Granulocyte colony-stimulating factor: Its role in gut-homing macrophage generation and colitis, and production by probiotics

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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(Thesis format: Integrated Article)

by

Shahab Meshkibaf

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The pleiotropic cytokine granulocyte-colony stimulatory factor (G-CSF) is mainly required for the generation of neutrophils, but its role in macrophage generation has also been reported. In addition, G-CSF is effective for the down-regulation of inflammatory cytokines and ameliorating gut disorders, such as colitis. However, the G-CSF function in macrophage generation and gut immunity remains unclear. The first focus of this thesis was to assess the role of G-CSF in macrophage generation and its contribution to gut immunity. G-CSF was found to promote the generation of Gr-1high/F4/80+ macrophages in macrophage (M)-CSF-treated bone marrow cells, most likely through suppressing cell death. Gr-1high macrophages showed anti-inflammatory/regulatory macrophage (M2)-like cytokine and surface marker profiles. G-CSF receptor deficient (G-CSFR−/−) mice harbored less gut macrophages, but had a similar number of neutrophils in the gut. In addition, adoptive transfer of G-CSF-treated bone marrow-derived macrophages (G-BMDM) showed a dominant gut-homing phenotype. G-CSFR−/− mice were also more susceptible to dextran sulfate sodium (DSS)-induced colitis than wild-type mice and adoptive transfer of G-BMDM protected these mice from DSS-induced colitis. The second focus of this thesis was to explore the signaling mechanism(s) controlling the preferential G-CSF production over inflammatory cytokines in probiotic bacteria-exposed BMDM. Lactobacillus rhamnosus GR-1 (GR-1) renders several immunomodulatory effects, at least in part, through preferentially inducing G-CSF in macrophages. However, the mechanism(s) by which GR-1 induces preferential G-CSF production in macrophages is still unknown. Among 84 genes tested, G-CSF was the cytokine induced at highest levels in GR-1-treated BMDM, but the induction of inflammatory cytokines, such as TNF-α, was minimal. The signaling pathway of GR-1-preferential G-CSF production was TLR2-, NF-κB-, ERKs- and PI3K/Akt-dependent. A secreted protein-like molecule(s) was found to be responsible for GR-1-preferential G-CSF production.

Collectively, these results demonstrated the immune regulatory function of G-CSF on macrophages in gut immunity and a potential mechanism of action of certain probiotics.
Keywords

Granulocyte colony-stimulating factor, macrophages, cytokines, gut homeostasis, colitis, probiotics
Co-Authorship Statement

In regards to the work presented in Chapter 3:


S. Meshkibaf designed and performed experiments, analyzed data, and wrote the manuscript. M. W. Gower contributed the data shown in figures 3.1 (C-D), 3.2, 3.4, and 3.9. G. A. Dekaban provided key reagents and helped to write the manuscript. S. O. Kim conceived the study, secured funding, designed and supervised experiments, analyzed data, and wrote the manuscript.

In regards to the work presented in Chapter 4:


S. Meshkibaf designed and performed experiments, analyzed data, and wrote the manuscript. A. J. Martins contributed the data shown in figures 4.1 and 4.2 (B-C). G. Henry contributed the data shown in figures 4.1A, 4.5 (A-B) and 4.6B. Dr. I. Welch provided expert assistance in histological assessment leading to the scoring presented in Figure 4.1 (B-C). All other experimental work presented in this chapter was performed by S. Meshkibaf. S. O. Kim conceived the study, secured funding, designed and supervised experiments, analyzed data, and wrote the manuscript.

In regards to the work presented in Chapter 5:

**S. Meshkibaf**, Marcelo Gottschalk and Sung O. Kim. Preferential production of G-CSF by a protein-like *Lactobacillus rhamnosus* GR-1 secretory factor through activating TLR2-dependent signaling events without activation of JNKs.

