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Macrophage Polarization and Function in Cystic Fibrosis

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&

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Abstract

If "*Diversity is the rule of nature*", macrophages, one of the innate immune cells of our body, have proven to be a true apostle. At the early stage of infection, they protect the host by exhibiting the pro-inflammatory phenotype (M1). Upon clearance of microbial threats, macrophages engage themselves in the repair process of the damaged tissue by showing anti-inflammatory or wound healing (M2) attributes. While incomplete microbial clearance leads to recurrent infections, defects in macrophage-mediated tissue repair mechanism result in immunopathology. The classical (M1) and alternatively (M2) polarized macrophages are the two extreme ends of a spectrum of *in vivo* macrophages phenotypes that dictate the nature, duration and severity of inflammation. Murine models of chronic inflammatory and autoimmune diseases showed the necessity of an adequate balance between macrophage subsets to maintain homeostasis, while imbalance is likely to lead exaggerated inflammation. In humans, an association between defective macrophage function and disease severity has been reported in many diseases including asthma, cystic fibrosis (CF), COPD, and atherosclerosis. Unfortunately, human M1 and M2 macrophages have not been well characterized. ***Firstly***, the lack of homologs for certain murine genes in humans makes murine markers of limited use in humans. ***Secondly,*** there was no consensus method for *in vitro* differentiation of human M1 and M2 macrophages. Therefore, the first part of this thesis aimed to develop a novel method to differentiate and characterize human M1 and M2 macrophages. Initial studies with THP-1 cell line derived macrophage-like cells demonstrated that they didn't fully represent human monocyte-derived macrophages (MDMs). MDMs were therefore chosen as starting materials for the rest of the study. MDMs were considered as uncommitted "M0" macrophages. A number of inducers were employed to polarize M0 macrophages into either M1s or M2s. LPS treated M1 macrophages showed $CD64^{+}CD80^{+}$ phenotype, whereas, IFN-γ induced M1s exhibited $CD64^{++}CD80^{-}$ phenotype. These M1s secreted pro-inflammatory cytokines including TNF-α, IL-1β, IL-8 and were highly phagocytic. On the contrary, IL-4/IL-13 induced M2 macrophages were identified as $CD11b^{+}CD209^{+}$ cell population and were endocytic. Once polarized, macrophages then were left in cytokine-deficient medium to assess the persistence of polarized phenotype over time. In cytokine-free condition, previously polarized macrophages reverted to M0 state by 12 days. Treatment with IL-13 on previously polarized M1 macrophages resulted in a switch to $CD209^{+}$ M2s and *vice versa* indicating the plasticity nature of human macrophages.

Excessive neutrophilic pulmonary inflammation is the hallmark of cystic fibrosis (CF). However, factors that trigger such dysregulated neutrophilic inflammation and why this is not switched off are not yet clear. Macrophages play crucial roles in initiation and resolution of pulmonary inflammation, however, surprisingly little research in CF had been dedicated to

macrophages. Defective phagocytosis by macrophages and its association with cystic fibrosis transmembrane conductance regulator (CFTR) gene had been reported in CF studies. Nevertheless, the influences of the mutated CFTR gene on macrophage polarization and thereby function had not previously been studied in CF. The model described above was utilized with blood samples from adults and children with CF. M2 polarization was significantly repressed in patients with CF. The number of cells expressing the human M2 marker CD209 was significantly low in CF [median (25^{th}-75^{th}%): healthy (n=9) 59(55-82)%; CF children (n=14) 41(30-52)% ($p<0.01$); CF adults (n=13) 46(25-60)% ($p<0.01$)]. Endocytosis was also decreased in both children and adults with CF ($P<0.001$). Inhibiting CFTR function with $CFTR_{Inh}$-172 and GlyH-101 in healthy cells recreated the CF macrophage phenotype, with a decreased number of cells expressing CD209 and decreased endocytosis. Following IL-13 treatment, both CF M0 cells and $CFTR_{Inh}$-172 inhibited M0 cells showed decreased surface expression of IL-13Rα1 compared to M0s from healthy volunteers indicating the inability to respond to IL-13 being associated with CFTR dysfunction. Furthermore, during acute pulmonary exacerbation (APE), but not in clinically stable CF, a greater proportion of M0 and IL-13 treated macrophages displayed surface expression of M1 marker, CD80 in both adults and children indicating both M0 and M2s during APE being prone to M1 polarization. Taken together, these data report a CFTR-dependent defective M2, but not M1, polarization of macrophages in CF which might be the possible underlying mechanism for exaggerated neutrophilic responses and impaired resolution of inflammation in CF.

In summary, two novel models had been developed during this Ph.D. study. The ***in vitro human macrophage polarization and characterization model*** described the differentiation, phenotypic and functional characterization of human M0, M1 and M2 macrophages as well their plasticity nature. Knowing the functional states of pathogenic macrophages has a huge impact on understanding the disease pathogenesis and developing novel therapeutic targets. The ***CF macrophage model*** was developed with CFTR inhibitors that recreated CF macrophage phenotypes *in vitro*. Using this model a previously unidentified CFTR-dependent defect of M2 macrophage polarization in CF had been reported.

Declaration by author

This thesis is composed of my original work and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed Articles

1. **Tarique AA**, Logan J, Thomas E, Holt PG, Sly PD, Fantino E., Phenotypic, functional and plasticity features of classical and alternatively activated human macrophages, American Journal of Respiratory Cellular and Molecular Biology, Vol 53, No. 5, Nov. 2015; DOI: 10.1165/rcmb.2015-0012OC.

Conference Abstracts

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3. Poster presentation at Children Health Queensland (CHQ) Research Day 2015 at Lady Cilento Children's Hospital (LCCH), Macrophages in Cystic Fibrosis Inflammation: The Forgotten Cells.
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5. Oral presentation at Queensland Children's Medical Research Institute (QCMRI) Student Expo 2014, at QCMRI, Royal Children Hospital, Brisbane, Phenotypic, functional and plasticity features of classical and alternatively activated human macrophages.

Publications included in this thesis

I have chosen to incorporate one peer-reviewed publication into my thesis as per UQ policy (PPL 4.60.07 Alternative Thesis Format Options)

Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E., Phenotypic, Functional and Plasticity Features of Classical and Alternatively Activated Human Macrophages, American Journal of Respiratory Cellular and Molecular Biology, Vol 53, No. 5, Nov. 2015 – incorporated as ***Chapter 3***.

Contributor	Statement of contribution
Author: Tarique AA (Candidate)	Conceptualized the study (70%) Designed and conducted experiments (80%) Data analysis (80%) Wrote the manuscript (80%)
Author: Logan J	Conducting experiment (20%)
Author: Thomas E	Conducting experiment (20%)
Author: Holt PG	Conceptualized the study (10%) Edited the manuscript (10%)
Author: Sly PD	Conceptualized the study (10%) Data analysis (10%) Edited the manuscript (10%)
Author: Fantino E	Conceptualized the study (10%) Data analysis (10%) Edited the manuscript (10%)

Contributions by others to the thesis

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Statement of parts of the thesis submitted to qualify for the award of another degree

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Macrophages, classical/alternative polarization, phagocytosis, endocytosis, cystic fibrosis, CFTR inhibitor, IL-13Rα1, IRF4, Cox-2, tobramycin.

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Contents

List of Tables

Lists of Supplementary Tables

List of Figures

List of Supplementary Figures

List of abbreviations used in the thesis

7-AAD	7-aminoactinomycin D
AM	Alveolar macrophage
AAM	Alternatively activated macrophage
AEC	Airway epithelial cells
AF	Alexa Fluoro
AHR	Airway hyper-responsiveness
APC	Allophycocyanin
APE	Acute pulmonary exacerbation
ARCBS	Australian Red Cross Blood Service
Arg 1	Arginase synthase 1
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
CAM	Classically activated macrophage
CD	Cluster of differentiation
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
CFTR-KO	Cystic Fibrosis transmembrane conductance regulator knock-out
CHRC	Children's Health Research Centre
CO_2	Carbon dioxide
Cy	Cyanine
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and drug administration
FEV_1	Forced expiratory volume in one second
FITC	Fluorescein isothiocyanate
GC	Glucocorticoid
GIT	Gastro-intestinal tract
GM-CSF	Granulocyte and macrophage colony stimulating factor
HBD	Human β-defensin
HPA	Hypothalamus-pituitary-adrenal
HREC	Human research ethics committee
HTS	High throughput screening

ICAM-1	Intercellular adhesion molecule 1
IC	Immune complex
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-13	Interleukin-13
IM	Intramuscular route
IN	Intranasal route
iNOS	Inducible NO synthase
IP-10	Interferon gamma-induced protein 10
IPA	Ingenuity Pathway Analysis
IRF	Interferon regulatory factor
IQR	Interquartile range
IV	Intravenous route
LCCH	Lady Cilento Children's Hospital, Brisbane
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
Mϕ	Macrophage
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte-derived macrophage
MPS	Mononuclear phagocyte system
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PE	Phycoerythrin
Pen-Strep	Penicillin-Streptomycin
PMN	Polymorphonuclear cells
QCMRI	Queensland Children's Medical Research Institute
RBC	Red blood cell

RCH	Royal Children's Hospital, Brisbane
RCT	Randomized clinical trial
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RV	Rhinovirus
SD	Standard deviation
SEM	Standard error or mean
SFM	Serum-free medium
TAM	Tumor-associated macrophages
TIP	Tobramycin inhaled powder
TNF-α	Tumor necrosis factor-α
URT	Upper respiratory tract
UQ	The University of Queensland
UWA	University of Western Australia

Chapter 1 Introduction and Literature Review

1.1 Monocyte/Macrophages Linage: The First Line of Immune Defence

The mammalian immune system consists of two arms: innate and adaptive immunity. Innate immunity is more ancient and constitutes the first line of defense against foreign pathogens (1). The mononuclear phagocyte system (MPS) is composed of three major cell types: monocytes, macrophages and dendritic cells (DCs). Although they have remarkable heterogeneity related to phenotype, functions, tissue localization, proliferative potentials, these features also overlap on all these cell types, therefore, no precise boundary had been reported (2). Monocytes and macrophages are the central players of innate immune system and are engaged in mounting immune responses to invading pathogens by phagocytosis, release of reactive oxygen species (ROS), inflammatory cytokines and chemokines. They also play critical roles in the resolution of inflammation and reparative functions to the damages tissues. DCs, on the contrary, present antigens to the players of adaptive immune system (3).

Monocytes originate from common myeloid progenitor cells in the bone-marrow and constitutes around 10% of peripheral white blood cells. After release into the peripheral blood, monocytes circulate for several days (3). Following immunological insults, pro-inflammatory or metabolic stimuli, circulating monocytes enter into tissue, under the influence of specific growth factors, such as, macrophage colony-stimulating factor (M-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF) differentiate into either tissue macrophages or specialized cell types, such as, dendritic cells (DCs) and osteoclasts and deal with the causative agents to maintain homeostasis. However, this traditional concept has been challenged by many recent studies showing that alveolar macrophages (AMs) of the lungs, microglia of brain, Langerhans cells of skin were seeded during embryonic development and are renewed by local proliferation (4-9).

Peripheral blood monocytes are heterogeneous in terms of size, granularity and nuclear morphology. Based on the differential expression of the co-receptor for lipopolysaccharide (LPS), CD14, Fcγ receptor (Fcγ), CD16 (also known as FcγR-III) and unique gene expression profile, human blood monocytes were divided into three subtypes: ***classical monocytes*** ($CD14^{++}CD16^{-}$), ***intermediate monocytes*** ($CD14^{++}CD16^{+}$) and ***non-classical monocytes*** ($CD14^{+}CD16^{++}$) (10, 11) (**Figure 1**), whereas, two subsets were identified in mice: ***resident monocytes*** ($CX_3CR1^{High}CCR2^{-}Gr1^{-}$) and ***inflammatory monocytes*** ($CX_3CR1^{Low}CCR2^{+}Gr1^{+}$) (12). In human, classical monocytes comprise the major portion (90%) of peripheral monocyte pool (10). They are able to sense microbial stress, perform chemotaxis, phagocytosis and release pro-inflammatory cytokines. They are considered as the counterpart of murine $Gr1^{+}$ inflammatory monocytes (13). Intermediate monocytes

were proposed as transitional population bridging between classical and non-classical monocytes (10). They have higher ability for antigen presentation and resembled tissue resident macrophages (11). Following macrophage colony stimulating factor (M-CSF) treatment, intermediate monocytes expanded earlier than non-classical monocytes (10, 11). Non-classical monocytes are involved in patrolling and sensing viruses and nucleic acids, however, their responses to LPS were poor (10, 11). Cluster analysis of microarray data demonstrated that the intermediate and non-classical monocytes were closely clustered, whereas, classical and non-classical monocytes were distally clustered (10). Both classical and intermediate monocytes express chemokine receptor 5 (CCR5), whereas, non-classical monocytes express CCR2 (3).

The physiological roles of human monocytes in healthy and disease conditions have not yet been fully revealed. Broadly, both classical and intermediate monocytes are able to exhibit inflammatory properties (14, 15). Elevated $CD16^{+}$ monocytes were observed during bacterial infections (14). Murine $Gr1^{-}$ monocytes patrol blood vasculature, enter to the tissue at later stage of inflammation and have been suggested to be associated with tissue repair (16, 17). However, clinical translation of such murine data was yet not confirmed by human studies (18).

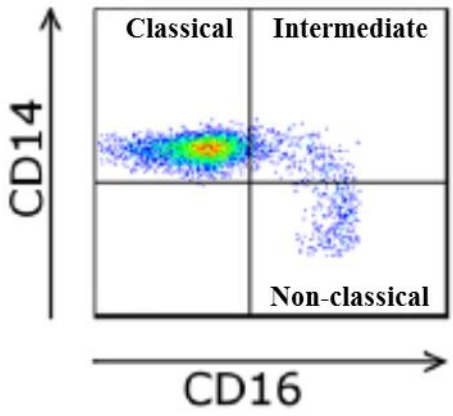


Figure 1: Phenotypic profile of human monocyte subsets. Figure was adapted from Cros *et al* (13). Human monocytes were stained with anti-human CD14 and CD16 antibodies.

1.2 Macrophage: A Multi-role Immune Cell

Macrophages constitute a heterogeneous cell population of innate immune system that regulate tissue immunity by orchestrating the initiation and resolution of immune responses against environmental or microbial agents and repair of damaged tissue. They were first described by a Russian-French biologist, Elie Metchnikoff in late 1884 as phagocytic cells engaging against invader

pathogens by elimination. The name "***macrophage***" came from the Greek words, "*makros*" large and "*phagein*' eat (15). In 1905, Hirsch *et al* proposed these phagocytic cells as resistant to certain bacterial infections and setting the basis for the concept (19). After 60 years of scientific efforts, it was gradually evident that antibacterial activity of macrophages is the immunological basis of acquired cellular immunity (20). Following infection, dead cells missing MHC-I antigens are first targeted by NK cells, then macrophages appear as the second earliest cell type found at the site of infection (4). By releasing ROS and pro-inflammatory cytokines, macrophages then exert their anti-microbial activity, clear invading pathogens and apoptotic polymorphonuclear cells (PMNs) by phagocytosis. In addition, macrophages communicate with the adaptive immune system by presenting antigens and cytokine storm. At the later stage of inflammation, macrophages play a reparative role to repair damaged tissues at the site of inflammation (21, 22).

Macrophage phenotypes and functions vary with their anatomical locations (15). Macrophages are found in lymphoid as well as non-lymphoid organs such as liver (Kuppfer cells), lung (alveolar macrophages), nervous system (microglia), reproductive organs, and in gut lamina propia (23). Kupffer cells, the resident macrophages in the liver, comprise the largest pool of tissue macrophages in the body. Based on location in the lungs, two different types of macrophages have been described: alveolar macrophages (AMs) and interstitial macrophages (IMs) (24). Both AMs and IMs were originated during fetal development, however, during inflammation, circulatory monocytes migrate to alveolar spaces and conducting airways. Compared to other tissue macrophages, AMs are more phagocytic and express high levels of CD11c and DEC-205 (25). AMs phagocytose potentially harmful air-borne pathogens, generate immune response to recruit other inflammatory cells from blood (26). Under resting conditions, AMs have a half-life of 1~2 months with a turnover rate of 40% in a year in mice (27), however, no studies till date reported about the half-life of human AMs. Microglia of brain are unique from tissue macrophages in many aspects. In particular, microglia arise from early yolk sac progenitor cells and have their own self-renewal process by local proliferation (7, 28). Unlike other tissue resident macrophages, tumor-associated macrophages (TAMs) abundantly found in tumor exhibit immunosuppressive nature (29).

1.3 Macrophage Polarization and Their Plasticity

Macrophage function varies with stimuli they received from their surrounding environment. Therefore, activation, also known as polarization, of macrophages has evolved as a new fundamental area of innate immunology. Over the last decades, the term "activation" or "polarization" of macrophages had been used to describe induction of certain sets of genes and proteins of recruited

macrophages by cytokines or toll-like receptor (TLR) agonists to display acquired tumoricidal or microbicidal activity. The origin of these terms was originated in the early 1990s when interferon-gamma (IFN-γ) and IL-4 were described to induce classical and alternative polarization of macrophages respectively (30-32). Later classical and alternative polarization were observed to be correlated with T_H1 and T_H2 paradigms, Mills then proposed the "***M1-M2***" terminologies for CAMs and AMMs respectively (32, 33). In this chapter, the words "***polarization***" and "***M1-M2***" were mostly used.

1.3.1 Classical (or M1) Activation of Macrophages

Macrophages polarized by IFN-γ or bacterial products, such as, LPS, are currently referred as *"**classically activated macrophages**"* or *"**M1 macrophages**"*. The understanding of this "classical" or "M1 polarization" dated from studies during the 1960s when Mackaness and colleagues described enhanced anti-microbial activities of macrophages in mice infected with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Listeria monocytogenes* (34). Soon after the discovery of IFN-γ and its cellular activity, it had been shown that IFN-γ produced by type I T helper (T_H-1) cells activates antimicrobial, cytotoxic and tumoricidal activities of macrophages (35-38). Later, studies with knockout mice and in humans with genetic defects of IFN-γ or its receptors validated that M1 polarization is crucial for host defense against microbial infection. Intracellular pathogens, TNF-α, IFN-γ, TLR4 ligands were known inducers of M1 polarization (39) though others have shown M1 polarization with GM-CSF (40, 41). Radiation (42), oxidized low-density lipid molecules (15) and a diet containing high salt (43) were able to trigger M1 polarization with enhanced pro-inflammatory cytokine secretion.

M1 phenotype is typically considered as being IL-12^{high} and IL-10^{low} (44, 45). M1 macrophages produce microbicidal enzymes, such as, inducible NO synthase (iNOS) to destroy invading pathogens, release pro-inflammatory cytokines, such as, IL-1β, IL-6, IL-12, IL-23, TNF-α, various chemokines to chemoattract other immune cells to the site of infection and matrix metalloproteinase (MMPs) and finally promote T_H1 immunity (44, 46). The panoply of cytokine and antimicrobial genes triggered during M1 polarization are under the regulation of a set of transcription factors. These include signal transducer and activator or transcription 1 (STAT1), nuclear factor-κ B (NFκ-B) and mitogen-activated protein kinases (MAPKs) pathway.

Although several studies showed the importance of M1 macrophages in host defense, a few concerns about these cells deserve particular attention. Few M1 cytokines, such as, IL-1, IL-6, IL-23 are able to efficiently trigger T_H17 cells. IL-17 from these T_H17 cells recruit PMNs that contribute autoimmune pathologies (22, 47-49).

1.3.2 Alternative (or M2) Activation of Macrophages

Inhibition of M1 polarization of macrophages was first reported by Abramson *et al* (50) who demonstrated inhibition of IFN-γ induced superoxide ($O2^-$) production by IL-4. The term "alternatively activated macrophages" was described by Gordon and colleagues in the 1990s following their observation that IL-4 induced mannose receptor expression on macrophages (31, 51). The term "alternative activation" initially caused confusion in the field since the term "activation" was generally associated with host defense which was not in the case here. Later, the concept of alternative activation of macrophages by type 2 cytokines, IL-4 and IL-13 gained credence and was accepted by many working groups with human cells. These "alternatively-activated macrophages were thought to be involved in tissue repair, tumor progression and fibrosis (52-54). M2 macrophages were characterized by the cell surface expression of the macrophage mannose receptor (MMR) CD206 (31), CD163 (55), CD23 (56) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)/CD209 (57) and the synthesis of soluble factors, such as the CC chemokine ligand 17 (CCL17) (58), CCL18 (59), TGF-β (60). Other than surface markers and cytokines, induction of *Arg 1*, Ym1 (also known as CHI3L3), Ym2, and FIZZ1 were reported as signature genes of M2 polarization (61). The enzyme arginase, *Arg 1*, is involved in the metabolism of arginine into ornithine that leads to the production of proline (62 452). *In vitro studies* showed proline production is directly associated with collagen deposition and production of extracellular matrix (ECM), thereby fibrosis (63). Production of other matrix building proteins, such as, chitinase and chitinase-like molecules, acidic mammalian chitinase (AMCase) and stabilin-interacting chitinase-like protein during M2 polarization support their wound healing property as well (22, 64-66). In contrast to M1, M2 macrophages demonstrate relatively poor antigen presentation, highly phagocytic, release anti-inflammatory cytokines, such as, IL-10, and promote regulatory T cell (Treg) development (55, 67, 68). Furthermore, M2 macrophages are key players in tissue remodeling, wound healing, chronic inflammation, angiogenesis and fibrogenesis (22, 69, 70).

Based on the stimuli to induce M2 polarization, M2 macrophages were further subdivided into three types: ***M2a*** induced by IL-4/IL-13, ***M2b*** induced by immune complexes (ICs) and TLR agonists or IL-1R and ***M2c*** by IL-10/TGF-β (44). Arginine pathway is induced by M2a and M2c, but

not in M2b. Macrophages exposed to IC and LPS (M2b) release high levels of pro-inflammatory cytokines with concomitant IL-10^{high} and IL-12^{low} (71). Despite their pro-inflammatory cytokine production, M2b macrophages promote T_H2 differentiation and humoral antibody production (44, 68). Interestingly molecular modeling of all these three M2 subsets demonstrated three distinct populations with discrete transcriptional profiling (72). It is possible that during the acute phase of infection, presence of huge amounts of LPS induces M2b phenotype and IL-10 release by macrophages; this secreted IL-10 later suppress the inflammation by polarizing M2c phenotypes. At the later stage of inflammation, M2a macrophages predominate and promote wound healing or fibrosis. However, this concept needs to be confirmed by systematic investigation approaches and to ratify that all these versions of M2 macrophages exist in the immune system and play their own role without eliminating others. This concept of diverse versions of M2 macrophages was later supported by Mosser *et al* though with different names: immune complex (IC) or glucocorticoid (GC) induced regulatory macrophages, IL-4/ IL-13 mediated pro-fibrotic/wound-healing macrophages, and tumor-associated macrophages (TAMs) (22, 44, 73). GCs released by adrenal cells at the hypothalamus-pituitary-adrenal (HPA) axis inhibit macrophage mediated inflammatory responses as well as host defense mechanisms, giving rise to regulatory macrophage population that abundantly produce tumor growth factor-β (TGF-β). Regulatory macrophages can also rise during late stages of inflammation with the key role of limiting inflammation though the molecular mechanism is yet to be identified (71). Tumor-associated macrophages (TAMs) are identified by their secretion of a variety of angiogenic factors (22, 73). Although previous literature has equated TAMs to M2s, increasing evidences suggest that TAMs are a blend of multiple distinct populations with overlapping features, rather than M2s (73).

1.3.3 Plasticity Nature of M1 and M2 Macrophages

Macrophages are able to exhibit an array of functions through the release of several factors. Their diversified pro- and anti-inflammatory roles associated with M1 and M2 polarized states raise important questions: ***1)*** whether the polarization of macrophages is stable, reversible or a state of terminal differentiation, ***2)*** upon withdrawal of inflammatory stimuli, whether polarized macrophages undergo apoptosis and ***3)*** whether reparative macrophages found during the healing phase originate from an entirely new emigrated population. Furthermore, the presence of other immune cells particularly lymphocytes should be taken into account their cytokine secretions are likely to alter macrophage polarization statuses and thereby functions. It is not yet known whether de-differentiation of once polarized macrophages is necessary to respond to new stimuli or immigration of new macrophages would replenish the once polarized macrophages. In murine models of inflammatory

bowel disease and asthma, adoptive transfer of M2 macrophages not only reversed the distribution of macrophage subsets but also reduced the disease severity, suggesting a dual role for macrophages in orchestrating both the onset and resolution of inflammation (74, 75). The ability of macrophages to adapt to changing cytokine environments has been demonstrated *in vivo* in tumour-bearing mice, where changing the microenvironment resulted in a switch of macrophage phenotypes (63, 76, 77).

Several hypotheses have been postulated to explain the mechanism of this plasticity issue (78). The ***first hypothesis*** considered specific monocyte subsets as precursors of specific macrophage phenotypes. For instance, $Ly6C^+$ monocytes become M1 macrophages and $Ly6C^-$ monocytes become M2s in tissues (16, 17, 78). However, differentiation of $Ly6C^+$ cells to M1, or $Ly6C^-$ cells to M2 (16, 79) and transactivation of $Ly6C^+$ M1 cells to M2 cells (80, 81) does not support this view. The ***second hypothesis*** proposed sequential recruitment of monocytes into the tissue during the course of inflammation. Therefore, monocytes entering at different phases of inflammation come across different microenvironments that can activate them into M1s during the acute phase and into M2s at the reparative phase (80). However, *in vivo* transactivation of M2 from M1 macrophages in sterile wounds (81) or injured kidney (82) weaken this argument. Based on the later data (81, 82), a ***third hypothesis*** postulated that macrophages can switch their functional states from one to another in response to sequential microenvironment changes, suggesting that the polarized state of macrophages is reversible (76, 83). However, apoptosis of M1 cells by its own NO does not fully support this hypothesis [Reviewed in (15)](84). Although the mechanism remains unexplored, an adequate balance between M1 and M2 subsets is required to maintain homeostatic milieu, and disequilibrium is likely to lead to unbalanced inflammation. The perception of adapting functions with changing environment has huge implications on understanding the role of macrophages in disease pathogenesis and development of therapeutic targets.

1.4 Human and Mouse M1/M2 Macrophage Characterization: Where Do Murine Models Diverge From Human?

Rodents and primates are being frequently used in studying human diseases to identify the basic pathophysiological mechanisms and to evaluate therapeutic approaches. Reasons to choose animal models include their availability, low housing cost, ease of modification according to interest and most importantly, they partly mirror the human immune system. Another advantage of using mouse models is that regular confounding factors can easily be controlled with animal housing conditions. However, there are no systematic studies that evaluate how well animal models mimic human pathological conditions. Absence of animal orthologs in humans is one of the main barriers to

translate animal data into humans. Another problem with animal models is that *in vivo* experiments are hardly able to exclude the effects of pathway redundancy, i.e., knocking out of a single gene may confirm its phenotype, but can't exclude the possibility of another functionally equivalent gene to take over to trigger the similar or opposing pathway of interest. For instance, although *in vivo* induction of M2 signature genes, i.e., Ym1, FIZZ1, were drastically halted in IL-4KO mice, a still significant expression of these two genes was observed. Such residual though significant induction might be as a result of IL-13 signaling (85). In the field of macrophage polarization and function, comparative gene expression analysis of mouse and human found ***Transglutaminase 2 (TGM2)*** as the single gene to be expressed by both species (21). This section, therefore, aimed to point out the paradigm and dichotomy of human and murine polarization of macrophages to better understanding the knowledge gap associated with species differences.

Macrophage responses are extremely dependent on ***1)*** cell types used, ***2)*** stimuli used to induce macrophage polarization. These two issues hugely contribute to the ultimate outcomes of the study, such as, gene expression, cytokine and chemokine secretion and phagocytosis of apoptotic cells. This section shows how the selection of these two factors determined the polarized macrophage attributes and led to discrepancies in earlier studies were discussed.

1.4.1 Starting Material Does Matter

Genetic background is important while studying host responses to pathogens. Studies using mouse macrophages as a model for macrophage polarization have shown that mouse strains or cell lines differ in their abilities in cytokine or chemokine production, bacterial killing and time of response. C57BL/6 mice were mostly used for macrophage polarization studies (41, 85-87). Macrophages of C57BL/6 mice contain a deletion in the promoter region of arginine transporter gene, *Slc7a2* making them genetically resistant to M2 polarization (88). Following TLR2 and TLR4 agonist challenges, macrophages from C57BL/6 mice preferentially produce high IFN-γ, TNF-α, IL-10, IL-12, IL-17 and low IL-4 and exhibit reduced systemic inflammatory responses (89, 90). These data suggest that M1 polarization would more easily achieve in C57BL/6 mice than BALB/c mice (33). Conversely, BALB/c showed higher cytokine and chemokine release in the plasma suggesting enhanced systemic responses to pathogens (***Table 1***). Macrophages from BALB/c mice showed lowered production of NO and lysosomal enzymes, resulting in lowered bacterial clearance by BABL/c mice than C57B/6 (89-92). NO production in BALB/c macrophage was significantly low than macrophages from C57BL/6 and was observed at an early stage following LPS challenge than at later stage of C57BL/6 mice (89). Phagocytic activity and bactericidal ability of alveolar

macrophages from the abovementioned mice strains were different too (93). Hence, C57BL/6 mice are regarded as T_H1-dominant mouse strains, whereas, BALB/c mice are considered as T_H2-biased mice strain (90, 91). Conducting macrophage polarization studies with C57BL/6 mouse strain inevitably bias the study outcomes toward classical macrophage phenotype, while it is opposite for BALB/c mice. Another inbred mouse strain, CBA is used as a model for studying immunobiology of cardiovascular and kidney diseases. Macrophages from CBA mice exhibited lowered bactericidal responses and failed to activate NFκB signaling pathway (94-96).

Cell lines are generally used to test the experimental hypothesis as they offer a homogenous population and ease to manipulate them based on the research questions. Macrophage researchers frequently use leukemic macrophage-like cell lines, such as J774.1, RAW 264.7 and P388D1 from murine sources and THP-1, U937, HL-60 for human. RAW 264.7 is the most commonly used murine macrophage cell line which was derived from BALB/c mice infected with Abelson leukemia virus. Following treatment with appropriate agents, RAW 264.7 cells express Fc receptors for immunoglobulins, release lysozymes and show phagocytic properties to zymogen and latex beads. However, RAW 264.7 is extremely sensitive to LPS. The proliferation ability of these cells was found to be severely halted by LPS stimulation, and they underwent morphological transformation by displaying DC like morphology (97). J774.1, on the other hand, is a murine monocytic cell line, mostly used to investigate the host-pathogen interaction studies. Both of these cell lines showed similar gene expression to TLR agonists, albeit the time to response to stimuli is shorter in RAW cells than JJ774.1 cells. However, the inflammatory responses of either of these two cell lines do not correspond the tissue macrophages (98). LPS treatment unable to induce IL-12p70, the bioactive form of IL-12, by either RAW 264.7 or J774.1 cells albeit IL-12p40 subunit was minimally induced in J774.1 cells, but not in RAW 264.7 cells (***Table 1***). Surprisingly, none of these two cell lines produced IL-12p35 subunit (98). These data suggest that these murine macrophage-like cell lines have distinct regulatory systems that differ from tissue macrophages when responding to inflammatory signals. Furthermore, continuous *in vitro* culture of leukemic cell lines may cause gene loss resulting in impaired macrophage phenotypes and functions, thereby erroneous interpretation of outcomes. Therefore, extensive care should be taken to extrapolate cell line data to tissue macrophages.

To date, the four major primary mouse macrophages, named by their sites that have been extensively used are peritoneal macrophages (PMs), bone marrow-derived macrophages (BMDMs), splenic macrophages (SMs) and alveolar macrophages (AMs). Although PMs, AMs and SMs represent tissue resident macrophages and morphologically and functionally they are very similar, they are markedly distinct in many aspects. For example, PMs with their larger size and higher

lysosomal content are considered as more matured macrophages than the other three mentioned above (99). Elevated expression of MHC II and CD86 ascribed higher antigen presentation ability of PMs. In response to microbial pathogens, PMs exhibited enhanced NO and IL-12 production, however, they lack proliferative ability, exhibited downgraded phagocytic and antigen presentation and scavenger receptor activity (100, 101). Recently Ghosn *et al* described two physically, functionally and developmentally distinct PMs in the peritoneal cavity (102). The first subset was comparatively larger in size and was described as resting PMs. These resting PMs were found to express high levels of canonical macrophage markers, F4/80 and CD11b. Following LPS challenge, these resting PMs exhibited rapid production of NO and thereafter disappeared. The second subset that enters into peritoneal cavity following LPS challenge was originated from blood monocytes and was described as recruited PMs. Recruited PMs were smaller in size, expressed a basal level of CD11b and F4/80 and exhibited higher phagocytic activity than resting PMs. This extended heterogeneity of PMs was not taken into account during earlier macrophage polarization studies. AMs are lung resident macrophages found in bronchoalveolar lavage (BAL) fluid and are critical for maintaining surfactant homeostasis in alveolar space. Because of the unique location in the lungs, AMs are the only cells among all phagocytes who are continually exposed to noxious materials and infectious pathogens of the air (103). AMs express a number of pattern recognition receptors (PRRs) and possess high phagocytic activity, which allows them to respond against respiratory pathogens (104). In addition, AMs exert anti-inflammatory features by releasing immunosuppressive mediators such as IL-10 (105) and are able to restrain antigen presenting ability of respiratory DCs (106, 107). During steady state, AMs don't proliferate (108). AMs are mostly $CD206^{+}$ cells, though AMs found in the airway linings are $CD169^{+}$, whereas, AMs those are found in the airway epithelia are $CD169^{-}$ (109). SMs are the most immunogenically active macrophages in the body and able to produce enormous amount of pro-inflammatory cytokines and highly cytotoxic (100). However, SMs are less phagocytic and require M-CSF for their survival *in vitro* (99). Nonetheless, AMs, PMs and SMs have a notable drawback. The multi-step isolation procedure from tissues is time-consuming and the yield varies with isolation approach and skills of the laboratory personnel. Furthermore, the environmental conditions of the animal facility severely affect the macrophage biology. On the contrary, M-CSF-treated BMDMs are fully differentiated macrophages from mouse bone marrow regardless of the health condition of the donor mice. BMDMs offer a homogenous population with longer life span. These advantages of BMDMs made them more accessible as macrophage model in immunological research in last decades. However, BMDMs are highly responsive to M-CSF, highly proliferative and phagocytic in nature and produce significantly less TNF-α following LPS challenge than PMs (100, 110). In resting condition, BMDMs produce significant amount of IL-10 and TGF-β indicating that *in vitro* grown BMDMs might be less mature and potentially be more likely to have M2 phenotype,

giving caution to use in macrophage biology (100). Some studies used BMDMs differentiated in conditioning media, for instance, L929, which is a rich source of GM-CSF and GM-CSF differentiated bone marrow cells were considered as a model of DCs (111, 112). Such discrepancies in the initial materials used in macrophage studies would expect to have a substantial impact on the study outcomes (***Table 1***).

Table 1: Issues to consider while choosing models for macrophage polarization studies.

Starting Materials			**Remarks**
Mouse	Animal model	C57BL/6	Due to deletion in *Arg* transporter promoter gene (*Slc7a2*), sensitive to M1 polarization
		BALB/c	Low NO and lysosomal enzyme production Lowered bacterial clearance Enhanced systemic responses
		KO model	Pathway redundancy
	Cell lines	RAW 264.7	Proliferation is halted after LPS stimulation Exhibit DCs morphology following LPS stimulation No IL-12p70 production
		J774.2	Minimal induction of IL-12p40 No IL-12p70 production
	Primary cells	PM	Lack of proliferation ability Less phagocytic Less Ag presentation ability Less scavenger receptor activity Two different subsets exist Time-consuming and laborious isolation protocol
		AM	Low Ag presentation ability High IL-10 release Lack of proliferation ability Expression of surface markers varies with locations

			Time-consuming and laborious isolation protocol
		SM	Highly inflammatory Less phagocytic Time-consuming and laborious isolation protocol
		BMDM	Highly proliferative Highly phagocytic Less inflammatory Require special conditioning medium
Human	Cell lines	TDMs	Inducer dependent macrophage differentiation Resistant to M-CSF and GM-CSF stimulation Lose proliferation ability No expression of CD206, MHC II DRα1 High expression of CD14 and IL-1β Attenuated TLR expression
		U937	Pro-inflammatory profiles following LPS challenge is different than MDM
	Primary cells	Monocytes	Less inflammatory than MDM Transcriptomically different than MDM
		MDM (GM-CSF)	Enhanced inflammatory cytokine release Higher HLA-DQ expression
		MDM (M-CSF)	Highly phagocytic More immunosuppressive (IL-10)

Ag: antigen; AM: alveolar macrophages; BMDM: Bone marrow derived macrophages; DC: Dendritic cells; MDM: Monocyte-derived macrophages; PM: Peritoneal macrophages; SM: spleen macrophages; TDM: THP-1 derived macrophage-like cells.

