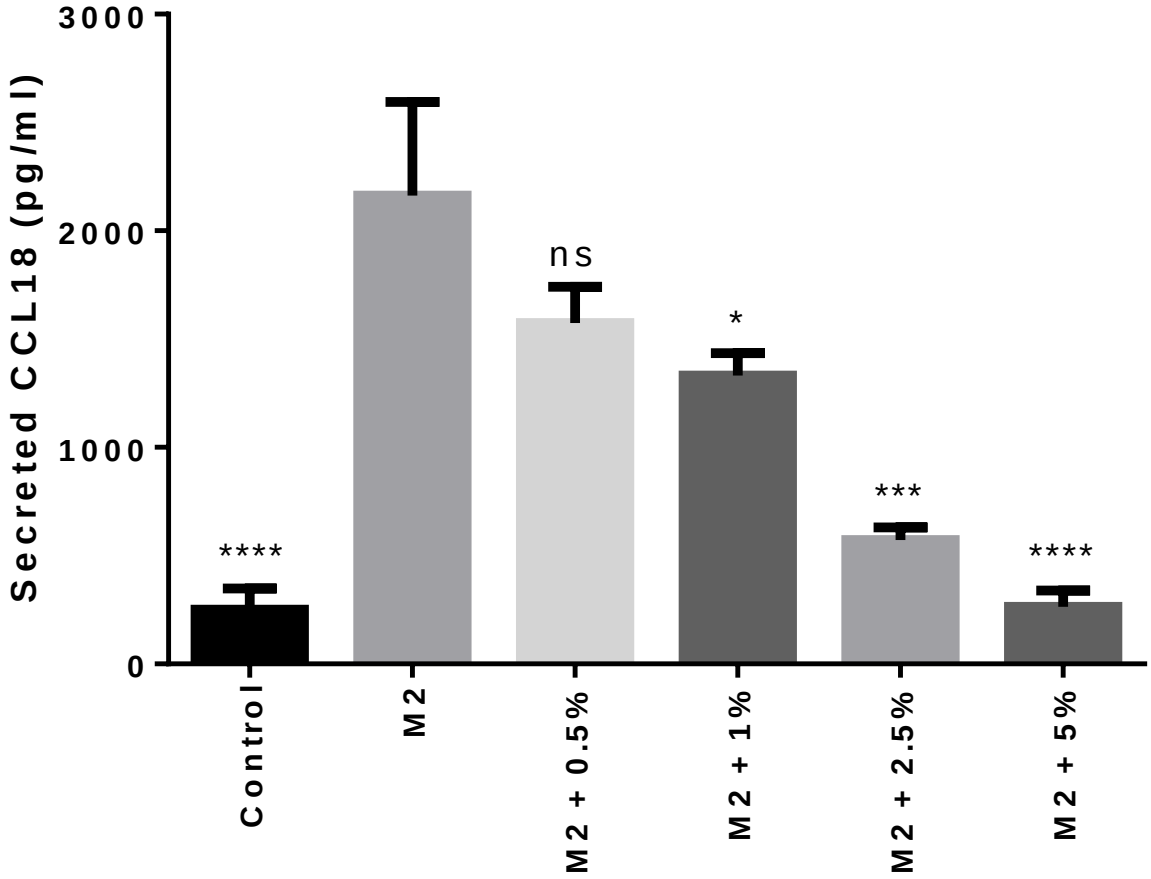

Figure 5: THP-1 cells were treated with M2 polarizing mixture for 72 hours with or without the addition of cigarette smoke extract and assayed for CCL18. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferonni correction. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

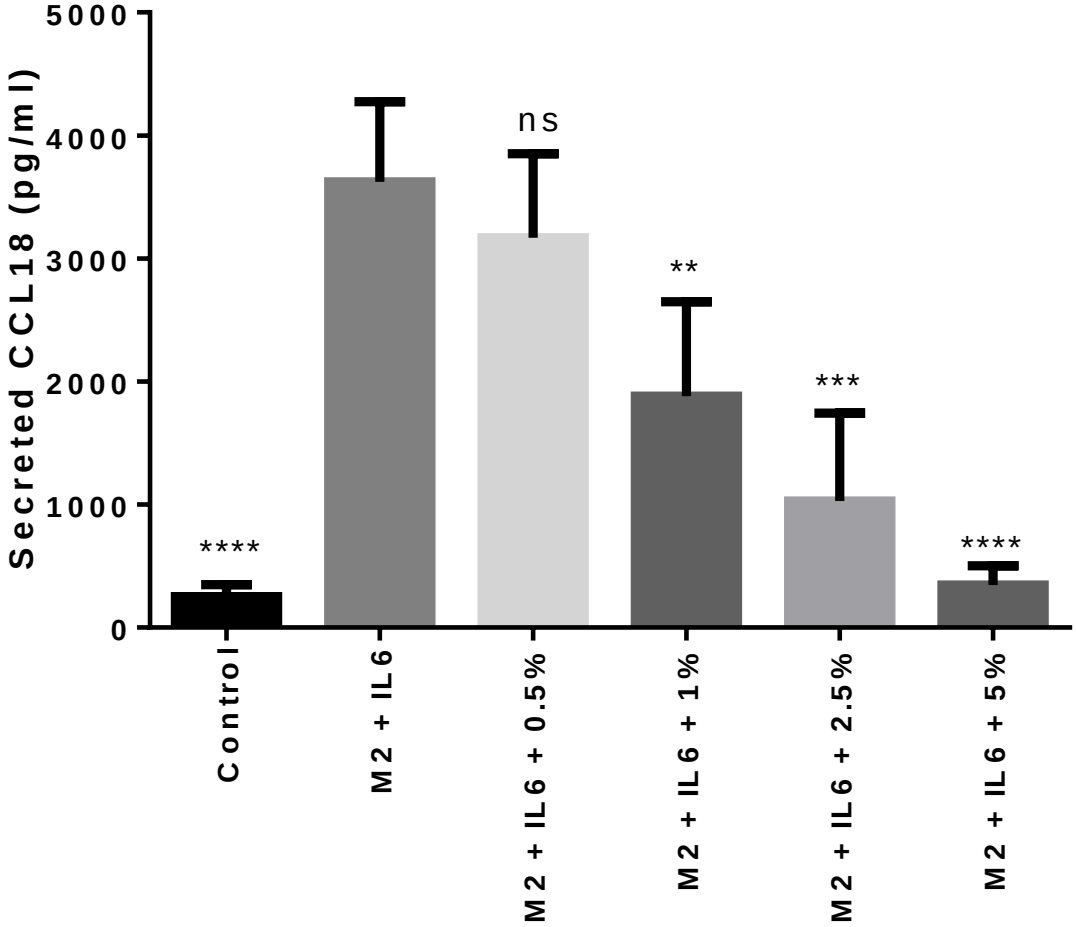

Figure 6: THP-1 cells were treated with M2 polarizing mixture with the addition of IL-6 for 72 hours with or without the addition of cigarette smoke extract and assayed for CCL18. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferonni correction. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

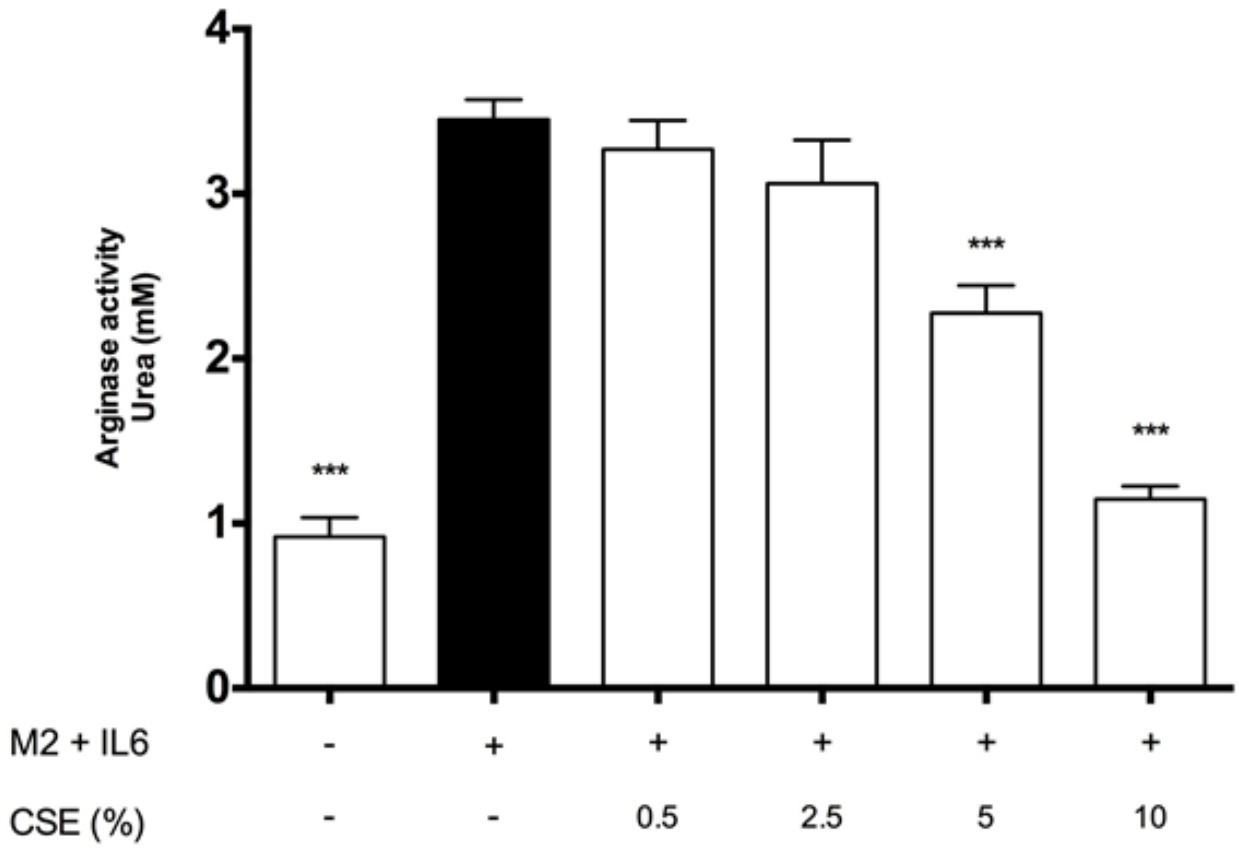

Figure 7: Bone Marrow Derived Macrophages (BMDMs) were treated with IL-4/IL-13 for 30h with M2 (IL-4/IL-13) plus IL-6 or M2 plus IL-6 with cigarette smoke extract. Statistical analysis was completed using One-way ANOVA, comparing the treatment groups with the positive control of alternatively activated macrophage. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