S. Meshkibaf designed and performed experiments, analyzed data, and wrote the
manuscript. H. I. Sheikh contributed the data shown in figure 5.3A, 5.4A and 5.5B. Dr. Marcelo Gottschalk provided bones from Toll-like receptor 2 deficient mice in Figure 5.4 (A). All other experimental work presented in this chapter was performed by S. Meshkibaf. S. O. Kim conceived the study, secured funding, designed and supervised experiments, analyzed data, and wrote the manuscript.
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Abbreviations

- Deficient
- ACAID Anterior Chamber Associated Immune Deviation
- ADAM17 A disintegrin and metalloproteinase domain-containing protein 17
- Akt Protein kinase B
- ANOVA Analysis of variance
- AP-1 Activation protein 1
- APC Antigen presenting cell
- ArgI Arginase type I
- ATF Activating transcription factor
- BHI Brain-Heart Infusion Broth
- BMCs Bone marrow cells
- BMDM Bone marrow-derived macrophages/M-CSF derived bone marrow-derived macrophages
- CCL C-C motif ligand
- CCR C-C chemokine receptor
- CD Cluster of differentiation
- CD Crohn’s disease
- C/EBP CCAAT/enhancer binding protein
- CFU Colony forming unit
- CLR C-type lectin receptor
- CMPs Common myeloid progenitor cells
- CNT Control
- cRPMI Complete RPMI1640
- CSF Colony stimulating factor
- CXCL C-X-C motif ligand
- CX3CR1 CX3C chemokine receptor 1
- DC Dendritic cell
- DSS Dextran sulfate sodium
- ECM Extra cellular matrix
- *E. coli* *Escherichia coli*
- EGF Epidermal growth factor
- EGFP Enhanced GFP
- ELISA Enzyme-linked immunosorbent assay
- ERKs Extracellular signal regulated kinases
- ETS E26 transformation-specific
- FACS Flow cytometry-activated cell sorting
- FBP Fibrinogen binding protein
- FBS Fetal bovine serum
- FIZZ1 Found in inflammatory zone 1
- FOXO1 Forkhead box protein O 1
- FoxP3 Forkhead box P3
- Gaddβ Growth arrest and DNA-damage-inducible, beta
- GALT Gut-associated lymphoid tissue
<table>
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<td>G-BMDM</td>
<td>G-CSF-treated BMDM</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>G-CSFR</td>
<td>G-CSF receptor</td>
</tr>
<tr>
<td>GI tract</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GM-BMDM</td>
<td>GM-CSF-treated bone marrow-derived macrophages</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony-stimulating factor</td>
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<td>GM-CSFR</td>
<td>GM-CSF receptor</td>
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<td>GMPS</td>
<td>Granulocyte-macrophage precursors</td>
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<td>G protein–coupled receptors</td>
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<td>GR-1</td>
<td><em>Lactobacillus rhamosus</em> GR-1</td>
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<td>Gr-1</td>
<td>Granulocyte receptor 1</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HB</td>
<td>Heparin binding</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IECs</td>
<td>Intestinal epithelial cells</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IRAK</td>
<td>IL-1 receptor-associated kinase</td>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNKs</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LGG</td>
<td><em>Lactobacillus rhamosus</em> GG</td>
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<tr>
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<td>Lipoteichoic acid</td>
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<tr>
<td>Ly6</td>
<td>Lymphocyte antigen 6</td>
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<td>Classically activated macrophages</td>
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<td>M2</td>
<td>Alternatively activated macrophages</td>
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<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MafB</td>
<td>V-maf musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP</td>
<td>MAPK-activated protein kinase</td>
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<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>M-CSFR</td>
<td>M-CSF receptor</td>
</tr>
<tr>
<td>MDPs</td>
<td>Macrophage/DC progenitors</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase</td>
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<tr>
<td>M KK</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
</tbody>
</table>
MHCII  Major histocompatibility complex-class II
MLNs  Mesenteric lymph nodes
MPO  Myeloperoxidase
MRS  deMan, Rogosa and Sharpe
MSK  Mitogen- stress-activated kinase
MTT  3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MyD88  Myeloid differentiation primary response gene 88
NF-κB  Nuclear factor kappa B
NLR  Nod-like receptor
NO  Nitric oxide
NOD  Nucleotide-binding oligomerization domain
Pam3CSK4  Triacylated lipopeptides
MAMPs  Microbial-associated molecular patterns
PBS  Phosphate buffered saline
PDL2  Programmed cell death 1 ligand 2
PI3Ks  Phosphoinositide 3-Kinases
PIP2  Phosphatidylinositol 3,4,5-biphosphate
PIP3  Phosphatidylinositol 3,4,5-triphosphate
PPs  Payer’s Patches
PRRs  Pattern recognition receptors
PU.1  Purine-rich nucleic acid binding protein 1
qRT-PCR  Quantitative real-time polymerase chain reaction
RIP1  Receptor-interacting protein 1
RLR  Rig-I-like receptor
RTKs  Receptor tyrosine kinases
SCA-1  Stem cell antigen-1
SCS  Spent culture supernatant
STAT  Signal transducer and activator of transcription
TAB  Transforming growth factor-β activated kinase-1-binding protein 1
TAK1  Transforming growth factor-β activated kinase 1
TGF-β  Transforming growth factor-β
Th  T helper
TIR  Toll-interleukin 1 receptor
TLR  Toll-like receptor
TNBS  Trinitrobenzene sulphonic acid
TNF-α  Tumor necrosis factor-α
TNFR  Tumor necrosis factor receptor
Tpl2  Tumor progression locus 2
TRAF  TNFR-associated factor
TRIF  TIR-domain containing adaptor-inducing interferon-β
UC  Ulcerative colitis
WT  Wild-type
XIAP  X-linked inhibitor of apoptosis protein
Ym1  Chitinase-like
1.1 THE IMMUNE SYSTEM