Current knowledge on human macrophage polarization is also limited and hampered by the lack of consistency in the experimental conditions used to study human macrophages *in vitro*, including the use of cell lines or different cell types for macrophage generation, diverse conditions for initial macrophage differentiation and subsequent polarization. THP-1 is the human leukemic monocytic cell line frequently used by human macrophage biologists which upon treatment of phorbol esters or vitamin D3 (Vit D3) mimic a number of cellular characteristics of peripheral monocytes derived macrophages (113, 114). Compared to other human myeloid cell lines, such as HL-60, U937, KG-1, or HEL, differentiated THP-1 cells behave more like native monocyte-derived macrophages in terms of both morphology and transcriptomically. Following LPS challenge, a different inflammatory gene expression signature was observed in U937 compared to MDMs (115). However, TDMs do not respond to M-CSF, a potent cytokine for macrophage lineage differentiation, but rather internalize and degrade it very quickly (116). Furthermore, disparate data have also been observed from THP-1 derived macrophage-like cells (TDMs). For instance, PMA treated TDMs do not express mannose receptor (CD206) and MHC class II DRα1, while CD14 and IL-1β were upregulated compared to monocytes or monocytes-derived macrophages (MDM) (117, 118). Furthermore, a subset of PMA treated THP-1 cells do not express scavenger receptors demonstrating heterogeneity in this cell line (119). Compared to primary cells, TLR expression and cytokine responses of THP-1 to LPS stimulation and to oxidized low-density lipoproteins were severely attenuated (120-122). Other significant differences have been recently reviewed by Qin (123). In addition, THP-1 expression of CD14 marker and cytokines was found to be very sensitive to culture conditions, in particular cell density and duration of stimulation (124). Taken together, TDMs data do not fully correspond to macrophage biology of physiological conditions (***Table 1***).

With the availability of commercial monocyte isolation kits, monocytes or monocytes-derived macrophages (MDMs) are now commonly being used in human macrophage studies. Blood monocytes were observed not to able to survive *in vitro* conditions unless they were given GM-CSF or M-CSF stimulation (103, 125). In response to LPS, pro-inflammatory cytokine release was found significantly higher in MDMs than their precursor monocytes (111). Although being classified as mononuclear phagocytic lineage, tissue macrophages and circulating monocytes are functionally different cell types and they both have their own independent self-renewal process during steady state (8). Comparative transcriptome analysis elegantly demonstrated monocytes, MDMs and dendritic cells (DCs) as three distinct cell populations and thereby questioned the interest to use monocytes themselves in macrophage studies (72). On the contrary, although both of the hemopoietic growth factors induce differentiation of morphologically and functionally distinct macrophages in *in vitro* conditions (126). GM-CSF differentiated macrophages (GM-Mϕ) express HLA-DQ and release

substantial amount of pro-inflammatory cytokines, including TNF-α and IL-6 (45). Macrophages differentiated by M-CSF (M-Mϕ) demonstrated higher Fcγ receptor-mediated phagocytic, bactericidal and immunosuppressive (IL-10) activities (125). Induction of IL-1β, IL-8, IL-12 and IL-23 were comparable among GM-Mφ and M-Mφ, however, in response to LPS, release of TNF-α was higher in GM-Mφ, while, IL-10 was found elevated in M-Mφ (111). Lacy *et al* elegantly demonstrated the comparative gene expression profiles of human GM-Mφ, M-Mφ and M-CSF and GM-CSF differentiated BMDMs (111). In human, 13% of genes were differentially expressed between GM-Mφ and M-Mφ. These genes were annotated to be involved in immune responses, receptor activity, cell adhesion and chemotaxis and endocytosis. Further analysis between human GM-Mφ and BMDMs showed 72% homology between species, however, 17% of these genes were found under different regulatory mechanisms between human and mouse macrophage systems. Later, pathway analysis showed that when cell functions were considered, pathways between human and mouse overlaps rather than individual gene expression or regulatory mechanisms (111). Higher yield of monocytes from large volume of donor blood and inter-donor heterogeneity are the two major pitfalls of using MDM.

1.4.2 Inducers Dictate Macrophage Polarization

Macrophage responses are extremely dependent on the environmental stimuli and stresses. Studies conducted till date on macrophage polarization demonstrated huge discrepancies with regard to both the nature of the vitro stimuli used and the duration of the stimulation. These discrepancies made it difficult to interpret and compare the outcomes from different studies to draw general conclusions on macrophages functions in homeostasis, disease pathogenesis and progression. IFN-γ and IL-4 are two prototypical inducers initially used for M1 and M2 polarizations (31, 35-37, 61). Later LPS, TNF-α as M1 and IL-13, IL-10, TGF-β as M2 inducers were added to the list (38, 39, 44). Even so, the study outcomes are still contradictory because of different sources and concentrations of cytokines used and the duration of maintaining stimulated conditions in different studies. For instance, *Arg1* was observed under regulation by IL-4, not by IL-10 (76). LPS at minimal amount is sufficient to induce iNOS, however, at high concentration, LPS also led to *Arg1* induction (127) indicating that the use of an appropriate inducer at an appropriate concentration is crucial while studying macrophage biology (***Table 2***). Furthermore, L-cell conditioned medium used as a source of M-CSF for BMDMs additionally contains type I interferons that would definitely have additive effects on subsequent polarizations (128). Endotoxin-free recombinant cytokines are therefore the best choice in macrophage research. Concentrations of cytokines used are another critical factor. 10

to 200nM of PMA were used for differentiation of THP-1 derived macrophage-like cells (TDMs). The concentration range of LPS used for M1 polarization varies from 10 to 100 ng/ml without taking into account the cytotoxic activity of LPS on the cells (expanded review in ***Table 5***, ***Chapter 3***) (72, 129, 130). Reporting the cytokine concentrations in different units is another issue to consider. While the reporting as "ng/ml" clearly gives an idea of the amount of cytokine(s) added to the culture system, the "U/ml" gives researchers no clue about the amount of cytokine used in the experiments. Duration of initial macrophage differentiation from monocytes and subsequent polarization varies from 3-8 days and 2-5 days among studies respectively (45, 72, 111, 129-131).

While GM-CSF and M-CSF are considered as hematopoietic factors for fully differentiation of macrophages from monocytes in human (132, 133), in the mouse, M-CSF is required for survival and proliferation of BMDMs (100) and GM-CSF treated cells were considered as model of DCs (111). In the last decade, some researchers ascribed GM-CSF derived macrophages as M1 (GM-M1) and M-CSF derived macrophages as M2 (M-M2) (40, 41, 45, 48, 111, 130, 134), however, there is still an ongoing debate on whether GM-CSF or M-CSF stimulated macrophages should be considered as fully polarized macrophages or post-differentiation signals with T_H1 or T_H2 cytokines are mandatory for achieving full polarization spectrum (32). In murine models, IL-12^{High}-IL-10^{Low} is considered as the hallmark feature of M1 polarization (61). Considering this as a prototypic definition, Verreck *et al* observed IL-12 induction in human GM-Mφ, but not in M-Mφ and described GM-Mφ as M1 and M-Mφ as M2 like macrophages (45). Krausgruber *et al* showed an association of interferon regulatory factor 5 (IRF5) with M1 phenotype and impaired M1 cytokine profile in IRF5 KO mice (48). Enhanced IRF5 induction was observed in human GM-Mφ, but not in M-Mφ (***Table 2***). Comparable induction of IRF4 was observed in both GM-Mφ and M-Mφ (48). IRF4 was later identified as a key regulator of M2 polarization (135). However, higher expression of IRF4 observed in human GM-Mφ and both IRF4 and IRF5 expression in M-Mφ not only pointed toward experimental inconsistency but also questioned the concept of GM-Mφ as M1 and M-Mφ as M2 (111). Pro-inflammatory cytokines, for instance, TNF-α was upregulated in unstimulated GM-Mφ, whereas, M2 associated anti-inflammatory cytokines, such as, IL-10 and CCL2 were induced in M-Mφ (41, 111). Induction of IL-1β, IL-8, IL-12 and IL-23 were comparable among GM-Mφ and M-Mφ (111). However, release of TNF-α, IL-6, IL-12, IL-23 was significantly higher in GM-Mφ than M-Mφ following LPS challenge (41, 111). Transcriptome analysis showed significant differences in macrophages generated with GM-CSF and M-CSF, however, no conclusive pattern associated with M1 and M2 polarization was observed (72, 111). Beyer *et al* reported M1 and M2 marker expression was independent of GM-CSF or M-CSF treatment, but depended on the presence of TLR agonists

and T_H1 or T_H2 cytokines (also reviewed in ***Table 5***, ***Chapter 3***) (136). Taken all together, although GM-CSF and M-CSF were able to induce M1 and M2 like phenotypes to some extent, lack of solid evidence supporting GM-Mφ as M1 and M-Mφ as M2 made macrophage biologists to conceptually abandon these terminologies (32).

1.4.3 Expression of Subset-Specific Markers and Functions in Polarized Mouse and Human Macrophages

Macrophages display polarization specific surface receptor expression, induction of signature genes and thereby perform corresponding functions. M1 and M2 polarizations of macrophages had been well defined in murine model by list genes, surface markers and soluble mediators. While translating the murine data into human, variations were expected due to species variability. This section describes differences where murine markers were not applicable to characterize polarized human macrophages. IL-12^{high}-IL-10^{low} is the prototypical characterization of murine M1 macrophages (44, 45). While elevated IL-12 was observed following M1 polarization in human, release of IL-10 was not observed following IL-4/IL-13 treatment but rather observed following LPS challenge in human macrophages. Therefore, IL-12^{low}-IL-10^{high} axis is not valid for human M2 polarization (61). Murine M1 condition demonstrated induction of iNOS gene which later induced release of NO to destroy invading microorganisms (137). Due to substantial nucleotide sequence differences in the promoter regions between human and murine iNOS gene, human iNOS gene demonstrated hyporesponsiveness to LPS or IFN-γ. Additionally, human NF-κB induced by IFN-γ binds to the iNOS promoter more weakly than murine NF-κB. Consequently, human macrophages do not produce substantial NO, therefore analyzing iNOS expression following M1 polarization became debatable (138). Different nomenclature for the same gene between human and mouse sometimes raise confusion. For instance, human co-stimulatory molecules CD80, CD86 and chemotactic cytokine IL-8 are known as B7-1, B7-2 and CXCL1 (KC) in mouse respectively (46).

The disparities between human and mouse were even more significant when comparing M2 macrophage markers. Induction of *Arg 1*, Ym1, Ym2, FIZZ1 genes and surface expression of CD206 and CD163 were associated with murine M2 polarization (31, 55, 61, 66, 85). However, humans do not have homologs of murine Ym1 and FIZZ1 genes. *Arg 1* assay had been reported as an ineffective assay for human MDM-derived M2 macrophages (139). CD206 and CD163 are the two prime murine surface markers for M2 macrophages (51). Stein *et al* first reported expression of mannose receptor

Table 2: List of M1/M2 genes differentially expressed in mouse and human macrophages.

Genes	Mouse (induced by)	Human (induced by)
Arg1	IL-4, LPS, not IL-10	Ineffective assay to assess human M2 polarization
Ym1	IL-4	No homolog
FIZZ1	IL-4	No homolog
iNOS	LPS IFN-γ	Nucleotide sequence variability in promoter and enhanced region Hyperresponsive to LPS, IFN-γ
IRF5	M-CSF, not by GM-CSF	Variable data in different studies GM-CSF, not by M-CSF
IL-1β	GM-CSF LPS IFN-γ	M-CSF LPS plus IFN-γ
IL-8 (KC)	LPS	GM-CSF LPS
IL-10	M-CSF IL-4/IL-13 Parasite infection	M-CSF (Basal) Not induced by IL-4/IL-13
IL-12	GM-CSF LPS	GM-CSF
CD16	Reported as M1 markers following stimulation with nematod Ag	Reported as M2 marker following stimulation with IL-10.
CD163 (RM3/1)	IL-4/IL-13	IL-10, not IL-4/IL-13
CD206	M-CSF, IL-4, parasitic infection IFN-γ (Basal) AMs (Basal)	GM-CSF Not by IL-4/IL-13
CD209	Not reported	Reported in Chapter 3
CD226	Not reported	IL-13, LPS
TGF-β	IL-4, IL-10	Not induced by IL-4/IL-13

(MR), CD206, following stimulation with IL-4 that mediate binding and ingestion of microorganisms with surface mannose residues (31). However, expression of CD206 was also observed on resident and elicited peritoneal and alveolar macrophages and at low on BCG- or IFN-activated macrophages (140). Maximal CD206 expression was observed in mice at an early stage of parasite infection (day 4) whereas M2 signature genes (Arg1, Ym1, FIZZ1, mMGL1, mMGL2) were substantially induced at the very late stage (day 14, 22, 29) (85, 141). Expression of CD206 also varied with *in vitro* experimental conditions. While M-CSF or IL-4 treatment showed significant expression of CD206 in mouse (86, 142), enhanced expression was observed on human GM-CSF treated MDMs compared to M-CSF treated MDMs (129, 143-146). Similar contradictory observations were found with CD163, another hallmark marker of murine M2 macrophages (51, 55, 70). CD163 was found to be highly expressed in some fibrotic diseases (147, 148), but not in asthma (143). *In vitro* differentiated macrophages showed CD163 expression following IL-10 treatment, but not following IL-4, in humans (129, 146, 149). Extensive transcriptome analysis did not report differential expression of CD163 following alternative polarization of human macrophages (72, 136, 150) indicating that CD163 expression varies with the experimental conditions (***Table 2***). These data questioned the acceptance of CD206 and CD163 as M2 surface markers for both human and mouse macrophages.

Current experimental protocols for macrophage polarization were inconsistent and imposed confusion while characterizing them. Therefore, polarization protocols of macrophages and the characterization of polarized subsets necessitated a complete revision as the diversity in experimental protocols led to inconsistencies of definition and difficulties in interpretation. Cell lines could be used to test hypotheses though cell line data need to be validated in primary cells. The importance of murine study in human biology is undeniable, however, extreme care should be undertaken when extrapolating conclusions from murine data to humans. Mouse strains should carefully be chosen based on the research questions of interest. Fully mature MDMs rather than their precursor monocytes should be used by human macrophage biologists. Incomplete reporting of macrophage isolation, differentiation and assay protocols hugely impact on the reproducibility across the laboratories. The facts elegantly demonstrated by earlier studies that monocytes and macrophages were two distinct types cell population and that GM-CSF and M-CSF were not true inducers of human macrophage polarization needs to be acknowledged by macrophage community. Researchers entering in this field have to keep in mind that the field is still expanding and should not consider that there are only “two types of macrophages.

1.5 Roles of Polarized Macrophages in Diseases

Polarized state of macrophages dictates the nature, duration and severity of inflammatory responses. M1 and M2 are the two functional states of macrophages which have been proposed to play a role in inflammation and fibrosis respectively. The number of macrophages markedly increases with the onset and progression of pathological conditions. Selective depletion of macrophages from inflamed tissue confirmed their crucial role in diseases pathogenesis. While dysregulated M1 macrophages elevated the disease score in autoimmune and chronic inflammatory diseases, aberrant activity of M2 macrophages can also be detrimental.

Analysis of *ex vivo* macrophage polarization demonstrated huge plasticity over various diseases. For instance, macrophages in the adipose tissues of obese individuals are considered to be pro-inflammatory M1 macrophages (151), whereas, important roles of M2 macrophages have been reported in many fibrotic diseases (52), insulin resistance (152), cardiac diseases (53) and tumor progression (54). A positive association of M2 macrophages with the aggravation of murine allergic inflammation and asthma was found (66, 153, 154).

Defective macrophage responses have been reported in patients with respiratory diseases, including asthma (155, 156), COPD (157, 158), CF (159-161). In agreement with murine studies, higher percentages of macrophages were present in BAL fluid and airway wall tissues from patients with asthma and were associated with disease severity (162-165). However, due to lack of appropriate M1/M2 subset specific markers, polarized states of these cells in disease condition are still poorly elucidated (26, 143). Knowing the functional states of pathogenic macrophages has a huge impact on developing novel therapeutic targets.

1.6 Cystic Fibrosis (CF): A Life-threatening Respiratory Disease

1.6.1 CF, CFTR Function and CF Inflammation

Cystic fibrosis (CF) is a life-limiting autosomal recessive disease with a high rate of premature mortality (166, 167). Mutation in the cystic fibrosis conductance regulator (CFTR) gene is the sole origin of CF and affects multiple organs including the lungs, gut, liver, pancreas and reproductive tissues, however, the lungs and the gastrointestinal tract are primarily affected. CF lung disease is characterized by chronic airway infections, inflammation, bronchiectasis leading to loss of lung function, repeated pulmonary exacerbations and eventually complete failure of the respiratory system. The highest frequency of CF was observed among the Caucasian population from developed

countries, such as, UK, Europe, North America and Australasia (168). However, the prevalence varies with genetic background. For instance, the disease occurs in roughly 1 in 3000 whites of Americans and Europeans, 1 in 4000–10,000 Latin Americans, 1 in 15,000–20,000 African Americans. Interestingly CF is rare or absent in Asians accounting 1 occurrence in 350,000 (169, 170). Several different possible mechanisms, including ethnic background, transfer of mutated allele from both parents to offspring, heterozygous advantage, multiple loci and reproductive compensation had been proposed to explain the high incidence of the disease among Caucasians, but not in the other parts of the world (171). In Australia, the frequency of cystic fibrosis is about 1 in 2800 live births, however, 1 in 25 Australians carry the genetically mutated gene, CFTR. With the advancement of diagnosis and treatment, the median survival time of patients with CF increased from 14 years in 1969 to 35 years in 2001 and to 37 years over the past decade (167, 172-175). Even more, improvement in the survival rate to 50 years was realistically predicted among children who born today with CF by a UK model (175).

CFTR protein is a cAMP-activated chloride (Cl^-) channel expressed at the apical membrane of many cell types, including epithelial cells, monocytes, mast cells (160, 176, 177). Following synthesis, the CFTR protein is glycosylated in the endoplasmic reticulum (ER). After passing the checkpoints for proper folding in ER, CFTR protein migrates to the Golgi apparatus for further glycosylation. The fully matured and functional CFTR protein is then transported to the plasma membrane to acts as chloride channel (178). In addition, CFTR plays regulatory roles on other channel activities, including, regulation of ATP channel, inhibition of sodium (Na^+) transport channel and calcium (Ca^{2+})-activated Cl^- channel, regulation of intracellular vesicle transportation and acidification of intracellular organelles. CFTR is also involved in bicarbonate (HCO_3^-)-chloride exchange. Deficiency of HCO_3^- secretion leads to poor solubility and accumulation of luminal mucin (179). Functional CFTR protein is required for fluid secretion in the airways as well as in intestine. Mutation in CFTR gene causes reduced transport of Cl^- and HCO_3^- ions in epithelial cells and blood cells (161, 176, 180, 181). In the lung, disturbed airway surface liquid homeostasis produces thick and viscous mucus that leads to mucus stasis, airway obstruction, persistence infection, chronic inflammation, a gradual decrease in lung function that ultimately results in limited life expectancy (168, 182, 183). Culture-independent pioneer studies showed the presence of complex and diverse microbiota in the airways of the patients with CF and a substantial shift in the airway microbiota composition associated with airway functions (184).

The underlying mechanisms of CFTR-mediated airway inflammation in CF are still under investigation. Several hypotheses regarding CFTR dysfunction and CF phenotype have been

postulated (185). The major hypotheses are described here. The ***low-volume hypothesis*** proposes that CFTR dysfunction leads to loss of inhibition of sodium (Na^+) ion channel in the epithelial cells, causing excessive reabsorption of Na^+ and water, resulting in dehydration of airway surfaces and reduction of lubricating layer between epithelium and mucus (185, 186). Bacteria, especially *Pseudomonas aeruginosa* harboring in the mucus layer trapped and cause infection (187). According to the ***high-salt hypothesis***, due to absence of functional CFTR surplus Na^+ and Cl^- retained in the airway spaces interrupt the regular functions of essential antibacterial peptides (such as, human β-defensin 1, LL-37) and allow bacterial colonization (185, 188). ***Dysregulated host inflammatory response hypothesis*** was supported by the unabated release of inflammatory mediators in BAL of children of 4 weeks of age (189) and uninfected ex-vivo tissue samples (190). ***Host susceptibility to CF associated pathogens***, particularly, *P. aeruginosa* and *Staphylococcus aureus*, is supported by the increased attachment of microbial pathogens to the host cell surface carbohydrate molecules, e.g., asialo-GM1, that initiate persistent inflammatory responses which is independent of CFTR (191). It would be possible that all four contribute to the pathogenesis of the diseases.

1.6.2 Onset of Inflammation in Cystic Fibrosis

Inflammation in CF is initiated at early stage of life (189, 192). Repeated and chronic infection with bacteria, particularly with *S. aureus* and *P. aeruginosa* are common in infancy (193). Other pathogens included: *H. influenza*, RSV, rhinovirus, adenovirus, parainfluenza virus type 3. Episodes of infection increase with age and are associated with neutrophilic inflammation leading to lung damage, bronchiectasis that leads to fibrosis during adulthood (193). This common pattern of disease inception and progression suggests intrinsic defects in host innate defense mechanism (194). The innate arm of host pulmonary defense comprises of three components: ***a)*** mucous layer as physical barrier that contributes to antioxidant defense, mucociliary transport and tight junctions, ***b)*** humoral immunity provided by surfactants and antimicrobial proteins, e.g., defensins, ***c)*** cell-mediated immunity provided by macrophages, neutrophils, epithelial cells, natural killer cells. Escape of pathogens from all these three innate arms activate the adaptive immune responses. (195). However, airways of infants with CF are susceptible to infection by *P. aeruginosa, Haemophilus influenza* or *S. aureus* which leads to inflammatory cytokine storm causing chemotaxis of neutrophils and PMNs (185, 196). Other pathogens associated with CF include *Burkholderia cepacia* (a complex of at least 9 different species), *Stenotrophomonas maltophilia*, meticillin-resistant *S. aureus* (MRSA), and atypical mycobacteria (172, 197).

Inflammatory responses in the CF airways are compartment-dependent. Inflammation in airway lumen is mostly mediated by macrophages, neutrophils, airway epithelial cells (AECs), NK cells whereas, recruitment and accumulation of lymphocytes was observed in the bronchial mucosa (194, 198). Neutrophils were hardly seen in CF and non-CF airways during fetal development (195). However, excessive neutrophil counts were observed in BAL of infected CF patients, but not in that of uninfected CF patients, compared to control (192, 196). Increased neutrophilic elastase and matrix metalloproteinase (MMP) activity, reduced antimicrobial activity and dysregulated cytokine production were reported in CF neutrophils (199, 200). Mouse models of CF showed an inverse relationship between dysfunctional CFTR protein and cellular redox state, ER stress and mucin overproduction by AECs (201). Pediatric studies in CF demonstrated increased oxidative stress in the airways of young children with CF (202). Reduced oxidative stress of AECs has been thought to be associated with defective bacterial clearance. Furthermore, many studies reported mitochondrial abnormalities, including oxidative phosphorylation, calcium homeostasis, oxidative stress associated with mutated CFTR (203).

Although macrophages are the professional phagocytes in the respiratory linings, the role of macrophages has largely been overlooked in CF. During fetal development, macrophages are the most abundant cells in respiratory linings of both CF and non-CF. However, the number of macrophages was significantly increased in CF airways with increasing fetal age while in non-CF macrophage count decreased (195). In accordance with the earlier study, significantly higher levels of CCL20, a chemokine involved in macrophage recruitment, were detected in BAL of CF infants (204). However, defective clearance of apoptotic cells (205) and blunted phagocytosis (160, 161) of macrophages were reported. Failure of bacterial clearance in the respiratory epithelia results in chronic infections that later lead to remodeling and thickening of airway lining and eventually failure of pulmonary function (206). Taken together it seems that macrophages in CF patients contain intrinsic defect (s). Furthermore, earlier studies demonstrated that AMs control inflammation in respiratory sites (207). Chronic inflammation in CF indicates that AMs failed to dampen pro-inflammation in CF lungs.

1.6.3 Types of Mutations in CFTR Gene

More than 1900 mutations have been so far reported in human CFTR gene which are broadly categorized into six different classes based on the mechanism of the degradation or absence of CFTR protein (***Table 3***). Mutations that introduce signals for premature termination of nascent CFTR polypeptide are known as class I mutations. Types of mutations include insertion of a stop codon,

frameshift due to insertion or deletion or nonsense mutations. Class I mutations leads to truncated or aberrant protein. Class II mutations are associated with impaired post-translational modification, such as, glycosylation. Despite the presence of full-length CFTR protein, class III mutations contribute to dysregulated CFTR channel activity resulting in lowered Cl- transport, whereas, class IV mutations cause reduced Cl^- permeability. Class V mutations lead to defective RNA splicing generating minimal copies of normal CFTR. Finally, class VI mutations negatively affect the protein stability causing accelerated turnover of CFTR protein at cell surfaces thereby reduce the quantity. In all cases, the abnormal CFTR protein rapidly undergoes proteolytic degradation in the ER (208).

Deletion of phenylalanine at position 508 in the CFTR protein, known as ΔF508 or Phe508Del, was the first identified mutation in CFTR gene (171, 209, 210) and the most common mutation among the patients with CF. Over 75% of patients carry at least one allele of ΔF508 (211, 212). Due to ΔF508 mutation CFTR protein is defectively folded and trapped in the endoplasmic reticulum (ER) and targeted for degradation, resulting in minimal amounts of CFTR at the cell surface (213-218). The small fraction of CFTR protein that was able to be delivered to the cell surface exhibits defective channel gating and increased turnover (219, 220). Loss of CFTR-mediated Cl^-ion transport cause an imbalance between fluid secretion and absorption, resulting in dehydration of airway epithelial spaces of CF lungs (186).

G551D, also known as S549N, is the second most common mutation, carried by around 5% of patients with CF (211). Despite the synthesis trafficking of full-length CFTR protein to the cells membrane, due to G551D mutation, the protein fails to open the channel, resulting in little or no ion transport (221).

Other than these two mutations, S549R and Trp1282X mutations are common in the United Arab Emirates (61.5%) and Israel respectively (211). Sharma *et al* recently reported 11 new rare CFTR mutations among patients of the Indian subcontinent that increased the spectrum of CFTR mutations worldwide (222).

1.6.4 Small Molecules That Modulates CFTR Functions

One of the major difficulties in CF research is lack of proper animal model of the disease. CFTR-knockout murine models experience low survival rate (223) and do not spontaneously develop chronic infection and demonstrate little or no airway inflammation making them a less appropriate surrogate model for conducting mechanistic studies for CF. However, they showed significant

inflammatory responses following *P. aeruginosa* infection. Recently ferret (224) and pig models (225) of CF were developed, albeit expression of CFTR mRNA showed variability with tissue distribution and the pathological phenotype of these CFTR-null animals have not been comprehensively studied. Hence, there was considerable interest in developing CFTR inhibitors to investigate CFTR-mediated Cl^- transport *in vivo* and to generate animal models of CF. Several high-affinity CFTR inhibitors have been introduced which have shown clinical potentials as therapy in secretory diarrhea, polycystic kidney disease and in inhibiting male fertility (226, 227). However, some of them block or inhibit all ion channels showing lack of CFTR specificity. For instance, diphenylamine-2-carboxylate (DPC) and 5-nitro-2(3-phenylpropyl-amino) benzoate (NPPB) inhibit CFTR in a nonspecific manner (228). Glibenclamide inhibits all Cl^- transporters as well as K^+ channels (226, 229).

Table 3: Class of CFTR Mutations

Class	Defects	Mutation Example	Cellular phenotype
I	Presence of nonsense mutations (i.e., stop codon), frameshift mutations, or abnormal mRNA splicing	W1282X	Synthesis of no or minimal functional CFTR protein.
II	Defect in protein processing and trafficking	ΔF508	Delivery of reduced copies of CFTR protein to the cell surface.
III	Defect in regulation of channel gating	G551D	Presence of CFTR protein at the cell surface with no channel activity
IV	Defect in ion selectivity and conductance	R117H	Surface CFTR protein unable to conduct ion transportation
V	Defect in RNA splicing	3849+ 10Kbc>T	Reduced copies of CFTR mRNA leads to minimal synthesis of CFTR protein
VI	Defect in protein stability	Q1412X	Accelerated turnover of CFTR protein at cell surfaces thereby reduce the quantity.

At 2002, Ma *et al* reported six high-affinity small molecule CFTR inhibitors that directly interact with CFTR expressed on epithelial cells: $CFTR_{Inh}$-020, $CFTR_{Inh}$-029, $CFTR_{Inh}$-172, $CFTR_{Inh}$-

185, $CFTR_{Inh}$-214 and $CFTR_{Inh}$-234 (226). Of them, ***$CFTR_{Inh}$-172*** was the most potent 2-thioxo-4-thiazolidinone chemical class CFTR inhibitor that is rapidly absorbed and selectively and efficiently blocked the Cl^- channel gate from the cytoplasmic side in a voltage-independent manner. $CFTR_{Inh}$-172 has also been shown to inhibit mitochondrial respiration and increases reactive oxygen species (ROS) production (230). N-(2-naphthalenyl) and 3,5-dibromo-2,4-dihydroxyphenyl glycine hydrazide, known as ***GlyH-101***, is an open-channel blocker, i.e., it binds at the external side of the CFTR channel, thereby, inhibits inward rectification of CFTR function in respiratory and intestinal epithelial cells in both human and mice, i.e., Cl^- influx from the intra- to the extracellular side of the cells is more rigorously attenuated than that in the opposite direction (227). Like $CFTR_{Inh}$-172, GlyH-101 is able to inhibit cholera toxin-induced fluid secretion in mouse model of diarrhea (227). ***MalH-2*** is another water soluble and non-absorbable glycine hydrazide type CFTR inhibitor (231). MalH2 rapidly, fully and reversibly blocked CFTR-mediated Cl^- current in mouse intestine (cholera toxin-induced model).

All these abovementioned CFTR blockers have advantages as well as pitfalls. $CFTR_{Inh}$-172 has a number of fascinating properties. In terms of potency, $CFTR_{Inh}$-172 readily inhibits CFTR-mediated Cl^- channel in less than 2 minutes (226). *Secondly*, $CFTR_{Inh}$-172 has no effect on other ion channels and transporters found in epithelial cells, including Ca^{2+}-activated and volume-regulated Cl^- channels and the ATP-binding cassette transporter P-glycoprotein showing specificity to CFTR-dependent Cl^- channels (226). GlyH-101, on the contrary, inhibits Ca^{2+}-dependent CL- channels. *Thirdly*, $CFTR_{Inh}$-172 showed longer mean channel closure time than GlyH-101 (231). *Fourthly*, by effectively inhibiting cholera toxin-induced fluid secretion in mouse diarrhea model $CFTR_{Inh}$-172 showed its therapeutic potential (226). Nevertheless, $CFTR_{Inh}$-172 suffers at least three drawbacks. It has limited solubility in water and intact cells and tissues reducing potency (227). Stahl *et al* reported a marked species difference in sensitivity to $CFTR_{Inh}$-172 and recommended using 20μM concentration to achieve 50% inhibition of CFTR in human oocytes (232). GlyH-101 has been considered as better CFTR inhibitor than $CFTR_{Inh}$-172 for three reasons (233). GlyH-101 is highly water soluble and remains as a monovalent anion between pH 6.0 and 8.0 (227). Submaximal concentration of GlyH-101 is enough to rapidly attenuate Cl- influx in a voltage-dependent manner (227). In animal models, $CFTR_{Inh}$-172 must be administered through the intraperitoneal route to inhibit CFTR activity, whereas GlyH-101 can be administered directly into the lumen of the small intestine (227). MalH-2 has even greater water solubility than GlyH-101 (231).

$CFTR_{Inh}$-172 and GlyH-101 have been widely used to inhibit CFTR activity and to study cellular mechanisms in CF inflammation. From 1 to 20μM concentrations of $CFTR_{Inh}$-172 and GlyH-

101 had no effect on cellular viability albeit higher concentrations, such as, 50μM, cause cell death (234, 235). 10μM was recommended for both $CFTR_{Inh}$-172 & GlyH-101 for *in vitro* cellular studies (159, 226, 234, 236). Studies mostly were conducted with epithelial cells to model CF and to evaluate the ability of CFTR inhibitors to mimic CF inflammation (234, 236). Both inhibitors induced a rapid increase in ROS level. Activation of nuclear translocation of nuclear factor-κB (NF-κB) was observed by $CFTR_{Inh}$-172, but not by GlyH-101. Spontaneous IL-8 release was slightly reduced by $CFTR_{Inh}$-172 though increased by GlyH-101 (235). IL-6 and GM-CSF release were not affected by $CFTR_{Inh}$-172 (236). Such varying data suggest that induction of ROS and NF-κB signaling cascade and IL-8 release are independent of CFTR function or regulation (234, 236). Effect of CFTR inhibition by $CFTR_{Inh}$-172 on Cl^- and phagocytosis in alveolar macrophages was first studied by Di *et al* (161). Later similar Cl^- channel attenuation and impaired bactericidal activity were demonstrated in monocyte-derived macrophages (MDM) from patients with CF (159, 160). Viability, IL-1β release and surface marker expression of CD14, CD16, CD64, TLR-2, TLR-4, TLR-5 on MDM were mostly unaffected following CFTR inhibition by $CFTR_{Inh}$-172 (159).

1.6.5 Current Treatment Options and Mutation Specific Therapies

1.6.5.1 Current Therapeutic Options

Inhaled dornase-α (recombinant human deoxyribonuclease, given daily) and inhaled tobramycin (300mg twice daily) are recommended by the US Cystic Fibrosis Foundation as treatment options for patients with moderate to severe CF. Hypertonic saline, macrolide antibiotics (e.g., azithromycin), ibuprofen, and inhaled β-agonists are recommended for patients over 6 years of age (174). Corticosteroids did not robust benefits to the patients rather impose adverse effects (174).

Tobramycin is an aminoglycoside that is used for gram negative infections. It is especially effective against *Pseudomonas* species. It binds on bacterial 30S and 50S RNA thereby prevent formation of 70S complex. As a result, mRNA can't be translated into protein ensuing cell death. However, tobramycin can't pass through the gastrointestinal (GIT) and is therefore administered intravenously (IV), intramuscularly (IM) or via inhalation. Tobramycin is approved antibiotic in the United States and highly recommended for patients with CF to delay colonization of *P. aeruginosa* in the lungs (237). Six different independent studies showed significant improvement of lung functions in patients with moderate to severe CF and decreased chronic *P. aeruginosa* count and weight gain following tobramycin treatment compared to placebo or standard treatment (174, 238-242). Tobramycin delayed biofilm formation of *P. aeruginosa* as well as disruption of already formed

biofilms thereby limits the growth of *P. aeruginosa* (243). *P. aeruginosa* count dropped in the infected lungs following intratracheal instillation of tobramycin (300µg/kg body weight) suggesting decreased pulmonary bacterial load in the infected animals (239). In addition, tobramycin limits LPS induced inflammation in human bronchial epithelial cells (244). On the contrary, chronic use of tobramycin has been shown to promote resistance and biofilm formation by surviving bacteria (239). However, modulatory effects of tobramycin on host immune surveillance cells, such as, macrophages had not been comprehensively studied.

1.6.5.2 Introduction of Small Molecules as Mutation-specific Therapeutics

Currently available therapeutic options are unable to repair or modulate the basic genetic defect of CF, i.e., the mutation(s) in CFTR gene. Therefore, researchers are seeking for mutation-specific drug that can specifically target mutant CFTR and restore normal CFTR function. Agents that increase the delivery of functional CFTR to the cell surface are called CFTR ***correctors***, whereas, agents that increase Cl^- channel gating are called CFTR ***potentiators***. ***Ivacaftor***, previously known as VX-770, is an FDA approved CFTR potentiator for the patients carrying G551D allele (245). Ivacaftor had been shown to enhance channel activity, improve lung function, lowers sweat chloride level thereby improve the clinical outcomes in patients carrying class III mutation (246-250). ***Lumacaftor,*** formally known as VX-809, is a promising investigational CFTR corrector that has been shown to correct ΔF508 mutation selectively, thereby improve CFTR processing, maturation in the ER and trafficking to the cell surface, finally enhance Cl^- secretion (249). Lumacaftor (VX-809) corrected ΔF508-CFTR protein was presented on the cell surface for longer period suggesting that ΔF508-CFTR protein was no longer recognized as defectively folded protein by the peripheral protein quality control machinery. The maximal effect has been observed after 24hr of treatment. Following washout of Lumacaftor, Cl^- transportation returned to an uncorrected level within 48hr (249).