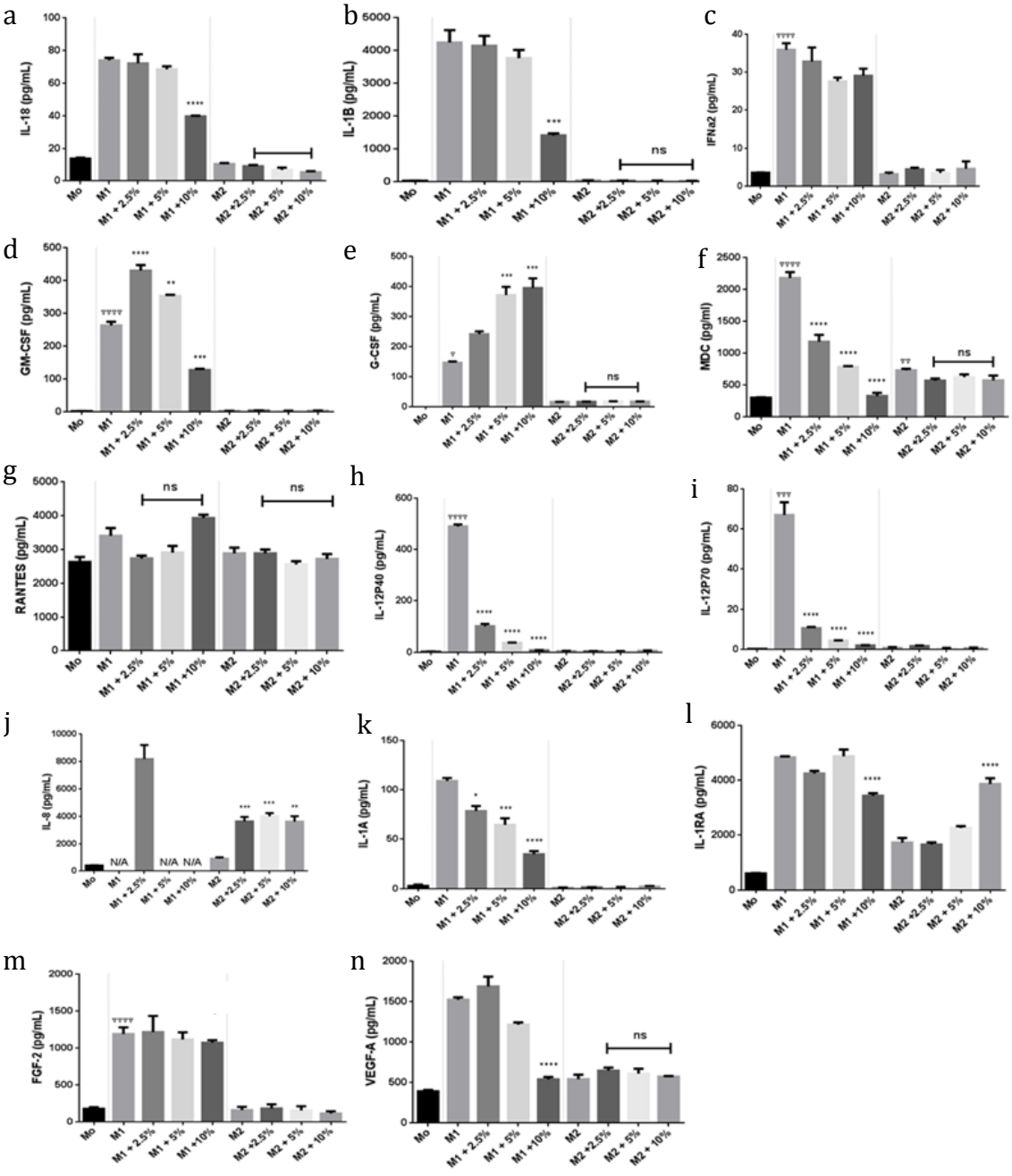

Figure 8: Measurement of chemokines in supernatant collected from THP-1 cells after 72h stimulation with MØ, M1 and M2 with various doses of cigarette smoke. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferonni correction (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

	M1 compared to MØ	M2 compared to MØ	M1 + CSE compared to M1	M1 + CSE + 60uM STF compared to M1	M1 + CSE + 30uM 4u8C compared to M1
CCL18	↑ (5.41)	↑ (13.34)	–	–	–
CCL2	↑ (35.02)	↑ (15.01)	–	↓ (-3.08)	↓ (3.70)
CCL22	↑ (13.45)	–	–	–	↓ (-3.95)
CD14	↓ (-2.76)	↓ (-2.34)	↑ (2.33)	↑ (1.97)	↑ (3.07)
CD163	↓ (-2.09)	–	↓ (2.59)	↓ (2.47)	↓ (-2.36)
CD68	–	↓ (4.90)	–	–	–
CLEC10A	↑ (2.19)	–	↓ (2.21)	↓ (2.94)	↓ (-3.11)
COL1A1	↑ (6.52)	–	–	–	–
COL3A1	↑ (12.52)	–	–	–	–
CXCL10	↑ (1058.60)	–	–	–	–
DDIT3	↑ (26.31)	–	–	–	–
FGF2	↑ (10.92)	–	–	–	↑ 3.38
FKBP2	↑ (7.12)	–	–	–	↓ (-2.57)
FN1	↑ (5.04)	↑ (4.14)	↓ (2.24)	↓ (2.90)	↓ (-2.30)
IL10	↑ (7.62)	–	–	–	↓ (-3.64)
IL1RL1	↑ (5.24)	–	–	–	–
IL33	–	–	–	–	–
IL4R	↑ (6.27)	–	–	–	↓ (-1.87)
IL6	↑ (6.00)	–	–	–	–
IL6R	–	↓ (-3.13)	↑ (2.02)	↑ (1.96)	–
ITGAM	↓ (-12.08)	↑ (4.35)	↑ (3.30)	–	↑ (2.48)
ITGAX	↑ (4.47)	–	–	–	–
MRC1	↑ (8.44)	–	–	–	–
MSR1	↑ (3.77)	–	–	–	↓ (-3.49)
NOS2	↑ (6.00)	–	–	–	–
OSM	↑ (5.40)	–	–	–	–
OSMR	↑ (6.21)	–	–	–	–
PDGFA	–	–	–	–	↑ (2.61)
TGFB1	↑ (1.72)	–	–	–	–
VEGFA	↑ (2.99)	–	–	–	–
XBP1	↑ (2.75)	–	–	–	↓ (-2.54)

Table 3: **Genetic analysis via nanostring of M1 compared to M2 and subsequent M1 macrophages exposed to CSE and IRE-1/XBP-1 inhibitors.** Arrows with green background represent upregulation and indigo with downregulation with increased expression associated with darker hues showing statistical significance ($p<0.05$), dashes represent no statistically significant change.

	M2 compared to MØ	M2 + IL-6 compared to M2	M2 + CSE compared to M2	M2 + CSE + 60uM STF compared to M2	M2 + CSE + 30uM 4u8C compared to M2
CCL18	↑ (13.34)	↑ (10.07)	↓ (-3.57)	↓ (-6.88)	↓ (-14.33)
CCL2	↑ (15.01)	–	↑ (1.71)	↑ (6.85)	↑ (4.19)
CCL22	–	–	–	–	–
CD14	↓ (-2.34)	↑ (3.44)	↑ (2.07)	↑ (3.26)	↑ (2.86)
CD163	–	–	–	–	–
CD68	↓ (-4.90)	–	–	–	–
CLEC10A	–	↓ (-2.26)	↑ (2.71)	↑ (2.10)	↑ (3.72)
CXCL10	–	↓ (-4.66)	–	–	↓ (-6.01)
FGF2	–	–	–	↑ (7.08)	↑ (7.41)
DDIT3	–	–	–	↑ (51.37)	–
FN1	↑ (4.14)	↑ (3.23)	↓ (-2.40)	↓ (-2.36)	↓ (-1.94)
HSP5a	–	–	–	↑ (2.76)	–
IL-4R	–	↑ (1.81)	–	↑ (1.63)	↑ (2.19)
ITGAX	–	–	–	–	–
IL-6R	↓ (-3.13)	–	–	–	–
ITGAM	↑ (4.35)	–	–	↑ (2.76)	–
MRC1	–	–	–	–	↑ (3.64)
OSM	–	–	–	–	↑ (3.58)
PDGFA	–	–	–	↑ (4.00)	↑ (2.27)
TGFB1	–	–	–	↑ (2.60)	↑ (2.26)
VEGFA	–	–	–	↑ (3.45)	–

Table 4: **Genetic analysis of M2 and M2+IL-6 exposed to CSE and IRE-1/XBP-1 inhibitors using nanostring.** Arrows with green background represent upregulation and indigo with downregulation with increased expression associated with darker hues showing statistical significance ($p<0.05$), dashes represent no statistically significant change.