The main and crucial function of the immune system is to prevent or limit microbial infections using an interactive network of organs and hematopoietic cells. The immune system consists of several organs and cell types that monitor foreign antigens, as well as abnormal tissues or cells, and function to eliminate those that are considered a health threat [1]. The immune system is divided into two arms: the innate immune system and the adaptive immune system.

The innate immune system is evolutionary ancient and consists of cells, such as macrophages, that respond rapidly to pathogens through recognizing the presence of conserved constituents of microorganisms. This pivotal subsystem of the body’s immune system comprises cells known as leukocytes that include natural killer cells, mast cells, eosinophils, basophils, neutrophils, dendritic cells (DCs) and macrophages. The innate immune system provides the first line of defense against infections and also initiates the development of an adaptive immune response. Cytokines play an important role in such innate immune response and those involved in this process are mainly produced by macrophages. In fact, macrophages regulate responses in both arms of immunity by participating in the anti-infectious function, antigen presentation and co-activation of lymphocytes that all employ cytokine release [2]. Macrophages, as a major source of many cytokines, are therefore important immune cells in shaping overall immune responses. In contrast, the adaptive immune system contains B and T lymphocytes and is more evolutionary recent and can provide lifelong immunity to the host, but this is not immediate [1, 3, 4]. The combined effects of innate and adaptive immune systems represent the main defense mechanism of the host against infectious agents [3].

Cells of both innate and adaptive arms of the immune system communicate with each other through cytokines, soluble mediators and cell surface ligands, to shape proper immune responses. To date, various types of cytokines have been found, including interleukins (ILs), chemokines, and growth factors (such as colony-stimulating factors; CSFs). ILs, which are mainly produced by immune cells, act on immune cells to regulate their function; however, chemokines, which are produced by many
different cell types, are involved in recruitment or movement of immune cells [5]. Growth factors stimulate the survival or development of lineage-specific precursor or differentiated cells. The effects of cytokines are complex, as each cytokine by itself or in combined with other cytokines has different functions on target cells. Abnormal production of cytokines has shown to be associated with various inflammatory diseases, such as inflammatory bowel diseases (IBD) [6].

1.2 MACROPHAGES

Macrophages, as key innate immune cells, fulfill a wide range of tissue- and organ-specific functions, including developmental, metabolic and inflammatory actions [7, 8]. These cells represent the mononuclear phagocytic system (MPS), which also comprises circulating monocytes, DCs and bone marrow progenitor cells [9].

Macrophages are distributed through all body tissues and acquire specific phenotypes to fulfill tissue-specific functions in response to the tissue cytokine milieu. They are located in the gut (lamina propria macrophages), lymph nodes, spleen, liver (Kupffer cells), kidney (mesangial cells), lung (alveolar macrophages), eye (intraocular macrophages), connective tissues (histocytes), serous cavities (pleural and peritoneum macrophages), bone (osteoclast), synovium, skin, and central nervous system (microglia) [10].