Unfortunately, monotherapy with either of these drugs failed to improve lung functions in patients who are homozygous for ΔF508 mutation (251, 252). In a double-blinded RCT with CF patients who carry homozygous mutation of ΔF508, Lumacaftor did not show any improvement in CFTR function in nasal epithelial cells or significant changes in lung functions (251). Furthermore, patients with heterozygous mutation for ΔF508/G551D mutations require treatment with both Ivacaftor and Lumacaftor to achieve clinical benefit (253). *In vitro* studies showed that combined treatment of Ivacaftor and Lumacaftor showed enhanced channel gating compared to corrector or potentiator alone (249). Likewise, in phase 2 clinical trial Ivacaftor-Lumacaftor combination therapy demonstrated improvement in lung function with a modest effect on sweat chloride concentration

among ΔF508 homozygous patients (254). Later phase 3 trials in two different multicenter studies demonstrated significant improvement in lung function with lowered rate of hospitalization and intravenous antibiotic administration in patients who received such combined treatment over 6 months compared to the placebo group (255). However, the study was conducted only in patients who had moderate to severe clinical symptoms. The effect of Lumacaftor-Ivacaftor treatment in patients with even poor pulmonary function has not been investigated yet.

Affordability of such Ivacaftor-Lumacaftor treatment is another key issue. Lumacaftor-Ivacaftor therapy is almost $300,000 per year which delays its introduction to the patients carrying G551D mutation. Other CFTR correctors, such as, SAHA, 4-phenylbutyrate, VRT-325, corr-4a have been reported in many studies, though they are less selective and insufficiently restore CFTR function (249). VX-325 is a promising small molecule capable of correcting ΔF508 mutation. VX-325 treated monocytes from patients carrying ΔF508 mutation showed higher expression of CFTR along with significant recovery of normal CFTR function (256). Patients who carry mutations other than ΔF508 mutation still had no choice other than conventional therapies.

1.7 Macrophages: The Missing Link in CF Inflammation?

Macrophages play critical roles in inception and resolution of pulmonary inflammation. Studies showed spontaneous infiltration of monocytes and macrophages in alveolar spaces as well as local tissues, such as, peritoneum (257). Depending on the post translational processing, three isoforms of CFTR proteins have been shown in earlier studies (176): isoform A of MW 130-140KD corresponds to an immature, incompletely-glycosylated form of CFTR. Isoform B represents the incompletely glycosylated form of CFTR protein expressed by CFTR-KO human bronchial epithelial cells. The fully glycosylated form of CFTR is known as isoform C. Monocytes from healthy individuals express all three isoforms. Presence of functional CFTR protein was demonstrated on the surface of human and murine AMs, peritoneal macrophages (161) as well as *in vitro* differentiated human macrophages (160). Conversely, the presence of isoform B was detected in monocytes from patients with CF (176, 180). Truncated forms of the protein appear to be loosely localized on the membrane. Macrophages from CF patients homozygous for ΔF508 mutation showed cytoplasmic localization of CFTR protein indicating trafficking defect due to class II CFTR mutation (160). Similar mislocalization of ΔF508 CFTR in cytoplasm had previously been reported primary airway and nasal epithelial cells (258, 259).

A recent study showed increased CFTR expression over AMs following LPS stimulation (260). Upon administration of CFTR inhibitor, MalH-2, wild-type mice showed a substantial increase in cytokine production following LPS challenge compared to corresponding controls suggesting regulation of CFTR on pro-inflammatory cytokine production during bacterial infection and how the lack of functional CFTR during bacterial infection worsen lung inflammation (260). Cellular phagocytosis activity encompasses the uptake and engulfment of foreign particles to a phagolysosomal compartment, also called phagosome and then destruction or killing of the foreign particles by phagolysosomal acidification – a combination of oxidative mechanisms including superoxide (O_2-), hypochlorous acid (HOCl), acidic pH and lysosomal enzymes (261). Using CFTR-KO mice Di and colleagues elegantly showed that uptake and phagosome formation were not defective in CFTR-KO mice, rather phagosomes from CFTR-KO AMs were less acidic, pH of 6.5 than their wild-type counterparts, pH of 4.5, to kill the invaded bacteria suggesting the role of CFTR in the acidification of phagolysosomal compartments (161). Monocyte-derived macrophages from patients with CF showed the similar altered bactericidal activity to *P. aeruginosa* indicating impaired clearance of pathogens by CF macrophages which later led to persistence infection and chronic exacerbated pro-inflammatory responses.(159, 160).

ΔF508 mutation bearing CF mice demonstrated M1 polarization with substantial induction of IL-1β in compared to wild-type mice even in the absence of M1 stimulation suggesting an association of M1 polarization and intrinsic CFTR defect (257). LPS challenge led to substantial induction of M1 signature genes in CF mice than in wild-type mice indicating that bacterial infection on a background of intrinsic CFTR mutation worsen the pulmonary inflammation. On the contrary, polarization of M2 signature genes as well as IL-10, hallmark M2 soluble marker in mouse, were not remarkably induced in AMs and PMs of CF mice (257). Taken together, this data suggests exaggerated pro-inflammatory responses in CF with basal or no induction of anti-inflammatory M2 macrophages. In human, increased expression of IL-13, but not IL-4, was observed in patients with CF (262). Nevertheless, subset specific macrophage responses have never been studied among patients.

Patients with CF are treated with antibiotics to prevent and eradicate respiratory infections (241). Aminoglycoside antibiotics such as tobramycin, gentamicin has been shown to restore expression of full-length functional CFTR and significant increase of cAMP-activated chloride channel (180, 263). In a mouse model Meyer *et al* analyzed the immunoregulatory effects of azithromycin on macrophage polarization (257). In wild-type mice, in the absence of M1 stimulation, NO production was not induced by azithromycin alone, however, azithromycin administration following M1 polarization led to substantial down-regulation of NO production. In the absence of

M2 stimulation, *Arg 1* activity was enhanced by azithromycin alone in a dose-dependent manner in wild-type mice, whereas, a combination of both M2 stimulation and azithromycin treatment considerably promoted M2 polarization in AMs and PMs. These data suggest that antibiotics downregulate pro-inflammatory macrophages and activate anti-inflammatory macrophages. Unfortunately, the authors did not analyze the effects of azithromycin on macrophage polarization in CF mice. In addition, the precise mechanism of action of azithromycin on macrophage polarization still remained unclear. A similar study was not carried out with human macrophages.

All these data reflect that CFTR dysfunction due to intrinsic defect is linked to macrophage polarization and function in CF. However, understanding of the contributions of macrophages in CF pathophysiology is lacking. Furthermore, the mechanism of actions of administered antibiotics on macrophage functions needs to be explored.

1.8 Hypothesis and Specific Aims

1.8.1 Hypothesis

Macrophage function is dependent on its polarization state. Both mouse and human studies showed a positive association of defective between CFTR function and impaired macrophage responses. Therefore, the hypothesis of this Ph.D. study was that dysfunctional CFTR leads to defective polarization of macrophages and thereby contribute to the chronic airway infections, and inflammation leading to loss of lung function in CF. Therefore, the general aim of this study is to investigate subset specific macrophage phenotype and function in young and adult patients with CF during their acute pulmonary exacerbation (APE) and clinically stable conditions.

1.8.2 Specific Aims

1.8.2.1 Development of in vitro Model to Characterize Human Classical and Alternatively Activated Macrophages

The first aim of this Ph.D. project was to develop a novel approach to characterize human M1 and M2 macrophages by surface markers, signature genes, inflammatory mediators and functional aspects. Additional interest was to analyze the persistence of polarized macrophages over time and to assess their reprogramming ability upon appropriate stimulation.

1.8.2.2 Study Subset-Specific Macrophage Responses in CF and Identify the Defective Macrophage Subset(s) in Cystic Fibrosis

Defective macrophage function has already been reported in murine CF models and human studies. Nevertheless, the dynamic changes of macrophage subsets during acute exacerbation and stable conditions of CF had never been studied among CF patients. A comprehensive analysis aimed to be conducted with the model mentioned in ***Aim 1*** to identify defective macrophage subsets in CF. The hypothesis here was that M2 polarization of macrophages might be defective which leads to incomplete resolution of inflammation in patients with CF. Since phagocytosis is under CFTR regulation, it was postulated that aberrant M2 polarization of macrophages might also be associated with a lack of CFTR function.

1.8.2.3 Effects of Tobramycin on Macrophage Polarization

Effects of tobramycin on macrophage polarization in CF was aimed to investigate in this Ph.D. study with the aim that how antibiotics modulate the host immune system.

Chapter 2 Methodology

2.1 Ethics Approval

The study protocols, consent forms and advertising materials related to healthy individuals considered as controls of this study had been approved by the human research ethics committee (HREC) of the Royal Children's Hospital (RCH), Brisbane and the University of Queensland (UQ), Australia. Permissions from the relevant Institutional Review Boards, i.e., Prince Charles Hospital and Children Health Queensland (Royal Children Hospital/Lady Cilento Children's Hospital), Brisbane, were obtained for collecting blood samples from patients with CF. Written consents from all adult patients or the parents of young patients were obtained.

2.2 Study Sites

The entire Ph.D. project was conducted as a research study at Queensland Children's Medical Research Institute (QCMRI), Royal Children's Hospital, Brisbane, and later at Children's Health Research Centre, UQ (CHRC-UQ). All patients' samples were collected from the CF clinics at the Prince Charles Hospital and Children Health Queensland (Royal Children Hospital/Lady Cilento Children's Hospital), Brisbane, Australia.

2.3 Study Populations

2.3.1 Patients Samples

Adult (n=13) and children (n=27) patients with verified CF carrying at least one ΔF508 allele were recruited from the CF clinics at the Prince Charles Hospital and Children Health Queensland (Royal Children Hospital/Lady Cilento Children's Hospital), Brisbane, Australia respectively. Paired blood samples from adult patients were collected by venepuncture during admission to hospital due to acute pulmonary exacerbation (APE) and prior to discharge from the hospitals. Blood from children were collected on admission to hospital (n=16) and/or at a clinic visit (n=14) when they are clinically stable. Paired blood samples were collected only from 2 children. Smoking habit of the enrolled adult patients or parents of young patients with CF was not taken into account this study.

2.3.2 Healthy Controls

A total of 29 buffy coats from healthy donors, aged from 18 to 40 years, were obtained from the Australian Red Cross blood services (ARCBS) (Brisbane, Australia). Buffy coats were processed within 18 hrs of collection. Buffy coats were initially used for optimizing and developing methods

for macrophage subset characterization. Later data from buffy coats were used as controls to compare with data from CF patients. Unfortunately, no demographic other than sex and age were inaccessible.

To validate the buffy data, similar experiments were conducted with freshly collected blood from volunteers. Volunteers at QCMRI or later at CHRC agreed and signed informed consent forms to be enrolled in the study. All volunteers were enrolled based on following selection criteria.

Inclusion criteria:

1. Individuals of either sex 18 years and older.
2. Individuals who were healthy at the time of enrolment.
3. Individuals who were not taking any medication in the last 24 hours.

Exclusion criteria:

1. Individuals who were suffering from any acute or chronic medical condition at the time of enrolment.
2. Individuals who were taking any medication for current cold and/or allergies.
3. Individuals who experienced any episode of allergy or asthma in the last three months.

Peripheral blood was collected from volunteers by experienced phlebotomists following WHO guideline (264). Following venous puncture, blood was collected in preservative free heparin containing tubes (20units/ml). To avoid clotting, blood containing tubes were inverted and kept standing at room temperature for 10-15min.

2.3.3 THP-1: Human Monocytic Cell Line

Due to lack of continuous supply or unavailability of human cells and less viability over longer periods, biologists tend to use continuous cell lines which mimic many characteristics of primary cells as a model system for investigating disease pathogenesis. At the very early time of this study, human monocytic cell line, THP-1, was used to establish macrophage culture. THP-1 cell line has been using for studying human macrophages and their involvement in diseases (115). Treatment with phorbol esters or vitamin D3 (Vit D3) enable THP-1 cells to differentiate into macrophage-like cells which mimic native monocyte-derived macrophages (MDM) in several respects, such as, morphology (e.g. adherent to the culture plate), secretory products, expression of membrane antigens (e.g., CD68, CD14) and the involvement of genes involved in lipid metabolism (113, 114). THP-1 cell line was brought from American Type Culture Collection (ATCC).

2.4 General Laboratory Methods

2.4.1 Differentiation of THP-1 derived Macrophages

Differentiation of THP-1 cells to macrophage-like cells was induced by overnight stimulation with 20nM of phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) or 6-days stimulation with M-CSF (50ng/ml) (Invitrogen, USA). THP-1 derived macrophage-like cells were referred as TDMs all through this thesis.

2.4.2 Isolation of Peripheral Blood Mononuclear Cells (PMBCs) and Purification of Monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or peripheral blood from patients or healthy volunteers by density gradient centrifugation using Lymphoprep (Axis-Shield, UK). PBMCs were washed twice with PBS to get rid of any serum, platelets or residual Lymphoprep. All the steps were performed in a biosafety cabinet to maintain sterility. Monocytes were purified from PBMCs using $CD14^+$ magnetic microbeads following manufacturer's instructions (Miltenyi Biotech, Germany). Briefly, cells were incubated with $CD14^+$ microbeads for 15 min at 4C. Cells were then washed and spun down at 300g for 10min. Cells were resuspended in MACS buffer (0.5mg BSA/ 2mM EDTA/ 100ml PBS) and passed through the MACS column (Miltenyi, Germany). After washing, cells magnetically retained in the column were recovered by flushing with a plunger. Purified monocytes were then counted by hemocytometer.

2.4.3 Analysis of Monocyte Subsets

$CD14^+$ monocytes isolated from buffy coats or from blood samples of patients with CF were subjected to monocyte subset analysis. Classical, intermediate and non-classical monocytes were analyzed by their surface expression of CD14 and CD16. Briefly, monocytes were incubated with Fc blocking solution or FACS buffer (FBS, 2%-BSA,0.1%-PBS, 100ml) for 20min at 4C. Cells were then spun down and resuspended in FACS buffer and stained with anti-human CD14-PE Cy 7 (BD, USA), CD16-PerCP Cy5.5 (BD, USA) and CD45-V450 (BD, USA). This analysis also demonstrated the purity of monocytes isolated by MACS separation. Data were acquired on BD LSR-Fortessa using BD FACS Diva software. All flow cytometric analysis were performed on Flowjo platform (Tree Star, USA).

2.4.4 In vitro Differentiation of Monocyte-derived Macrophages

Both M-CSF and GM-CSF are potent hematopoietic growth factors for macrophage differentiation from monocytes. M-CSF is abundant in the circulation, while the respiratory lining is rich in GM-CSF. GM-CSF, therefore, was mostly used in this study to define respiratory macrophages. M-CSF was used to confirm that M-CSF differentiated macrophages exhibited similar phenotypes and functions to GM-CSF differentiated macrophages.

For macrophage differentiation, monocytes were cultured in RPMI-1640 (Lonza, USA), supplemented with 10% heat inactivated FBS (Life Tech, USA), 1% Penicillin-streptomycin-fungizone (Lonza, USA) and rhGM-CSF (50ng/ml) (BioLegend, USA) or rhM-CSF (50ng/ml) (Invitrogen, USA) for 6 days. Medium was refreshed on day 3 with appropriate amount of cytokines. These 6-day differentiated macrophages were considered as uncommitted macrophages and referred as M0 throughout this thesis.

2.4.5 Phenotypic Characterization of M1-M2 Macrophages

Flow cytometry has been widely used as a powerful tool to quantify any cell population by size, granularity and the presence of specific markers. In this study, this technique has been extensively used to characterize human macrophages and their subsets. For phenotypic characterization of polarized macrophages, cells were incubated with Fc blocking solution or FACS buffer (FBS, 2%-BSA,0.1%-PBS, 100ml) for 20min at 4C. Cells were then spun down and resuspended in FACS buffer and stained with appropriate antibodies. 7-AAD (BD, USA) staining was performed to exclude dead cells for further analysis. Differentiation of macrophages was assessed by their intra-cellular expression of CD68 antigen using anti-human CD68-AF647 antibody (BD, USA). For intracellular staining, cells were fixed and permeabilized with CytoFix solution (BD, USA) following manufacturer's instructions. Characterization of M1 and M2 macrophages performed by staining with a panel of surface markers: CD1a, CD1b, CD11b, CD14, CD23, CD64, CD68, CD80, CD163, CD200R, CD206, CD209, CD226 (***Suppl Table 1*** and ***Suppl Table 2***). Data were acquired on BD LSR-Fortessa using BD FACS Diva software. All flow cytometric analysis were performed on CD68 positive cells on Flowjo (Tree Star, USA).

Unfortunately, phenotypic characterization of macrophages is complicated by their inherent autofluorescence (AF) property (265-267). AF is associated with cellular complexity (268). Macrophages are granulated and contain substantial amounts of NAD and FAB. These molecules can be excited by distinct wavelengths, especially 488nm in flow cytometry (269). This signal obscures

fluorescent signals generated by standard fluorescent dyes, such as, fluoresceine isothiocyanate (FITC) or phycoerythrine (PE) (265, 270). Irregular shapes of macrophages is another contributing factor for AF (270). Cigarette smoking was reported as an external factor that is also involved in AF of macrophages though the underlying mechanism is not known (271). Therefore, antibodies with fluorochromes at far blue, red and violet dyes were chosen for this study. Quality control of the flow cytometer including fluorescence standardization, linearity assessment, and spectral compensation were performed on daily basis to ensure identical operation from day to day.

2.4.6 Quantification of Inflammatory Cytokines and Chemokines

To quantify cytokines present in the culture supernatant, supernatants were collected and spun down to get rid of any residual cells. Cell-free supernatants were immediately stored at -20^{0}C. IL-1β, TNF-α, IFN-γ, IL-8, RANTES, IP-10, IL-10 and IL-13 in cell-free culture supernatants were quantified in duplicate wells by alphaLISA (Perkin Elmer, USA). TGF-β, CCL17 (BioLegend, USA), CCL18, CCL22 (R&D Systems, USA) in the culture supernatant were quantified using conventional ELISA. IL-13Rα2 was measured using sandwich ELISA (Ray Biotech, USA).

2.4.7 Total RNA Extraction and Gene Expression Analysis by Quantitative PCR (qPCR)

RNA was extracted using RNeasy Kit, reverse transcribed to cDNA using Quantitect RT kit (Qiagen, USA). qPCR was performed using pre-designed TaqMan primer/probe combinations for human IRF4, IRF5, APOL3, TNF-α, CXCL11, CCL18, FN1, COX-2, and β-actin genes in at least duplicate wells (LifeTech, USA) on ABI 7900HT (Applied-Biosystem, USA). Primer sequences are available on the manufacturer's website. After normalizing the data with β-actin, relative gene expression was calculated by considering M0 as control (272). ΔΔCT was calculated by the formula below:

$$2^{(-\Delta\Delta CT)}=[CT(target,untreated)-CT(ref,untreated)]-[CT(target,treated)-CT(ref,treated)]$$

2.4.8 Networking Analysis of M2 Macrophages by Microarray

Microarray analysis requires genomic DNA (gDNA) free high-quality RNA. To get gDNA free RNA, RNA for microarray experiments were extracted using RNeasy Plus Kit (Qiagen, USA). RNA integrity number (RIN) were analyzed with Agilent Bioanalyzer 2100 in the sequencing facility of

the Institute for Molecular Bioscience (IMB), University of Queensland. RIN numbers for all samples were above 8.0. Microarray was conducted using U219 array plate (Affymetrix, USA) containing more than 36,000 gene probes at the Ramaciotti Centre for Genomics, University of New South Wales (UNSW), Australia. To identify biological processes whether upregulated or downregulated in CF macrophages compared to macrophages from healthy individuals, network analysis was performed using Ingenuity Pathway Analysis (IPA) software by Professor Anthony Bosco, Telethon Kids Institute, University of Western Australia.

2.5 Novel Methods Developed During This Ph.D. Study

2.5.1 In vitro Model for Human M1 and M2 Macrophages Polarization and Characterization

Initial experiments at the early stages of this Ph.D. study were commenced with non-adherent monocytic cell line THP-1. To get adherent macrophage-like cells, THP-1 cells were treated them with 20nM of PMA (Sigma, USA) or M-CSF (50ng/ml, Invitrogen, USA), or GM-CSF (50ng/ml, BioLegend, USA). Differentiation to macrophage-like cells was analyzed by cell adherence to the tissue culture plate. Overnight PMA treatment showed full adherence of THP-1 derived macrophage-like cells (TDMs). However, M-CSF treated cells did not adhere at all (117), while GM-CSF treatment exhibited partial adherence. PMA treated TDMs were then treated with LPS (20ng/ml, Sigma, USA) plus rhIFN-γ (20ng/ml, Life Technologies, USA) for M1 polarization and with IL-13 (20ng/ml, Life Technologies, USA) for M2 polarization. Whether TDM data can truly reflect MDM data, monocytes from healthy donors were differentiated with either M-CSF (50ng/ml, Invitrogen, USA) or GM-CSF (50ng/ml, BioLegend, USA) and polarized with abovementioned protocol and compared with TDM data. Expression of CD80 as M1 marker and CD163 and CD206 as M2 markers were analyzed by flow cytometry.

TDMs showed partial induction of CD80 following LPS treatment compared to LPS treated MDMs (**Suppl Figure *1***). In the case of M2, no or basal induction of CD206 (**Suppl Figure *2***) and CD163 (**Suppl Figure *3***) were observed on IL-13 treated TDMs. Induction of these two murine M2 markers were not substantial on IL-13 conditioned MDMs compared to medium control. Taken together, ***firstly,*** TDMs demonstrated disparate data than MDMs, therefore did not mimic the phenotypes of MDMs. ***Secondly,*** murine M2 markers CD163 and CD206 failed to distinguish IL-13 treated M2s from medium controls and therefore were off use in human indicating the necessity of new markers for human macrophage study. No further experiment was conducted with TDMs.

Available human data on M1/M2 polarization were inconsistent in the experimental conditions used to generate *in vitro* human macrophages and stimuli those were used for macrophage polarization (reviewed in ***Chapter 1***). Based on earlier studies (72, 136) 6-day GM-CSF or M-CSF stimulated and fully differentiated MDMs were chosen for experiments onwards. 6-day fully differentiated macrophages were termed as uncommitted (M0) macrophages in this entire thesis. All possible combinations for M1 and M2 inducers were employed and later compared the phenotype and functions to define the best *in vitro* inducers for corresponding polarized states. Following 6-day stimulation with GM-CSF or M-CSF, fully differentiated macrophages were washed with sterile PBS to get rid of any residual GM-CSF or M-CSF. Medium containing either *E. coli* LPS alone (20ng/ml) (Sigma, USA), recombinant IFN-γ alone (20ng/ml) (Invitrogen, USA) or LPS plus IFN-γ were added to M0 cells for next 2 days for M1 polarization. M2 polarization was induced by using recombinant IL-4 alone (20ng/ml) (Invitrogen, USA), IL-13 alone (20ng/ml) (BioLegend, USA) or IL-4 plus IL-13 for 2 days (***Figure 2***).

Although earlier studies mostly focused on gene expression profiles of M1 and M2 macrophages, differential expression of a few number surface markers for human M1 and M2 macrophages had also been reported (72, 130, 136). Since those markers were shared by both subsets, a single population for each subset was hard to identify. Therefore I was looking for markers those were not shared and would clearly differentiate M1 or M2 macrophages as a single population. That's why a panel of surface markers including CD1a, CD1b, CD11b, CD14, CD23, CD64, CD80, CD163, CD200R, CD206, CD209, CD226 were tested by flow cytometry (***Suppl Table 1*** and ***Suppl Table 2***).

To validate this model, expression of subset-specific genes reported by previous studies were analyzed by qPCR. For instances, genes associated with M1 polarization included Cox-2, APOL3, CXCL11, TNF-α and IRF5 (48, 72, 130, 136), whereas IRF4, FN1 and CCL18 were found to be associated with M2 polarization (111, 130, 135, 273). These genes were chosen based on their highest expression reported in the abovementioned studies. Release of inflammatory mediators and functional attributes of M0, M1 and M2 macrophages were also studied. This method ultimately allowed to characterize M0, M1 and M2 macrophages based on their surface markers, signature genes, secreted inflammatory mediators and functions.

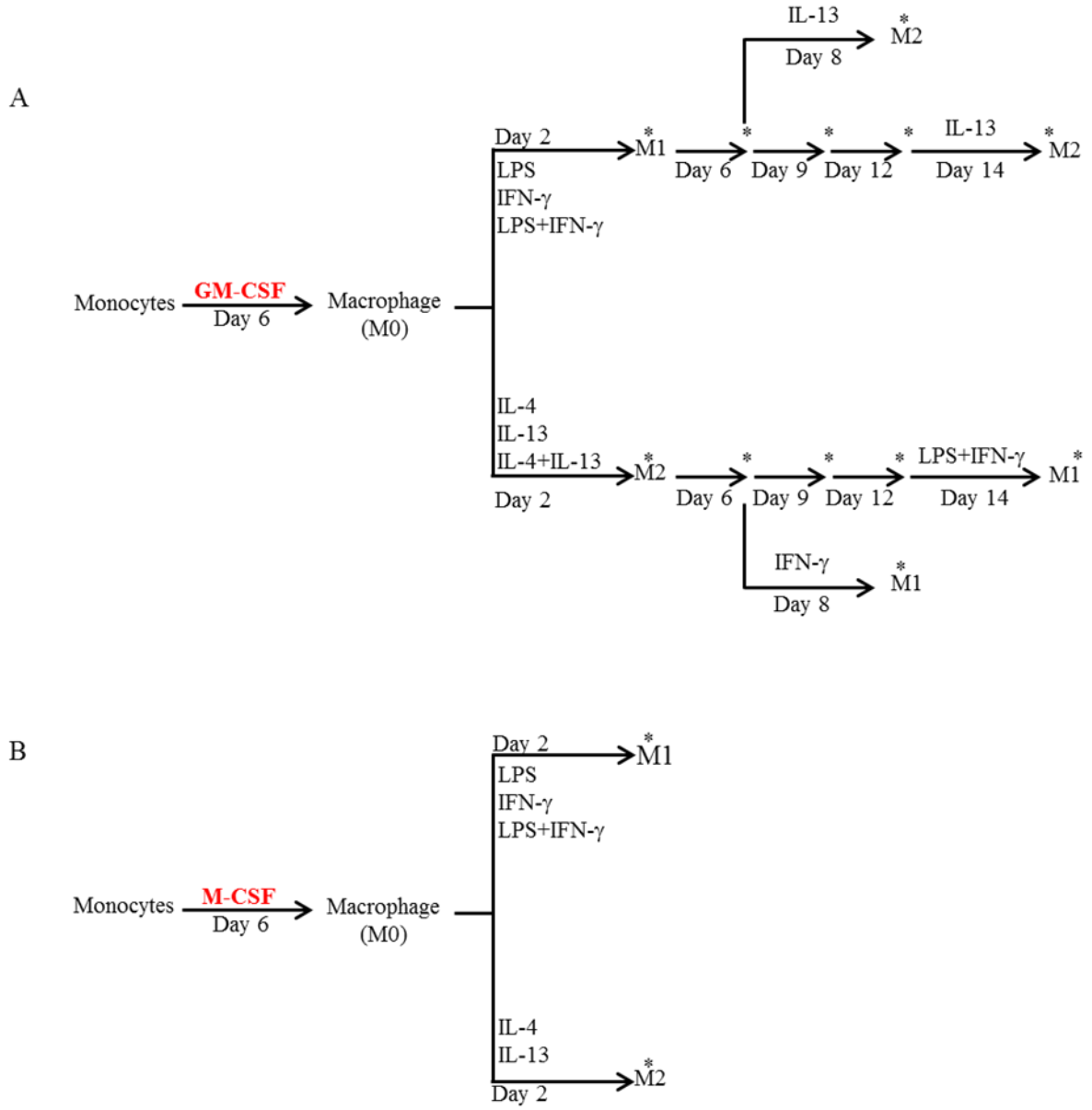


Figure 2: *In vitro* model for human macrophage polarization. Human $CD14^{+}$ monocytes were differentiated into macrophages (M0) by either GM-CSF (A) or M-CSF (B) and further polarized into M1 or M2 macrophages using either LPS/IFN-γ or IL-4/IL-13 for two days respectively. To study the stability of the phenotype, after polarization to M1 or M2, the cells were left in cytokine-free medium and analyzed after 6, 9 or 12 days. To test reprogramming into the opposite functional state, after 6 or 12 days in cytokine-free medium, M1 cells were exposed to M2 stimulus, and M2 cells were exposed to M1 stimulus for 2 days and analyzed. (B) Similar differentiation and polarization were performed with M-CSF derived macrophages. * denotes the time of cell harvest and phenotypic analysis by flow cytometry.

2.5.3 Phagocytosis and Endocytosis: Functional Properties of M1 and M2 Macrophages

Phagocytosis and endocytosis represent mechanisms by which M1 and M2 macrophages, respectively engulf and eliminate foreign particles (31). These functional properties have not been fully studied in human macrophage subsets. Commercially available kits for phagocytosis and endocytosis were used to demonstrate the subset-specific functional attributes of human M0, M1 and M2 macrophages. Internalization of AF-647 labeled dextran (10KD) (LifeTech, USA) and pHrodo green *E. coli* bioparticles (LifeTech, USA) were used to assess endocytic and phagocytic abilities of human M0, M1 and M2 macrophages. Phagocytosis process involves detection and attachment of the foreign microorganisms, engulfment of microbes and formation of phagosomes, fusion of phagosomes with lysosomes and finally killing of engulfed microbes by lysosomal acidic enzymes. pHrodo® green conjugates are sensitive to pH. They are non-fluorescent outside the cell at neutral pH, but fluoresce brightly green at acidic pH such as in phagosomes.

Macrophages were incubated with either 5μg/ml of dextran for endocytosis or recommended numbers of pHrodo green *E. coli* bioparticles for phagocytosis. Earlier studies recommended 90min incubation at 37^0C for these assays (274). A washing step after 90-min incubation with pHrodo conjugates had been reported to result in a decrease in fluorescence signals. Therefore any wash step was avoided in phagocytosis assay (274). However, cells subjected to the endocytosis assay were washed after incubation as recommended by the supplier. Uptake and internalization were measured by mean fluorescence intensities (MFIs) of corresponding fluorochromes by flow cytometry. Cells without bacteria or dextran were set as negative control (NC), M0 macrophages were set as a baseline control.

Phagocytosis or endocytosis index (%) = (MFI, experimental well - MFI, NC) / (MFI, baseline control well - MFI, NC) ×100%.

2.5.4 In vitro CF Macrophage Model

This study developed an *in vitro* model for CF macrophages using two CFTR inhibitors, $CFTR_{Inh}$-172 and GlyH-101. Using $CFTR_{Inh}$-172, earlier study had shown that the inhibition of normal CFTR function alter phagocytosis ability of human macrophages (159), however, the macrophage differentiation protocol was different. In the study described here, monocytes from buffy coats were exposed to CFTR inhibitors $CFTR_{Inh}$-172 (10μM) (Sigma, USA) or GlyH-101 (10μM)

(Santa-Cruz, USA) during macrophage differentiation or polarization or both (***Suppl Figure 8***). These inhibitors work by different mechanisms, with $CFTR_{Inh}$-172 binding CFTR intracellularly, while GlyH-101 blocks the channel on the cell surface (226, 227). The rationale was to study whether blocking CFTR function in two different mechanisms would provide same or similar macrophage phenotypes and to choose an *in vitro* CF macrophage model that mostly mimic physiological functions and phenotypes of CF macrophages.

2.5.5 Effects of Tobramycin on Macrophage Polarization and Functions

Peak concentration of tobramycin in the BAL and sputum samples of CF patients was reported as 1mg/ml/ (243, 275, 276). No cytotoxic effect was observed in human bronchial epithelial cells with this concentration of tobramycin. Therefore, this concentration of tobramycin was chosen for *in vitro* macrophage differentiation and polarization to maintain clinical relevance. Tobramycin was added every 3rd day in the culture media.

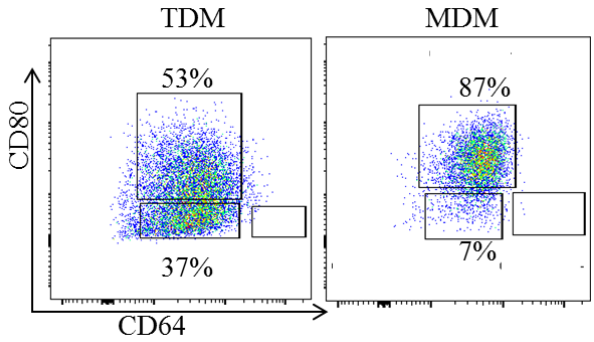
2.6 Data Handling and Storage

All patients and volunteers information were collected at the time of the enrolment and were entered into a database in a non-identifiable way and stored on a secure system at The University of Queensland. All data from laboratory analyses were stored in the same location.

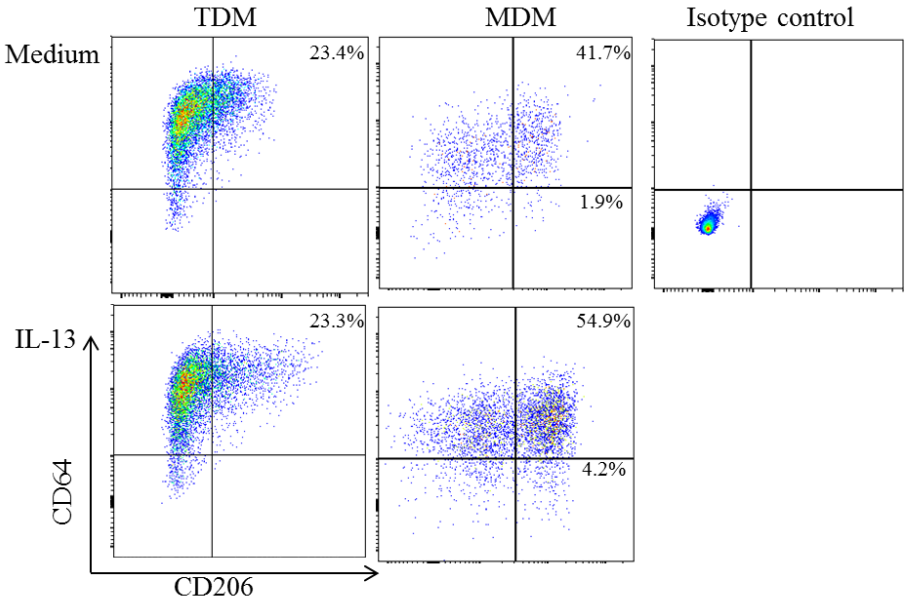
2.7 Statistical Analysis

All statistical analysis were performed using GraphPad-Prism (version 6.07) software (San Diego, CA). Statistical tests were chosen based on the research questions to be addressed and the experimental designs. In the Chapter 3, one-way nonparametric ANOVA was performed among M0, M1 and M2 groups. Data in Chapter 4 were shown as group mean±SD for flow cytometry and median and IQR unless stated otherwise. Wilcoxon paired signed ranked test was performed to determine the differences of inflammatory parameters amongst adult patients between hospital admission and discharge. Mann-Whitney test was performed to assess statistical significance of inflammatory parameters between control and CF groups. Two-way ANOVA was used in time-course studies, with group (CF/control) and time entered as main effects. Statistical significance was considered with $p<0.05$.

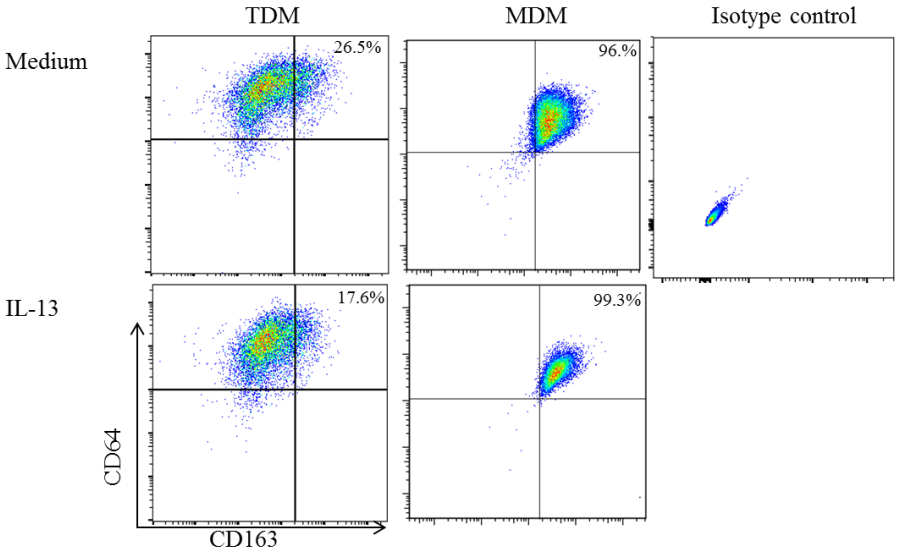
2.8 Supplementary Data



Suppl Figure 1: Partial induction of CD80 in PMA treated THP-1 macrophages (TDMs) following LPS stimulation. TDMs and GM-CSF treated MDMs were given LPS (20ng/ml) stimulation for 2 days. Cells were harvested washed and stained with CD64 PE Cy7 (X-axis) and CD80 AF 700 (Y-axis). At least 5000 events were acquired on BD LSR Fortessa. Data were shown from at least 2 experiments.