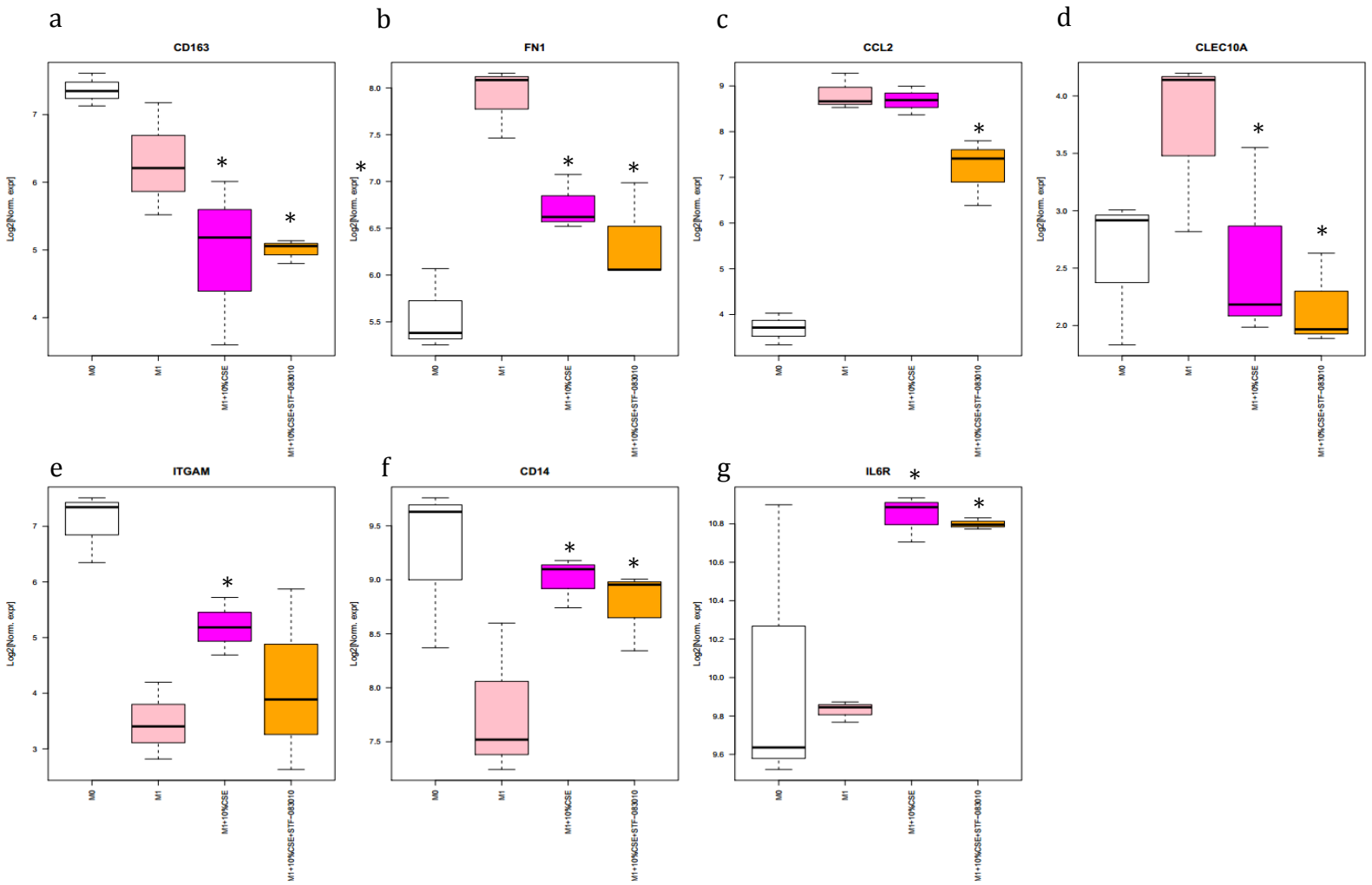

Figure 9: Boxplot representation of select regulated genes from cigarette smoke exposure using expression based on log2 expression from cigarette smoke treated M1 macrophages with IRE-1/XBP-1 pathway inhibitor. Groups include (from left to right: MØ, M1, M1+10%CSE and M1+10%CSE+STF-083010), * represents ($p<0.05$).

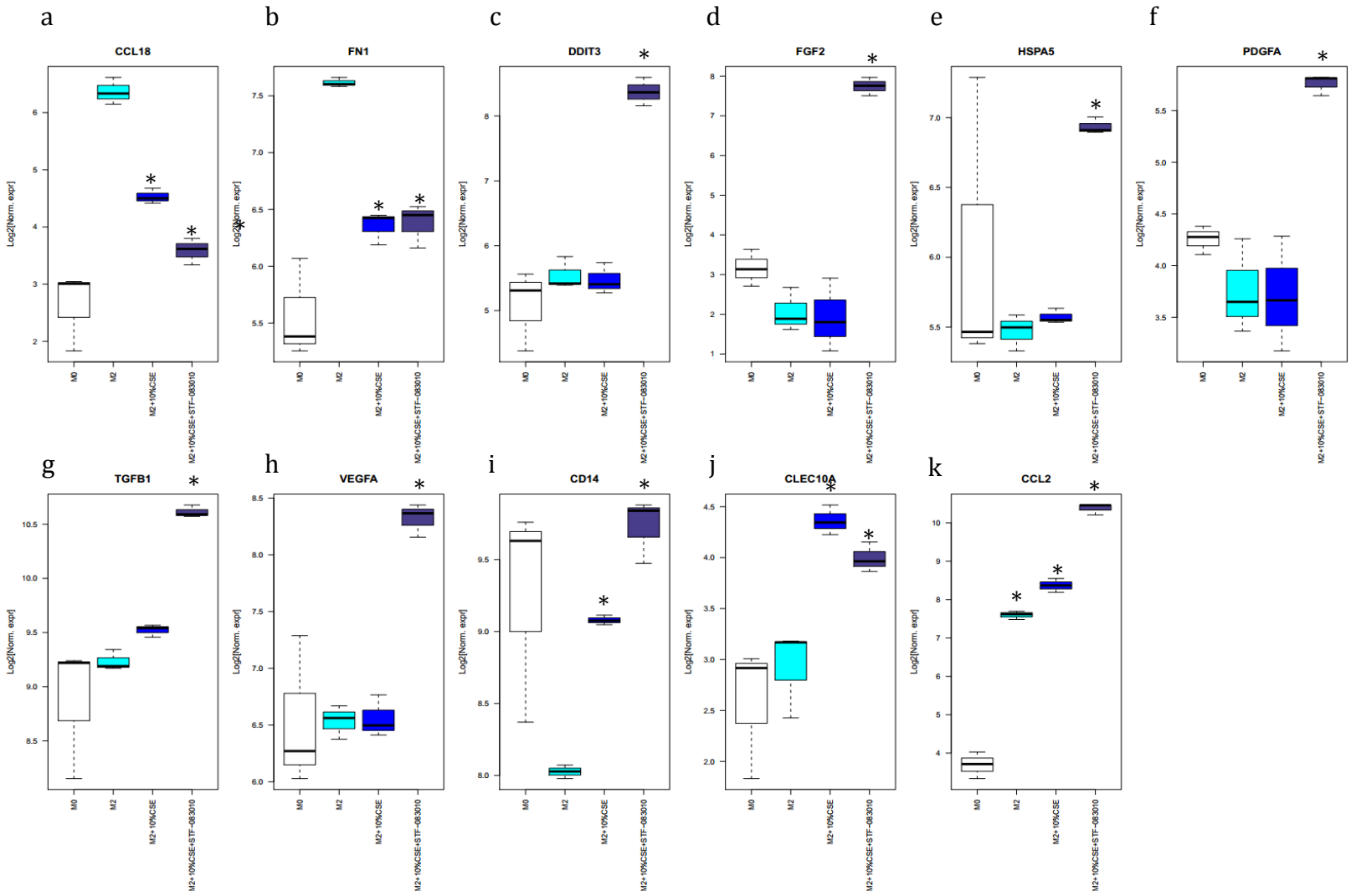

Figure 10: Boxplot representation of select regulated genes from cigarette smoke exposure using expression based on log2 expression from cigarette smoke treated M2 macrophages with IRE-1/XBP-1 pathway inhibitor. Groups include (from left to right: MØ, M1, M1+10%CSE and M1+10%CSE+STF-083010) , * represents ($p<0.05$).