1.2.1 Macrophage Origin and Development

In adults, tissue macrophage populations are maintained by self-renewal of local precursor cells, which initially arise from the yolk sac and/or fetal liver progenitors in development, and recruitment of circulating monocytes, which are originated from common myeloid progenitors in the bone marrow [9, 11, 12]. In the bone marrow, hematopoietic stem cells (HSCs) give rise to the multipotent common myeloid progenitor cells (CMPs; defined by surface marker expression: cluster of differentiation (CD) 34+ and stem cell antigen-1 (SCA-1-)) [13, 14], granulocyte-macrophage precursors (GMPs; CD34+, CD16+ and CD32+) [13], and macrophage/DC progenitors (MDPs; CD115+, CX3C chemokine receptor 1
(CX3CR1)^+ and CD135^+). MDPs further develop to monocytes/macrophages, conventional/monocyte-derived DCs or plasmacytoid DCs without monocytic intermediates [15]. Recent studies also showed that MDPs give rise to a new subset of cells known as common monocyte progenitors (cMoPs; CD115^+, CX3CR1^+ and Ly6C^+), which differentiate to Ly6C^{high} monocytes (CD115^+, CX3CR1^{low}, C-C chemokine receptor type 2 (CCR2)^{high} and CD62L^{high}) upon leaving bone marrow and then to Ly6C^{low} monocytes (CD115^+, CX3CR1^{high}, and CCR2^{low} and CD62L^{low}) after arriving local tissues or organs and differentiating to macrophages [16].

Macrophage lineage survives, proliferates and differentiates under the influence of CSFs, mainly macrophage-CSF (M-CSF) and granulocyte-macrophage-CSF (GM-CSF), which regulate expression of transcription factors that are important in monopoiesis.

Purine-rich nucleic acid binding protein 1 (PU.1) transcription factor belongs to the E26 transformation-specific (ETS) transcription factor family and is involved in the development of myeloid lineages [17]. Different expression levels of PU.1 in progenitor cells were shown to direct such development; where high levels of PU.1 expression favor DC development and moderate levels of PU.1 expression promote macrophage over neutrophil development [18, 19]. One of the mechanisms underlying such a graded response to PU.1 expression levels is its competitive interaction with other transcription factors such as CCAAT/enhancer binding protein (C/EBP) α. C/EBPα is crucial for neutrophil development and competes with the PU.1 macrophage promoting activity. Indeed, the ratio of PU.1 to C/EBPα expression levels directs macrophage or neutrophil development [19-21]. M-CSF and GM-CSF both have been shown to induce PU.1 and therefore macrophage development [22, 23]. M-CSF also induces other transcription factors such as interferon regulatory factor (IRF) 8, which is also important in the development of monocytes/macrophages [24, 25]. In contrast, granulocyte-CSF (G-CSF) has been shown to induce the expression of C/EBPα and growth factor independent (GFi) 1 transcription factors, favoring neutrophil development [19, 26]. Overall, these observations demonstrated that CSFs induce interaction of a pool of transcription
factors, where expression levels dictate lineage commitment and subset specification.

1.2.2 Macrophage Cell-Surface Markers

Monocytes/macrophages express several specialized cell-surface molecules at different stages of development and activation states. These molecules, including CD11b, F4/80, CD11c, Ly6G, and Ly6C are often used as markers to distinguish monocytes/macrophages from other cell types [10]. Of note, the expression levels of these macrophage markers may differ among various tissues. Therefore, combinations of these markers should be used to identify macrophages in different stages of development and activation, and in different tissues.

F4/80

F4/80 glycoprotein, a member of the epidermal growth factor (EGF)-seven transmembrane (EGF-TM7) family, is a well-known specific cell-surface marker for murine macrophages [27, 28]. The human homologue is known as EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1). F4/80 is less expressed in monocytes than most tissue macrophages, including intestinal macrophages, Kupffer cells, red pulp macrophages of the spleen, epithelial macrophages, endothelial macrophages, microglia cells and Langerhans cells [29, 30]. However, expression of F4/80 in alveolar macrophages and myeloid DC is low, and absent in osteoclasts [29]. Expression of F4/80 was also observed on eosinophils [31], but not in neutrophils. Therefore, the presence of F4/80 has been used to distinguish macrophages from neutrophils. Interferon (IFN)-γ has been shown to down-regulate F4/80 expression [32]. GM-CSF driven macrophages, in contrast to their M-CSF driven counterparts, also showed reduced F4/80 expression [33, 34].