Suppl Figure 2: Expression of CD206 is not induced by IL-13 in PMA treated THP-1 derived macrophages (TDMs). TDMs and M-CSF or GM-CSF treated MDMs were given IL-13 (20ng/ml) stimulation for 2 days. Cells were harvested washed and stained with CD64 PE Cy7 (Y-axis) and CD206 PE-Cy 5 (X-axis). At least 5000 events were acquired on BD LSR Fortessa. Data were shown from at least 2 experiments.



Suppl Figure 3: Expression of CD163 is not induced by IL-13 in PMA treated THP-1 derived macrophages (TDMs). TDMs and M-CSF or GM-CSF treated MDMs were given IL-13 (20ng/ml) stimulation for 2 days. Cells were harvested washed and stained with CD64 PE Cy7 (Y-axis) and CD163 PE CF594 (X-axis). At least 5000 events were acquired on BD LSR Fortessa. Data were shown from at least 2 experiments.

Chapter 3 Phenotypic, Functional and Plasticity Features of Classical and Alternatively Activated Human Macrophages

3.1 Summary

Identification of the polarized state of macrophages is the cornerstone in understanding disease pathogenesis. Therefore, the first aim of this chapter was to develop an *in vitro* model for human M1 and M2 polarization and their characterization. Macrophages were differentiated from monocytes isolated from buffy coats by 6-day stimulation with GM-CSF and M-CSF. These uncommitted macrophages were termed as M0. A number of stimuli were employed to polarize M0 macrophages to M1 and M2 macrophages to find out the best *in vitro* stimulus for the corresponding polarized state. A panel of surface markers was tested to get single population for M0, M1 and M2 macrophages. The model was verified by subset-specific genes reported by earlier studies. Subset-specific cytokine and chemokine secretion and functional characteristics were analyzed.

The second aim of this chapter was to assess the persistence of polarized phenotypes over time. Once polarized M1 and M2 macrophages were left in the cytokine-free medium for next 12 days and subset-specific surface marker expression was monitored every 3rd day by flow cytometry.

Plasticity is another hallmark feature of macrophages. Murine model showed that upon appropriate stimuli, once polarized M1 macrophages were able to switch to M2 and vice versa. Unfortunately, this concept had not been clearly shown in human. Therefore the final aim of this chapter was to assess the reprogramming ability of once polarized macrophages into another.

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3.2 Abstract

Macrophages are dynamic cells that mature under the influence of signals from the local microenvironment into either classically (M1) or alternatively (M2) activated macrophages with specific functional and phenotypic properties. While the phenotypic identification of M1 and M2 macrophages is well established in mice, this is less clear for human macrophages. In addition, the persistence and reversibility of polarized human phenotypes is not well established. Human peripheral blood monocytes were differentiated into macrophages (M0) and then polarized to M1 and M2 phenotypes using LPS/IFN-γ and IL-4/IL-13, respectively. M1 and M2 were identified respectively as $CD64^{+}CD80^{+}$ and $CD11b^{+}CD209^{+}$ by flow-cytometry. Polarized M1 secreted IP-10, IFN-γ, IL-8, TNF-α, IL-1β and RANTES while M2 secreted IL-13, CCL17 and CCL18. Functionally, M2 were highly endocytic. In cytokine deficient medium the polarized macrophages reverted back to the uncommitted M0 state within 12 days. If previously polarized macrophages were given the alternative polarizing stimulus after 6 day resting in cytokine deficient medium, a switch in polarization was seen, i.e., M1 macrophages switched to M2 and expressed $CD11b^{+}CD209^{+}$ and vice versa. In summary, we report phenotypic identification of human M1 and M2 macrophages, their functional characteristics and their ability to be re-programmed given the appropriate stimuli.

Keywords: Macrophage, classically activated macrophages (CAM/M1), alternatively activated macrophages (AAM/M2), phagocytosis/endocytosis, phenotypic stability, reprogramming of polarization.

3.3 Introduction

Macrophages are widely distributed innate immune cells that play central roles in host defense against invading pathogens and in maintaining immunological homeostasis. Heterogeneity and plasticity are hallmarks of macrophages (61). Macrophages have broadly been characterized as either classical activated (M1) or alternatively activated (M2) based on surface receptors, gene signatures and secretion of inflammatory mediators (61, 277). Studies with knock-out mice made it possible to identify a number of signature genes (iNOS, Arg 1, Ym1, FIZZ1) along with chemokines that discriminate between different polarization pathways (141, 278, 279). However, due to the substantial physiological and immunological disparities between mouse and human, murine models poorly mimic human inflammatory biology (150, 280, 281) and murine markers are of limited use in human studies. For instance, murine studies are usually performed using either bone-marrow derived macrophages or peritoneal macrophages, whereas human studies are carried out using monocytes or monocytes derived macrophages (MDMs). Additionally, murine M1 or M2 signature genes iNOS, Ym1, FIZZ1 have no homologs in humans. The expression of the surface markers that define mouse M1 and M2 macrophages is different in human. The mannose receptor, CD206, a prototypic mouse M2 marker, is highly expressed in M-CSF treated macrophages in mouse, while in human monocytes CD206 is substantially induced by GM-CSF (86, 146). CD163, scavenger-R type A, another mouse M2 marker is highly expressed after IL-10 treatment in human, but not in IL-4 condition (146). Transaminoglutamase 2 (TGM2) had been found as the sole gene induced in both murine and human M2 macrophages (150). These data cast doubt on whether murine M1/M2 definition really mimics human macrophage biology.

Available human data on M1/M2 polarization are limited and hampered by the lack of consistency in the experimental conditions used to study human macrophages *in vitro*, including the use of different cell types or cell lines for macrophage generation, different culture conditions and different stimuli for macrophage differentiation and polarization. Monocytic cell lines, such as THP-1 are commonly used by human macrophage biologists. However, while THP-1 is recognized as a good model, some significant differences have been identified when comparing to primary cells. For instance, PMA treated THP-1 macrophage-like cells do not express mannose receptor (CD206) MHC class II DRα1, while CD14 and IL-1β were upregulated compared to monocytes or monocytes-derived macrophages (MDM) (117). Furthermore, a subset of PMA treated THP-1 cells do not express scavenger receptors demonstrating heterogeneity in this cell line (119). Compared to primary cells, the responses of THP-1 to LPS stimulation (121) and oxidized low-density lipoproteins (120) were attenuated and finally TLR3 and TLR5 mRNA expression were lower (122). Other significant differences have been recently reviewed by Qin (123). In addition, THP-1 expression of CD14 marker

and cytokines was found to be very sensitive to culture conditions, in particular cell density and duration of stimulation (124). Therefore monocytes or monocytes-derived macrophages (MDM) are now more frequently being used in human macrophage studies. Transcriptome-based network analysis revealed that macrophages are clearly distinct cell types from their progenitor monocytes (72). While using monocytes, some investigators induce M1 and M2 polarization with GM-CSF and M-CSF, respectively (48), whereas others used type II interferon and/or TLR agonists for M1 and T_H2 cytokines, e.g., IL-4, IL-13 for M2 polarization, showing no consensus protocol for human macrophages polarization. Beyer *et al* reported a few surface markers for human M1 and M2 macrophages; the expression level of these markers was independent of GM-CSF or M-CSF treatment, but depended on the presence of T_H1 or T_H2 cytokines (136). This study not only demonstrated the necessity of using MDM for human macrophage research as well as the importance of cytokines or TLR agonists for macrophage polarization, but also challenged the concept of GM-CSF and M-CSF mediated M1 and M2 polarization in human studies. However, the differentially expressed markers reported by Beyer *et al* were not adequate to distinguish between uncommitted (M0), M1 and M2 macrophage subsets.

The current literature is not definitive on whether the polarized subsets of macrophages are stable, whether the shift from pro- to anti-inflammatory conditions or vice versa required removal of one subset and replacement by a new subset or whether depolarization followed by repolarization could occur *in situ*. An adequate balance between M1 and M2 subsets maintains the homeostatic milieu, whereas disequilibrium is likely to lead to unbalanced inflammation. In murine models of inflammatory bowel disease and asthma, adoptive transfer of M2 macrophages not only reversed the distribution of macrophage subsets but also reduced the disease severity, suggesting a dual role for macrophages in orchestrating both the onset and dissolution of inflammation (74, 75). The ability of macrophages to adapt to changing cytokine environments has been demonstrated *in vivo* in tumor-bearing mice, where changing the microenvironment resulted in switch of macrophage phenotypes (63, 76, 77). Such shifts between macrophage subsets have not been studied using human macrophage models.

Both M-CSF and GM-CSF are potent hematopoetic growth factors for macrophage differentiation from monocytes. M-CSF is abundant in the circulation, while the respiratory lining is rich in GM-CSF. We utilized GM-CSF in most parts of our study to define respiratory M1 and M2 macrophages. However, we also used M-CSF to confirm that, in humans, M-CSF derived macrophages exhibited similar functional phenotypes following M1 and M2 polarization. Therefore our findings would not be limited only to respiratory macrophages exposed to a GM-CSF-rich

environment. In the present study, we used MDMs to determine combinations of surface markers that distinguished between populations of M0, M1 and M2 macrophages and described their functional signatures. We also analyzed the phenotypic stability of each polarized state over time, and demonstrated the reprogramming ability of one polarized state into another.

3.4 Materials and Methods

3.4.1 In vitro Polarization of M1 and M2 Macrophages

Buffy coats from eight healthy blood donors were obtained from the Australian Red Cross blood services (Brisbane, Australia) and PBMCs isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, UK). Monocytes were purified using CD14+ magnetic microbeads (Milteny Biotech, Germany). For macrophage differentiation, monocytes were cultured in RPMI-1640 (Lonza, USA), supplemented with 10% heat-inactivated FBS (Life Tech, USA), 1% Penicillin-streptomycin-fungizone (Lonza, USA) and rhGM-CSF (50ng/ml) (BioLegend, USA) or rhM-CSF (50ng/ml) (Invitrogen, USA) for 6 days. Uncommitted macrophages (M0) were polarized into M1 using *E. coli* LPS alone (20ng/ml) (Sigma, USA), rhIFN-γ alone (20ng/ml) (Invitrogen, USA) or LPS plus IFN-γ and into M2 using rhIL-4 alone (20ng/ml) (Invitrogen, USA), rhIL-13 alone (20ng/ml) (BioLegend, USA) or IL-4 plus IL-13 for 2 days (**Figure *3***). To assess the persistence of polarized phenotype, M1 and M2 cells were washed with PBS and left in medium without any cytokine for up to 12 days. Cells that previously received M1 stimuli were then given M2 stimuli (IL-13) and vice versa. Less than 2% cell death was observed in all conditions.

3.4.2 Phenotypic Characterization of M1/M2 macrophages

Cells were harvested by TrypLE (Invitrogen, USA), washed with PBS and incubated with Fc blocking solution (FBS, 2%-BSA,0.1%-PBS, 100ml). Cells were stained with a panel of surface markers: CD1a, CD1b, CD11b, CD14, CD23, CD64, CD68, CD80, CD163, CD200R, CD206, CD209, CD226 (***Suppl Table 1*** and ***Suppl Table 2***). 7-AAD (BD, USA) staining was performed to gate out dead cells. Data were acquired on BD LSR-Fortessa using BD FACS Diva software. Cell populations were identified on CD68 positive cells on Flowjo (Tree Star, USA).

3.4.3 Gene Expression Analysis by Real-Time PCR

RNA was extracted using RNeasy Kit, reverse transcribed using Quantitect RT kit (Qiagen, USA). qPCR was performed using TaqMan primer/probe combinations for human IRF4, IRF5, APOL3, TNF-α, CXCL11, CCL18, FN1, COX-2 and β-actin genes in at least duplicate wells (LifeTech, USA) on ABI 7900HT (Applied-Biosystem, USA). After normalizing the data with β-actin, relative gene expression was calculated by considering M0 as control (272).

3.4.4 Quantification of Cytokines and Chemokines

IL-1β, TNF-α, IFN-γ, IL-8, RANTES, IP-10, IL-10, IL-13 in culture supernatant were quantified in duplicate wells by alphaLISA (Perkin Elmer, USA). TGF-β, CCL17 (BioLegend, USA), CCL18, CCL22 (R&D Systems, USA) were quantified using conventional ELISA.

3.4.5 Functional Assays: Phagocytosis and Endocytosis

Internalization of AF-647 labeled dextran (10KD) (LifeTech, USA) and pHrodo green *E. coli* bioparticles (LifeTech, USA) were used to assess endocytic and phagocytic abilities of macrophages. Briefly, macrophages were incubated with either dextran (5μg/ml) or green *E. coli* for 90min at 37C. Uptake and internalization were measured by mean fluorescence intensities (MFIs) by flow cytometry. Cells without bacteria or dextran were set as negative control (NC), M0 macrophages were set as baseline control.

Phagocytosis or endocytosis (%) = (MFI, experimental well − MFI, NC) / (MFI, baseline control well − MFI, NC) × 100%.

3.4.6 Statistical Analyses

Statistical analysis was performed using GraphPad-Prism software. One-way nonparametric ANOVA was performed among M0, M1 and M2 groups. Statistical significance was considered with $p<0.05$ and data presented as means ± SEM.

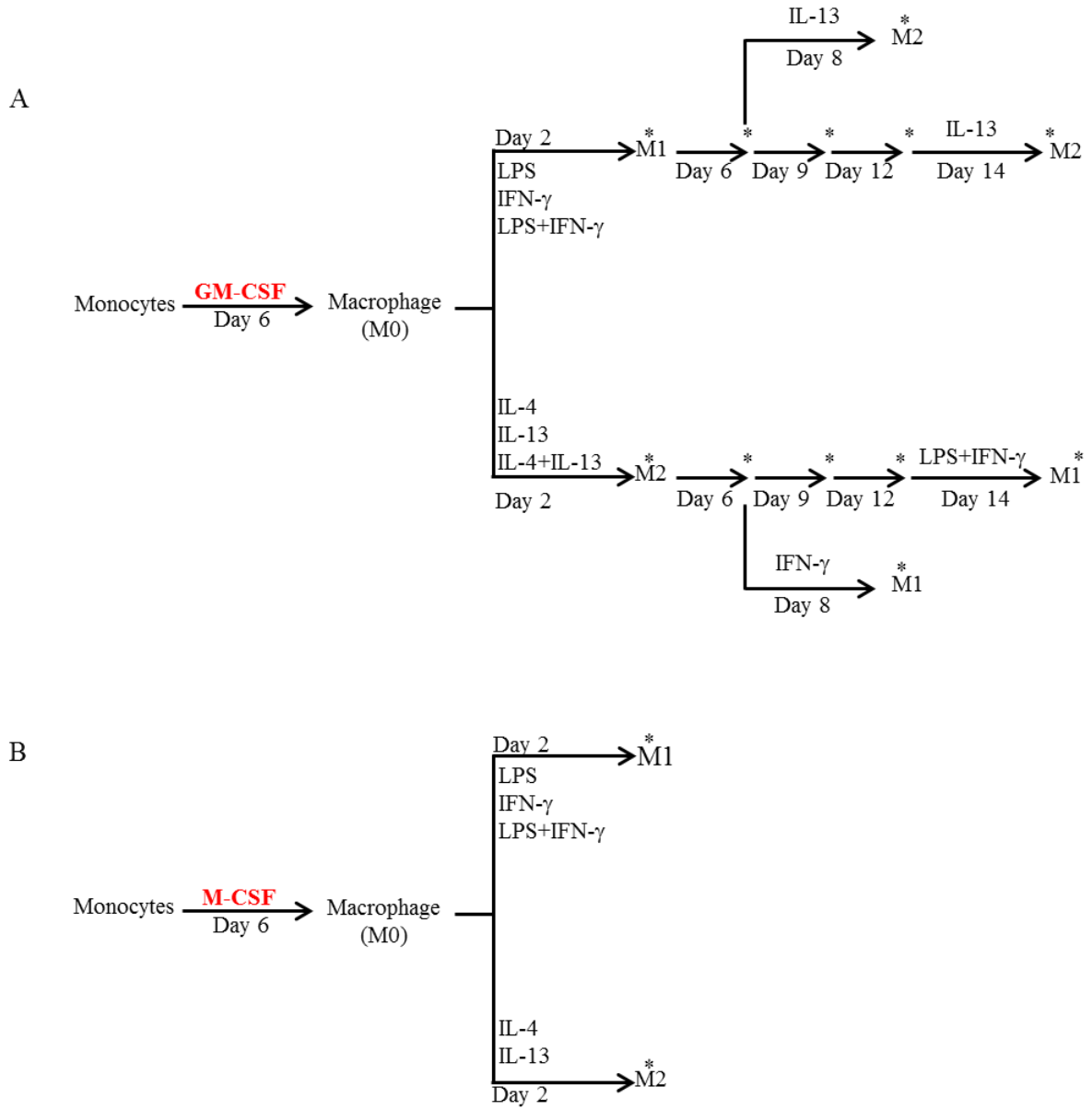


Figure 3: In vitro model for human macrophage polarization. Human CD14^{+} monocytes were differentiated into macrophages (M0) by either GM-CSF (A) or M-CSF (B) and further polarized into M1 or M2 macrophages using either LPS/IFN-γ or IL-4/IL-13 for two days respectively. To study the stability of the phenotype, after polarization to M1 or M2, the cells were left in cytokine-free medium and analyzed after 6, 9 or 12 days. To test reprogramming into the opposite functional state, after 6 or 12 days in cytokine-free medium, M1 cells were exposed to M2 stimulus, and M2 cells were exposed to M1 stimulus for 2 days and analyzed. (B) Similar differentiation and polarization were performed with M-CSF derived macrophages. * denotes time of cell harvest and phenotypic analysis by flow cytometry.

3.5 Results

3.5.1 M1s are $CD64^{Hi}CD80^{-}$ or $CD64^{+}CD80^{+}$ while M2s are $CD11b^{+}CD209^{+}$

Macrophages differentiated in the presence of GM-CSF for 6 days maintained the regular morphology while macrophages differentiated in the presence of M-CSF were elongated with numerous vacuoles (***Suppl Figure 4A***). We did not find any substantial difference in CD68 expression between GM-CSF and M-CSF derived macrophages (***Suppl Figure 4B***). Following 2-day polarization of mature M0 macrophages with M1 and M2 stimuli, we investigated the expression of a broad panel of surface markers by flow cytometry (***Suppl Table 1***). The markers that best characterized M1 macrophages were CD64 (the high-affinity Fcγ receptor I) and CD80 (T-lymphocyte activation antigen). However, the levels of induction of these two markers were different depending on the nature of the M1 stimulus: M1-IFN-γ cells exhibited a robust upregulation of CD64 ($CD64^{hi}$) in 6 out of 8 donors (***Figure 4A***). Co-stimulatory molecule, CD80 was expressed mainly when cells received LPS. A distinct $CD64^{+}CD80^{+}$ population was detected when M1 polarization derived from LPS (72%) or LPS plus IFN-γ (63%) stimulation (***Table 4***). Both M0 and M2 cells were $CD64^{+}CD80^{-}$.

M2 macrophages were identified based on the expression of CD209, [dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN)] a C-type lectin. M2 macrophages polarized with IL-4, IL-13 or both all expressed $CD11b^{+}CD209^{+}$ (***Table 4***, ***Figure 4B***). In addition, 50% of these $CD209^{+}$ M2 cells expressed the inhibitory receptor CD200R. M2 cells also expressed either T-cell surface glycoproteins CD1a or CD1b both (***Table 4***, ***Suppl Figure 4C***), with 50% of the M2 cells in a $CD1a^{+}CD1b^{+}$ population. CD23 was solely expressed in M2 macrophages, though the magnitude varied among donors.

M-CSF differentiated macrophages showed a similar pattern of expression of $CD64^{+}CD80^{+}$ for M1 cells and $CD11b^{+}CD209^{+}$ for M2 cells (***Suppl Figure 5***). In contrast to GM-CSF derived macrophages, M-CSF derived IFN-γ polarized M1 cells contained a subpopulation that expressed the $CD64^{hi}CD80^{+}$ surface phenotype.

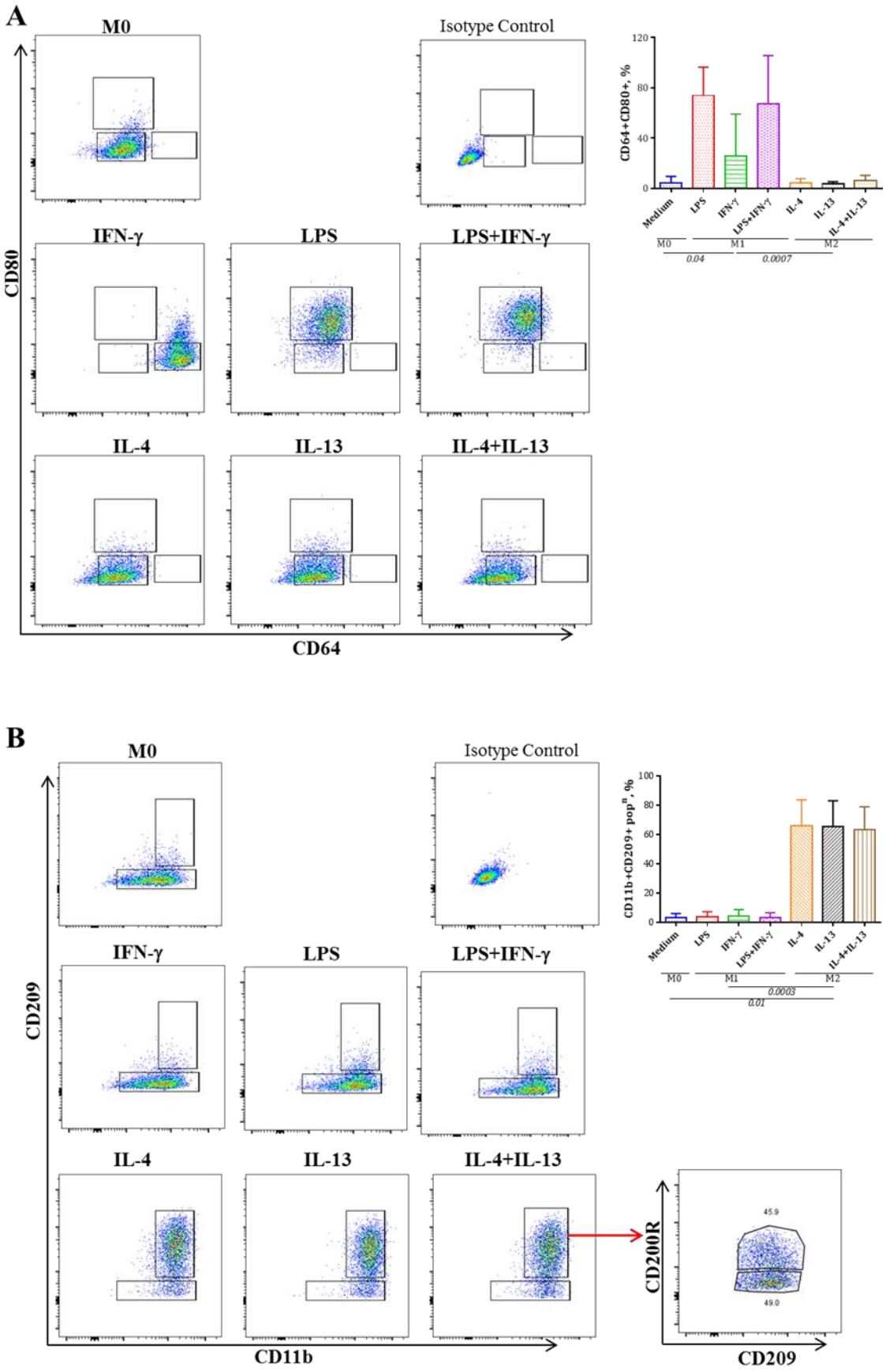


Figure 4: Phenotypic characterization of human M1 and M2 macrophages. Monocytes from healthy donors were cultured with GM-CSF containing medium for 6 days to differentiate into uncommitted macrophages (M0). M1 macrophages were then generated by stimulating with LPS

alone, IFN-γ alone and LPS plus IFN-γ. M2 polarization was performed with IL-4 alone, IL-13 alone and IL-4+IL-13 for 2-days. Population frequencies of M0, M1 an M2 conditioned cells were assessed with two marker sets; A: CD64 and CD80, and B: CD11b and CD209. A & B were representative flow cytometric plots of 1 donor out of 8 donors. Bar graphs (C & D) represent the mean and SD of population frequencies of $CD64^{+}CD80^{+}$ and $CD11b^{+}CD209^{+}$ respectively for at least 8 different individual subjects. Statistical significance was calculated using nonparametric one-way ANOVA.

Table 4: Population frequencies of human M0, M1 and M2 macrophages

	M0	M1			M2		
	Medium	LPS	IFN-γ	LPS+IFN-γ	IL-4	IL-13	IL-4+ IL13
CD64+CD80+	4.5 ± 5.7	71.6 ± 23.7	25.4 ± 36.8	63.4 ± 40.2	3.4 ± 1.2	3.1 ± 1.8	5.5 ± 3.7
CD11b+CD209+	3.6 ± 3.1	4.6 ± 3.2	4.7 ± 4.8	3.8 ± 3.4	68.4 ± 18	68.5 ± 17	65.5 ± 15.9
CD209+CD200R+	1.4 ± 2.6	0.6 ± 1.0	0.8 ± 1.5	0.7 ± 1.5	35.7 ± 13.2	34 ± 15.6	25.4 ± 18.8
CD1a+CD1b+	6.7 ± 4.4	7.7 ± 4.1	8.5 ± 2.7	7.2 ± 2.6	54 ± 9.6	39.8 ± 17.2	43.8 ± 18.6

Data were representative of at least 5 individual donors. Mean ± SD were shown.

3.5.2 Transcriptional Profiles of M1 and M2 Macrophages

Considering the M0 as controls, we observed upregulation of Cox-2, APOL3, CXCL11, IRF5 and TNF-α in all M1 cells (***Figure 5***), however, the magnitude varied with the polarizing stimulus. Polarization with LPS plus IFN-γ demonstrated the maximal induction of these genes compared to individual treatments. These genes were suppressed in M2 macrophages. Conversely, IRF4, FN1 and CCL18 were significantly induced in M2 cells and suppressed in M1 cells.

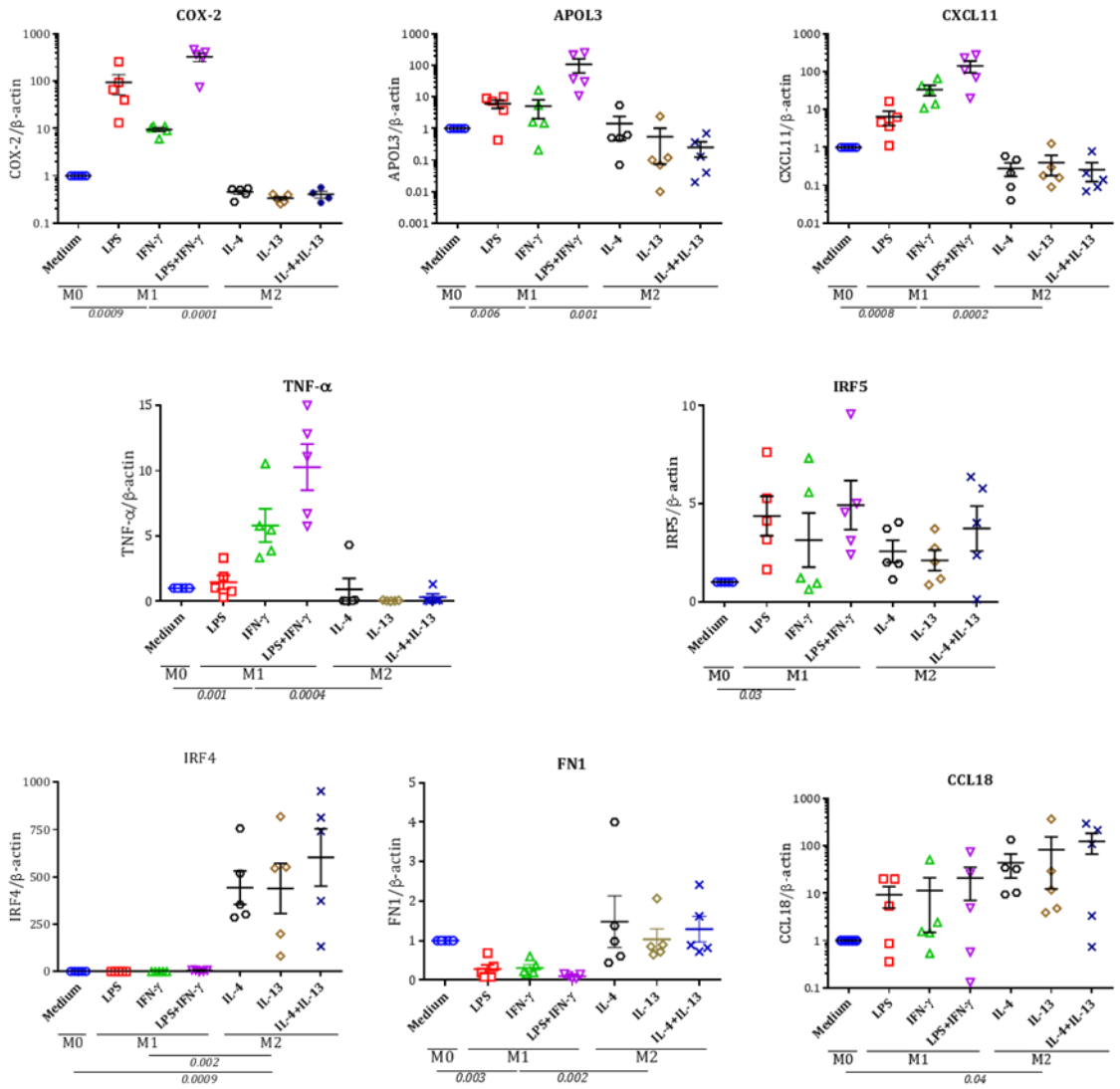


Figure 5: Gene expression profile of human M0, M1 and M2 macrophages. M1 and M2 macrophages harvested after polarization with either LPS alone, IFN-γ alone, LPS plus IFN-γ or IL-4 alone, IL-13 alone and IL-4 plus IL-13 for qPCR analysis. Each symbol represents results from an independent subject (n=8). Group mean and SEM are shown. Statistical significance was calculated using nonparametric one-way ANOVA.

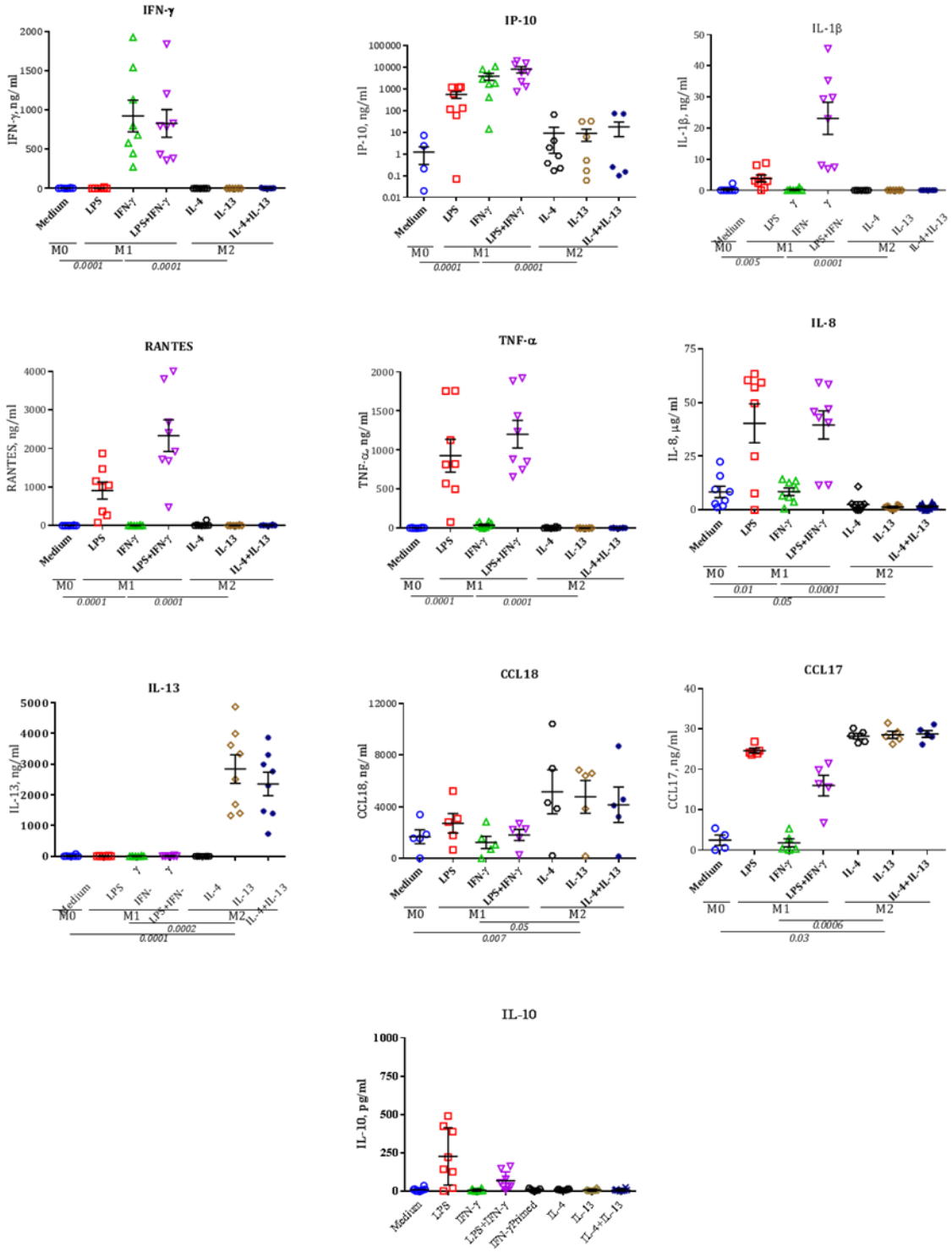


Figure 6: Unique inflammatory profile of M1 and M2 macrophages. GM-CSF derived uncommitted macrophages (M0) were induced with LPS alone, IFN-γ alone or LPS+ IFN-γ. M2 polarization was performed with IL-4 alone, IL-13 alone and IL-4+IL-13 for 2-days. Each symbol represents an independent experiment. Error bars represent the SEM for n=8. Statistical significance was calculated using non-parametric one-way ANOVA.

3.5.3 *Unique Inflammatory Profiles of M1 and M2 Macrophages*

Similar to the phenotypic data, we observed two different cytokine and chemokine patterns among M1 cells. IP-10 and IFN-γ were produced at much higher levels by M1-IFN-γ macrophages indicating an autocrine feedback induction of these cytokines by IFN-γ itself (***Figure 6***). LPS induced IL-8, TNF-α, RANTES and IL-1β secretion. IL-8 was the most abundantly secreted M1 cytokine measured at microgram (μg/ml) level, whereas, others were within the nanogram (ng/ml) range. These cytokine data support the presence of two sub-types of M1 macrophages. Conversely, CCL17 and CCL18 were induced in M2 macrophages. Induction of CCL17 was observed in LPS treated M1 cells although not statistically differently than in M2 cells. We observed significantly elevated IL-13 production by IL-13 treated M2 macrophages, but not by M0, M1 and IL-4 treated M2 macrophages. IL-10 was only induced by LPS. LPS treated and LPS+IFN-γ treated M1 cells showed higher IL-10 production than M0 and M2 macrophages (*P* 0.0009, 0.0002). All macrophage subtypes secreted similar amounts of TGF-β and CCL22 (data not shown). Comparable cytokine and chemokine profiles were observed in M-CSF derived M1 and M2 macrophages (***Suppl Figure 6A***).

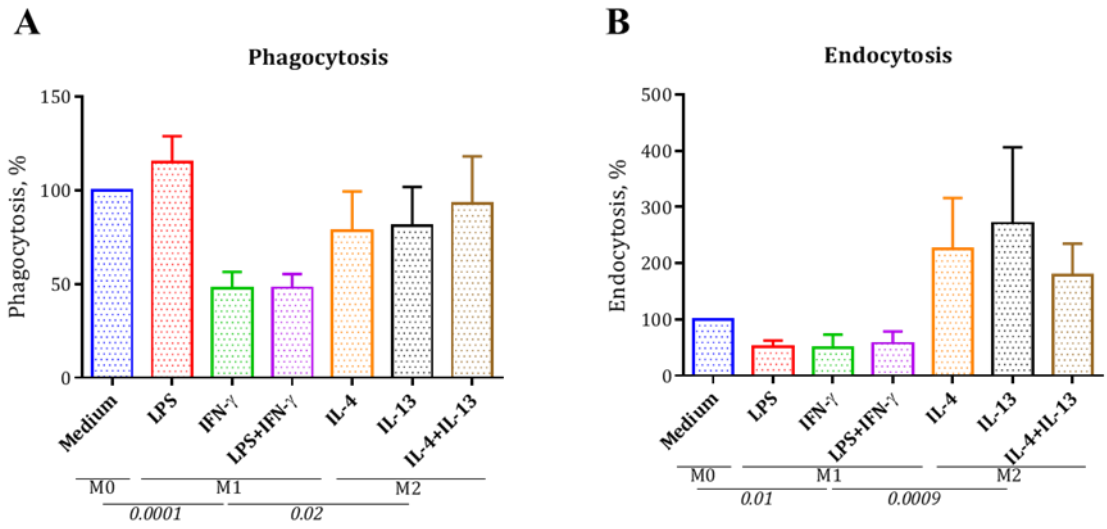


Figure 7: Phagocytosis and endocytosis of polarized M1 and M2 macrophages. M0, M1 and M2 polarized macrophages were treated with either (A) pHrodo green *E. coli* bioparticle for phagocytosis or (B) AF-647 labeled dextran (10KD) for endocytosis (B) for 90 mins at 37°C. Percentages of phagocytosis and endocytosis were calculated by normalizing the data to M0. Data reflect 7 independent experiments from 7 independent donors. Error bars represent the SEM. Significance was determined using nonparametric one-way ANOVA among M0, M1 and M2 groups.