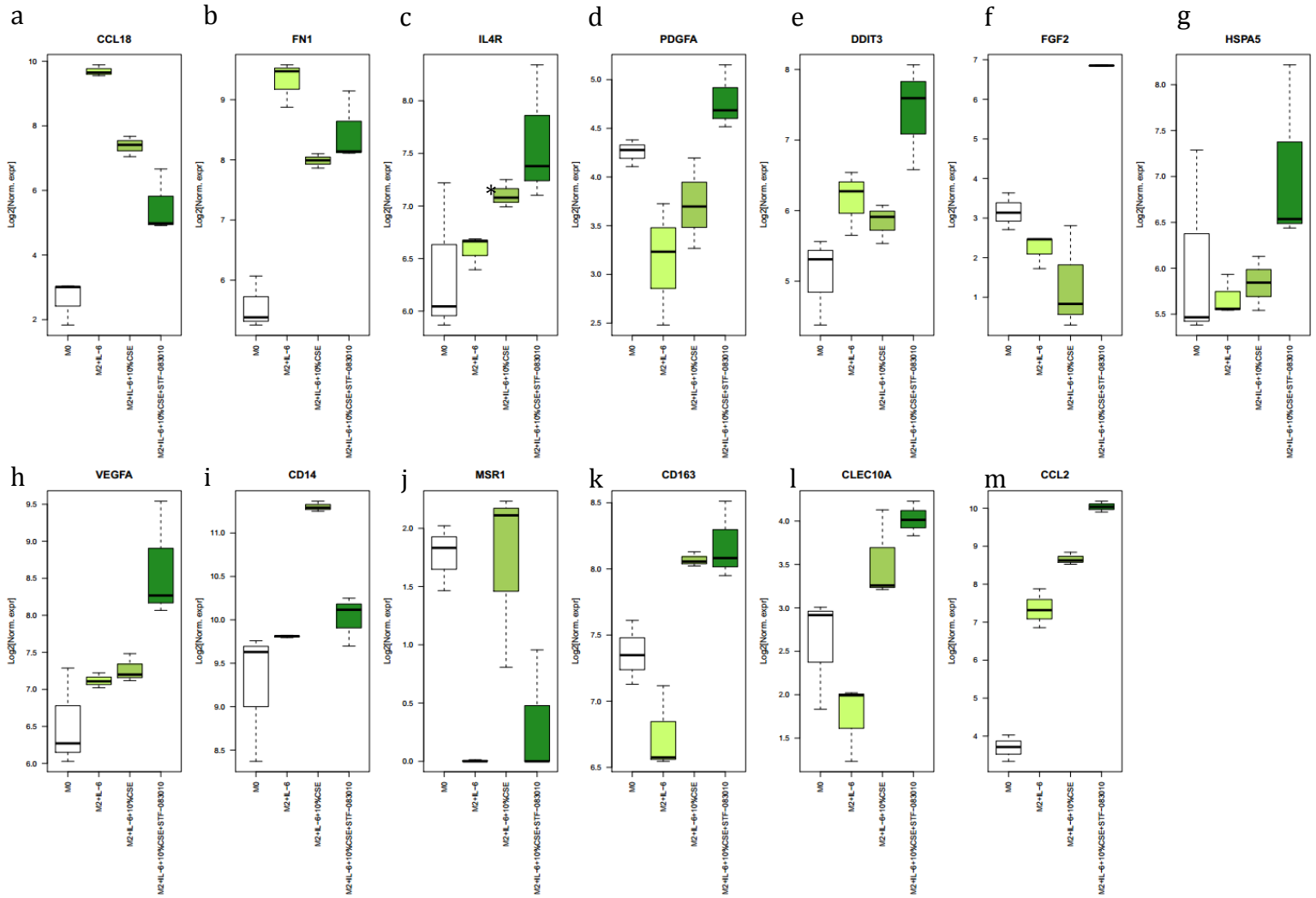

Figure 11: Boxplot representation of select regulated genes from cigarette smoke exposure using expression based on log2 expression from cigarette smoke treated M2 + IL-6 macrophages with IRE-1/XBP-1 pathway inhibitors. Groups from left to right: MØ, M1, M1+10%CSE and M1+10%CSE+STF-083010), * represents ($p<0.05$).

Chapter 4— Discussion

Main Findings

The investigation studied the effects of the IRE-1/XBP-1 pathway on cigarette smoke affected macrophage polarization *in vitro.* Overall, cigarette smoke directly activated the IRE-1/XBP-1 pathway in macrophages leading to a dampening in immune phenotype and polarization of macrophages. Furthermore, use of IRE-1/XBP-1 pathway inhibitors, STF-083010 and 4μ8C, exacerbated the observed changes rather than reversing the polarization as hypothesized via changes in markers (Table 3-4). The presented data demonstrates CSE, present under both M1 and M2 polarization mixtures, results in an unconventional polarization state. Cigarette smoke exposed M1 macrophages exhibit a downregulation in pro-inflammatory cytokines while an increase in GM-CSF was observed. Furthermore, M2 macrophage markers were downregulated following addition of cigarette smoke extract. Utilization of IRE-1/XBP-1 inhibitor did not reverse the downregulation of M2 macrophage markers. Given these findings it suggests in cigarette smoke the downregulation of M1 and M2 markers, a conventional polarization program leads to lung disease caused by cigarette smoke. Thus, requirement for further study of macrophage behaviour in the context of cigarette smoke and the UPR is required.

Summary

Utilizing classical macrophage polarization mixtures, LPS and IFN-γ, we observed an increase in IL-12p40 and p70. Part of this work was focused on the effect of CSE on the classical M2 activation cocktail using IL-4 and IL-13, while we also examined the effect of CSE on the added effect of IL-4/IL-13 and IL-6 hyper-activation of M2 macrophages.

Following the multiplex analysis of 42 proteins analyzing the changes of cigarette smoke in macrophage polarization, 12 candidate proteins, a mixture of cytokines,

chemokines and growth factors, identified to be highly regulated by cigarette smoke. Cigarette smoke significantly decreased secretion of M1 related inflammatory cytokines, suggesting regulation by cigarette smoke. These cytokines are important because of their importance in promoting adaptive immune responses that combat bacterial and viral infections, a cause of acute exacerbations in COPD [238]. These cytokines include IL-12p40, IL-1beta, IL-18, and IL-1alpha (Figure 9). It was previously reported that M1 polarized macrophages secrete IL-12p40 and p70 when stimulated with IFN-γ [157]. IL-12p40 represents a subunit of the mature secreted IL-12 protein expressed by antigen-presenting cells, such as macrophages— which is associated with the measurement of the mature form, IL-12p70 [239]. In our experience, the addition of cigarette smoke reduced both IL-12p40 and IL-12p70 in a dose dependent manner (Figure 9). The maximum dose of 10% cigarette smoke decreased secretion to levels of negative control. No increase of IL-12p40 was identified in M2 polarized macrophages and subsequent addition of cigarette smoke did not affect the change in secretion (Table 3-4). An increase in IL-12p40 and p70, representing M1 polarization, between M1 and MØ with statistical significance confirmed that M1 polarization induces the production of IL-12p40 [157]. This finding is consistent with earlier observations examining mature human dendritic cells stimulated with LPS *in vitro,* where the authors showed that cigarette smoke addition prevented the release of IL-12p40 and p70 [240]. Moreover, e*x vivo* stimulation of chronic cigarette smoke exposed dendritic cells were impaired in producing IL-12p40 and p70 [240]. It is known that IL-12p40 also binds to IL-23 promoting a Th17 response demonstrating pleiotropic effects [239]. This may be consistent with the observation of a dampened Th17 response observed

in PBMC of COPD patients and *ex vivo* stimulation of monocyte derived dendritic cells in response to cigarette smoke [214].

Next, we showed that CSE both reduced the IFN-γ/LPS mediated release of IL-1β and IL-18. IL-1β and IL-18 are both endoplasmic reticulum derived proteins known to be cleaved by the NLRP3 inflammasome before they reach their mature form [241]. A previous *in vivo* study showed that cigarette smoke induced IL-1β, contrary to our findings [242]. Similarly, IL-18 was found to be increased in mice exposed to cigarette smoke [243-245]. The specific inhibition of IL-1β and IL-18 observed in our study outlines a difference between these *in vivo* studies, suggesting that multiple exposures to cigarette smoke and chronic cigarette smoke exposure to an intact lung may regulate IL-1β and IL-18 differently than a single administration of CSE directly to LPS/IFN-γ activated macrophages *in vitro*.

Further, we determined that macrophages exposed to CSE and M1 polarizing LPS/IFN-γ reduced the secretion of IL-1α. IL-1α is an alarmin that produced in the cytoplasm has transcription factor properties in cells [246]. The release of IL-1α acts as a potent inflammatory mediator in the context of cigarette smoke induced inflammation. Specifically, knockout mice for IL-1α were protected against cigarette smoke induced neutrophilia which leads to emphysema [247, 248].

The chemokines affected by cigarette smoke represent a change in the type of cellular infiltration orchestrated by macrophages. These findings also determined an increase in IL-8 consistent with the results demonstrating that cigarette smoke causes the increase of IL-8, a potent chemokine for neutrophils [207, 249]. Macrophage derived chemokine (MDC) produced by macrophages and dendritic cells allows for the infiltration of monocytes, differentiating into macrophages, Th2 cells and dendritic cells and NK-cells

[250]. A decrease of macrophage-derived chemokine was also determined in M1 macrophage population in a dose dependent manner; however, there was no effect on M2 macrophage derived MDC. These demonstrate novel findings as other studies have shown MDC has not been released from cigarette smoke exposure and from primarily an M2 polarization source from macrophages [251].

Another neutrophil chemokine, granulocyte/macrophage colony-stimulating factor, increased from low dose (2.5%) of cigarette smoke with M1 mixture. However, from increasing doses of cigarette smoke, sharp decreases were determined of M1 derived GM-CSF. In *in vivo* studies, GM-CSF shows an increase and contributes to inflammation leading to emphysema [252]. However, with granulocyte-colony stimulating factor (G-CSF) we observed a dose dependent increase in the release of the chemokine representing the infiltration of granulocytes such as neutrophils, fibroblasts and consistent with cigarette smoke exposure in mice [253].

Recent findings in human and mouse studies, *in vitro*, support M2 macrophage polarization in response to cigarette smoke extract [216, 218, 254]. The M2 macrophage polarization may be beneficial as a response to toxic airborne xenobiotics such as cigarette smoke, promoting tissue healing from the emphysema caused by cigarette smoke as seen *in vivo [217]*. Moreover, after *in vivo* chronic cigarette smoke exposure, lymphocyte derived IL-10 can induce M2 macrophage polarization [254]. The increased expression of Mannose Receptor (MR) is evidenced in M2 macrophages differentiated from the B-cell lymphocyte derived IL-10 [254]. In addition, these scientists lacked an assessment of arginase, an important M2 marker while our lab has confirmed *in vivo* that cigarette smoke fails to express arginase expression at 2 week, 8 week and 4 month cigarette smoke exposure

(unpublished findings). Complementary to these findings, we utilized *in vitro* IL-6 stimulated M2a (IL-14 and IL-13 stimulated) macrophages known as "hyperpolarized M2 macrophages", which produce high levels of arginase, to identify the response to cigarette smoke during polarization and determined an inhibition in its expression in a dose-dependent manner (Figure 9) [152]. Simultaneous work showed a 10-fold increase in CCL-18 secretion in when IL-6 was added (Figure 6). Our data clearly indicate that macrophage exposed to CSE were not able to secrete similar amounts of CCL-18 as cells macrophages exposed to IL-4/IL13 or IL-4/IL-13 and IL-6, where 5% CSE completely blocked CCL-18 secretion (Figure 5). This observation was not species dependent as the same effect was observed on BMDM's isolated from C57BL6 mice. Figure 9-11 shows genes that were regulated by cigarette smoke and further affected through knockdown of IRE-1/XBP-1 pathway from STF-083010. A comprehensive list of the genes analyzed in the experiment shows statistical significance summarized in Tables 3 and 4 and Figure 14 (Appendix). Overall, it was determined from cigarette smoke treatment, a decrease in FN1, CD163, CCL2 and CLEC10 expression observed in M2 macrophage polarized cells and increase in expression of IL-6 and CD14 expression. After incorporation of IRE-1 inhibitor, a statistically significant increase in CCL2, ITGAM and CD14 expression was observed. A statistically significant decrease in CCL18 and FN-1 were observed when cigarette smoke was added. However, IRE-1 inhibitor increased DDIT3, HSP5a, FGF2, PDGFA, VEGFA, CD14 and CCL2, while a decrease in CLELC10 was observed from addition of IRE-1 inhibitor. The final group tested was M2 macrophages treated with IL-6. Cigarette smoke addition increased CD14, CD163, CLEC10, CCL2, IL4R and MSR expression (Figure 11). A decrease in expression of CCL18 and FN-1 was observed from cigarette smoke exposure. Upon addition

of IRE-1 inhibitor, increases in IL-4R, DDIT3, HSP5A, VEGFA, CCL2 and CLEC10 were observed. Furthermore, the use of IRE-1 inhibitor, STF-083010, caused a decrease in CCL18 and FN-1.