Studies with F4/80 deficient mice have shown that F4/80 plays an important role in peripheral immune tolerance [35, 36]. These studies demonstrated that F4/80 deficient mice fail to induce antigen-specific efferent regulatory T cells and result in Anterior Chamber Associated Immune Deviation (ACAID). To date, functions of F4/80 are not yet fully understood and await further characterization.
CD11b and CD11c

CD11b, also termed macrophage-1 antigen (Mac-1) or complement receptor type 3 (CR3), is a heterodimeric integrin comprised of an integrin αM (CD11b) subunit and a common integrin β2 (CD18) subunit. CD11b is found on leukocytes, including monocytes/macrophages, granulocytes such as neutrophils and natural killer cells (NKs) [37, 38]. CD11b binds to endogenous ligands, such as extracellular matrix (ECM) proteins [39], intercellular adhesion molecule (ICAM)-1 receptor [40] and complement C3bi factor deposited on the microbial surface [41]. It also binds to exogenous microbial and fungal ligands, such as lipopolysaccharide (LPS), mannose and zymosan [42, 43]. CD11b mediates adhesion, migration, chemotaxis, cellular activation in coordination with other receptors such as CD14, and phagocytosis during immune responses [38, 40].

Similarly, CD11c, also known as CR4, consists of a CD11c subunit and a CD18 subunit [44]. Different cell types express CD11c, including DCs, monocytes/macrophages, granulocytes (at lower level) and some lymphocytes [45]. Similar to CD11b, CD11c is involved in phagocytosis [46], adhesion and migration of cells [46, 47] as well as binding of cells to C3bi factor [48] and fibrinogen [49].

Ly6C and Ly6G

The lymphocyte antigen 6 (Ly6) family of murine proteins are tethered to the cell membrane via a C-terminal Glycophosphatidylinositol (GPI) anchor [50]. Ly6C is expressed in monocytes/macrophages, neutrophils, subsets of CD4+ and CD8+ lymphocytes, and endothelial cells [51]. Ly6C is involved in co-stimulatory activation of T cells [52] and endothelial adhesion of leukocytes [53]. Recently, it was observed that monocytes released from mice bone marrow into peripheral blood express high levels of Ly6C, which gradually decrease [54, 55]. In a steady state, Ly6C– monocytes migrate to different tissues and give rise to tissue-resident macrophages or DCs with anti-inflammatory characteristics [55]. However, under inflammatory conditions, Ly6C+ monocytes are recruited to inflammatory sites and differentiate into macrophages or DCs [55-57]. The murine blood ratio of Ly6C– to Ly6C+ monocytes
in a steady state is 4:6; however, inflammatory conditions favor Ly6C⁺ monocytes [55, 58, 59]. In human, CD14⁺/CD16⁺ monocytes correspond to mouse Ly6C⁻ monocytes and CD14⁺[high]/CD16⁻ monocytes correspond to mouse Ly6C⁺ monocytes [60].

Ly6G, also known as granulocyte receptor 1 (Gr-1), is another member of Ly6 family expressed on granulocytes, mainly neutrophils [61, 62]. Gr-1 is a specific marker for distinguishing neutrophils from other leukocytes; however, its function is poorly understood. It has been speculated that Gr-1 is involved in myeloid expansion [63] and neutrophil migration [64]. Gr-1 associates with CD11a and CD11b and involves in adhesion and crawling of neutrophils, respectively [64, 65], leading to optimal emigration of neutrophils out of the vasculature [65]. However, migratory effects of Gr-1 are controversial, as others have shown no migratory effect of Gr-1 on neutrophils [66].

1.2.3 Macrophage Activation States

Signals from cytokines, microbial components and damaged tissues can affect polarization of monocytes/macrophages towards one of the two distinct macrophage activation states: classically activated macrophages (M1) and alternatively activated macrophages (M2) [67]. This classification of macrophages mirrors the T helper 1 (Th1) (cell-mediated immunity) and Th2 (humoral immunity) polarization; where Th1 cells induce bactericidal activity of macrophages by producing IFN-γ and Th2 cells induce alternative states of activation through producing cytokines such as IL-4 [60, 68].