3.5.4 M2 Macrophages Are Highly Endocytic and Partially Phagocytic

LPS treated M1 cells exhibited the highest level of phagocytic activity (***Figure 7A***). Enhanced phagocytic activity was observed in M0 and M2 macrophages (M0 vs M1, *p<0.0001;* M1 vs M2, *p=0.02;* M0 vs M2, *p=0.5*) (Figure 5A). Conversely, robust endocytic activity was observed by M2 macrophages when compared to M1s (*p<0.001*) (***Figure 7B***). M2 macrophages exhibited enhanced dextran uptake compared to M0 cells, although this failed to reach statistical significance (*p=0.07*). M1s showed less dextran uptake suggesting lower endocytic ability than uncommitted M0 cells. Similar phagocytic and endocytic indices were observed with M-CSF derived M1 and M2 macrophages (***Suppl Figure 6B***).

3.5.5 Stability and Reversibility of the Polarized M1 and M2 Phenotypes

In cytokine deficient medium, both M1 and M2 cells lost their polarized phenotype over time. After 6-day resting in cytokine-free medium, the percentages of $CD64^{+}CD80^{+}$ M1 cells or $CD11b^{+}CD209^{+}$ M2 cells were reduced by at least 50% with concomitant increases in $CD64^{+}CD80^{-}$ or $CD11b^{+}CD209^{-}$ M0 cells. These cells fully reverted to uncommitted M0 state by day 12 (***Figure 8***). As expected, the corresponding genes and cytokines declined accordingly (***Figure 9A & B***).

Upon exposure to IL-13, previously polarized M1 cells showed the distinctive $CD11b^{+}CD209^{+}$ M2 phenotype (Figure 6A). However, we did not observe CD1a, CD1b and CD200R expression in these switched (previously M1) M2 cells. Similarly, following LPS plus IFN-γ treatment, previously M2 cells exhibited the M1 phenotype of $CD64^{+}CD80^{+}$ (***Figure 8B***). When rested in cytokine-free medium for 6-days prior to being exposed to the alternative stimulus, previously polarized M1 and M2 cells showed the similar switch in phenotypes, i.e., following IL-13 treatment previously M1-IFN-γ cells switched to the characteristic $CD11b^{+}CD209^{+}$ M2 phenotype and following IFN-γ treatment previously M2 cells switched to M1 phenotype demonstrating the representative M1-IFN-γ population, $CD64^{Hi}CD80^{-}$ (***Suppl Figure 7A***).

Induction of M1 signature genes, IRF5, APOL3, CXCL11, TNF-α, was observed in switched M1 (previously M2) cells (***Figure 9A, Suppl Figure 7B***). IFN-γ, IP-10, TNF-α, RANTES, IL-8, the hallmark cytokines and chemokines for M1 cells were also induced in switched M1 cells. Similarly, M2 signature genes, i.e., IRF4 and FN1 and cytokines, such as, IL-13, CCL17 and CCL18 were substantially induced in switched M2 (previously M1) cells, at similar levels to 2-day polarized M2s (***Figure 9B***). However, we did not see any switch in the functional features within the time frame of

the experiments. Switched M2 (previously M1) cells gained phagocytic activity similar or slightly higher than the original M2 cells, however, their endocytic ability stayed at original M1 levels (***Figure 9C***). Switched M1 (previously M2) cells lost their endocytic but not their phagocytic activity.

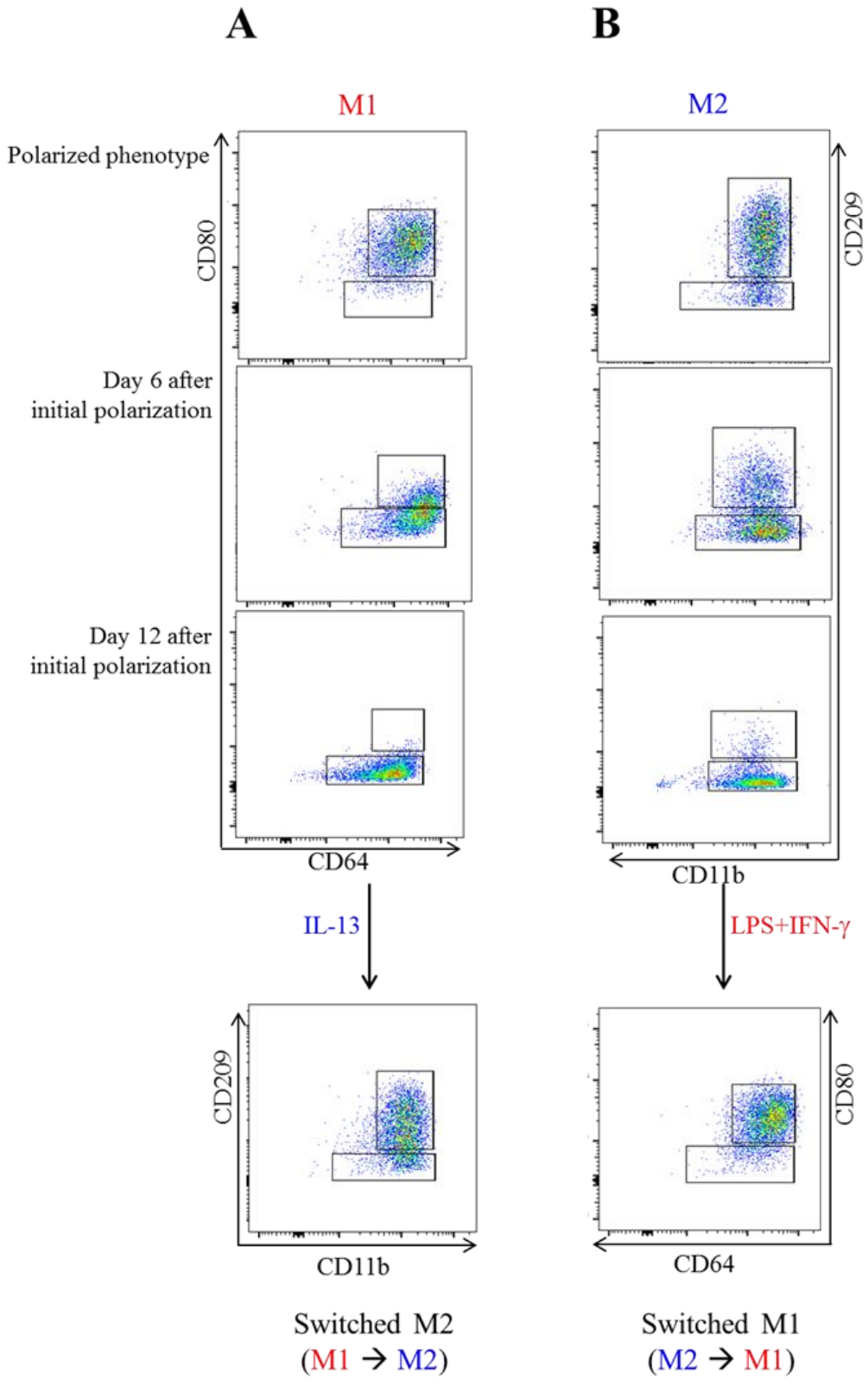


Figure 8: Phenotypic stability and reversibility of polarized macrophages. Previously LPS plus IFN-γ treated M1 and IL-13 treated M2 cells were left in cytokine-free medium till 12 days after initial polarization. IL-13 stimulus was then given to M1 cells and LPS plus IFN-γ was given to M2 cells for next 2 days. Population frequencies of switched M1 (M2→M1) and switched M2 (M1→M2) cells were assessed using two previously described markers; A: CD64 and CD80, and B: CD11b and CD209. Data were representative of at least 5 different individual donors.

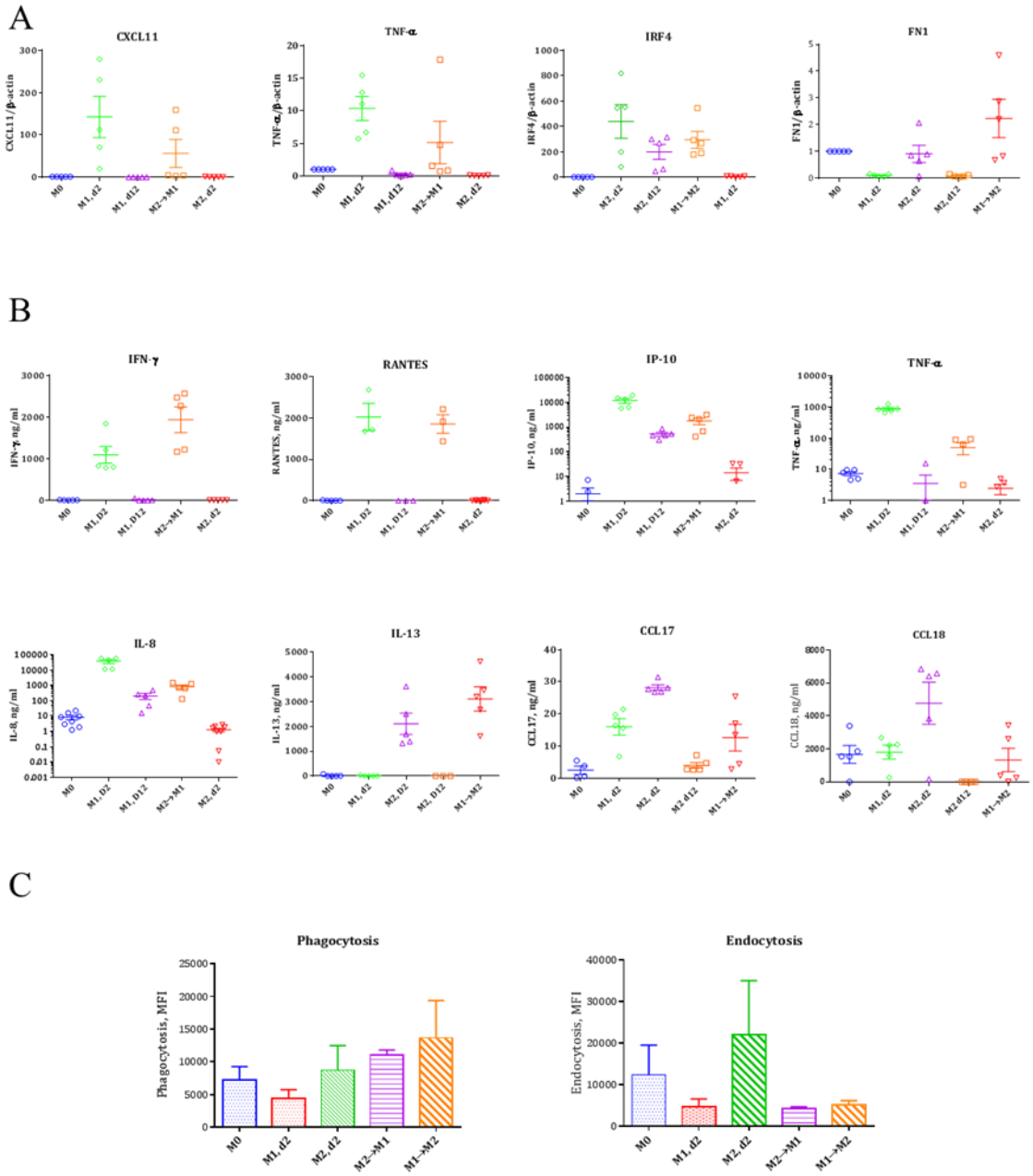


Figure 9: Gene expression, inflammatory and functional profiles of reprogrammed M1 and M2 macrophages. Previously M1 and M2 cells were left in cytokine-free medium for 12 days after initial polarization. M2 stimulus was then given to M1 cells (M2→M1) and M1 stimulus was given to M2 cells (M1→M2) for next 2 days. Gene expression (A), cytokine/chemokine secretion (B) and

phagocytic/endocytic ability (C) of reprogrammed M1 (M2→M1) and reprogrammed M2 (M1→M2) macrophages were analyzed. Results are mean± SEM (A and B) or SD (C) from at least 5 independent experiments.

3.6 Discussion

Previous human macrophage studies particularly focused on the differential gene expression profiles of M1 and M2 macrophages as well as identification of functional surface markers (51, 130, 136, 146, 282). However, a clear phenotypic characterization of human M1 and M2 subsets is important for better understanding their biological functions and their roles in diseases. Here, we present the results of a systematic study analyzing definitive separation of human macrophages into M1 and M2 phenotypes using surface markers, specific gene expression, secreted inflammatory profiles and functional activity. Furthermore, we demonstrated that these polarized subsets exhibit plasticity in that they can depolarize to uncommitted (M0) macrophages or repolarize into the opposite phenotypes (M1 to M2 and vice versa) depending on the cytokines present in their local environment. We highlighted the major findings of our studies along with three other macrophage studies to provide an overview on the recent progresses of human M1 and M2 macrophage characterization (***Table 5***). Xue *et al* (72) demonstrated monocytes-derived macrophages (MDM) as a distinct cell type than monocytes or DCs in regard to surface marker expression and cytokine secretion. Beyer *et al* (136) demonstrated that induction of macrophage functional states relies on T_H1 and T_H2 cytokines, not on the growth factors like GM-CSF or M-CSF. Finally, extensive differential gene expression profiles by Martinez *et al* (130) facilitated the understanding of two functional states of macrophages.

Macrophage responses to stimuli are variable across species and are highly sensitive to culture conditions. For instance, the mannose receptor (CD206) and the scavenger receptor type A (CD163) are considered as prototypic murine M2 markers (31, 142). CD206 expression in murine macrophages is upregulated by M-CSF (86) or IL-4 and inhibited by LPS, IFN-γ and GM-CSF (142). The scenario for these two receptors is opposite in human macrophages, i.e., GM-CSF treated human monocytes are reported to show higher CD206 expression than M-CSF treated monocytes (146). CD163 is induced in murine macrophages by IL-6, IL-10 and by immunosuppressants (146, 149), but not by IL-4 (143). These data raised two essential questions: ***firstly***, whether GM-CSF and M-CSF are true inducers of M1 and M2 polarization of human macrophages, and ***secondly***, whether CD163 and CD206 are useful human M2 markers.

To date, there is no widely agreed upon *in vitro* culture conditions for human M1 and M2 polarization. Previous human studies mostly used M-CSF, the most abundant circulating growth factor, for macrophage differentiation, and then polarized to M1 and M2 by T_H1 and T_H2 cytokines respectively (130, 136) The respiratory lining is rich in GM-CSF and GM-CSF derived macrophages phenotypically and functionally resembled lung macrophages (126). Therefore, in this study, we

primarily used GM-CSF to define M1 and M2 macrophages. We then employed a number of stimuli (such as IL-13, bacterial product lipopolysaccharide LPS and inflammatory cytokine IFN-γ) to model M1 and M2 polarization of respiratory macrophages. IL-13 is associated with wound repair and fibrosis (52). Enhanced IL-13 had been reported in patients with asthma and cystic fibrosis (66, 283). M-CSF was used to confirm that following M1 and M2 polarization human M-CSF derived macrophages exhibited similar functional phenotypes than GM-CSF derived macrophages.

M1 macrophages polarized by LPS or IFN-γ alone or in combination could be best described by surface expression of CD64 and CD80. Upregulation of these receptors was previously reported following M-CSF and IFN-γ treatments, but not with GM-CSF in humans (146). The differential expression of CD64 and CD80 we observed was related to the M1-polarizing stimuli rather than to GM-CSF or M-CSF. Interestingly, we observed two distinct phenotypic patterns for M1 macrophages: $CD64^{hi}CD80^{-}$ population for IFN-γ treated M1s and $CD64^{+}CD80^{+}$ population when LPS was employed, either alone or in conjunction with IFN-γ. Such differential expression of CD64 and CD80 would permit the separation of IFNγ treated M1 cells from LPS treated M1 cells. In contrast, M2 macrophages polarized by IL-4 or IL-13, alone or in combination showed analogous phenotypes in all three conditions, suggesting a single M2 phenotype in these conditions. M2 cells expressed CD209, CD200R, CD1a and CD1b. CD209 (DC-SIGN), a human C-type lectin, has previously been reported to be expressed on both immature DC (284) as well as M-CSF, IL-4 or IL-13 treated monocytes, but not on GM-CSF treated monocytes (285). IFN-γ has been reported as a negative regulator of CD209 (284, 285). In agreement with previous studies, we observed a distinctive population of $CD11b^{+}CD209^{+}$ amongst IL-4/IL-13 polarized M2 macrophages, demonstrating that IL-4/IL-13 was able to dominate over the GM-CSF effect and induce the M2 pathway. Since DCs express CD83, M2 macrophages, therefore, can be discerned from DCs as a $CD83^{-}CD209^{+}$ population. Expression of CD1a and CD1b was significantly induced in our M2 conditions (57, 136, 284). In agreement with previous reports, we did not observe induction of these two markers on GM-CSF or M-CSF differentiated uncommitted M0 macrophages. Similar to the previous study, we observed enhanced CD226 expression following IL-4/IL-13 treatment, (136); however, because of its shared expression over all M0, M1 and M2 cells, it was not a useful marker for phenotypic discrimination between subsets. CD226 could rather be a differentiation marker of GM-CSF versus M-CSF macrophage differentiation (111).

Table 5: Major Findings of three recent human M1/M2 macrophage studies.

	Chapter 3 (286)	**Martinez *et al.* (130)**	**Beyer *et al.* (136)**	**Xue *et al.* (72)**
Cell types used	Human MDM	Human MDM	Human MDM	Human MDM
In vitro macrophage differentiation	GM-CSF (50ng/ml) or M-CSF (50ng/ml) (6 days) (M0)	M-CSF (100ng/ml) (7 days) (M0)	GM-CSF (500 U/ml) or M-CSF (100 U/ml) (3days) (M0)	GM-CSF or M-CSF (3days) (M0)
M1/M2 polarization	M1: LPS (20ng/ml), IFN-γ (20ng/ml) M2: IL-4 (20ng/ml), IL-13 (20ng/ml)	M1: LPS (100ng/ml) + IFN-γ (20ng/ml) M2: IL-4 (20ng/ml)	M1: LPS (10μg/l), IFN-γ (200 U/ml), TNF-α (800 U/ml) M2: IL-4 (1000 U/ml), IL-13 (100 U/ml)	29 different stimuli.
Methodology	C, FC, GE, functional assays	C, GE, TP, WB	FC, GE, NA, TP, WB	C, FC, GE, NA, TP, WB
Differential surface markers expression	M0: CD64 M1: $CD64^{Hi}$, CD80 M2: CD1a, CD1b, CD200R, CD209	M0: Not described. List of differentially expressed M1/M2 markers at mRNA level.	M0: Not described. M1: CD14, CD64, CD89, CD120B, TLR2, SLAMF7, M2: CD1a, CD1b, CD11b, CD23, CD93, CD200R, CD226	M0: Not described. M1: CD14, CD86 M2: CD23, CXCR7 CD14, CD23, CD25, CD86, CD197
Signature population	M0: $CD64^{+}CD80^{-}CD209^{-}$ M1: $CD64^{+}CD80^{+}CD209^{-}$ M2: $CD64^{+}CD80^{-}CD209^{+}$	Not studied	Not studied	Not studied

Genes	M0: Basal induction M1: Cox-2, TNF-α, APOL3, CXCL11, IRF5 M2: FN1, IRF4, CCL18	List of differentially expressed M1/M2 genes.	List of differentially expressed M1/M2 genes.	A list of differentially expressed M1/M2 genes.
Soluble factors release	M0: Basal secretion M1: IFN-γ, IP-10, TNF-α, IL-1β, IL-8, RANTES M2: IL-13, CCL17. CCL18	M0: Not described. M1: IP-10, CXCL13, CXCL15, CXCL19, CXCL20 M2: CCL13, CCL14, CCL17, CCL18, CCL23	Cytokines mRNA expression only.	M0: Not described. M1: CXCL5, IL-1α .
Functional Features	M0: Phagocytic M1-LPS: Phagocytic M2: Phagocytic; Highly endocytic	Not studied	Not studied	Not studied
Stability of polarized phenotypes over time	Yes	Not studied	Not studied	Not studied
Reprogramming to opposite polarizing states	Yes	Not studied	Not studied	Not studied

C: Cytokine/chemokine secretion, FC: Flow cytometry; GE: subset-specific gene expression validation by real-time PCR; MDM: Monocyte-derived macrophages; NA: Network analysis; TP: Transcriptional profiling; WB: Western blot.

Since earlier studies have extensively studied the transcriptional regulation of macrophage polarization, we chose a several subset-specific, differentially-expressed genes to validate our M1/M2 model (72, 130, 136). Genes associated with lipid metabolism were induced in M1 macrophages, such as, Cox-2, APOL3. APOL3 is the one of the gate keepers for eliminating tissue cholesterol, while Cox-2 has long been associated with arachidonic acid metabolism (287). Biological functions of M2 signature genes, i.e., fibronectin (FN1) and IRF4, reported in this study are involved in wound repair mechanism. FN1 is involved in an extracellular matrix formation, whereas IRF4 is pivotal to negatively regulating TLR signaling thereby downregulating inflammation (111, 130, 273).

M1 signature cytokines and chemokines represent an immuno-stimulatory state. Similar to the phenotypic data, we observed two distinct inflammatory patterns in M1 macrophages: IFN-γ polarization predominantly induced IFN-γ and IP-10 suggesting an autocrine feedback loop where IFN-γ induces further IFN-γ secretion. LPS polarized M1 macrophages produced the minimal or basal level of IFN-γ or IP-10 but secreted TNF-α, RANTES, IL-1β and IL-8. In agreement with previous reports (130, 282), we observed high levels of CCL17 and CCL18 in all M2-polarizing conditions. To our knowledge, no distinct cytokine profile has previously been reported for human M2s (32). In the present study only human M2 macrophages secreted IL-13. Although IL-10 is a hallmark M2 cytokine in mouse (71), as we and others have shown that, in human, IL-10 is induced in LPS treated M1 cells (273, 282). Such distinct cytokine/chemokine profiles of M1 and M2 macrophages may contribute to pathophysiology.

Phagocytosis and endocytosis represent mechanisms by which M1 and M2 macrophages, respectively, engulf and eliminate foreign particles (31). Binding and ingestion of microorganisms during non-opsonic phagocytosis had been reported via mannose receptor (CD206) (31). IFN-γ is reported to downregulate CD206 expression, and thereby suppress phagocytic activity (288), whilst, LPS enhanced the engulfing of phagocytic cells (289). In line with these reports, we observed suppressed phagocytic activity by IFN-γ induced M1 cells compared with M0 and M2 cells and enhanced phagocytic activity by LPS induced M1 cells. In the present study, we used non-opsonized bacteria to calculate a phagocytic index in unpolarized (M0) macrophages. M0 macrophages have previously demonstrated the comparable phagocytic ability for either IgG-opsonized or non-opsonized bacteria (290), suggesting that our experimental conditions are unlikely to have adversely influenced our results. In addition, the demonstration of suppressed endocytic ability of M1 cells is consistent with previous reports that LPS and other pro-inflammatory cytokines inhibit

macropinosome formation, thereby modulating antigen uptake and downregulating endocytosis both in human and mouse macrophages (291, 292).

We observed antagonistic effects of M1 and M2 inducers on polarized macrophages i.e., M1 conditions were able to downregulate M2 phenotype, gene expression, inflammatory mediator secretion and functional features and vice versa. Furthermore, our observation of two distinct M1 phenotypes induced by either LPS or IFN-γ fully corresponded with a previously reported network analysis (72) suggesting heterogeneity within the M1 phenotype. A similar transformation of functional phenotypes of macrophages has been reported for murine macrophages (76, 77) although these studies did not analyze the fate of polarized macrophages when stimulatory cytokines were removed. The present study provides an in-depth analysis of stability and reversibility of macrophage phenotypes and confirms that polarization does not represent terminal differentiation. Such a shift from M1 to M2 state or vice versa may indicate how the innate immune balance is maintained by macrophage subsets during inflammatory and homeostatic conditions. Taken together, this model of macrophage subset characterization can be used to investigate macrophage-related disease pathogenesis and facilitate developing therapeutic interventions where restoring the subtle balance of macrophage subsets would become beneficial for patients.

We observed qualitatively similar phenotypic, signature gene expression, inflammatory and functional profiles of M1 and M2 macrophages when differentiated by either GM-CSF or M-CSF. Our data thereby questions the validity of inducing M1 and M2 polarization by GM-CSF and M-CSF in humans. We recognized one limitation in our study. We assume that our donors were healthy and this is supported by the relative consistency in results obtained between donors, however, we do acknowledge that direct information concerning the clinical status of the donors was very limited.

In summary, we have defined *in vitro* conditions for differentiating human monocytes into macrophages and then polarizing them into M1 and M2 subsets. Our data provides a way to characterize human M1 and M2 macrophages which will aid the study of human macrophage biology as well as the analysis of macrophage profile in clinical samples.

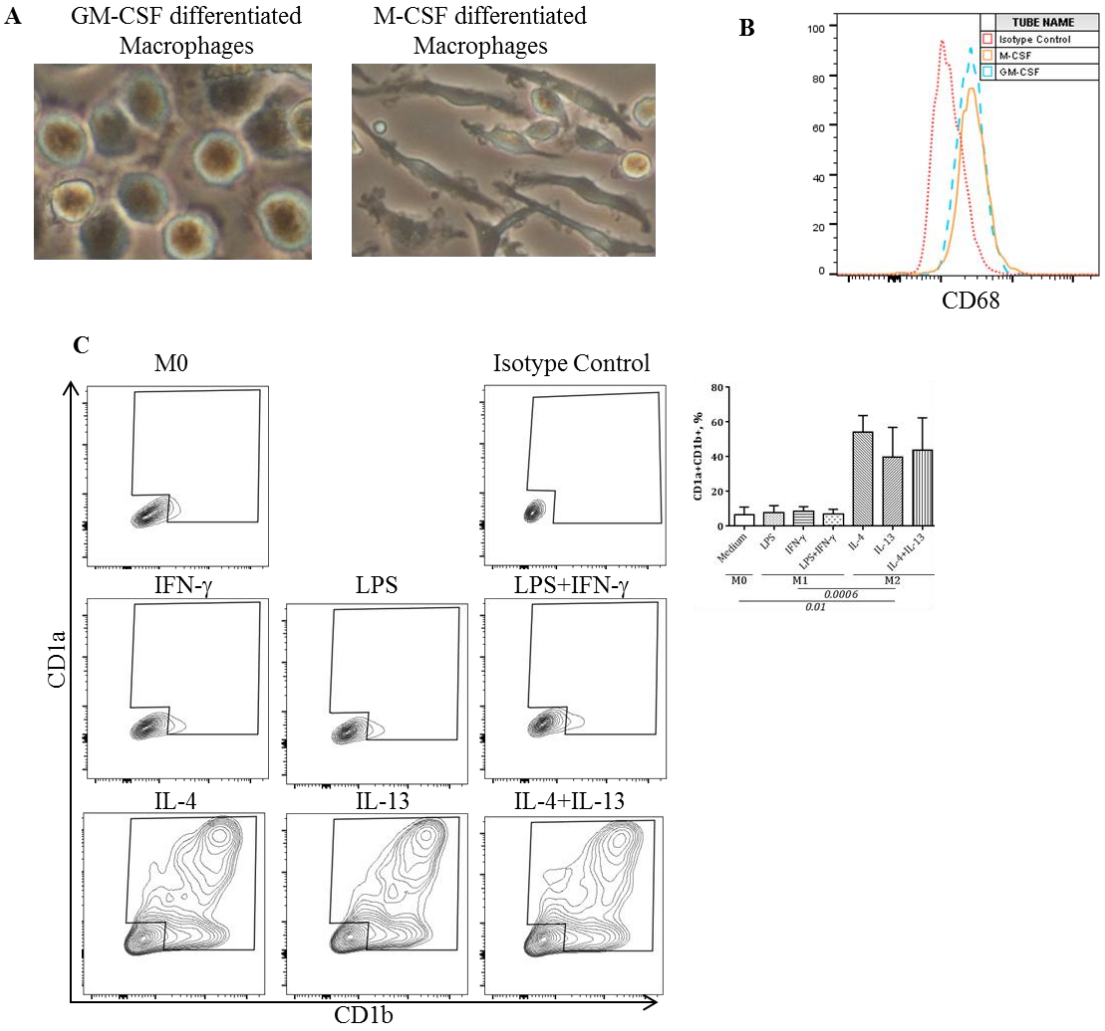
3.7 Supplementary Data

Suppl Table 1: List of monoclonal antibodies, clones and manufacturers.

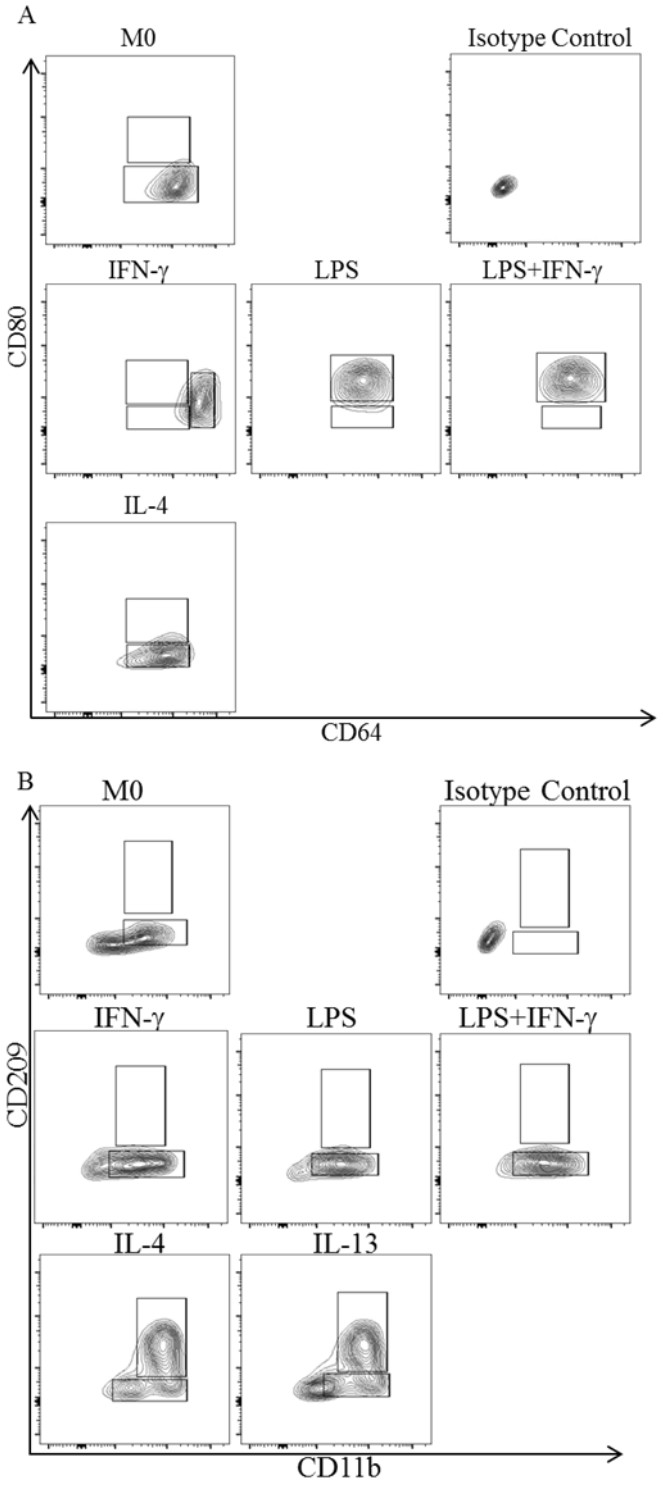
CD antigen	Clone	Fluorochrome	IgG class	Source
CD1a	HI149	BV421	M IgG1, k	BioLegend
CD1b	SN13	PE	M IgG1, k	BioLegend
CD11b	ICRF44	APC	M IgG1, k	BD
CD14	HCD14	PE Cy 7	M IgG1, k	BioLegend
CD16	3G8	PerCP Cy5.5	M IgG1, k	BD
CD23	EBVCS-5	PerCP Cy5.5	M IgG1, k	BioLegend
CD64	10.1	PE Cy7	M IgG1, k	BD
CD68	Y1/82A	AF488	M IgG2b, k	BioLegend
CD68	Y1/82A	AF647	M IgG2b, k	BD
CD80	2D10	BV421	M IgG1, k	BioLegend
CD80	2D10	PE	M IgG1, k	BioLegend
CD163	GHI/61	PE-CF594	M IgG1, k	BD
CD200R	OX108	FITC	M IgG, k	AbDSerotec
CD206	19.2	PE Cy 5	M IgG1, k	BD
CD209	DCN46	BV450	M IgG2b, k	BD
CD213α1 (IL-13Rα1)	SS12B	APC	M IgG1, k	BioLegend
CD226	11A8	APC	M IgG2b, k	BioLegend
7-AAD				BD

Suppl Table 2: Antibody panels for macrophage phenotyping.

Panel 1	Panel 2	Panel 3
CD68 AF488	CD68 AF488	CD11b APC
CD64 PE Cy7	CD1a V421	CD209 V450
CD80 V421	CD1b PE	CD200R FITC
CD206 PE Cy5	CD163 PE-CF594	CD23 PerCP Cy5.5
	CD226 APC	

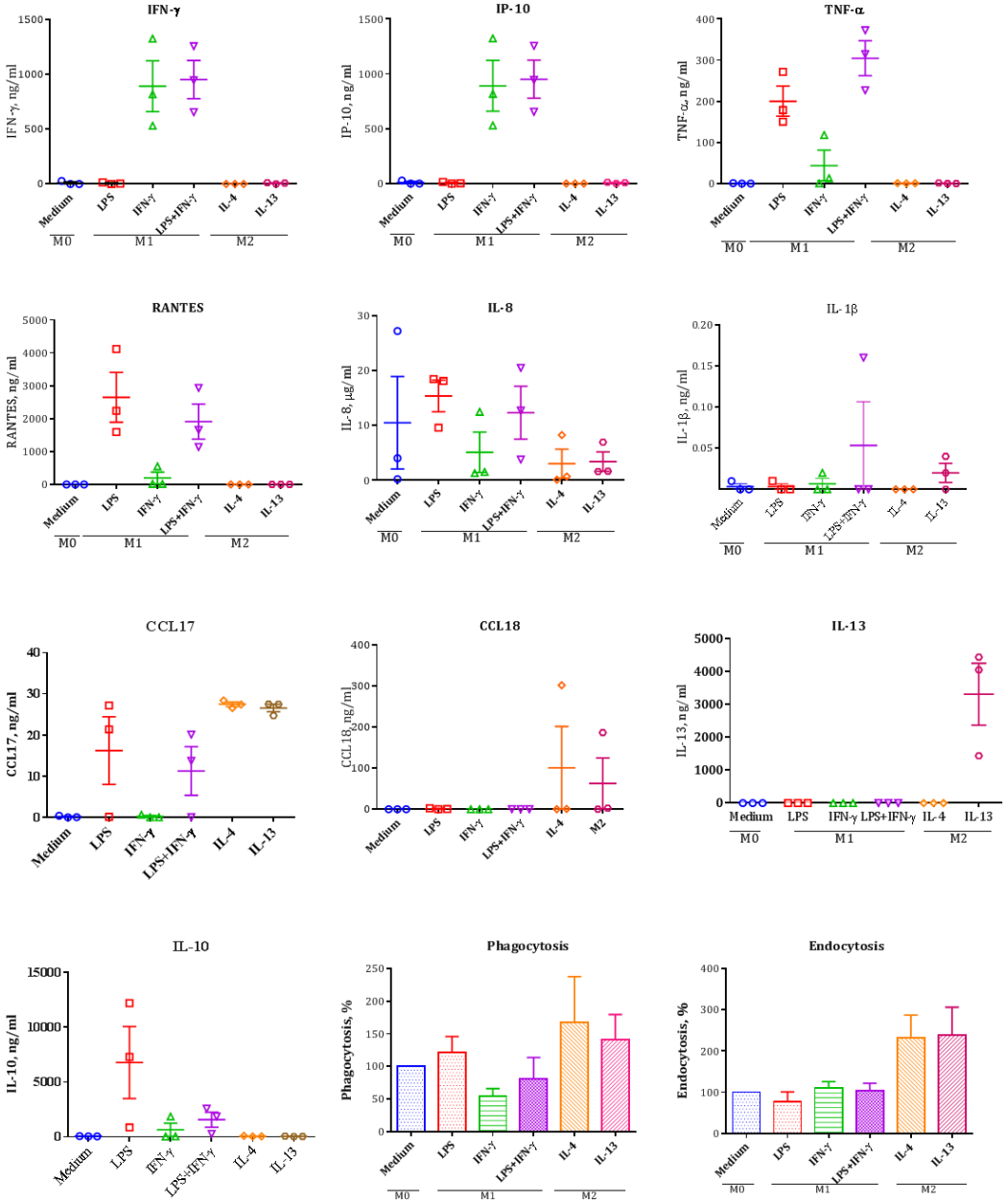


Suppl Figure 4: Morphology and CD68 expression of GM-CSF and M-CSF derived macrophages. $CD14^+$ Monocytes from healthy donors were cultured with GM-CSF or M-CSF containing medium for 6 days to differentiate into uncommitted macrophages (M0). (A) Morphology was observed under microscope. (B) Intracellular expression of CD68, a macrophage marker in human, was analyzed by flow cytometry. M1 macrophages were then generated by stimulating with LPS alone, IFN-γ alone and LPS plus IFN-γ. M2 polarization was performed with IL-4 alone, IL-13 alone and IL-4+IL-13 for 2-days. Population frequencies of M0, M1 an M2 conditioned cells were assessed by CD1a and CD1b marker combination (C). Bar graph represents the mean and SD of population frequencies of CD64/CD80 and CD11b/CD209 respectively. Data were representative of at least 5 different individual subjects. Statistical significance was calculated using nonparametric one-way ANOVA.