CCL18 remains an important indicator of M2 macrophage polarization and has been described as a chemokine attracting T-cells, B-cells and dendritic cells [255]. Additionally, CCL18 can further polarize MO to M2 macrophages in an autocrine manner [256]. Our findings are in parallel with Kollert et al. (2009), who demonstrated that cigarette smoker's alveolar macrophages stimulated with LPS developed a decreased CCL18 secretion compared to non-smokers. Additionally, BAL fluid CCL18 was decreased in cigarette smokers. Additionally, the scientists discussed that other markers of M2 macrophage polarization were presumably downregulated from cigarette smoke and following a unique genetic alteration rather than an M2 macrophage polarization [255].

Although our primary interest in this work was to study the effect of CSE on M2 polarization, in the context of IRE-1/XBP-1 activation, we also included the conventional M1 polarization mixture (IFN-γ/LPS) and examined the effect of CSE on the secretion of cytokines and the gene regulation on both M1 and M2 polarization cocktails.

Comparison to Literature

The 2014 Surgeon General's report outlined the involvement of cigarette smoke and an overactive UPR as potential cause for the inflammatory damage leading to progression of lung disease [1]. Given seminal studies in XBP-1 KO plasma cells unable to expand the ER, the main indicator of a UPR, it was hypothesized that inhibition of the IRE-1/XBP-1 pathway would reduce the ER stress response in macrophages responding to cigarette smoke [56].

The effect of IRE-1/XBP-1 knockdown in cigarette smoke treated macrophages led to an increase in the UPR (Table 3-4). These findings could be exclusive to the cell type or confirm Peter Walter's postulation that UPR compensation occurs when one arm of the ER stress response becomes non-functional leading to a heightened response [257]. This is supported by the ATF-6/ATF-4 pathway activating the same promoter that IRE-1/XBP-1 affects and inducing ER expansion in CHO cells [258]. Thus, the idea that the UPR has detrimental effects from its upregulation in response to CS requires validation and viewing the UPR as maintaining cellular homeostasis and regulating physiology will help understand its role in chronic lung pathology.

From our findings, cigarette smoke inhibited M1 IL-10 secretion, increased M2 macrophage IL-10 secretion and inhibited IL-12 secretion (Figure 5). M2c macrophages express high levels of CD14+ cells, where M2a polarization using IL-4 and IL-13 downregulated its expression. In fact, M-CSF, a polarizer of M2c macrophages, induces high secretion of CCL2 in murine and human macrophage populations [259]. However, cigarette smoke extract increased the expression of CD14 and use of IRE-1/XBP-1 inhibitors further activating the expression. Interestingly, other markers of M2a expression were downregulated. Thus, the phenotype based on the cytokine profile resembles the markers of the anti-inflammatory, *"deactivated"*, M2c subset that actively promotes tissue remodeling from MMP-9 release and diminished cytokine release [180, 260].

The importance of identifying cigarette smoke blocks the polarization of M2 macrophages, provides a plausible explanation to the mechanism behind cigarette smoke induced changes of lung pathology. Macrophage dysfunction caused by cigarette smoke may be the reason for development into COPD as aged lungs develop lung pathology

readily [261]. COPD represents a heterogeneous disease consisting of differing symptoms in patients. In addition, cigarette smoke represents 80% of COPD cases, 90% of lung cancer and remains the most important risk factor for Idiopathic Pulmonary Fibrosis (IPF). The M2c macrophage readily releases MMP-9, an ER derived protease that causes remodeling of the lungs [262]. More interestingly, the activation of TGF-beta from cigarette smoke suggests that tissue remodeling from cigarette smoke and loss of IRE-1/XBP-1 pathway promotes COPD, tumour growth and even IPF.

These conclusions point at downregulation of M2 polarization with CCL18 and FN1 and furthermore outlined the IRE-1/XBP-1 pathway, activated upon CSE administration, enhances this effect seen through CCL18. It directs a need for further investigation of the IRE-1/XBP-1 pathway in macrophage polarization *in vivo* and ER stress. Future findings are needed to fully determine the effects of sXBP-1 given various stimuli [216, 218].

Limitations and Future Directions

The investigation outlined the effects of cigarette smoke on human and mouse macrophages over a 3-day period using *in vitro* culture techniques. A limitation in the study design, relating to these findings in human macrophages, results from poor indication of M2 polarization *in vitro* in human macrophages using arginase as a marker [263]. Compensating for the lack of arginase, we utilized BMDMs and assayed them in the presence of cigarette smoke to determine if their activity was increased (Figure 8). As previously mentioned, *in vivo* analysis of arginase also demonstrated a lack of arginase expression, suggesting cigarette smoke inhibits arginase and prevents M2a macrophage polarization.

Furthermore, the role of the ER stress pathway's other arms must be better studied in the context of M2 macrophage polarization determined previously by Oh et al. (2009)— who demonstrated tunicamycin induces M2 macrophage polarization in a dose dependent manner. A potential target is HSP90, the main receptor for glucocorticoid activation, which couples with CDC37 to stabilize IRE-1alpha activation— where inhibition using geldanamycin attenuated the stabilization activity [161, 264-266]. Furthermore, a relationship with the ER and M2 macrophage polarization is seen by induction of M2 polarization by estrogen which induces polarization through the G protein coupled receptor (GPER) located in the endoplasmic reticulum, suggesting additional physiological correlates with polarization [267].

The findings demonstrate cigarette smoke downregulates production mediators of M1 and M2 polarization programming suggesting their functions are dampened *in vivo*. A bleomycin model for fibrosis, which contains a tissue healing component leading to scar formation in the lungs could be used having a group exposed to cigarette smoke along with bleomycin to understand the change in disease phenotype. Hamsters have been exposed to cigarette smoke and bleomycin showing an increase in fibrosis, however, the role of the macrophage within the system remains to be understood [268, 269]. Presumably, the M2 macrophage becomes activated from the indirect effects of cigarette smoke or a certain subset seen *in vivo* rather than *in vitro* mediates the tissue healing.

The effects cigarette smoke most accurately depicts pollution; the two have particular matter, oxidative species and carbon monoxide from incomplete combustion [270, 271]. Utilizing diesel exhaust collected from trucks and used *in vitro* we can confirm if a similar effect could be seen in our model. LaBranche (2017) identified adding DEP to the

polarization mechanism did not affect IL-1beta or MRC expression, indicative of M1 and M2 macrophage polarization, respectively. Additionally, pollution could be used *in vivo* and specifically the polarization of macrophages could be determined in the context of the IRE-1/XBP-1 pathway.

Cigarette smoke also activates an autophagy response that sXBP-1 can control [272]. Thus, this could suggest that activation of the ER stress pathway from cigarette smoke acts as a compensatory mechanism of macrophages phagocytizing tar particles. Based on findings of macrophages in COPD patients contain tar stored in vacuoles, unable to digest the tar, demonstrates perhaps infiltrating macrophages are M2 polarized clearing up the debris of dead cells and tar in the lungs [124]. However, our experiments in bone derived macrophages, which produce arginase during M2 polarization suggest differently (Figure 9), arginase was inhibited by increasing doses of cigarette smoke. These observations fall in line with literature that supports cigarette smoke causes polarization different than of M1 and M2 polarizations. Recent studies support that cigarette smoke causes M2 macrophage polarization in human cells causing upregulation of receptors indicative of M2 polarization.

Overall, we were able to demonstrate that cigarette smoke affects both M1 and M2 polarization, consistent with our initial hypothesis. However, given the activation of XBP-1 from cigarette smoke *in vitro* the hypothesis of IRE-1/XBP-1 pathway being activated was supported. However, given that our model of cigarette smoke did activate the IRE-1/XBP-1 pathway, the specificity of cigarette smoke induced ER stress activating M2 macrophage polarization contradicts previous findings of M1 polarization and IRE-1/XBP-1 inflammation [206, 207]. These previous findings suggest TLR-4 activated NFκB secretion of IL-8, where ligation of TLR-4 using an antibody abrogated IL-8 secretion by cigarette

smoke via NFκB in an LPS independent fashion [207]. These findings contradict other nfκb related cytokines being released such as IL-1beta, IL-1alpha, IL-1RA, IL-12 and IL-18. In M1 macrophages, we observed a decreased secretion of IL-8 and pro-inflammatory cytokines and chemokines, presumably due to IRE-1 activated translational control [273-275]. Contrary to these findings, cigarette smoke increased IL-8 expression from M2 and MØ macrophages suggesting that ER stress activation could be using a differential pathway to produce IL-8, an important chemokine in COPD pathology [276-278].