M1 macrophages are mainly induced by the Th1 cytokine IFN-γ with or without microbial stimuli, such as LPS [67, 69]. Other cytokines such as tumor necrosis factor (TNF)-α and GM-CSF can also polarize macrophages towards the M1 phenotype [70-72]. These macrophages are characterized by high production of IL-12, IL-23, IL-1β, TNF-α, IL-6, CXCL9 and CXCL10, and low production of IL-10. M1 macrophages also efficiently produce toxic intermediates, such as nitric oxide (NO) and reactive oxygen intermediates (ROI), and have high capacity of antigen presentation by the
expression of co-stimulatory molecules, such as CD80 and CD86 (Table 1.1) [69, 72, 73]. M2 macrophages are further divided into three subsets, M2a, M2b and M2c, based on their stimuli and subsequent gene expression profiles. The M2a subset is induced by IL-4 or IL-13; however, the M2b subset is induced by immune complexes in combination with TLR/IL-1R ligands, such as LPS. The M2c subset is induced by IL-10, transforming growth factor (TGF)-β or glucocorticoid hormones [72, 74]. Cytokines such as M-CSF and chemokines such as C-C chemokine ligand (CCL) 2 can also polarize macrophages towards the M2 phenotype [70, 71, 75]. M2 macrophages are generally characterized by high production of IL-10, low production of IL-12 and IL-23, less efficient in production of TNF-α, and high expression levels of scavenger receptors (CD163), transferrin receptor (CD71), mannose receptors (CD206), and programmed cell death 1 ligand 2 (PDL2), Chitinase-3-like protein 3 (Ym1), found in inflammatory zone 1 (FIZZ1), TGF-β, chemokines such as CCL17, CCL22, CCL24, and shifted arginine metabolism to ornithine and polyamines (Table 1.1) [69]. M2 macrophages are mainly involved in immune suppression and tissue remodeling [72].

1.2.4 Macrophage Pattern Recognition Receptors

Macrophages express pattern recognition receptors (PRRs): a group of innate immune receptors involved in sensing unique microbial molecules, termed microbial-associated molecular patterns (MAMPs) and host-derived signals from damaged cells, known as danger-associated molecular patterns (DAMPs). Receptors in this family include membrane-associated receptors such as toll-like receptors (TLRs) and cytoplasmic receptors such as the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [76].

To date, more than 10 TLRs (13 in mice) and 23 NLRs have been identified in humans. All TLRs are plasma membrane-associated receptors, except TLR3, TLR7, TLR8 and TLR9 which are endosomal membrane-associated. In addition, all TLRs except TLR10 have at least one known MAMP ligand, including double-strand RNA (TLR3), LPS (TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), and bacterial/viral unmethylated CpG DNA (TLR9). All TLRs homodimerize or
Table 1.1. Characteristics for M1 and M2 macrophage populations
<table>
<thead>
<tr>
<th>Macrophage</th>
<th>M1</th>
<th>M2a</th>
<th>M2b</th>
<th>M2c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stimulation</strong></td>
<td>IFNγ/LPS TNF-α</td>
<td>IL-4/IL-13</td>
<td>ICs/TLR or IL-1R ligands</td>
<td>IL-10 TGF-β GHs</td>
</tr>
<tr>
<td><strong>Expression</strong></td>
<td>CD80 CD86 MHCII iNOS CCR7</td>
<td>CD23 CD71 CD163 CD206 YM1 FIZZ1 Arginase I PDL2 CXCR1 CXCR2 MHCII Decoy IL-1RII IL-1RA</td>
<td>CD80 CD86 Arginase I MHCII</td>
<td>CD163 CD206 Arginase I CCR2 CD150</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td>TNF-α IL-1β IL-12 high IL-23 IL-6 IL-10 low</td>
<td>IL-10 TGF-β</td>
<td>IL-1 IL-6 IL-10 high TNF-α IL-12 low</td>
<td>IL-10 TGF-β</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td>CCL2 CCL5 CCL3 CCL9 CCL10 CXCL8 CXCL9 CXCL10 CXCL11 CXCL16</td>
<td>CCL17 CCL18 CCL22 CCL24</td>
<td>CCL1</td>
<td>CCL16 CCL18 CXCL13</td>
</tr>
</tbody>
</table>