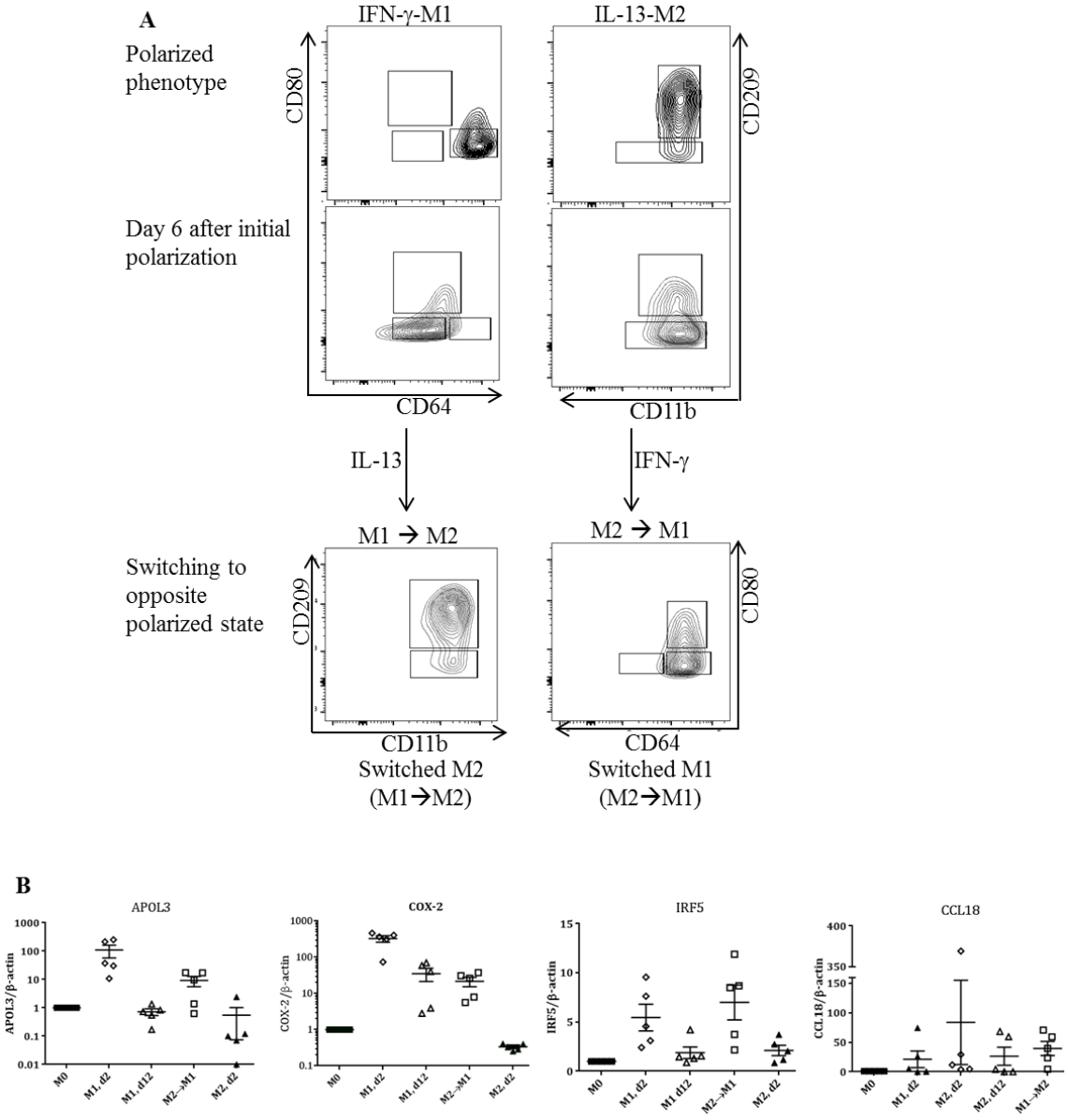
Suppl Figure 5: Phenotypic characterization of M-CSF derived human M1 and M2 macrophages. Monocytes from healthy donors were cultured with M-CSF containing medium for 6 days to obtain uncommitted macrophages (M0). M1 macrophages were then generated by stimulating with LPS alone, IFN-γ alone and LPS plus IFN-γ. M2 polarization was performed with IL-4 alone, IL-13 alone and IL-4+IL-13 for 2-days. Population frequencies of M0, M1 an M2 conditioned cells were assessed for two different panels; A: CD64 and CD80, and B: CD11b and CD209. Bar graphs (C & D) represent the mean and SD of population frequencies of CD64/CD80 and CD11b/CD209

population respectively. Data were representative of 3 different individual donors. Statistical significance was calculated using nonparametric one-way ANOVA.



Suppl Figure 6: Inflammatory and functional profile of M-CSF derived human M1 and M2 macrophages. M-CSF derived uncommitted macrophages (M0) were induced with LPS alone, IFN-γ alone or LPS+IFN-γ. M2 polarization was performed with IL-4 alone, IL-13 alone and IL-4+IL-13 for 2-days. (A) A panel of cytokines and chemokines were analysed in the supernatants of M0, M1 and M2 macrophages. Each symbol represents an independent experiment. Error bars represent the SEM for n=3. Statistical significance was calculated using non-parametric one-way ANOVA. (B) M0, M1 and M2 polarized macrophages were treated with either pHrodo green *E. coli* bioparticle for

phagocytosis or AF-647 labeled dextran (10KD) for endocytosis for 90 mins at 37C. Data reflect 3 independent experiments from 3 independent donors. Error bars represent the SEM. Significance was determined using nonparametric one-way ANOVA among M0, M1 and M2 groups.



Suppl Figure 7: Phenotypic and gene expression reversibility of reprogrammed M1 and M2 macrophages. Previously IFN-γ treated M1 and IL-13 treated M2 cells were left in cytokine-free medium till 12 days after initial polarization. IL-13 stimulus was then given to M1 cells and IFN-γ was given to M2 cells for next 2 days. (A) Population frequencies of switched M1 (M2→M1) and switched M2 (M1→M2) cells were assessed using two previously described markers; A: CD64 and CD80, and B: CD11b and CD209. (B) Gene expression profile of switched M1 (M2→M1) and switched M2 (M1→M2) cells were analyzed by qPCR. Data were representative of 5 individual donors. Mean and SEM are shown. Statistical significance was calculated using nonparametric one-way ANOVA.

Chapter 4 CFTR-Dependent Deficiency in Alternatively-Activated Macrophages in Cystic Fibrosis

4.1 Summary

Roles of macrophages in CF inflammation had largely been overlooked. However, a few studies demonstrated that impaired macrophage functions in CF were associated with dysregulated function exhibited by mutated CFTR. Since there was no valid marker for human M1/M2 macrophages, no comprehensive studies have been conducted to analyze subset specific macrophage responses in CF. Using the model described in ***Chapter 3***, this chapter aimed to study the subset-specific macrophage responses in CF, to identify defective macrophage subset(s) in CF and finally to investigate the underlying mechanism.

The manuscript was submitted to the New England Journal of Medicine.

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4.2 Abstract

Background: Exaggerated neutrophil-dominated inflammation is an integral component of cystic fibrosis (CF) lung disease. Why this occurs and is not switched-off normally is unclear. Macrophages play important roles in initiation and resolution of pulmonary inflammation but have not been extensively studied in CF.

Methods: Using an ex vivo model we developed for differentiating and polarizing monocytes into uncommitted (M0), classically-activated (M1) and alternatively-activated (M2) macrophages, we determined macrophage phenotype and function in patients with CF and compared them with healthy adults. The effect of inhibiting CFTR function in healthy monocytes was also determined.

Results: Macrophages deficient in CFTR function, naturally or experimentally-induced, showed an reduced ability to respond to IL-13 with a significant reduction in the expression of the M2 marker CD209 [median(25th-75th%): healthy (n=9) 59(55-82)%; CF children (n=14) 41(30-52)% ($p<0.01$); CF adults (n=13) 46(25-60)% ($p<0.01$)]. Endocytosis was also decreased in both children and adults with CF compared with healthy adults (both $p<0.001$). CF M2s showed a marked reduction in expression of the archetypal M2 gene IRF4, decreased surface expression of IL-13Rα1 but increased secretion of IL-13. M1 phenotype and pro-inflammatory cytokine secretion was normal or increased in children and adults with CF, especially during an acute pulmonary exacerbation. However, CF M1s were resistant to repolarization to M2 by IL-13 and inhibition of CFTR function in healthy macrophages markedly reduced phagocytosis.

Conclusions: We have demonstrated a CFTR-dependant deficiency in the ability of macrophages to respond to IL-13, limiting the development and function of alternatively-activated macrophages in CF.

4.3 Introduction

Progressive destructive inflammation is the hallmark of lung disease in cystic fibrosis (CF); with most studies reporting an exaggerated neutrophilic response to infective or inflammatory stimuli (189, 192, 293). The extent of that response is a major risk factor for the onset and progression of structural lung disease (294, 295). However, the factors initiating an exaggerated inflammatory response in the lungs of young children with CF or why this is not switched off normally are not clear. While most research into CF inflammation has concentrated on neutrophils, we have previously shown that the number of macrophages, together with the levels of CC macrophage attractant chemokines, present in the bronchoalveolar lavage (BAL) of young children with CF were higher than in non-CF disease controls (296). As macrophages play important roles in initiation and resolution of pulmonary inflammation, these earlier data raise the possibility that some macrophage functions may be abnormal in CF.

Elegant studies in murine models of acute lung injury have given insight into the roles macrophages play (297, 298). The resident pulmonary macrophage population, alveolar macrophages (AM), are thought to populate the lung during fetal development, replicate locally and not repopulate from the bone marrow. Macrophages recruited to the lungs by inflammatory stimuli are of bone marrow origin, differentiate from monocytes (monocyte-derived macrophages (MDM), M0) and polarize into pro-inflammatory (classically-activated or M1) or inflammation-resolving (alternatively-activated or M2) phenotypes in both mice (297) and humans (286). M1s secrete pro-inflammatory cytokines (TNF-α, IFN-γ, IL1β) and have anti-microbial and phagocytic function while M2s secrete cytokines (IL-4, IL-13) that inhibit M1 function and are endocytic (51). The initial murine response to acute lung injury is an influx of neutrophils, peaking on day 2-3, followed by recruitment of M1 macrophages, peaking on day 6-12. Resolution of inflammation is facilitated by M2 macrophages over the next 10+ days (297, 298), including clearance of apoptotic cells by efferocytosis to remove inflammatory stimuli from the lungs (299).

Given the importance of macrophages in pulmonary inflammation, there has been surprisingly little research in patients with CF dedicated to these cells. Major differences between mouse and human macrophages (286) made interpretation of murine study outcomes difficult, necessitating human studies. Abnormal function of human macrophages in CF has been reported with: increased secretion of IL-1β and TNF-α; decreased surface expression of CD11b, and TLR-5; decreased phagocytosis (159) and decreased killing power against *P. aeruginosa* related to defective chloride

channel function resulting in a failure to acidify the phagolysosome (160). Data on macrophages apoptosis and efferocytosis in CF are conflicting (300).

We have recently described a technique for differentiating and polarizing human monocytes into MDMs and characterizing their phenotypes and function (286), allowing us to examine M0, M1 and M2s and their response to external stimuli. The present study was undertaken to test the hypothesis that alternatively-activated M2 macrophages are abnormal in CF, especially when collected during an acute pulmonary exacerbation requiring admission to hospital (APE). We also aimed to determine whether any deficiencies in macrophage function were related to defective CFTR function, i.e. intrinsic to CF or secondary to acute inflammation during an exacerbation.

4.4 Methods

4.4.1 Study Participants

Thirteen adults and 27 children with verified CF carrying at least one ΔF508 allele were recruited from the CF clinics at the Prince Charles Hospital and Children Health Queensland (Royal Children Hospital/Lady Cilento Children's Hospital), Brisbane, Australia. Demographic and clinical characteristics are shown in the ***Table 6***. Paired blood samples were collected by venepuncture from adult patients on admission to hospital with an acute pulmonary exacerbation and prior to discharge after ceasing treatment. Blood was collected from children on admission to hospital (n=16) and/or at a clinic visit (n=14) when clinically stable, with paired bloods only available from 2 children. Permission was obtained for the study from the relevant Institutional Review Boards and written consent obtained from patients and/or their parents, as appropriate. As the study reported in ***Chapter 3*** buffy coats (n=9) from healthy donors, aged 18 to 40 years, obtained from the Australian Red Cross Blood Services, Brisbane were used as controls (286). No demographic data apart from age were available for the controls. These cells adequately represent peripheral blood monocytes obtained from healthy adult volunteers (***Suppl Figure 11***).

Table 6: Demographic and clinical characteristics of the patients with CF.

	Adults (n=13)	Children (n=27)
Age, years (range)	23-46	9 -15
Sex, male (%)	11 (84.6%)	17 (62.9%)
Genotype		
ΔF508 homozygous	5 (38.5%)	11 (40.8%)
ΔF508 heterozygous	8 (76.5%)	16 (59.2%)
Lung function		
FVC (L) (mean±SD)	2.62±0.9	2.9±0.75
FEV_1 (L) (mean±SD)	1.78±0.93	2.4±0.72
***P. aeruginosa* infection status**		
Chronic	13 (100%)	2 (7%)
Intermittent	-	16 (59%)
Once	-	4 (34%)

4.4.2 Flow Cytometric Analysis of Monocyte Subsets in CF

Monocytes were isolated by $CD14^+$ magnetic microbeads (Miltenyi, Germany) from blood samples of patients with CF, as previously described (***Chapter 3***) (286). Classical, intermediate and non-classical monocytes were analyzed by their surface expression of CD14 and CD16 as described in ***Chapter 2***.

4.4.3 In vitro Macrophage Differentiation and Polarization

Macrophages were differentiated (M0) and polarized into M1 and M2 subsets, as previously described (286) (***Figure 10***). M1/M2 surface markers were analyzed using antibodies to CD64, CD80, CD209 (286). Following M2 polarization by IL-13, $CD209^+$ cells sorted by CD209 magnetic microbeads following manufacturer's instruction (Miltenyi, Germany). Kinetic expression of IL-13 receptor α1 (IL-13Rα1) was analyzed following IL-13 stimulation on M0 macrophages. Data were acquired on BD LSR-Fortessa using BD FACS Diva software. Subsequent analysis was performed on $CD68^+$ macrophages using Flowjo (Tree Star Inc. USA).

4.4.4 Cytokines and Chemokines Quantification

IL-1β, IL-8, IL-13, IP-10, TNF-α, IFN-γ, RANTES were quantified in the culture supernatant by alphaLISA (Perkin Elmer, USA). Soluble IL-13Rα2 in the culture supernatants was measured by sandwich ELISA (RayBiotech, US).

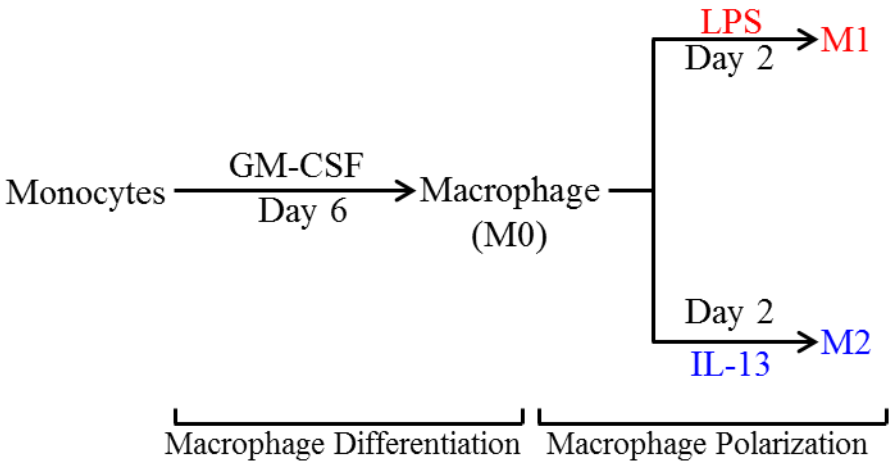


Figure 10: Schematic representation of monocyte-derived macrophage differentiation (M0) and subsequent M1 and M2 polarization.

4.4.5 Phagocytosis and Endocytosis

pHrodo green *E. coli* bioparticles (LifeTech, USA) and internalization of AF-647 labeled dextran (10KD, LifeTech, USA) were used to assess phagocytic and endocytic abilities of M1 and M2 macrophages, respectively. Briefly, macrophages were incubated with either dextran (5μg/ml) or green *E. coli* at 37C for 90min. pHrodo dye fluoresces only in acidic environments which mimic physiological phagolysosomal compartments. Specific mean fluorescence intensities (MFIs) were recorded by flow cytometry. MFI of cells without bacteria or dextran were subtracted from experimental samples.

4.4.6 M1-M2 Subset-Specific Gene Expression

RNA was extracted using RNeasy Kit (Qiagen, USA), reverse transcribed using Quantitect RT kit (Qiagen, USA). qPCR was performed using TaqMan primer/probe combinations for human Cox-2 (M1 specific), IRF4 (M2 specific) and β-actin genes (LifeTech, USA) on ABI 7900HT (Applied Biosystem, USA). After normalizing the data with β-actin, relative gene expression was calculated by considering M0 as control (272).

4.4.7 CFTR Inhibition During Macrophage Differentiation/ Polarization

Monocytes obtained from healthy controls (buffy coat) were exposed to the CFTR inhibitors $CFTR_{Inh}$-172 (10μM, Sigma, USA) or GlyH-101 (10μM, Santa-Cruz, USA) during macrophage differentiation and/or polarization (***Suppl Figure 8***). These inhibitors work by different mechanisms, with $CFTR_{Inh}$-172 binding CFTR intracellularly and while GlyH-101 blocks the channel on the cell surface (226, 227).

4.4.8 Networking Analysis of M2 Macrophages by Microarray

Microarray analysis requires genomic DNA (gDNA) free high-quality RNA. To get gDNA free RNA, RNA for microarray experiments were extracted using RNeasy Plus Kit (Qiagen, USA). RNA integrity number (RIN) were analyzed with Agilent Bioanalyzer 2100 in the sequencing facility of the Institute for Molecular Bioscience (IMB), University of Queensland. RIN numbers for all samples were above 8.0. Microarray was conducted using U219 array plate (Affymetrix, USA) containing more than 36,000 gene probes at the Ramaciotti Centre for Genomics, University of New South Wales (UNSW), Australia. To identify biological processes whether upregulated or downregulated in CF macrophages compared to macrophages from healthy individuals, network analysis was performed

using Ingenuity Pathway Analysis (IPA) software by Professor Anthony Bosco, Telethon Kids Institute, University of Western Australia.

4.4.10 Effects of Cox-2 Inhibition on Macrophage Polarization

Monocytes obtained from healthy controls (buffy coat) were exposed to indomethacin alone (inhibitor of Cox-1 & Cox-2) (100μM) (Cayman, USA) or NS-398, a Cox-2 specific inhibitor alone (50μM) (Cayman, USA) or in combination with CFTR inhibitor, $CFTR_{Inh}$-172 (Cayman, USA) during their differentiation to macrophages and polarization to M1 and M2 macrophages.

4.4.11 Statistical Analysis

Data are shown as median and interquartile range (IQR) unless stated otherwise. Wilcoxon paired signed ranked test was performed to determine the differences of inflammatory parameters amongst adult patients between hospital admission and discharge. Mann-Whitney test was performed to assess differences in inflammatory parameters between control and CF groups. Two-way ANOVA was used in time-course studies with group (CF/control) and time entered as the main effects. Statistical analysis was performed using GraphPad Prism 6.7 (San Diego, CA). Statistical significance was set at $p<0.01$ to allow for multiple comparisons.

4.5 Results

4.5.1 Monocyte Subsets in CF is Similar to Healthy Controls

No difference in the frequencies of classical and intermediate monocyte subsets was observed between CF patients and healthy controls (***Figure 11***). The percentages of non-classical monocytes were very minimal in both controls and CF patients. Therefore data were not shown.

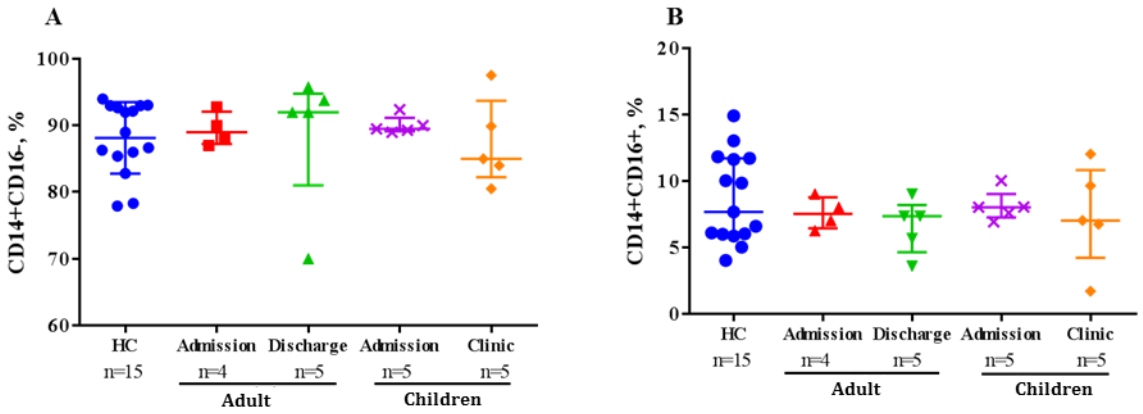


Figure 11: Monocyte subsets in CF. Isolated $CD14^{+}$ monocytes from buffy coats and patients with CF were Fc blocked and stained with anti-human CD14 PE-Cy 7 and CD16 PerCP Cy5.5 antibodies. Data were shown as median and IQR.

4.5.2 Deficient M2 Polarization in CF

Following M2 polarization with IL-13, the proportion of cells expressing CD209 was significantly reduced in children [median (25^{th}-75^{th}%) healthy (n=9) 59(55-82)%; CF children (n=14) 41(30-52)%; CF adults (n=13) 46(25-60)%; all comparisons $p<0.01$]. Data from representative individuals (flow cytometry plots) and from the group are shown in (***Figure 12***). Endocytosis by M2s was decreased in all CF groups (***Figure 12***). The group median (25^{th}-75^{th}%) MFI for controls was 22,711(10,639-34,253) compared to 3,769(2,350-8,101) and 10,104(1,922-2,685) for adults with CF on admission ($p<0.001$) and discharge ($p<0.001$) from hospital, respectively and to 9,031(2,003-16,667) and 5,896(3,818-7,376) for children with CF on admission to hospital ($p=0.01$) and when clinically-stable ($p<0.001$), respectively. Secretion of IL-13 was higher in all CF groups ($p<0.001$), than in controls (***Figure 12***).

To determine whether the decreased M2 function seen in CF was due to a reduced number of normally-functioning M2 or to a failure of CF M0 to respond to IL-13, we sorted CF and control cells

by CD209 expression following exposure to IL-13. Expression of M2-associated gene IRF4 was essentially absent in CF M2 macrophages, regardless of surface expression of CD209 (***Figure 13***).

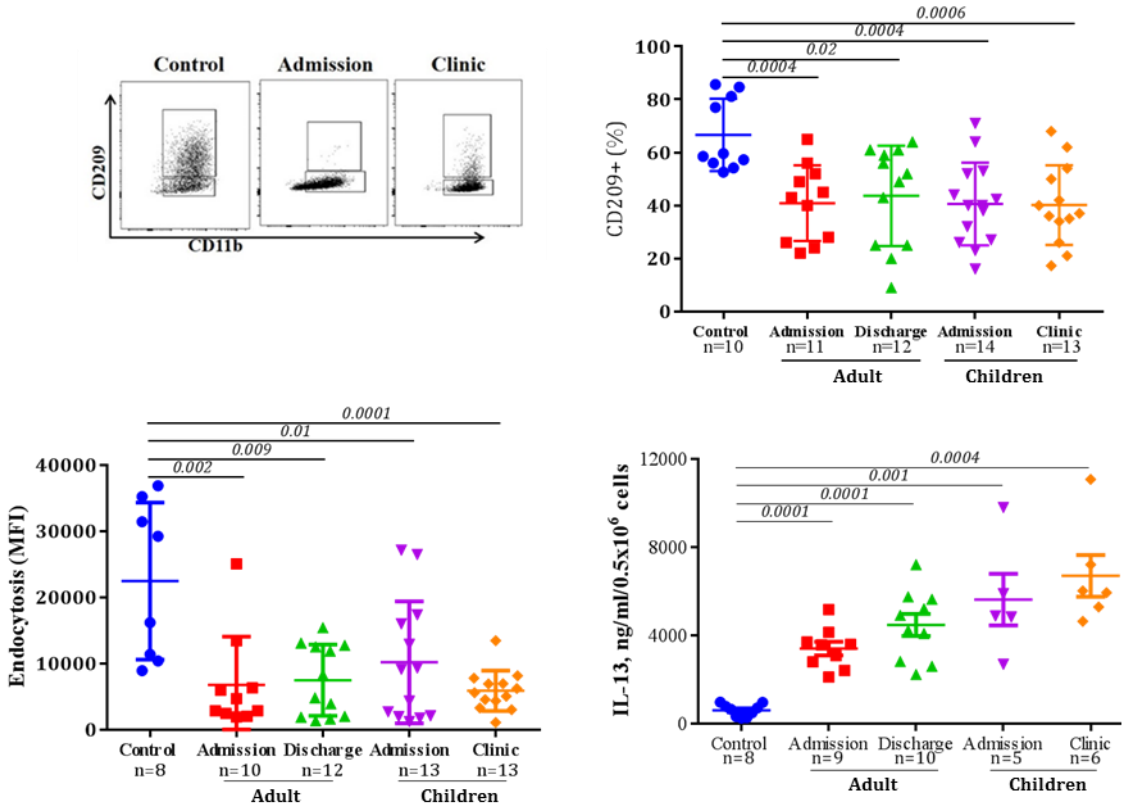


Figure 12: Deficient M2 polarization in CF. A representative flow cytometry plot from an adult with CF showing surface expression of the M2 marker CD209 following differentiation by GM-CSF and polarization to M2 by IL-13 of monocytes obtained on admission to hospital for an acute pulmonary exacerbation and when clinically stable. Group data are shown as median and interquartile range as well as individual data. Groups sizes are as follows: Control (n=10); Adult CF on admission (n=11) and on discharge (n=12); children with CF on admission (n=14) and at clinic (n=13).

4.5.3 CF M0 and M1 Macrophages Exhibit Comparable Phenotypes to Controls

Following differentiation but prior to polarization a similar proportion of M0s from all groups showed expression of the M1 marker CD80 (***Suppl Table 3***). The surface expression of CD80 was more variable in both children and adults with CF, especially during APE (***Suppl Figure 9***).

Following M1 polarization with LPS the proportion of cells expressing CD80 was not significantly different between healthy controls and children and adults with CF, both during periods

of clinical stability and with an APE (***Suppl Table 3***). Surface expression of CD80 was more variable in patients with CF (***Suppl Figure 9***).

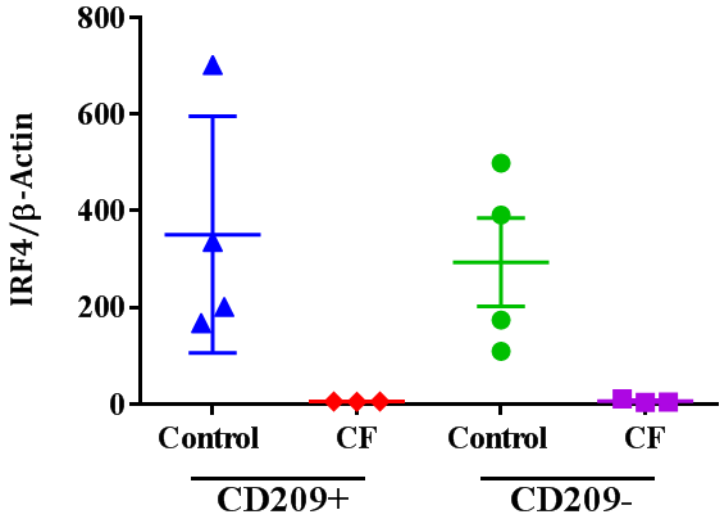


Figure 13: Expression of the archetypal M2 gene IRF4 in CF and Control macrophages following stimulation with M2-polarizing cytokine IL-13. Cells are stratified by surface expression of the M2-marker CD209. RNA was extracted from sorted cells and induction of M2 signature gene, IRF4 was analysed with real-time PCR. After normalization by β-actin, gene expression is shown as fold-change compared to the relevant M0 gene expression (median and IQR).

Phagocytic activity of M1 macrophages was similar in children and adults with CF both when clinically stable and on admission to hospital, to that seen in heathy controls (***Suppl Figure 10***). There was no obvious difference in the pattern of secretion of pro-inflammatory cytokines from M1 macrophages in patients with CF (***Suppl Figure 10***). Secretion of RANTES was higher is all CF groups than in controls ($p<0.01$ for all comparisons) but no consistent pattern was seen for IL-1β. Secretion of IFN-γ was also higher in all CF groups ($p<0.01$ for all comparisons).

Unexpectedly, surface expression of the M1 marker CD80 was higher following polarization with IL-13 (***Figure 14***) in children ($p=0.006$) on admission to hospital than healthy controls (***Suppl Table 3***). A similar trend was seen in adults with APE that was not statistically significant ($p=0.08$). The proportion of "M2" cells expressing CD80 was not different when clinically stable in children or adults (***Suppl Table 3***) (acute vs stable $p=0.60$, $p=0.50$, respectively).

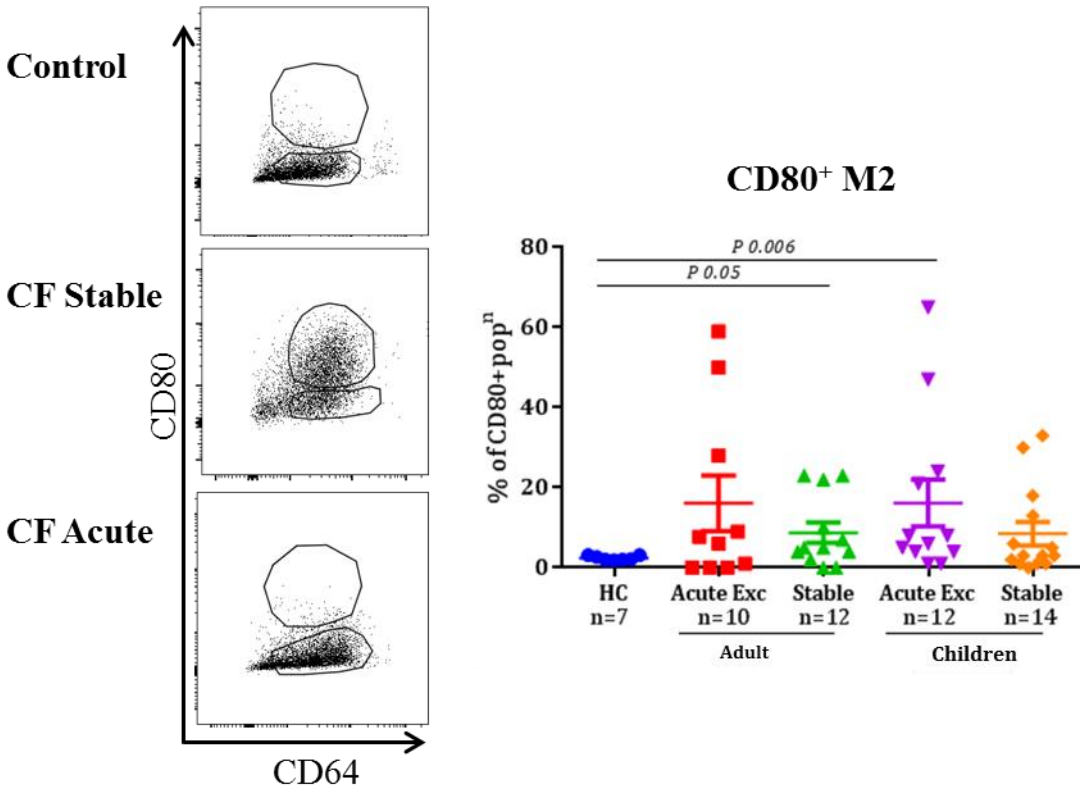


Figure 14: Expression of CD80 on M2 following polarization with IL-13. A representative flow-cytometry plot from an adult with CF showing surface expression of the M1 marker CD80 following differentiation by GM-CSF and polarization to M2 by IL-13 of monocytes obtained on admission to hospital for an acute pulmonary exacerbation and when clinically stable. Group data are shown as median and interquartile range as well as individual data. Groups sizes are as follows: Control (n=10); Adult CF on admission (Acute n=11) and on discharge (n=12); children with CF on admission (Acute n=14) and at clinic (n=13).

4.5.4 CF Macrophage Model: Inhibition of CFTR Function in Healthy Macrophages Mimics CF Phenotypes

When control MDMs were differentiated and polarized in the presence of CFTR inhibitors they adopted a phenotype seen in CF. Following polarization with IL-13 the expression of the M2 marker CD209 was markedly reduced (p=0.002) but the expression of CD80 on M1s was not affected (p=0.80) (***Figure 15***). This effect was seen with both inhibitors. CFTR-inhibition reduced phagocytosis by M1s (p=0.03) and endocytosis by M2s (p=0.006) (***Figure 15***). Using IL-4 to polarize M2 did not result in an increase in CD209 expression in the presence of $CFTR_{Inh}$-172 (***Suppl Figure 13***).

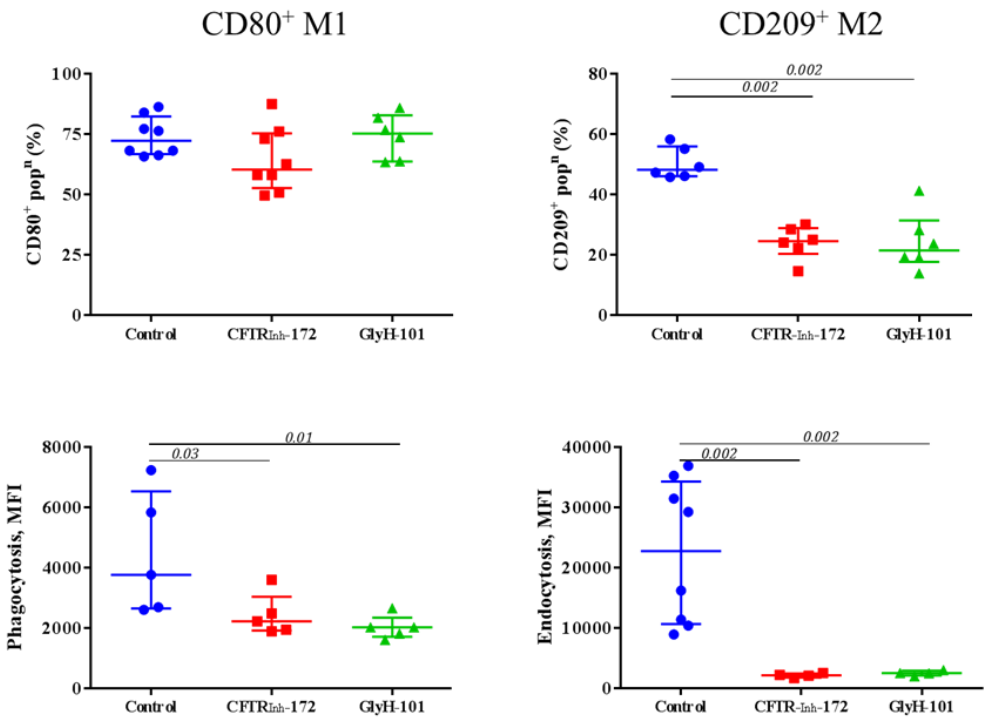


Figure 15: Effect of CFTR inhibition on macrophage phenotype and function. Group median (IQR) expression of the M1 marker CD80 and the M2 marker CD209 following differentiation and polarization in the presence of the CFTR inhibitors ($CFTR_{Inh}$-172 or GlyH-101). The effects of CFTR inhibition on phagocytosis and endocytosis are also shown as group median and interquartile range.