Zhao et al. (2017) outlines the inhibitory effects of cigarette smoke extract directly on murine macrophages. Utilizing the Ana-1 macrophage cell line, 20 cytokines and chemokines were assayed when exposed to CSE [279]. They observed a decrease in pro-inflammatory cytokines and chemokines: IL-5, IL-6, IL-12, TNF-α, VEGF, IP-10, MCP-1, MIP-1α and VEGF representing the M1 macrophage polarization milieu. MØ macrophages treated with M1 polarizing mixture and CSE displayed a similar downregulation in cytokines. Due to technical limitations (out of range values) IL-6 and MCP-1 was not able to be analyzed at the cytokine level. At the gene level, CCL2 did not show statistical significance when CSE was added, while IRE-1/XBP-1 inhibitor decreased its expression compared to M1. Additionally, at the gene level IL-6R showed an increased expression from addition of CSE and IRE-1/XBP-1 inhibitor, however, specific cytokine analysis must be compared to gene expression of IL-6. To fully understand the immunomodulatory effects of cigarette smoke functionality assays (phagocytosis) must be completed to confirm cigarette smoke.

Other aspects of the UPR, in terms of immune response impairments, caused by cigarette smoke could be cell death rather than polarization. We have shown higher doses

of cigarette smoke causing cell death via MTS assays and flow cytometry analysis of 7AAD/Annexin V (data not shown). The downstream pathways of IRE-1/XBP-1 may be required for survival of macrophages from cigarette smoke exposure, demonstrated by the cytotoxic effects of cigarette smoke, through a caspase independent mechanism [280]. In fact, loss of IRE-1 activation can lead to cell death through increased ROS species production [48]. These ROS can worsen the misfolded protein load in the ER due to oxidative stress [48]. Interestingly, IRE-1 mediated ROS production, caused by initiation of anti-bactericidal activity, was inhibited using STF-083010 and 4μ8c [111].

Therefore, the control of IRE-1 in terms of production of ROS may be dependent on the stimulus. In addition, the previous study completed by Bodas & Vij et al. (2015) did not identify if altering calcium or ROS mediated by the UPR or altering the ER stress response was involved in the cell death mechanism—all aspects of the IRE-1/XBP-1 pathway [48, 211]. However, one downstream transcription factor of IRE-1/XBP-1 pathway, CHOP, from *in vivo* cigarette smoke exposure is activated [8, 224]. CHOP activates the apoptotic cycle of cell death causing cells to undergo programmed cell death. Interestingly, it was found that IRE-1 activation prevented permeabilization of the ER contents, along with promoting survival from ER stress [35]. However, under unresolved UPR, the use of sXBP-1 mediated through CHOP will induce necrosis through a caspase-3 independent pathway, further causing inflammation [76, 77]. Interestingly, IRE-1/XBP-1 has been shown to converge at CHOP along with ATF6 inducing inflammatory cell death [76, 281-284].

As a result, the specific physiological differences of macrophage polarization should be better studied in the context of endoplasmic reticulum stress. As previously mentioned, M1 macrophages utilize Hypoxia Inducible Factor 1 alpha (HIFalpha), downstream from

nfκb activation, controlled by IRE-1/XBP-1, to induce inflammation and glycolysis [62, 123, 197]. NO and ROS production also are associated with M1 macrophages, mediated by the mitochondria [197]. M2 macrophages, undergo mitochondria biogenesis and are capable of greater degrees of beta oxidation for energy through ER stress [197]. Thus, depending on how energy is utilized by macrophages, the phenotype will be changed [197]. Interestingly, M2 macrophages have an increased ER stress response in comparison to M1 macrophages [154]. With many M1 and M2 cytokines produced in the ER, a role for IRE-1/XBP-1 may exist for governing macrophage polarization [104, 157].

Thus, the IRE-1/XBP-1 pathway likely can affect polarization. Loss of the IRE-1/XBP-1 pathway led to increased M2 macrophage related gene expression using nanostring analysis (Figure 10). We identify that the ER stress pathway compensates for the loss of IRE-1/XBP-1 pathway by increasing ER stress observed in M1 + CSE treated with IRE-1 inhibitors that increased FKBP13 and HSP5a (Figure 10). Interestingly, M1 macrophages rely on HIF-1alpha, induced by IRE-1/XBP-1, and a subset of M2 macrophages rely on IL4R signaling inhibited by IRE-1/XBP-1 downstream signaling through C/EBPbeta [157, 160, 285]. This is supported from increased insulin sensitivity of XBP-1 expressive cells, where insulin normally stimulates glycolysis, increasing inflammation through XBP-1 and FOXO1 [286]. In addition, mitochondrial biogenesis by M2 macrophages which is controlled via ER crosstalk will be reduced, presumably shifting macrophages to a more quiescent state [197].

In fact, loss of IRE-1 activation can lead to cell death through increased ROS species production [48]. These ROS can worsen the misfolded protein load in the ER due to

oxidative stress [48]. Interestingly, IRE-1 mediated ROS production, caused by initiation of anti-bactericidal activity, was inhibited using STF-083010 and 4μ8c [111].

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The impairment of these macrophages to clear the debris could be a cause for constant inflammation and constant wound healing, with a continuous positive feedback loop [202]. Damaged lung lining causes type II epithelial cells to replicate making it a risk factor for lung cancer, where the wound healing properties associated with replication of these cells can continue uncontrollably leading to small lung carcinoma [4, 202]. Alveolar macrophages can aid in the replication of these cells by producing growth factors that further induce inflammation in early tumour stages and tissue remodeling that allows for tumour growth and angiogenesis later causing metastasis [4].

The importance of these findings in the context of other diseases such as IPF may understand the Similarly, in cigarette smoke the various diseases that result from it such as

lung cancer, fibrosis and COPD can all be attributed to the facts that the resolution of inflammation and wound healing are not done. To understand what types of environments in the lungs that cigarette smoke are causing would require an *in vitro* model of the lungs and analyze the entire proteome along with the genetic changes to understand how progression to these various types of diseases occur.

Overall Conclusion

Combined, our data shows that cigarette smoke exposure to macrophages undergoing polarization affect their immune phenotype. Cigarette smoke exposure can directly activate the UPR and prevent M2 polarization. During M1 polarization process, cigarette smoke exposure resulted in a differential response in cytokine secretion and gene expression, with a strong increase in selected cytokines and a complete abolition of others. These *in vitro* observations suggest that cigarette smoke exposure may prevent an effective innate immune response towards various pathogens. It is warranted that the specific immune phenotype of these macrophages should be investigated further. Additionally, the effects of cigarette smoke on macrophage survival and phagocytic capacity, should be further studied, given the severity of acute exacerbations in COPD patients [146].

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Appendix

Protein Code	Protein Name
EGF	Epidermal Growth Factor
FGF-2	Basic Fibroblast growth factor
Eotaxin-1 or CCL11	Eotaxin-1 or chemokine C-C motif 11
TGF-a	Transforming growth factor alpha
G-CSF	Granulocyte colony-stimulating factor
Flt-3L	Fms-related tyrosine kinase 3 ligand
GM-CSF	Granulocyte macrophage colony-stimulating factor
Fractalkine or CX3CL1	Fractalkine or chemokine C-X3-C motif ligand 1
IFNa2	Interferon alpha-2
IFNy	Interferon gamma
GRO alpha or CXCL1	Growth-related oncogene-alpha or chemokine C-X-C motif ligand 1
IL-10	Interleukin-10
MCP-3 or CCL7	Monocyte-chemotactic protein 3 or chemokine C-C motif ligand 7
IL-12P40	Interleukin-12 subunit 40
MDC or CCL22	Macrophage derived chemokine or chemokine C-C motif ligand 22
IL-12P70	Interleukin-12 subunit 70
PDGF-AA	Platelet-derived growth factor AA
IL-13	Interleukin-13
PDGF-BB	Platelet-derived growth factor BB
IL-15	Interleukin-15
sCD40L	Soluble cluster of differentiation 40 ligand
IL-17A	Interleukin-17A
IL-1RA	Interleukin-1 receptor antagonist
IL-1a	Interleukin-1 alpha
IL-9	Interleukin-9
IL-1b	Interleukin-1 beta
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IP-10	Inhibiting protein-10
MCP-1	Monocyte chemoattractant protein-1
MIP-1a	Macrophage inflammatory protein-1a
MIP-1B or CCL4	Macrophage inflammatory protein beta or chemokine C-C motif ligand 4
RANTES or CCL5	Regulated on activation, normal T-Cell expressed and secreted or chemokine C-C motif ligand 5
TNFa	Tumour necrosis factor alpha
TNFB	Tumour necrosis factor beta
VEGF-A	Vascular epithelial growth factor-alpha
IL-18	Interleukin-18

Table 5: List of 42 proteins assayed using multiplex analysis described in Methods & Materials

Gene Code	Gene Name
CCL18	Chemokine (C-C motif) ligand 18
CCL2	Chemokine (C-C motif) ligand 2
CCL22	Chemokine (C-C motif) ligand 22
CD14	Cluster of Differentiation 14
CD163	Cluster of Differentiation 163
CD68	Cluster of Differentiation 68
CLEC10A	C-Type Lectin Domain Family 10 Member A
COL1A1	Collagen Type I Alpha 1 Chain
COL3A1	Collagen Type III Alpha 1 Chain
CXCL10 (IP-10)	C-X-C motif chemokine 10 or Interferon gamma-induced protein- 10
DDIT3 (CHOP)	DNA Damage Inducible Transcript 3 or CCAAT/enhancer-binding protein
FGF2	Fibroblast Growth Factor 2
FKBP2/FKBP13	FK506 Binding Protein 2, 13kDa
FN1	Fibronectin 1
HSPA5	Heat Shock Protein Family A (HSP70) Member 5
IL10	Interleukin-10
IL1RL1	Interleukin 1 Receptor-Like 1
IL33	Interleukin-33
IL4R	Interleukin-4 Receptor
IL6	Interleukin-6
IL6R	Interleukin-6 Receptor
ITGAM (CD11b)	Intergrin Subunit Alpha M
ITGAX (CD11c)	Integrin, alpha X (complement component 3 receptor 4 subunit) or CD11c
MRC1	Mannose Receptor C 1
MSR1	Macrophage Scavanger Receptor 1
NOS2	Nitric Oxide Synthase 2
OSM	Oncostatin-M
OSMR	Oncostatin-M Receptor
PDGFA	Platlet-derived growth factor subunit A
TGFB1	Transforming growth factor beta 1
VEGFA	Vascular endothelial growth factor
XBP1	X-box binding protein 1