IFNγ, interferon γ; LPS, lipopolysaccharide; IL, interleukin; IL-1R, IL-1 receptor; ICs, immune complexes; TGF-β transforming growth factor β; TLR, Toll-like receptor; GHs, glucocorticoid hormones; CD, cluster of differentiation; YM1, Chitinase-3-like protein 3; FIZZ1, found in inflammatory zone 1; PDL2, programmed cell death 1 ligand 2; CCR, C-C chemokine receptor; CXCR, C-X-C chemokine receptor; MHCII, major histocompatibility complex class II; iNOS, inducible nitric oxide synthase; Decoy IL-1RII, type II decoy receptor of IL-1; IL-1RA, IL-1 receptor antagonist; TNF-α, tumor necrosis factor α; CCL, chemokine (C-C motif) ligand; CXCL, C-X-C motif ligand
heterodimerize upon binding to their ligands. TLR2 heterodimerizes with TLR6 by diacylated lipopeptides (Pam$_2$CSK$_4$), zymosan and lipoteichoic acid (LTA) and with TLR1 by triacylated lipopeptides (Pam$_3$CSK$_4$) and lipoarabinomannan [77-81]. Among NLRs, NOD1 and 2 recognize the units of peptidoglycan cell wall components, diaminopimelic acids and muramyl dipeptide, respectively [82, 83].

1.2.4.1 Signaling Pathways of TLRs

TLRs, as type I transmembrane proteins, contain an ectodomain with leucin-rich repeats, a transmembrane domain, and an intracellular Toll-interleukin 1 receptor (TIR) domain [84]. Ligation of TLRs by their respective ligands leads to the activation of downstream signaling pathways that have been divided into two major distinct pathways depending on the TIR-domain-containing cytoplasmic adaptor proteins: myeloid differentiation primary response gene 88 (MyD88)-dependent and TIR-domain containing adaptor-inducing interferon-β (TRIF)-dependent pathways. [85]. MyD88 is the main adaptor molecule for all TLRs with the exception of TLR3, which exclusively utilizes TRIF, and TLR4, which uses both MyD88 and TRIF [85, 86]. Triggering TLRs by MAMPs induces an engagement of TIR domains of TLRs with TIR domain of MyD88 or MyD88-adaptor-like protein (MAL), resulting in the recruitment of interleukin (IL)-1 receptor-associated kinases (IRAKs) and subsequent interaction with tumor necrosis factor receptor (TNFR)-associated factor (TRAF) 6. This leads to the activation of the transforming growth factor β-activated kinase 1 (TAK1). TAK1 activation in turn couples to the nuclear factor kappa-light-chain-enhancer of B cells (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. The other pathway that is activated upon the activation of TLRs is the phosphoinositide 3-Kinases (PI3Ks) pathway [85, 87].

The NF-κB signaling pathway is an important signaling cascade for cell proliferation, differentiation and survival, as well as expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12 p40 [88-90]. The NF-κB family includes RelA (p65), RelB, c-Rel, P50, and P52 members that are capable of binding to DNA as well as inhibitor of NF-κB (IκB) [91]. Upon activation, TAK1 phosphorylates and activates the inhibitor kappa B kinase (IKK) complex that comprise IKKa, IKKβ and
IKKγ (also known as NEMO). Activated IKK phosphorylates IκB, resulting in proteasome-mediated degradation and translocation of NF-κB complexes, predominantly the P50/RelA dimer, to the nucleus [85, 87].

The MAPKs are a family of protein kinases involve in diverse cellular functions, such as cytokine production and cell survival. The MAPK family comprises p38 MAPK, extracellular signal regulated protein kinases (ERKs), and c-Jun N-terminal kinases (JNKs). Activation of MAPKs is mediated by two families of upstream protein kinases: the MAP kinase kinase kinase (MKKK) and MAP kinase kinase (MKK). Each MAPK is activated by a specific MKK: MKK 3/6 activates p38 MAPK, MKK 1/2 activates ERKs and MKK4/7 activates JNKs. Phosphorylation of MAPKs leads to activation of transcription of their effector genes [92].