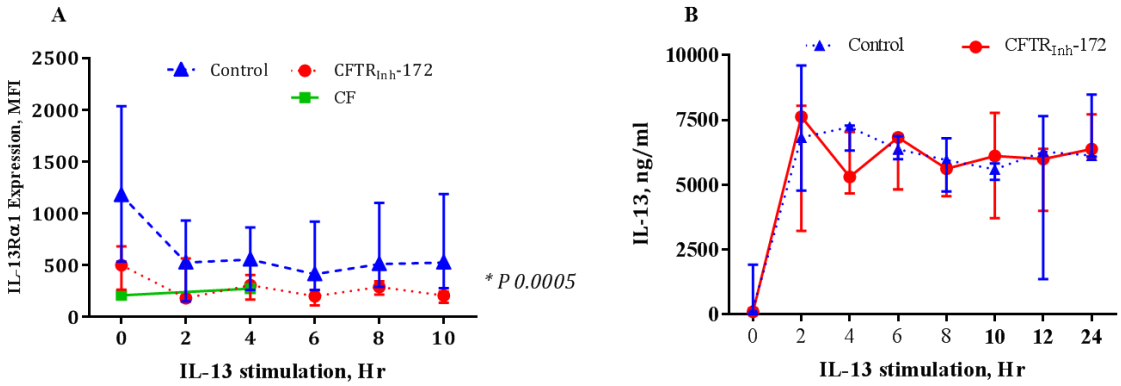


Figure 16: Effect of CFTR inhibition on macrophage surface expression of IL-13Rα1 and secretion of IL-13. MDMs from healthy donors were stimulated with IL-13 (20ng/ml) in the presence (n=5) or absence (n=4) of $CFTR_{Inh}$-172. CF monocytes (n=5) were differentiated to MDMs. Kinetic expression of surface IL-13Rα1 (A) and secretion of IL-13 (B) following IL-13 stimulation were measured by flow cytometry and alphaLISA respectively. Data showed median and IQR.

Surface expression of IL-13Rα1 was reduced in CF M2 and when CFTR function was inhibited in control cells by $CFTR_{Inh}$-172 (***Figure 16***). When control M0s were incubated with IL-13 to induce M2 polarization there was a time-dependent reduction in surface expression of IL-13Rα1 (***Figure 16***). The surface expression of IL-13Rα1 also decreased over time in cells with lacking CFTR function, either naturally or experimentally-induced with incubation with IL-13 (p=0.001) (***Figure 16***). There was no reduction in surface expression of IL-4Rα in CF or with $CFTR_{Inh}$-172. Despite no reduction in the ability of either control or CFTR-inhibited cells to secrete IL-13, neither CF M1s nor experimentally-induced "CF" M1s were responsive to IL-13 and did not re-polarize into the M2 phenotype (***Suppl Figure 12***). No soluble IL-13Rα2 was detected in the supernatants of IL-13 treated CF, CFTR inhibited and control cells.

4.5.5 Cox-2 Inhibition Downregulates M1 and M2 Polarizations

Preliminary analysis of microarray data showed significant upregulation of prostaglandin E receptor 2 (PGE2) signaling pathway. M1 signature gene, Cox-2, had been reported as a regulator of PGE2 pathway while functional CFTR negatively regulates the COX-2-PGE2 positive feedback loop (301). That's why, we hypothesized that inhibition of Cox-2 would restore M2 polarization in CF or CFTR inhibited condition. Cox-2 inhibition did not have any effect on M1 or M2 polarization in control cells. However, a marked reduction of CD80 expression was observed on NS-398 plus $CFTR_{Inh}$-172 treated M1 cells compared to control ($p = 0.02$) and $CFTR_{Inh}$-172 treated cells ($p = 0.02$) (***Figure 17A***). No effect of Cox-2 inhibition was observed on CD209 expression in control and $CFTR_{Inh}$-172 treated cells (***Figure 17B***). Interestingly, inhibition of Cox-1/2 by indomethacin showed downregulation of both CD80 (M1) and CD209 (M2) expression compared to controls.

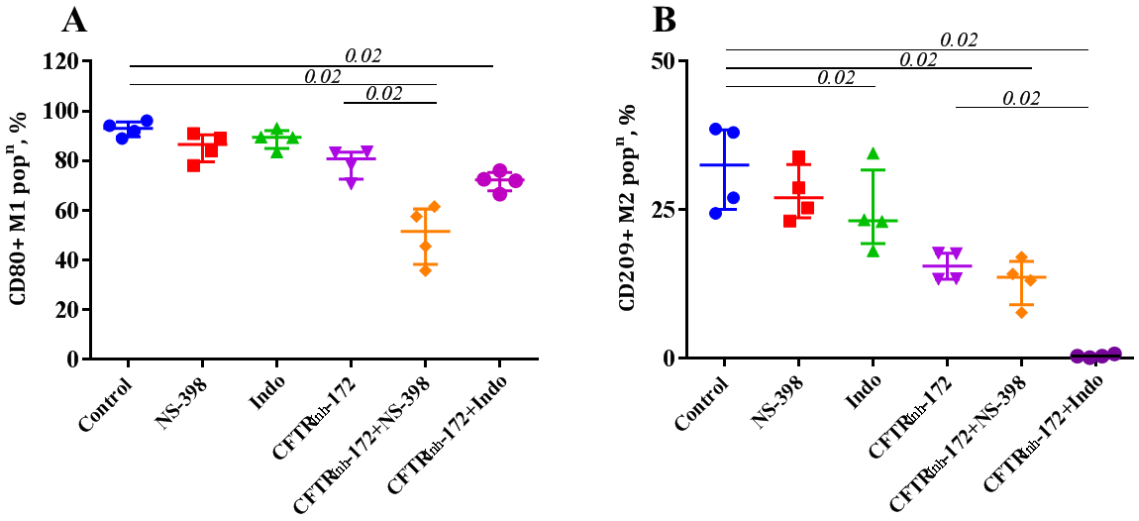


Figure 17: Effects of Cox-2 inhibition of macrophage polarization. $CD14^+$ monocytes from buffy coats (n=4) were differentiated and polarized into M1 and M2 macrophages in presence or absence of indomethacin (Cox-1/2 inhibitor) and NS-398 (Cox-2 inhibitor). Percentage of $CD80^+$ and $CD209^+$ cells were analyzed for M1 and M2 polarization respectively.

4.6 Discussion

Exaggerated neutrophil-dominated inflammation is a hallmark of CF lung disease, especially in early life and is associated with an increased risk of developing structural lung disease (293, 295). However, the precise mechanism underlying this phenomenon is not known. Similar to earlier studies (302, 303), no difference in monocyte compartments was observed between CF and healthy controls. However, MDMs showed impaired functions (159-161). The results of the present study demonstrate a CFTR-dependent defect in MDMs to respond to IL-13 that results in reduced ability of M0s to polarize into the M2 phenotype and of M1s to repolarize to M2s. However, inhibition of CFTR function resulted in a marked reduction of the ability of M1s to acidify the phagolysosome, as previously reported (161). This potentially means that macrophages in CF can initiate inflammatory responses to infective or inflammatory stimuli but may not contribute normally to bacterial killing or inflammation resolution.

The data from the present study point to defective macrophage function in the resolution phase of inflammation. Macrophages act to limit neutrophil-dominated inflammation in numbers of ways including: directly engulfing neutrophil granular molecules and enzymes such as myeloperoxidase (304); efferocytosis of apoptotic neutrophils and those expressing surface "eat-me" markers (304); and participating in the killing of bacteria contained within efferocytosed neutrophils (304). Using the *ex-vivo* model we developed (286), we have previously shown that the phenotype of polarized macrophages is not "fixed" and they can be induced to re-polarize to the opposite phenotype. If re-polarization occurs in vivo this provides an advantage for resolving inflammation as the pro-inflammatory M1 cells can be reprogrammed into M2s in situ by locally-secreted IL-13 or IL-4. The data from the present study suggest that this is unlikely to happen in CF, where incubation with IL-13 fails to induce the M2 phenotype (***Figure 13***), especially during an APE where cells that should be M2 express the M1 surface marker CD80 (***Figure 14***). In addition, experimentally-induced "CF" M1s were not able to re-polarise to the M2 phenotype.

The lack of CFTR function, both in patient cells and in our experimentally-induced "CF" macrophages has several important consequences on macrophage phenotype and function. Pro-inflammatory CF M1s readily produce inflammatory cytokines but do not have the normal ability to acidify the phagolysosome, which will inhibit bacterial killing. M0s lacking CFTR function, either naturally or experimentally, have a markedly lower surface expression of IL-13Rα1 and fail to respond to IL-13 despite more than adequate IL-13 production. Lack of CFTR function also results

in marked reduction of expression of the archetypal M2 gene, IRF4, irrespective of surface expression of CD209. Why CF CD209^{+} M2s do not express IRF4 is unclear, but the relative kinetics of expression may be involved. Why lack of CFTR function should decrease expression of IRF4, surface expression of IL-13Rα1 and how this contributes to the failure to response to IL-13 is not clear but warrants further investigation.

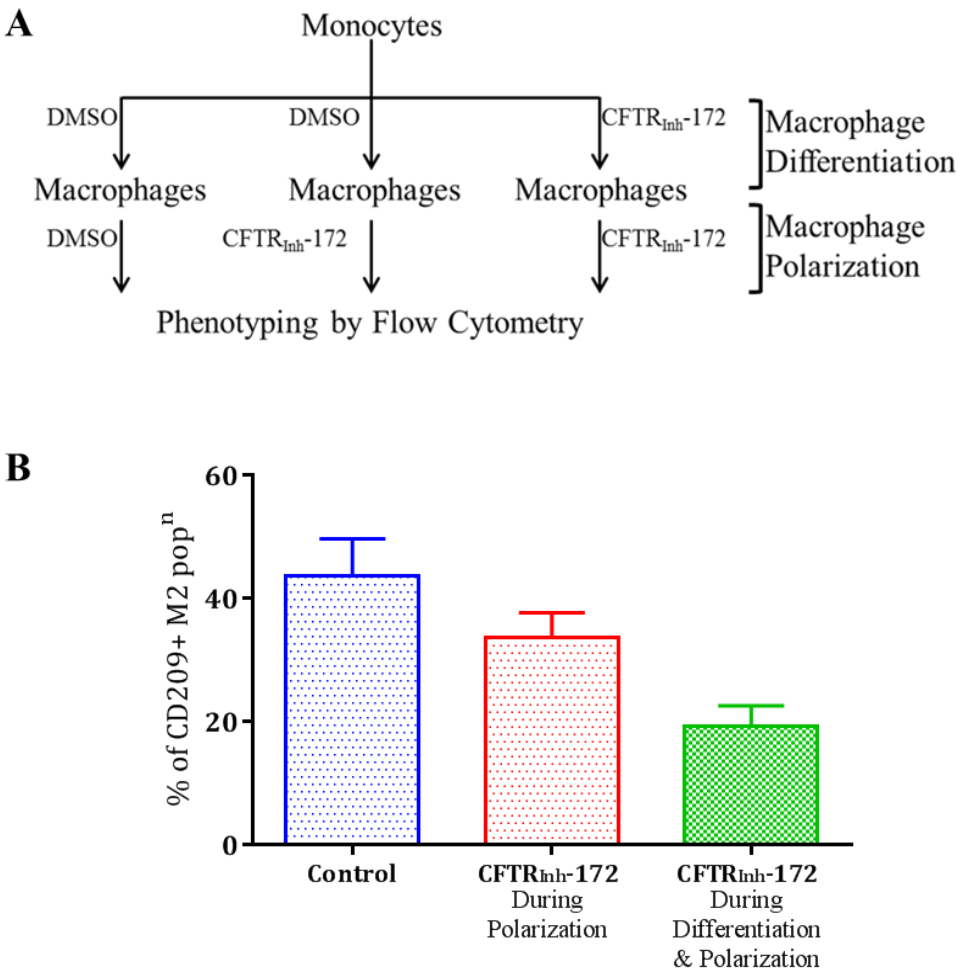
Cox-2 had been shown to be upregulated following LPS stimulation and suppressed following IL-13 stimulation in MDMs (72, 130, 136, 286). Induction of Cox-2 was shown to be associated with upregulation of NF-κB signaling cascade, thereby promoting inflammation. Drugs for instance ibuprofen that non-specifically inhibit Cox-1/2 had been beneficial for patients with CF (305). Chen *et al* have reported functional CFTR as a negative regulator of Cox-2 in AECs (301). However, how inhibition of Cox-1 or -2 modifies macrophage function and polarization still remained unexplored. It was assumed that inhibition of Cox-2 might suppress M1 and restore M2 polarization. Similar to AECs study (301), simultaneous inhibition of Cox-2 and CFTR exhibited a reduction in CD80 expression (M1 polarization). Such reduction in CD80 was not observed in control Cox-2 inhibited cells which indicates an associated with inhibition or dysregulation of CFTR function and exaggerated CF inflammation. Increased expression of CD80 in CF M0 is consistent with this. However, inhibition of Cox-2 in CFTR inhibited cells does not restore CD209 expression (M2 polarization) suggesting a link between normal CFTR function and the molecular machinery involved in M2 polarization. In accordance with earlier study (305), inhibition of both Cox-1/2 resulted in decreased expression of CD80 (M1) and CD209 (M2). Taken together, this study indicates that inhibition of Cox-2 might suppress inflammation in CF but would not be able to trigger anti-inflammatory mechanism.

There are several limitations with the present study that need to be acknowledged. We have collected peripheral blood monocytes to differentiate and polarize into MDMs ex vivo. Studies on acute lung injury in mice suggest that these are the cells that are attracted to the lungs to respond to inflammatory or infective stimuli (297, 298). Certainly, such monocyte-lineage cells are important components of the acute inflammatory response seen in acute asthma (306, 307). However, whether these are the cells responsible for resolution of pulmonary inflammation in CF remains to be proven. In addition, while we have paired samples from adults with CF collected during APE and when clinically stable, we do not have paired samples from the children with CF. Clearly using paired samples from individual patients would have allowed us to determine with more certainty how

macrophage function changed with an APE. While trends were seen for uncommitted M0 macrophages to be pre-primed towards the M1 phenotype this response was variable and the study did not have sufficient power to address this question.

Despite the limitations, the results of the present study have some clear clinical implications and give direction for future study. If, as indicated by the present data, CFTR function is essential for the development of the inflammation-resolution M2 phenotype then this poses a major problem for patients with CF. Studies from the AREST CF group in Australia have demonstrated the predominance of neutrophil-dominated inflammation, even in the absence of clinical symptoms and detectable infection, that increases the risk for bronchiectasis in infants as young as 3 months of age (293, 295). A CFTR-dependant deficiency in M2 macrophages could be the underlying reason for these findings. An understanding of the links between deficient CFTR function and M2 polarization may lead to novel therapeutic targets aimed at delaying the onset and progression of structural lung disease in CF. If macrophage function can be restored by correcting CFTR dysfunction it will become imperative to study the effects of CFTR potentiators and correctors on the inflammatory airway milieu and structural and functional disease outcomes in early life as by 5 years of age 50-70% already have bronchiectasis (255, 295, 308).

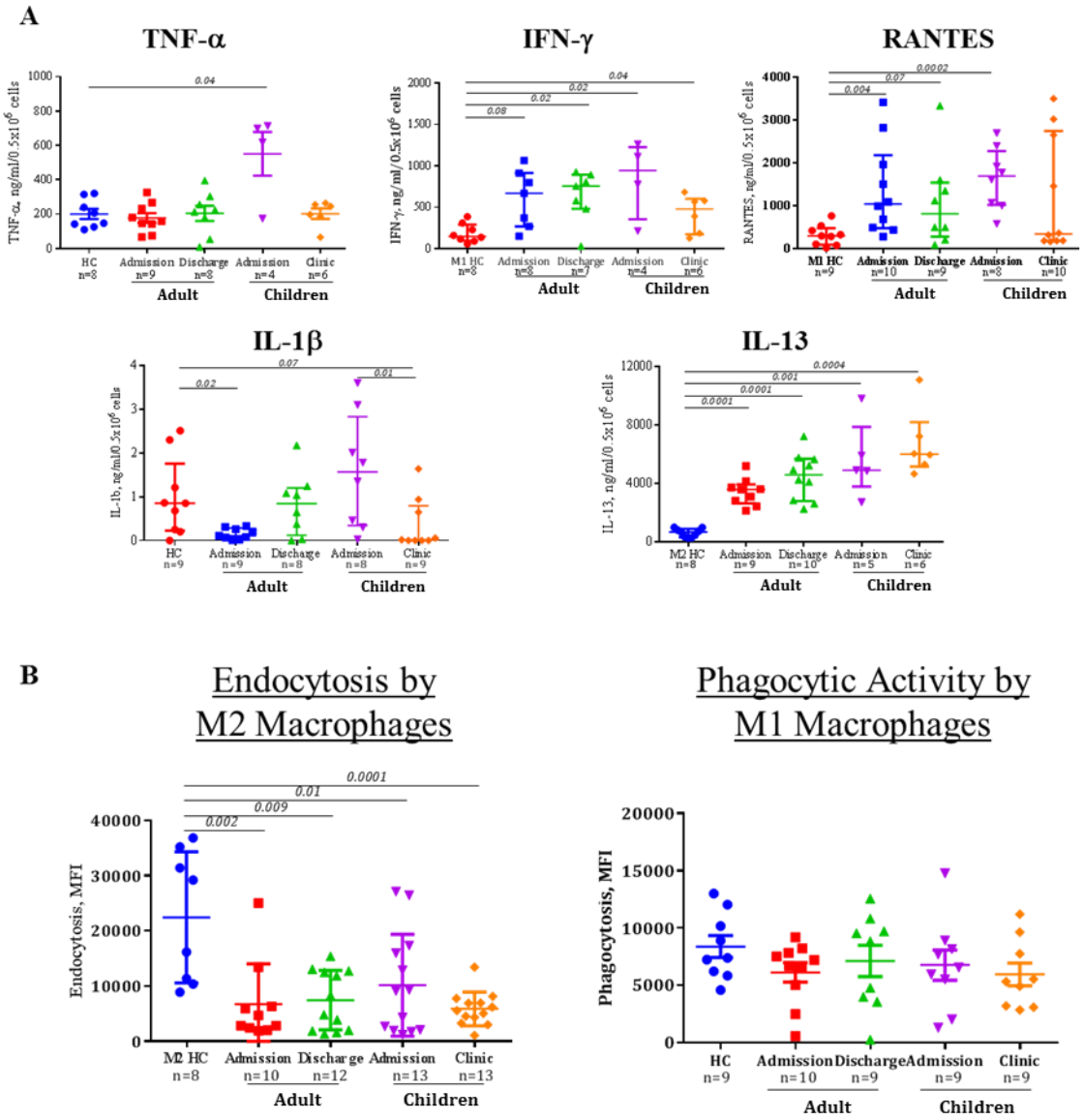
4.7 Supplementary Data



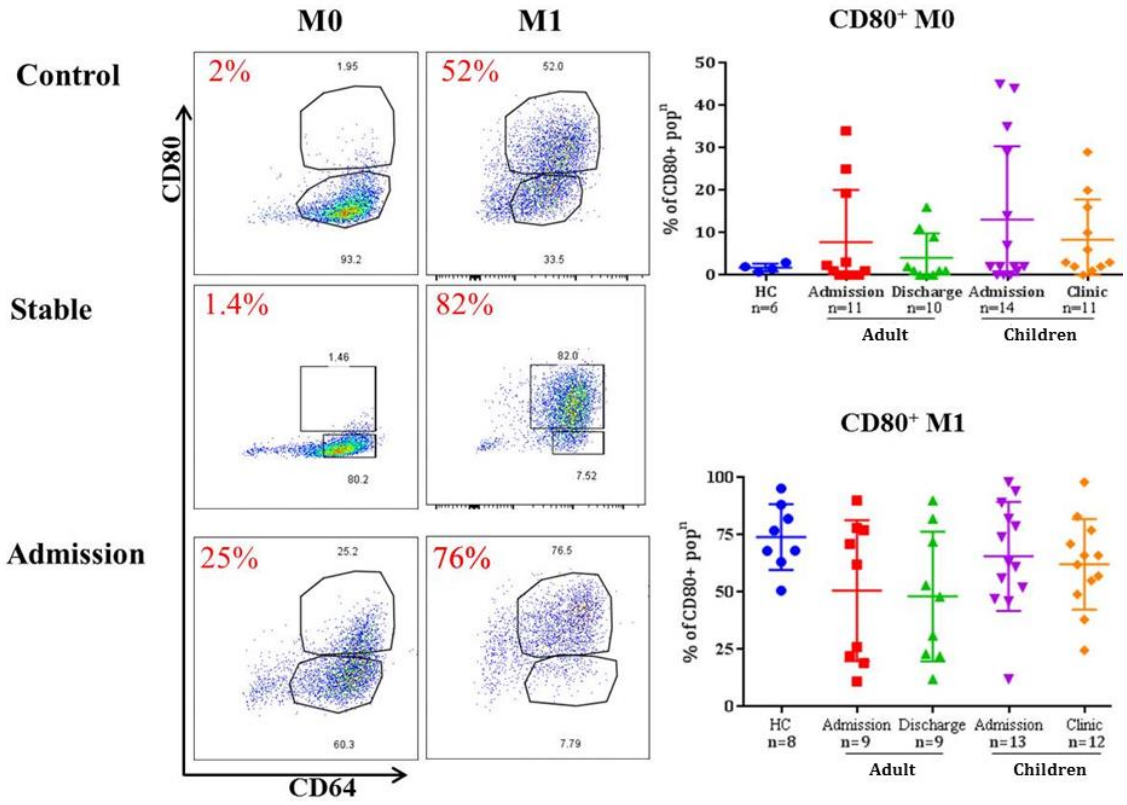
Suppl Figure 8: In vitro model for CF macrophage differentiation and polarization. Schematic representation of developing CF macrophage model with CFTR-inhibitors (n=3). A: CFTR inhibitor, $CFTR_{Inh}$-172 or GlyH-101 was added either during polarization only or during differentiation and polarization. B: Frequency of $CD209^{+}$ cells were analyzed as M2 macrophages by flow cytometry. Data were from at least three individuals and shown as mean±SD.

Suppl Table 3: Surface expression of CD80 in macrophages subtypes. Data are shown as median and 25th – 75th percentile; comparisons are CF group against control.

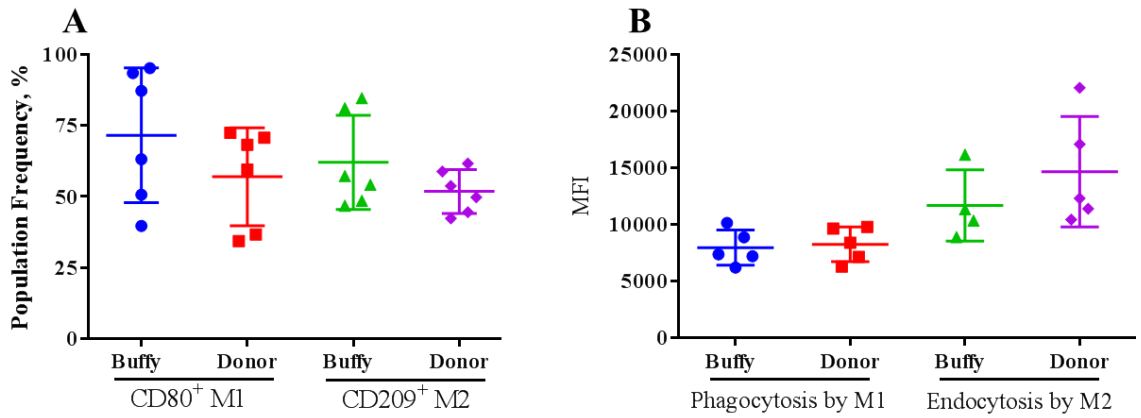
CD80^{+} (%)	Controls	Adults with CF		Children with CF	
		APE	Discharge	APE	Clinic
M0	2.5 (1.3-3.9)%	1.0 (0.0-19.3)% p=0.6	1.0 (0.0-9.5) % p=0.6	2.0 (0.8-30.5)% p=0.6	2.0 (2.0-16.0)% p=0.2
M1	76.9 (64.4-87.9)%	62.0(20.5 -77.5)% p=0.19	48(22.5-77.0)% p=0.07	63.0(49.5-85.5)% p=0.42	66.0(50.5-75.5)% p=0.17
M2	2.2 (2.0-3.15)%	7.8 (0.0-28.0)% p=0.36	5.0 (2.0-13.0)% p=0.08	8.0 (4.0-25.7)% p=0.006	3.0(1.0-15.5)% p=0.62



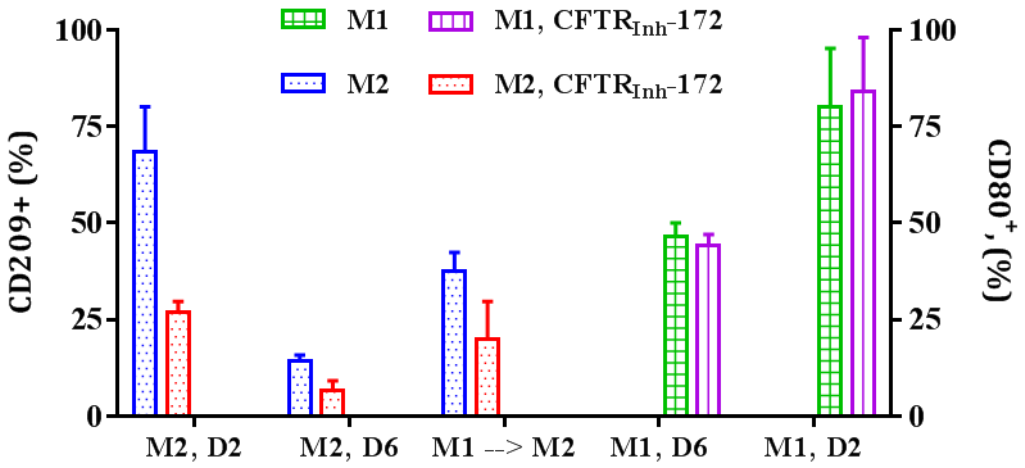
Suppl Figure 9: Function of M1 and M2 macrophages from children and adults with CF, on admission to hospital and when clinically stable) compared to healthy controls. Individual data as well as group median and IQR are shown.



Suppl Figure 10: Surface expression of M1 marker, CD80, following M1 polarization. Monocytes from buffy coats from healthy donors, clinically stable patients with CF and hospital admitted patients with CF for acute pulmonary exacerbation were differentiated to macrophages by 6-day stimulation with GM-CSF. M1 polarization was induced with LPS (20ng/ml). % of $CD80^+$ cells was analyzed by flow cytometry. Group data are shown as mean and standard deviation as well as individual data.

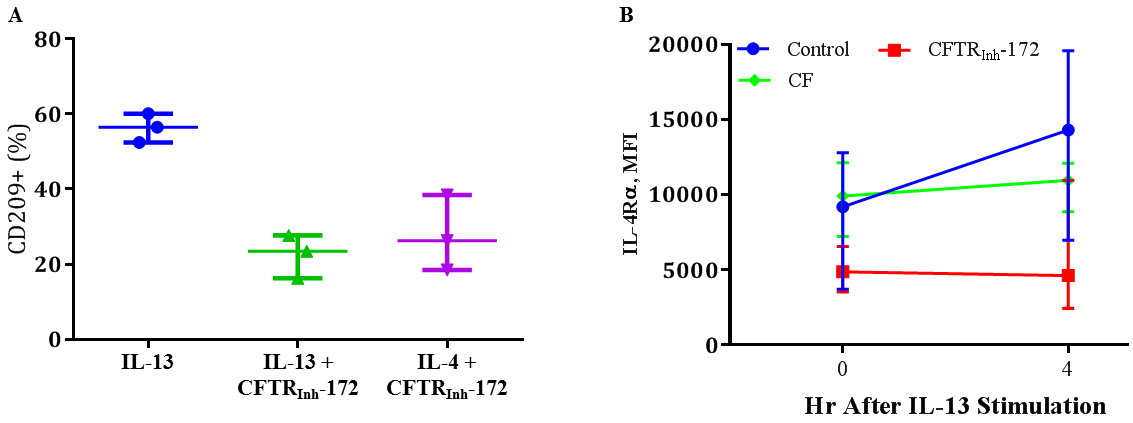


Suppl Figure 11: Comparison of phenotype and functions of polarized macrophages derived from buffy coat and freshly isolated monocytes. $CD14^+$ monocytes were isolated from either buffy coats provided by ARCBS or freshly isolated donor blood samples (n=5). 6-day GM-CSF (50ng/ml) was given to get fully mature macrophages. M1 and M2 polarization was induced by LPS (20ng/ml) and IL-13 (20ng/ml) respectively. Percentage of $CD80^+$ and $CD209^+$ cells were assessed for M1 and M2 polarization (A). Phagocytic ability of M1 cells and endocytic ability of M2 cells were analyzed by flow cytometry (B). Data represents mean±SD.



Suppl Figure 12: Reprograming ability of CFTR inhibited macrophages. M1 and M2 polarizations were performed with monocytes from buffy coats (n=4) in absence or presence of $CFTR_{Inh}$-172 (See method section). M1 and M2 cells were then left in cytokine-free medium for 6 days. M1 cells were then given to IL-13 for next 2 days. Expression of CD209 (left Y axis) and CD80 (right Y axis) were analyzed at d2, d6 and 2-day after IL-13 stimulation given to M1 cells. Ability of M1 cells to respond to IL-13 was analyzed by CD209 expression. Mean and IQR were shown. On each testing occasion the surface expression of CD209 was significantly lower in cell that had been

incubated with the $CFTR_{Inh}$-172 than in control cells; after 2 days incubation (D2) p=0.004, after 6 days in cytokine-free medium (D6) p=0.01 and after previously polarized M1s were incubated with IL-13 (M1→M2), p=0.01.



Suppl Figure 13: IL-4 mediated M2 polarization and surface expression of IL-4Rα in CFTR inhibited cells. MDMs were differentiated using monocytes from buffy coats (n=3) in presence or absence of CFTR inhibitor, $CFTR_{Inh}$-172. (A) CFTR inhibitor, $CFTR_{Inh}$-172 treated or control M0 cells were exposed to IL-4 for M2 polarization. CD209 expression was analyzed. (B) Surface expression of IL-4Rα on M0 cells from clinically stable patients with CF (n=4), $CFTR_{Inh}$-172 treated (n=4) and controls (n=4) were analyzed at time 0 and after 4 hours stimulation with IL-13. Data were shown as median and IQR.

Chapter 5 Effects of Tobramycin on Macrophage Polarization

5.1 Introduction

Antibiotic treatment for patients with CF is directed to prevent and eradicate respiratory infections. Aminoglycosides, such as, tobramycin, gentamicin has been shown to restore expression of full CFTR and cAMP-activated chloride channel in HeLa cells transfected with mutated CFTR gene (263). A recent article reported restoration of functional CFTR with a significant increase in CFTR-dependent chloride efflux in monocytes following azithromycin, ciprofloxacin (180). Azithromycin treatment demonstrated suppressed M1 polarization and enhanced M2 polarization in murine alveolar and peritoneal macrophages (257). However, the mechanism of action of antibiotics on the restoration of CFTR function and macrophage polarization were yet to be explored.

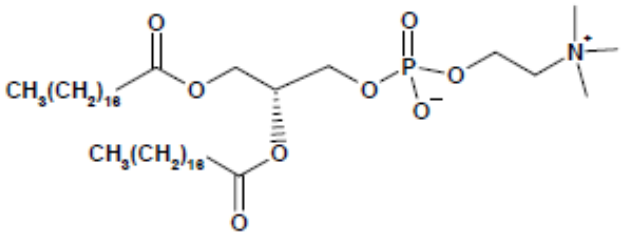


Figure 18: Chemical structure of tobramycin.

Tobramycin is a water-soluble aminoglycoside antibiotic produced by *Streptomyces tenebrarius* (***Figure 18***). It is an approved bactericidal drug and highly recommended for patients with CF to suppress of *P. aeruginosa* infection in the lungs that result in improved lung function and prevention of pulmonary exacerbations (237). Since intravenous tobramycin poorly penetrates into the bronchoalveolar spaces, a frequent and high dose of tobramycin is required to achieve the optimal therapeutic concentration at the site of infection (237, 309). To exclude the possibility of systemic toxicity due to its high dose, tobramycin inhaled solution or dry powder inhaler is of the choice of clinicians for treating patients with CF. Inhaled tobramycin has a longer half-life in the lung tissues and remained detectable for at least 4 weeks after exposure ceased. After administration of aerosolized tobramycin, the mean peak concentration of tobramycin measured was 1,237µg/g in sputum and 0.95µg/ml in serum (276). Similar concentration (1mg/ml) of tobramycin had been shown to prevent biofilm formation of *P. aeruginosa* as well as disrupt already formed biofilms (243, 275, 310, 311). Neither group reported any cytotoxicity of such higher concentration of tobramycin on cells.

Although studies demonstrated significant improvement in lung function in patients with moderate to severe CF, immunomodulatory effects of tobramycin at cellular level is scarce. A very recent study demonstrated that tobramycin treatment on THP-1 derived macrophage-like cells was shown to suppress LPS induced TNF-α, IL-1β, CXCL1, but produced significant IL-8 (312). Lack of an appropriate CF macrophage model was an obstacle to study the mechanism by which tobramycin modulate host immune responses. Using the CF macrophage model reported in ***Chapter 4***, I aimed to study the effect of tobramycin on macrophages in CF.

5.2 Methods

5.2.1 In vitro Differentiation and Polarization of Macrophages

Monocytes from buffy coats or patients from CF were differentiated into monocyte-derived macrophages (MDMs) and then polarized into M1 and M2 macrophages as previously described in ***Chapter 3***. Tobramycin (Sigma, USA) dissolved in water was added to the medium at day 0, 3 and 6 (***Figure 19***). To optimize the dose of tobramycin for macrophage differentiation and polarization, initially 0.1, 1.0 and 10mg/ml of tobramycin were used.

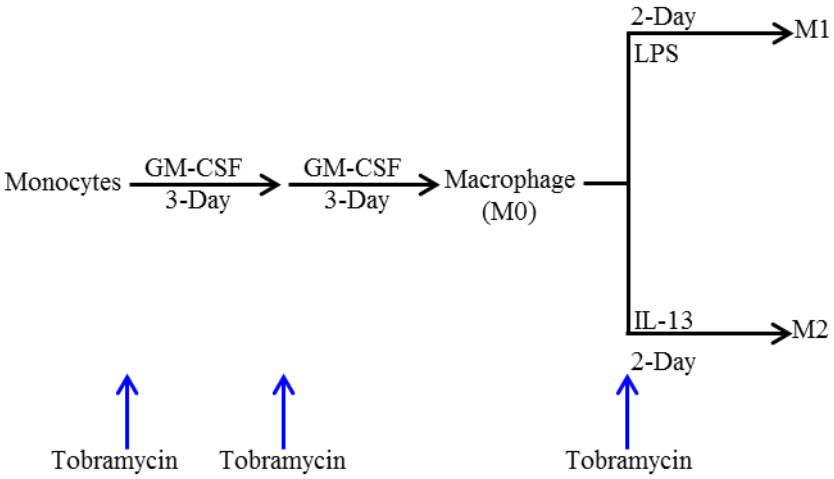


Figure 19: *In vitro* differentiation and polarization of human macrophages in the presence of tobramycin. Human $CD14^{+}$ monocytes were differentiated into macrophages (M0) by GM-CSF (50ng/ml) and further polarized into M1 and M2 macrophages using LPS (20ng/ml) and IL-13 (20ng/ml) respectively. Tobramycin was added to the culture media at day 0, 3 and 6. Phenotype was assessed by flow cytometry.

5.2.2 Cell Viability Assay of Tobramycin Treated Macrophages

To assess the cytotoxicity of different tobramycin doses used, the viability of macrophages was tested using 7-aminoactinomycin D (7-AAD) staining on M0 macrophages (BD, USA). 7-AAD is a fluorescent derivative of actinomycin D that selectively binds to GC regions of the DNA (313). 7-AAD is unable to pass through the intact cell membranes of live cells. Rather, it can easily enter through the disrupted membrane of dead cells and stain the DNA. Fully differentiated macrophages (M0) were harvested at day 6 by TrypLE (Invitrogen, USA), washed with PBS and incubated with Fc blocking solution (FBS,2%-BSA,0.1%-PBS,

100ml) for 20min at 4^0C. Cells were then spun down and resuspended in FACS buffer and stained with 7-AAD (5µg/ml) 20min at 4^0C (313). After a second washing step, cells were analyzed on BD LSR-Fortessa using BD FACS Diva software. Subsequent analysis was performed using Flowjo (Tree Star Inc. USA).