Table 6: List of 32 genes assayed using nanostring analysis described in Methods & Materials

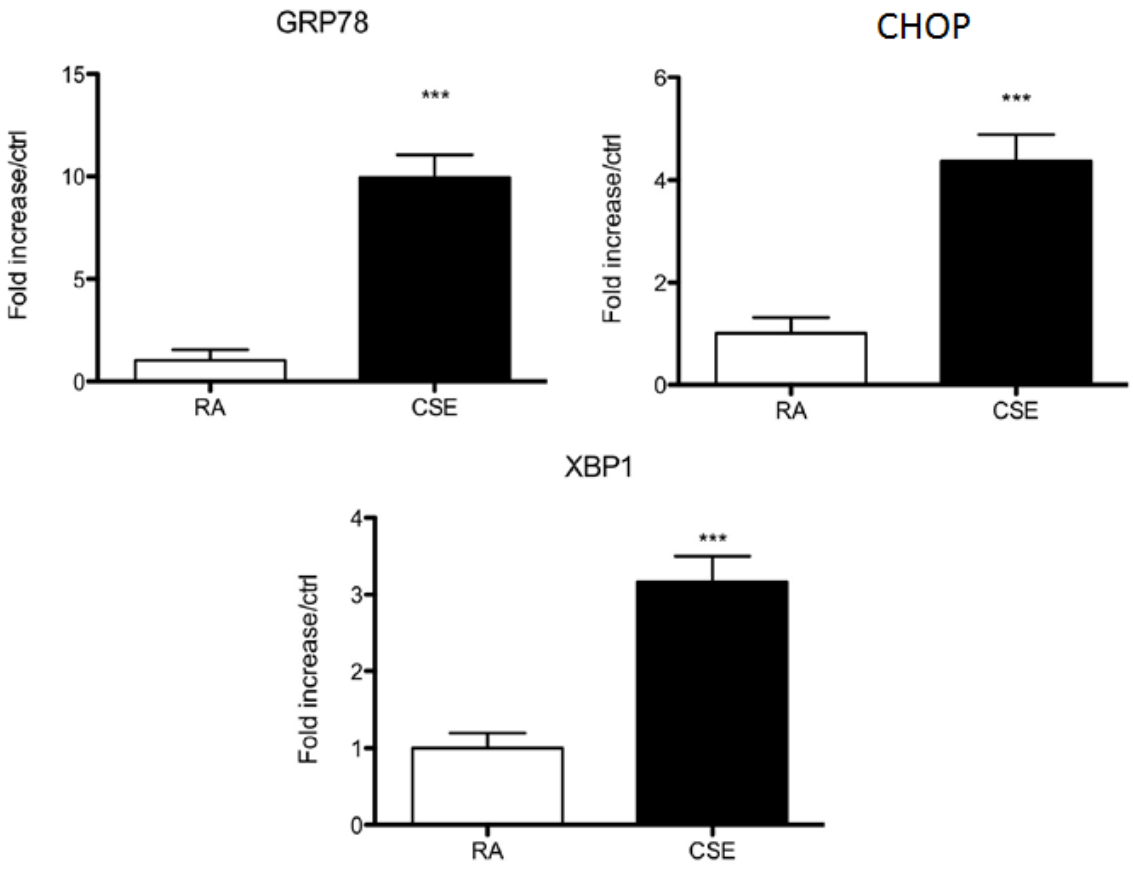

Figure 12: ER stress related genes measured from lung homogenates from 2 week cigarette smoke exposed C57Bl/6 mice (n=5). Data presented are fold induction over control samples and normalized to 18s expression. The data presented in the graph are expressed as mean ± SEM (n = 5-10 per group). Statistical significance was established using GraphPad, Prism 5.0 with 1way Anova using Bonferroni's Multiple Comparison Test, assessing the difference between control mice and smoke exposed groups (*$**p < 0.01, *p < 0.05$*).

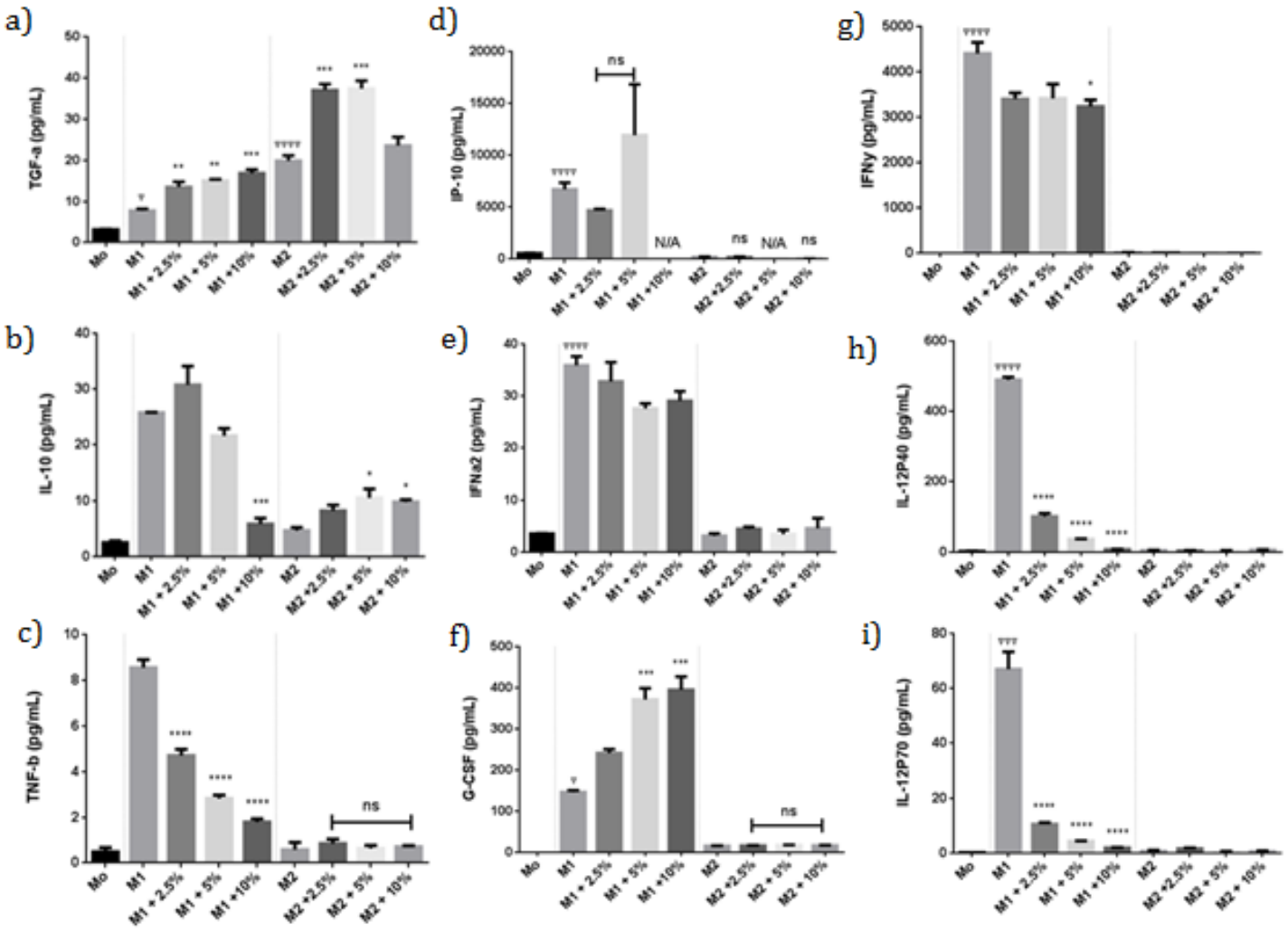

Figure 13: Measurement of cytokines in supernatant collected from THP-1 after 72h stimulation with MØ, M1 and M2 with various doses of cigarette smoke. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferroni. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