5.2.3 Effect of Tobramycin on CF Macrophages

To study how tobramycin modulates macrophage polarization and thereby function in CF, tobramycin (1mg/ml) was added in the CF macrophage model described in ***Chapter 4***. Briefly, monocytes obtained from buffy coats or patients from CF were exposed to the CFTR inhibitor $CFTR_{Inh}$-172 (10µM) (Sigma, USA) alone, tobramycin alone (1mg/ml) or in combination during macrophage differentiation and/or polarization.

5.2.4 Phenotypic Characterization of Tobramycin Treated M1/M2 Macrophages

For assessing M1/M2 marker expression on tobramycin-treated M0, M1 and M2 macrophages, cells were harvested and stained with anti-human CD64, CD80, CD209, as previously described (***Chapter 3***) (286). Surface expression of IL-13 receptor α1 (IL-13Rα1) was examined on M2 macrophages using flow cytometry. Data were acquired on BD LSR-Fortessa using BD FACS Diva software. Subsequent analysis was performed on $CD68^+$ macrophages using Flowjo (Tree Star Inc. USA).

5.3 Results

5.3.1 Dose Selection

1- and 10mg/ml of tobramycin resulted in an immediate increase in pH of the culture medium from 7.0 to 6.2. Therefore tobramycin-containing medium was left in the CO_2 (5%) incubator for 60-90min to bring the pH to 7.0. At 0.1mg/ml conc. no change in medium pH was observed. The percentages of live cells with 0.1 and 1mg/ml of tobramycin were similar to the untreated macrophages. However, viability significantly declined at the higher dose of tobramycin, with only 16.7% of cells surviving (***Figure 20***). Since 1mg/ml concentration of tobramycin was found in the BAL of CF patients, this concentration was chosen for experiments afterward (276).

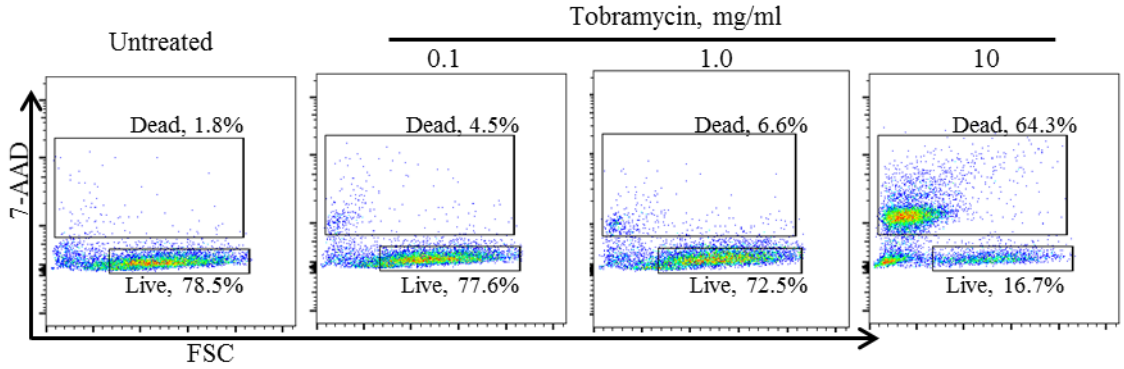


Figure 20: Cytotoxicity assay of tobramycin-treated macrophages. 6-day GM-CSF differentiated macrophages (M0) were harvested, Fc blocked and then stained with 7-AAD (5µg/ml) for 20min at 4^0C. Forward scatter (FSC) versus 7-AAD plot shows the distribution of the live and dead cells.

5.3.2 Tobramycin Downregulates Macrophage Polarization

Tobramycin alone showed downregulation of both M1 ($p = 0.009$) and M2 ($p = 0.002$) polarizations assessed by $CD80^+$ and $CD209^+$ cells respectively when compared to untreated controls (***Figure 21***). As previously reported in ***Chapter 4***, $CFTR_{Inh}$-172 treated cells showed comparable frequency of $CD80^+$ cells with medium control cells. Combined effect of CFTR inhibitor, $CFTR_{Inh}$-172 and tobramycin showed synergistic inhibitory effect on M1 polarization

assessed by the percentage of $CD80^+$ cells compared to untreated control ($p = 0.002$) or $CFTR_{Inh}$-172 treated cells ($p = 0.009$).

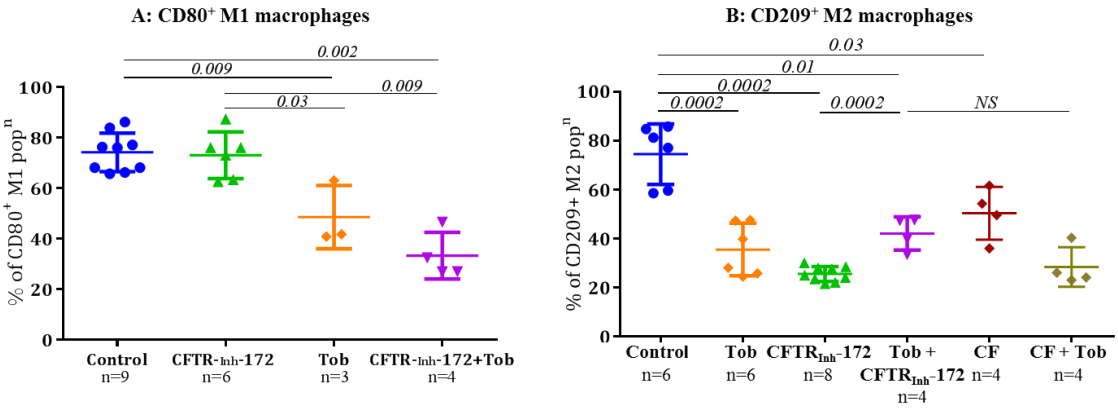


Figure 21: Expression of M1/M2 markers on tobramycin-treated M1 and M2 macrophages. $CD14^+$ monocytes were isolated from buffy coats (A & B) or patients with CF (n=4) (C). Monocyte-derived macrophages (MDMs) were differentiated by 6-day stimulation with GM-CSF in presence or absence of $CFTR_{Inh}$-172 alone, tobramycin (Tob) (1mg/ml) alone or in combination. M1 (A) and M2 (B & C) polarizations were induced by *E. coli* LPS and IL-13 respectively. Error bars represent the SD. Statistical significance was calculated using non-parametric t-test.

M2 polarization assessed by $CD209^+$ cells was significantly lower in $CFTR_{Inh}$-172 and tobramycin-treated cells compared to controls. Interestingly combining tobramycin with $CFTR_{Inh}$-172 significantly upregulated the percentage of $CD209^+$ cells compared to $CFTR_{Inh}$-172 ($p = 0.002$). However after stimulation with IL-13, monocytes derived macrophages from CF patients did not show similar restoration of $CD209^+$ cells following tobramycin treatment. The difference in M2 polarization between $CFTR_{Inh}$-172 inhibited cells and cells from patients with CF following tobramycin treatment raised a possibility of competitive binding between tobramycin and $CFTR_{Inh}$-172 which remained unexplored. Due to insufficient patient material, it was not possible to analyze the effect of tobramycin on M1 polarization.

5.3.3 Tobramycin Induces IL-13Rα1 Expression

Kinetic expression of IL-13Rα1 following IL-13 stimulation was analyzed to investigate the mechanism of observed M2 restoration in $CFTR_{Inh}$-172 inhibited plus tobramycin-treated

cells. Initially kinetic expression of surface IL-13Rα1 was analyzed on M0 cells following IL-13 stimulation. Tobramycin treated M0 cells displayed comparable receptor expression with untreated M0 cells (***Figure 22***). Significant reduction of IL-13Rα1 expression was observed on $CFTR_{Inh}$-172 inhibited cells compared to controls ($p = 0.0005$) and tobramycin-treated cells ($p = 0.001$). Enhanced IL-13Rα1 expression was observed following synergistic effect of tobramycin and $CFTR_{Inh}$-172 that was not significant. IL-13Rα1 expression was then analyzed on differentiated M0 cells from CF monocytes (n=4) and 4h after IL-13 stimulation (***Figure 22B***). Differentiated M0 cells from CF monocytes exhibited similar IL-13Rα1 expression to $CFTR_{Inh}$-172 treated cells. Due to unavailability of patient samples, it was not possible to study the effect of tobramycin on IL-13Rα1 expression on CF monocytes differentiated macrophages.

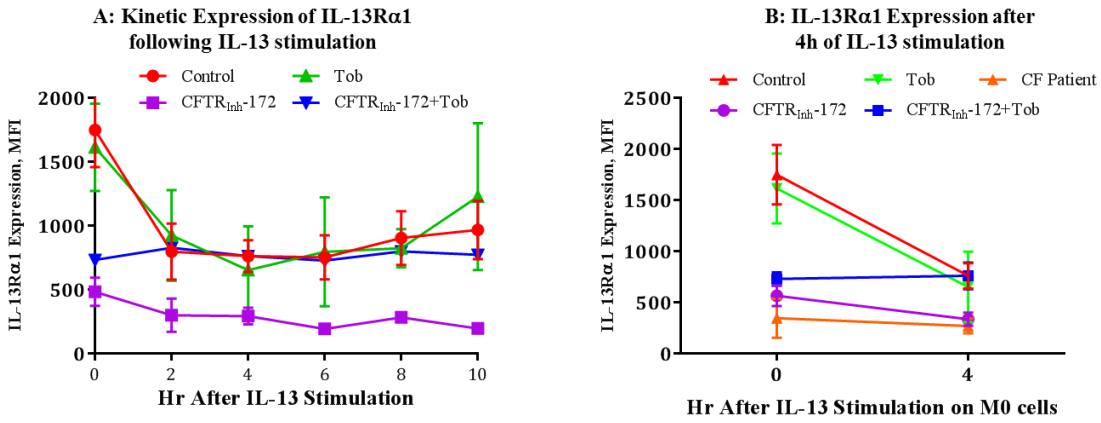


Figure 22: Effect of tobramycin on IL-13Rα1 expression. Human monocyte-derived macrophages (MDMs) were differentiated by 6-day stimulation with GM-CSF in presence or absence of $CFTR_{Inh}$-172 alone (n=4), tobramycin (1mg/ml) alone (n=3) or in combination (n=3). IL-13 (20ng/ml) was added to induce M2 polarization. Surface expression of IL-13Rα1 was analyzed by flow cytometry at 0, 2, 4, 6, 8 and 10 hours after IL-13 treatment. Error bars represent the SD. Statistical significance was calculated using non-parametric repetitive measure test.

5.4 Discussion

Aminoglycoside antibiotics such as tobramycin and gentamycin were the first drug that has been approved to treat patients with CF (263, 314). Expression of functional CFTR and restoration of chloride channel on PMNs following IV tobramycin treatment has been demonstrated (180). Clinical trials showed successful eradication of *P. aeruginosa*, significant improvement of lung functions and weight gain in patients with moderate to severe CF following tobramycin treatment compared to placebo controls (174, 238-242). However, these studies did not assess the immunomodulatory effects of tobramycin on host immune cells, thereby the mechanism of improved lung function. In this chapter, the effect of tobramycin on macrophage polarization was investigated.

After adding tobramycin in the culture media, immediate increase in pH of the medium was observed. This might be a mode of action how tobramycin exerts its anti-bacterial activity in the tissues. Like in an earlier study (312), cytotoxicity of administered tobramycin on MDMs was observed in a dose-dependent manner. Low to medium doses of tobramycin had no effect on cell viability, however, macrophages hardly survive at the high dose of tobramycin (10mg/ml).

A significant decrease in numbers of circulatory PMNs was observed after IV antibiotic treatment including tobramycin among patients with acute exacerbation of CF (180). THP-1 derived macrophage-like cells showed a considerable reduction in LPS-induced TNF-α, IL-1β, IL-8 and CXCL1 release following tobramycin administration (312). Consistent with those observations, significant suppressions of both M1 and M2 polarization were when tobramycin was added during macrophage differentiation and polarization. Since the abovementioned study (312) focused on the pro-inflammatory macrophage responses, modulation of anti-inflammatory responses of macrophages by tobramycin in CF (CFTR inhibited) got more attention in this study. To identify the mechanism of suppressed M2 polarization by tobramycin, IL-13Rα1 expression was analyzed following IL-13 stimulation. No effect of tobramycin was observed on IL-13α1 expression suggesting tobramycin might use different signaling pathway to arrest M2 polarization.

Tobramycin with inhibition of CFTR by $CFTR_{Inh}$-172 resulted in a further reduction of M1 polarization and a partial restoration of M2 polarization. It was not assessed that whether

tobramycin physically or chemically binds to $CFTR_{Inh}$-172 and thereby compete and rule out CFTR inhibition. Tobramycin mediated enhanced IL-13Rα1 expression in CFTR inhibited cells correlates with enhanced M2 polarization in synergistic condition of $CFTR_{Inh}$-172 and tobramycin. Lowered expression of IL-13Rα1 during CFTR inhibition and restoration of CFTR function by tobramycin suggest that tobramycin in CF (CFTR inhibited cells) restores CFTR function which later leads to improved IL-13Rα1 expression thereby improved M2 polarization.

Differentiated macrophages from CF monocytes did not show similar restoration of M2 polarization with no upregulation of IL-13Rα1 expression following tobramycin treatment. An earlier study showed suppression of two disease-associated stop codon mutations (G542X and R553X) (type I mutations) by aminoglycoside antibiotic G-418, thereby resulting in expression of full of length CFTR and restoration of Cl channel activity (263). Gentamycin treatment was also reported to express full-length CFTR in HeLa cells carrying these mutations (263). Expression of full-length CFTR and restoration of chloride channel activity was observed in monocytes of patients with CF following intravenous administration of a combination antibiotics including tobramycin (180). Effect of tobramycin on macrophages among patients carrying ΔF508 mutation is still unknown. $CFTR_{Inh}$-172 were reported to enter into the cells, binds to the intracellular portion of CFTR protein and thereby block the channel activity (226, 227). CFTR inhibition by $CFTR_{Inh}$-172 is therefore supposed to exhibit similar impaired channel activity displayed by mutated CFTR protein due to ΔF508 mutation. In this study, $CFTR_{Inh}$-172 showed similar repressed M2 polarization with differentiated macrophages from CF monocytes. However, tobramycin effect on M2 polarization in CFTR inhibited cells was different from macrophages differentiated from CF monocytes. The mechanism by which tobramycin modulates macrophage polarization in CFTR inhibited cells and macrophages from CF monocytes remained unexplored. However, this data provide some important clues regarding the mode of action of tobramycin on host innate immune responses. Furthermore, observed restorations of M2 polarization in CFTR inhibited cells as well as in differentiated macrophages from CF monocytes validates the CF macrophage model reported in ***Chapter 4***.

Taken together, these data indicates the multifunctional properties of tobramycin. Tobramycin is not only able to limit bacterial growth but also able to modulate the host innate immune response. Flow cytometry data of this study pointed toward potential effects of

tobramycin in damping inflammation and initiating resolution of inflammation in CF. However, suppression of M1 genes, induction of M2 genes, cytokine profile as well as phagocytosis or endocytosis were not evaluated following tobramycin-treated. The molecular mechanism whether tobramycin restores CFTR function and how also remained unexplored. Further studies are needed to minimize the knowledge gap.

Chapter 6 Discussion and Future Recommendations

6.1 Key Research Findings and Their Clinical Implications

Historically macrophages are known for their phagocytic activity and responses to microbial insults. With recent advancements, macrophages are now recognized as critical players not only in the initiation of inflammation but also in the resolution of inflammation. These two opposing actions are governed by their two different polarized states: pro-inflammatory *classical* or M1 polarization and wound healing *alternative* or M2 polarization. Murine models of inflammatory, autoimmune diseases have elegantly demonstrated that the polarized state of macrophages dictates the nature, duration and severity of disease pathogenesis. Therefore, understanding the polarized state of macrophages is important in studying inflammation. The easiest way to assess their polarized status is by analyzing their surface markers and measuring the inflammatory mediators present in the body fluid or *in vitro* culture supernatants. Due to substantial interspecies variability, murine markers are not suitable to characterize human macrophages (reviewed in ***Chapter 1***). Therefore a clear phenotypic characterization of human M1 and M2 macrophages was needed. Additionally, monocytes and differentiated MDMs have been used to study human macrophage biology. Responses to stimuli by monocytes and MDMs had never been systemically investigated. Comparative transcriptome analysis elegantly demonstrated two different gene expression profiles by monocytes and MDMs suggesting that MDMs, not precursor monocytes, should be used while studying macrophage biology (72). During this Ph.D. study, an elegant model for *in vitro* differentiation of human M1 and M2 macrophages and their characterization had been developed (***Chapter 3***) (286). This *in vitro* model systematically demonstrated the phenotypes and functions of three states of macrophage: uncommitted M0, classical M1 and alternative M2 macrophages. The human macrophage polarization and characterization model described in ***Chapter 3*** can be used to investigate potential roles of macrophage in disease pathogenesis and facilitate developing therapeutic interventions where restoring the subtle balance of macrophage subsets would become beneficial for patients.

Since respiratory linings are rich in GM-CSF, GM-CSF was used to differentiate macrophages from monocytes. However, this study as well as Beyer *et al* (136) showed that M-CSF differentiated macrophages showed similar phenotype, gene expression and functions to GM-CSF differentiated macrophages. Therefore, this M1-M2 model is not limited only to respiratory macrophages. The model can also be used to study roles of macrophages from other tissues. Although M2 macrophages were subdivided into three groups: M2a, M2b and M2c

(44), no subtype of M1 macrophages had previously been reported. However, the phenotype and function of M1 cells were observed to vary with the inducers used to activate the M1 state (***Chapter 3***) (286)**.** M1 cells activated by LPS or IFN- γ or LPS plus IFN-γ exhibited three distinct phenotypes and functions. LPS induced M1 cells demonstrated surface expression of CD80 with a population of $CD64^{+}CD80^{+}$ cells. M1-LPS cells were highly phagocytic, were found to release pro-inflammatory cytokines including TNF-α, RANTES, IL-8 with induction of Cox-2 gene. M-IFN-γ cells demonstrated significant expression of CD64, minimal phagocytic activity, release of IFN-γ and IP-10 and induction of CXCLL11 gene. Cells those received both LPS and IFN-γ for M1 polarization displayed similar phenotype with M1-LPS cells, however, these M1-LPS plus IFN-γ cells were not phagocytic. Such subdivisions of M1 macrophages corresponded with the previously reported pathway analysis study (72) suggesting heterogeneity within the M1 macrophages is a real phenomenon. This area needs proper attention from experts in this field. Since, many respiratory diseases, such as asthma and CF, are dominated by T_H2 cytokines, IL-13 conditioned M2a macrophages received the most attention in this thesis. However, the term "M2" was used throughout the thesis for the sack of simplicity.

Plasticity may not be very important during homeostasis, but becomes more important during inflammation. The ability to switch from one polarized state to another had been demonstrated in mice (77, 315), however, the fate of once polarized macrophages following the withdrawal of inducers had not been studied. Using MDM, I provided an in-depth analysis of macrophage behavior following polarization and of the reversibility of phenotype following alteration of cytokine stimuli (***Chapter 3***) (286). Polarized macrophages were observed to revert to uncommitted M0 state by 12 days following the removal of the polarizing stimulus. Antagonistic effects of M1 and M2 inducers on once polarized macrophages, i.e., downregulating M1 phenotype by M2 inducer, thereby polarization toward M2 and *vice versa* was observed. Such a shift from M1 to M2 state or *vice versa* indicates how the innate immune balance could be maintained by macrophage subsets *in vivo*.

Exaggerated neutrophilic inflammation has been reported to lead to permanent damage of lungs in CF. However, the mechanism by which neutrophils get triggered and why they do not turn off normally is still unknown. Very few studies showed defective macrophage responses and functions in CF (159-161). ΔF508 KO mice demonstrated exaggerated M1

induction along with minimal or no induction of M2 genes (257). However, engagement of reparative M2 macrophages at the end of pulmonary exacerbation of CF was not thoroughly investigated. Due to lack of markers for human M1/M2 macrophages, no comprehensive studies have been conducted to analyze subset specific macrophage responses in CF.

Using the *in vitro* human macrophage polarization and characterization model described in ***Chapter 3***, an intrinsic defect of M2 polarization in CF has been identified. Percentage of $CD209^+$ cells, a hallmark of M2 polarization has been found to be significantly lower among adults and children with CF (***Chapter 4***). In addition, induction of M1 phenotype ($CD80^+$ cells) was observed in both uncommitted M0 and IL-13 treated cells during acute pulmonary exacerbation (APE) of CF, but not in clinically stable patients. IL-13 treated CF macrophages showed lowered endocytosis as well, however, IL-13 secretion was significantly higher in both patient cohorts compared adult healthy controls. Interestingly, in both patient cohorts, the levels of IL-13 was observed to be slightly higher at the time of discharge than APE. It is possible that during inflammation M1 macrophages take control to initiate inflammation and that at the later stage of inflammation macrophages release large amounts of IL-13 to initiate the reparative phase. Together, these data suggest enhanced M1 responses by all three macrophage compartments during APE and relentless suppression of M2 polarization as underlying contributors to chronic inflammation in CF. These observations were fairly similar with CF mouse model data (257) and indicated that CF monocytes are prone to M1 polarization. Nevertheless, monocyte compartment (classical, intermediate and non-classical) of CF was found similar to healthy controls.

To study the mechanism of such aberrant macrophage responses in CF, a CF macrophage model had been developed using CFTR inhibitors, $CFTR_{Inh}$-172 and GlyH-101. Inhibiting CFTR function in healthy cells recreated the CF phenotype with decreased M2 polarization and lowered phagocytosis and endocytosis. The model allowed further investigation of defective M2 polarization in CF. Wu *et al* showed the role of IL-13 on CFTR expression and Cl^- secretion in epithelial cells (316). In agreement with this study, we observed decreased surface expression of IL-13 receptor, IL-13Rα1, on CFTR-inhibited macrophages. Similarly to CFTR inhibited M0 cells, differentiated M0 cells from CF monocytes showed lowered IL-13Rα1 expression following IL-13 stimulation, indicating a direct regulation on the expression of IL-13Rα1 by functional CFTR. Functional CFTR is therefore essential for

M2 polarization. IRF4, the key gene in M2 polarization, was not induced at all in differentiated CF M0 macrophages following IL-13 stimulation. These data explained the inability of CF M0 macrophages to respond to IL-13, a key cytokine that inhibits M1 and promotes M2 polarization in CFTR-dependent manner. Such CFTR-dependent imbalance in the innate immune system might explain the defective resolution of pulmonary inflammation during CF which eventually leads to exaggerated neutrophilic responses. Apart from macrophages, IL-13 induces IgE secretion by B cells and proliferation of T_H2 cells. Similar to earlier studies (262), IL-13 release was observed to be substantially higher in CF macrophages compared to macrophages from healthy controls. It is very likely that IL-13 would induce other immune cells, such as, T cells, B cells or structural cells including AECs to initiate pulmonary fibrosis. However, this mechanism has to be tested with a systematic study.

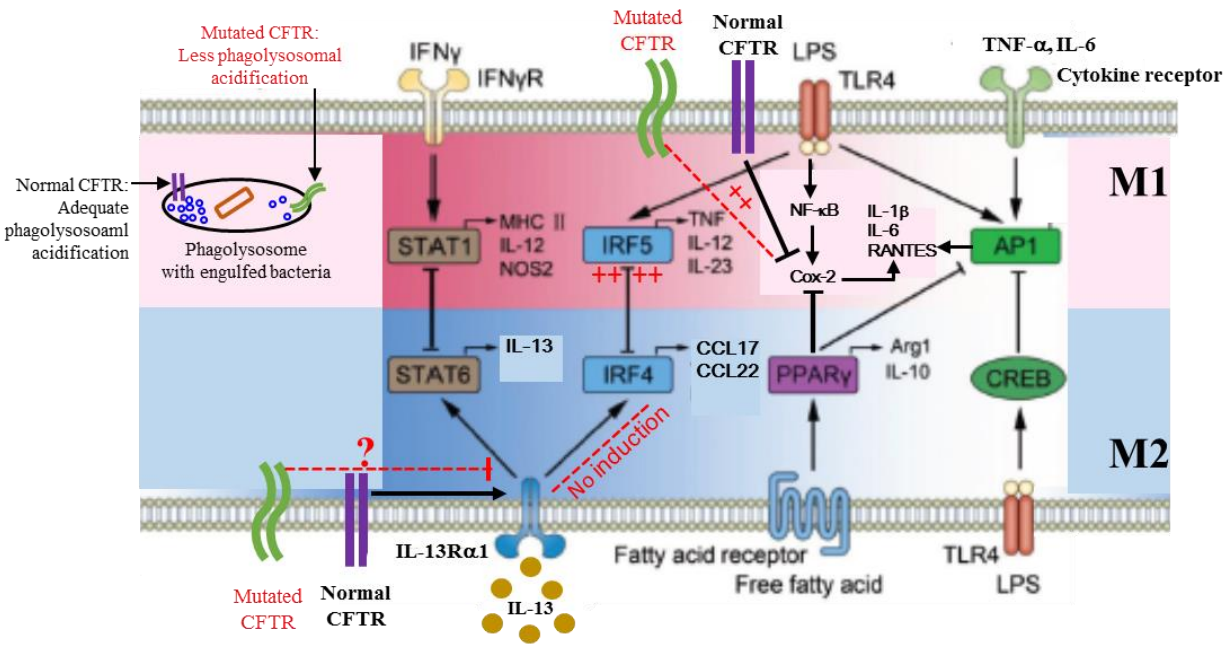


Figure 23: Proposed signaling pathways of macrophage polarization in CF. The figure illustrates several mechanisms underlying macrophage polarization and the feedback regulation between M1 and M2 signal pathways in CF (text, dashed lines and signs in red) and healthy control. Due to dysfunctional CFTR protein, phagolysosomal acidification fails, resulting in less killing and clearance of microbes. Functional CFTR is a negative regulator of Cox-2. In presence of defective CFTR, Cox-2 pathway remains activated. Lack of CFTR function due to mutation or inhibition ($CFTR_{Inh}$-172) leads to reduced IL-13Rα1 expression, resulting in no or minimal induction of IRF4, the key gene of M2 polarization. IRF4 and IRF5 are under feedback regulation. Absence of IRF4 results in the continuous induction of IRF5,

another key gene of M1 polarization. In summary, lack of anti-inflammatory machinery and constant induction of pro-inflammatory Cox-2 and IRF5 pathways might be the underlying reason of CF inflammation. The figure was adapted from Liu *et al* (317).

IL-13 secreted by T_H2 and mast cells is the key cytokine that promotes M2 polarization. Two different IL-13 receptors, IL-13Rα1 and IL-13Rα2 have been reported. IL-13Rα1 is present on monocytes, macrophages, B cells, basophils and endothelial cells. Following IL-13 stimulation IL-13Rα1 forms heterodimer with IL-4Rα and activates JAK1 and JAK3 (30, 318). Activation of JAK leads to phosphorylation of STAT6 and translocation to the nucleus. STAT-6 activation is required for IL-13 production and the development of mucus production and AHR in animal models of allergic airway diseases (319). IL-13 also activates IRF4, a crucial transcription factor for M2 polarization (135). IRF4 is expressed in macrophages, DCs, lymphocytes, downregulates TLR signaling pathway to trigger inflammation and promotes differentiation of T_H2 (320) and T_H17 cells (321). Our data indicates that IL-13Rα1 induction is under CFTR control. Absence of functional CFTR due to either mutation or chemical inhibition ($CFTR_{Inh}$-172, GlyH-101) leads to a reduction of IL-13Rα1 expression resulting in reduced induction of IRF4 and ultimately lowered M2 polarization (**Figure *23***). However, it is not clear about the molecular mechanism of substantial IL-13 production in CF. Phosphorylation of STAT6 is required for IL-13 production (30, 318). Similar IL-4Ra expression was observed in natural (CF) and experimental (CFTR inhibited) cells to controls which raised a valid question that whether IL-4Ra alone is enough to activate STAT6 pathway. Further study is needed to explore the molecular mechanism of low M2 polarization, but high IL-13 production.

Chronic bacterial infection in CF causes non-stop induction of Cox-2 and IRF5 signaling cascades which later contributes to M1 polarization and thereby relentless production of pro-inflammatory cytokines (317). Like earlier studies (301, 305), this study reported that suppression of Cox-2 pathway may possibly be beneficial in CF, however, deactivation of Cox-2 pathway might not be enough to induce the anti-inflammatory machinery in CF macrophages. IRF4 and IRF5, activated by LPS maintains a feedback regulation to initiate and resolve inflammation (317). Since IRF4 isn't induced in CF, it is possible that activation of IRF5 stays uncontrolled (**Figure *23***). Taken together our data indicates that lack of anti-inflammatory mechanism and constant induction of pro-inflammatory Cox-2 and IRF5 pathways might be

the underlying reason of CF inflammation. It is also possible that other pathways are involved in the dysregulated inflammation in CF. Further study is needed to explore the molecular networks underlying the CF inflammation.

These two novel models described in *Chapter 3* and *Chapter 4* are likely to be useful for biomedical research. The ***first model*** that described the phenotypic identification of human M0, M1 and M2 macrophages will be able to identify the defective macrophage subset in pathogenic conditions. Knowing the pathogenic macrophage subset has a huge impact on developing novel therapeutic targets. This model was successfully applied to patients' samples of CF to analyze subset specific macrophage responses in CF and identified a fundamental defect in CF macrophages. The ***CF macrophage model*** allowed us to identify a CFTR-dependent fundamental defect of CF macrophage. This model would also allow CF researchers to conduct extensive investigations on macrophage functions without any patient samples.

Lastly, the ***CF macrophage model*** was used to gain a better understanding of the possible effects of tobramycin, a regular treatment for CF patients, on host innate immune systems, especially macrophages (***Chapter 5***). As an FDA approved bactericidal drug, tobramycin is commonly used for patients with CF. Tobramycin had been shown to delay colonization of *P. aeruginosa* in the lungs and significant improvement of lung functions in patients with moderate to severe CF (174, 237-242). However, due to lack of appropriate CF macrophage model, the mode of action of tobramycin on host immune cells had never been studied. Using the ***CF macrophage model*** reported in ***Chapter 4***, here I studied the effect of tobramycin on healthy macrophages as well as macrophages in CF (CFTR inhibited cells). Administration of tobramycin along with CFTR inhibitor exerted superfluous abolition of M1 polarization. Such synergistic effect of tobramycin and CFTR inhibitor was observed to be beneficial toward M2 polarization. These data indicate that tobramycin not only restrained M1 polarization but also restored M2 polarization in CF.

6.2 Limitations

The limitations of this study need to be acknowledged. Blood donation in Australia is an unpaid and volunteer service. Eligibility of the donors is screened by standard protocols by ARCBS. Donors of the buffy coats used in this study were assumed to be healthy and not carriers of mutated CFTR gene. No data regarding the life-style or smoking habits of the donor was accessible. While screening for infections that could be transmitted by blood transfusion, not all conditions that could influence macrophage function can be excluded. Only tested and infectious agent free buffy coats were supplied by ARCBS and used in these studies. No demographic data apart from age, sex and blood group were available for ARCBS blood donors. Any clinical information of the donors was inaccessible. However, phenotype or functional attributes observed with buffy coat derived macrophages were similar to those obtained with macrophages differentiated from freshly isolated monocytes from healthy adult donors.

M2 macrophages were subdivided into three groups: M2a, M2b and M2c (44). Since elevated IL-13 level had been reported in many respiratory and fibrotic diseases, IL-13 treated M2a macrophages received the most attention during this study. Hence, the thesis lacks any data on human M2b or M2c macrophages.

Data in ***Chapter 4*** were generated from cohorts of children and adult patients with CF and compared with adult healthy controls. Due to lack of ethical permission, it was not possible to collect blood samples from healthy children non-CF controls, hence children CF group lacked appropriate age-matched control group. Children healthy or patients with non-CF respiratory diseases would be the appropriate control for children CF group.

P. aeruginosa is the most commonly found pathogen among patients with CF. However, all the experiments in ***Chapter 4*** were conducted with commercially available *E. coli* LPS or *E. coli* bioparticles which might not reproduce the macrophage responses against *P. aeruginosa* clinical isolates. It is reasonable to surmise that CF macrophage responses would be more pronounced with *P. aeruginosa* clinical isolates.

Although *in vitro* macrophage models reported in ***Chapter 3*** and ***Chapter 4*** showed significant promises, they both lacked the physical influences of lung environment.

Macrophages in the BAL and sputum would be the most pertinent macrophages that would reflect the real scenario of CF inflammation. Studying the sputum macrophages would give some extra weight in this study. However, BAL and sputum from patients with CF mostly contained neutrophils, rather than macrophages (189, 322, 323). Earlier studies reported that due to chronic inflammation CF PMNs are likely to be exhausted (324). It is also possible that macrophages in alveolar spaces are ready exhausted, therefore, might not exhibit the proper phenotype. Di *et al* elegantly showed that engulfment and phagolysosome formation were not defective in AMs of CFTR-KO mice, rather phagolysosomes of AMs in CFTR-KO mice were less acidic (161). In this study, phagocytosis was monitor by pH-sensitive fluorescence dye pHrodo green, engulfment and formation of phagolysosome were not analyzed. Macrophage response studied during this Ph.D. study was correlated with any clinical parameters including lung function (FEV1), infection status, medication taken by patients before venous blood collection.

Although $CFTR_{Inh}$-172 and GlyH-101 treated macrophages displayed similar phenotypes with differentiated macrophages from CF monocytes, the detailed mechanism of dysfunctional or inhibited CFTR and repressed M2 polarization was not elucidated in the thesis. No comparison was conducted among patients those carry ΔF508 homozygous and heterozygous mutations or had intermittent and chronic infection with *P. aeruginosa.* Due to insufficient cells numbers, expression of M1 signature gene, Cox-2 was not able to analyze in M0 cells from APE patients with CF. Furthermore, monocytes from CF patients were not thoroughly studied. It is possible that CFTR-dependent or independent defects may already be concealed in monocytes of CF patients.

6.3 Future Directions

There are huge opportunities for conducting further research using the macrophage models described in ***Chapter 3*** and ***Chapter 4***. Since polarized status of macrophages dictates the nature and duration of inflammation and contributing to disease severity, the human macrophage polarization and characterization model described in ***Chapter 3*** can be used as the foundation for further investigating the roles of polarized macrophages in fibrotic diseases including asthma, atherosclerosis, renal fibrosis as well as obesity and during pregnancy.

A prospective and longitudinal study can be undertaken to study macrophage polarization and function during APE prior to treatment, following treatment and at routine follow-up and find the association of macrophage responses with clinical outcomes. Such study would include the following aims:

1) Investigating the role of CFTR genotypes (ΔF508 homozygous vs heterozygous vs G551D) is an excellent opportunity to study how CFTR genotype effect(s) would effect on macrophage functions.
2) In murine model, the role of MMP28 has been shown to be associated with M2 polarization (325). Expression of MMP28 on monocytes was analyzed in this study. It would be worthy to analyze expression of MMP28 on monocytes from CF patients. Such analysis should be performed during APE and clinical stable condition of CF. The data might put a light on the underlying mechanism of reduced M2 polarization in CF.
3) Restoration of functional CFTR on macrophages should be tested following tobramycin or other antibiotic administration.
4) With the CF macrophage model, it is possible to investigate the effect of potentiators/correctors (Ivacaftor/Lumacaftor) on macrophage responses and whether these mutation-specific drugs are able to alter the ability of monocytes to differentiate and polarize into M2 macrophages and correlate clinically.
5) Pathway analysis of next generation sequencing data is an advanced tool in biomedical research (326). It would be extremely worthy to use this recently developed tool to identify the molecular defects in CF M0, M1 and M2 macrophages. Such analysis would also explore the underlying mechanism of expression of CD80 on M0 and M2 macrophages during APE, but not in clinically stable condition of CF. Similar analysis on CFTR inhibited M0, M1 and M2 cells

would not only validate the model but also allow researchers to study macrophage-related CF inflammation without tissue samples from patients with CF.

6) Macrophage responses should be correlated with lung function of patients with CF.
7) CF macrophage data reported in ***Chapter 4*** should be validated by the most pertinent macrophages, i.e. sputum macrophages. Phenotype of macrophages from sputum of HC and CF patients should be studied. Analyzing the sputum macrophages from CF and non-CF patients would reveal intriguing information regarding their involvement in the lung inflammation during CF.

It is also possible to develop CF AEC model, using CFTR inhibitors, $CFTR_{Inh}$-172 and GlyH-101.

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Appendices

Following items were attached:

1. Manuscript version of Chapter 4 which was submitted to the New England Journal of Medicine was added here.