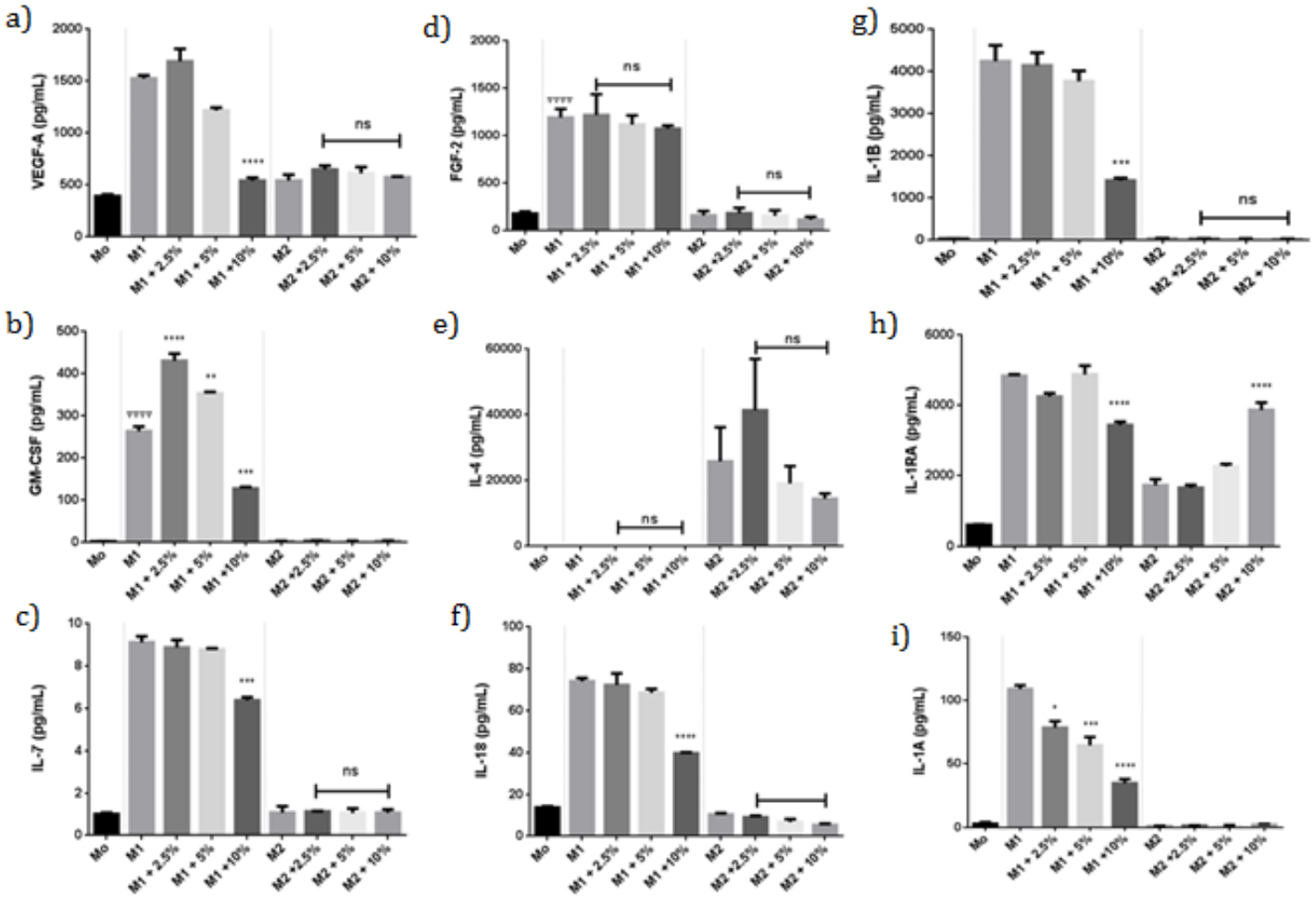

Figure 13 (continued 1): Measurement of additional secreted proteins (cytokines and growth factors) in supernatant collected from BMDM after 72h stimulation with MØ, M1 and M2 with various doses of cigarette smoke. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferroni. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

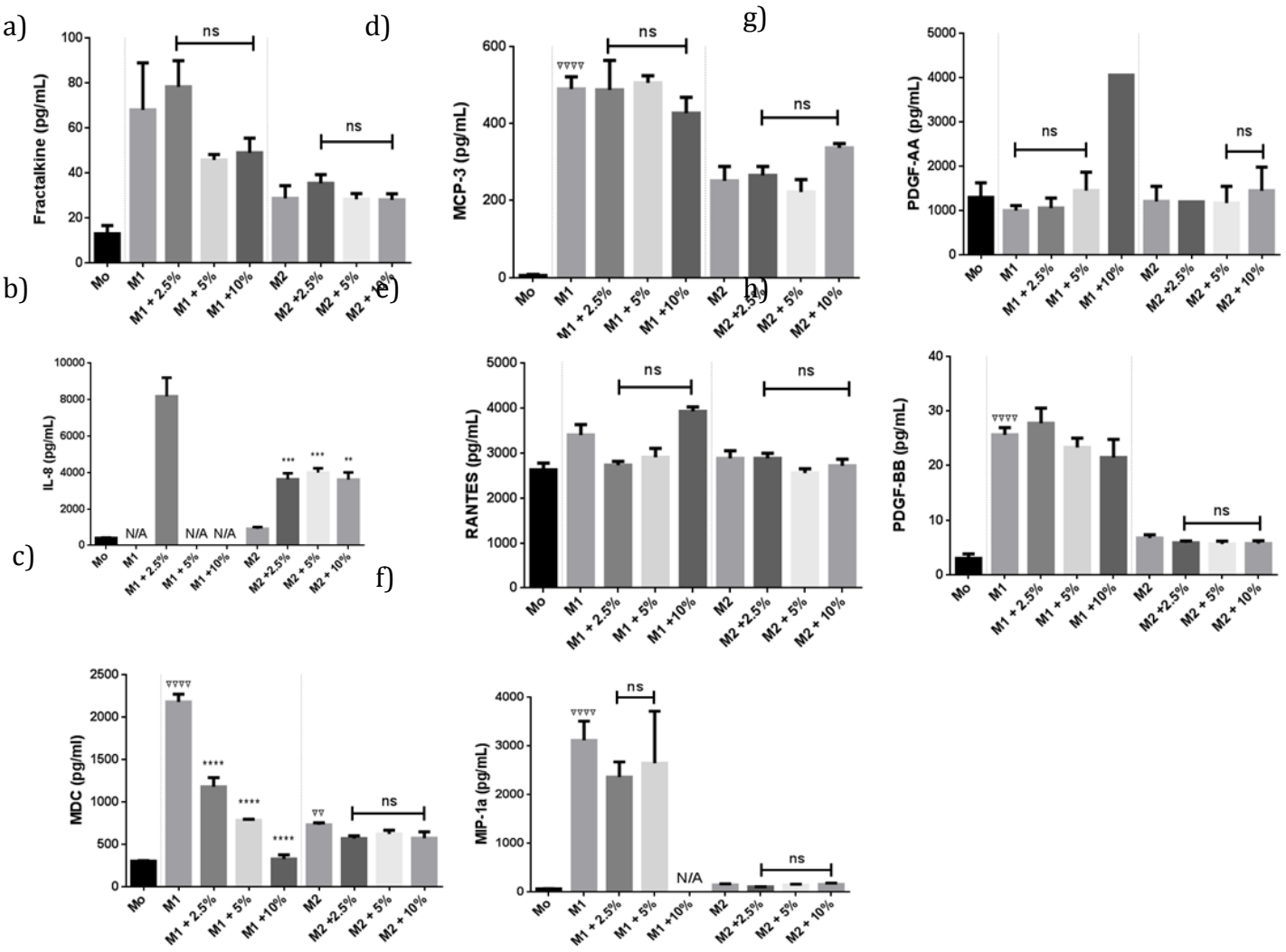

Figure 13 (continued 2): Measurement of chemokines in supernatant collected from BMDM after 72h stimulation with MØ, M1 and M2 with various doses of cigarette smoke. Results were graphed using mean with SEM. Statistical analysis was completed using ANOVA and Bonferonni correction (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

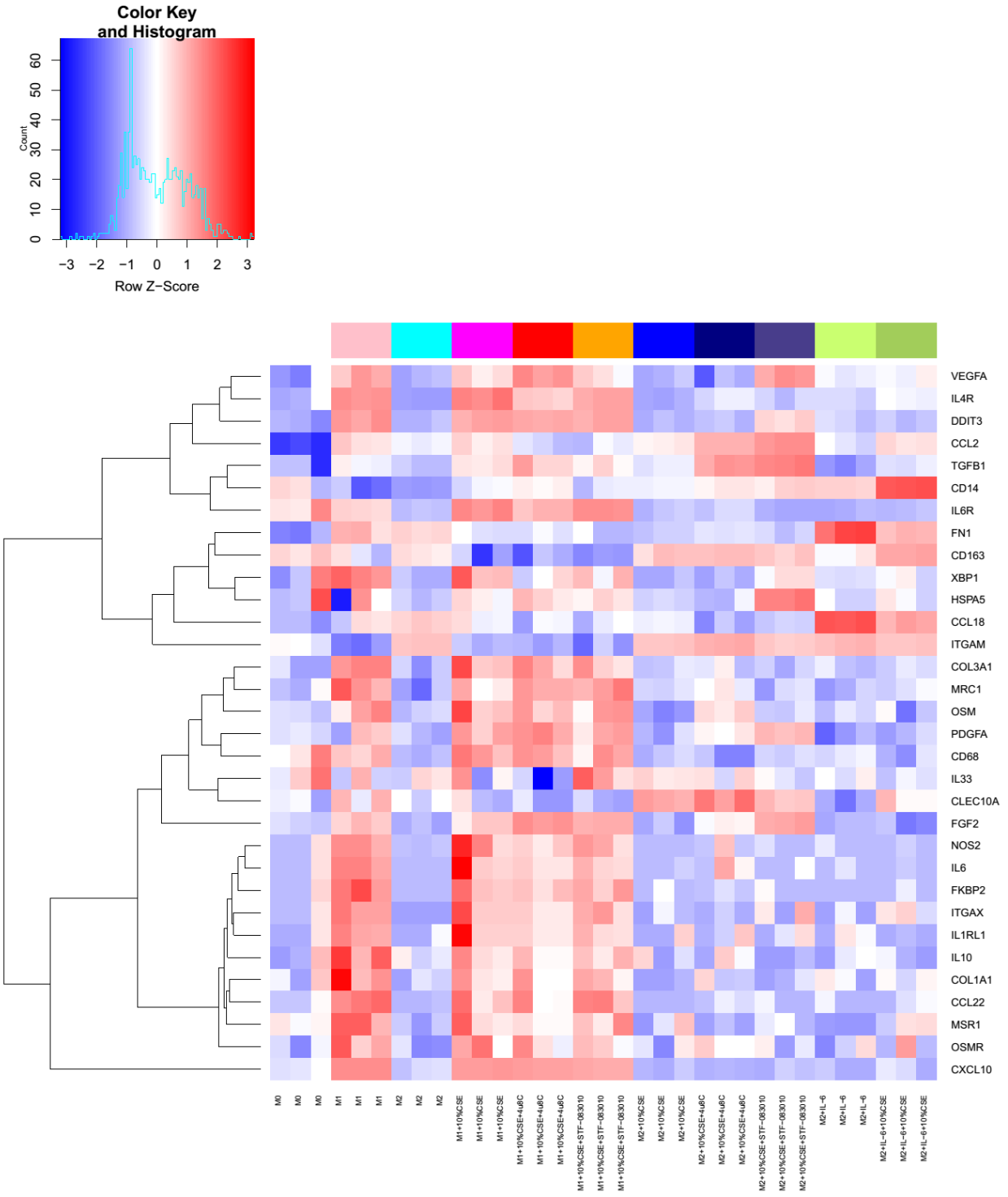

Figure 14: Heatmap representing gene expression from nanostring analysis. Average Log2 expression of each gene represented by colours: White (no change), blue (decrease) or red (increase) and dendrogram expressing the RNA patterns for each cell type group.