The p38 MAPK is strongly activated by environmental stressors, cytokines and other receptors [93-96]. There are four members of p38 MAPK family: α, β, γ, and δ. p38α is ubiquitously expressed in most cells including macrophages, whereas p38β, p38γ and p38δ are expressed in brain, skeletal muscle and endocrine glands, respectively [97]. TAK1 and apoptosis signal-regulating kinase 1 (ASK1) phosphorylate MKK3 and MKK6 which are upstream kinases for p38 MAPK. Activation of p38 MAPKs then leads to the activation of downstream protein kinases such as MAPK-activated protein kinase (MAPKAP) 2/3, mitogen- and stress-activated kinase (MSK) 1/2, as well as transcription factors such as activating transcription factor (ATF) 1/2/6, C/EBPβ, and P53 [93, 97]. In macrophages, the activation of p38α MAPK has been associated with expression of key inflammatory cytokines such as TNF-α, IL-6 and IL-1β [98, 99], and inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) 2 [100]. p38α MAPK also plays a central role in cell survival [101] of osteoclasts [102] and chemotactic-induced macrophage migration [103]. Altogether, these findings suggest that p38 MAPK is essential for inflammatory cytokine production and many other cellular processes.

The ERKs, comprising ERK1, ERK2, ERK3/4, ERK5 and ERK7, are expressed ubiquitously and activated by a variety of extracellular agents. Following TLRs
activation, the tumor progression locus 2 (Tpl2) complex, containing NF-κB subunit precursor protein p105, A20-binding inhibitor of NF-κB activation (ABIN) 2 and Tpl2, becomes activated through phosphorylation of the p105 inhibitory subunit by TAK1-dependent activation of IKKβ of IKK complex [104, 105]. Consequently, the Tpl2 is released from the complex and activates MKK1/2 leading to activation of ERKs [86, 106]. Following ERKs activation, MSKs, subfamilies of ribosomal protein S6 kinases (RSKs), MAPK-interacting kinases (MNKs) and other cytoplasmic and nuclear substrates are activated [86]. Activation of MSKs induces expression of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1RA) protein [107-109] and expression of the dual-specificity protein phosphatase (DUSP) 1/2/4, which in turn, limits MAPKs activation [86]. Overall, ERKs are involved in anti-inflammatory cytokine production and limiting inflammation. ERKs activity have also been implicated as a crucial mediator of cell proliferation [110] and myeloid cell differentiation [111].

The JNK family comprises JNK1, JNK2 and JNK3. Of these, JNK1 and JNK2 are ubiquitously expressed while JNK3 is largely restricted to the brain, heart and testis [112, 113]. JNKs are activated in response to a wide variety of stimuli including cytokines, ultra violet irradiation, growth factors, DNA-damaging chemicals and other receptors such as TLRs [114-118]. JNKs activate a large number of transcription factors including ETS domain-containing protein Elk-1/3 (Elk1/3) and AP-1 complex (composed of c-Fos, c-Jun, ATF and Jun dimerization partner), and involved in the expression of various pro-inflammatory cytokines [119]. JNKs are also implicated in apoptosis of macrophages and inhibition of M-CSF-dependent differentiation of bone marrow cells (BMCs) into macrophages [120]. Other studies have also shown that JNK2 is required for TNF-α production [121] and phagocytosis [122] in macrophages.

The PI3Ks are lipid kinases that mediate conversion of phosphatidylinositol 4,5-biphosphate (PIP₂), which is normally located in the inner leaflet of the plasma membrane, to phosphatidylinositol 3,4,5-triphosphate (PIP₃) [123]. The dephosphorylation of PIP₃ is mediated by the lipid phosphatase activity of
phosphatase and tensin homolog (PTEN), which is a negative regulator of PI3K signaling [124]. PI3Ks are divided into three subclasses: I (A and B), II, and III. Class IA subtypes consist of a catalytic subunit (p110α, p110β, or p110δ) and a regulatory subunit (p85α or p85β). Class IB consists of p110γ, which associates with either p87 or p101 regulatory subunit [85]. Although p110α and p110β are expressed ubiquitously, p110δ and p110γ are primarily expressed in leukocytes [125, 126]. However, p110δ is found as the major enzyme involved in the production of PIP₃ in myeloid cells [127].

The signaling mechanism involved in PI3K activation by TLRs is not well understood. Several studies indicate that MyD88 [128], MAL [129], TRIF [130, 131] and scaffold protein B-cell adaptor for PI3K (BCAP) [132] can induce PI3K. Once PI3K is activated, it mediates conversion of PIP₂ to PIP₃. PI3K production of PIP₃ leads to the activation of downstream signaling molecules such as protein kinase B (PKB), also known as Akt through the phosphatidylinositol-dependent kinases (PDK) 1/2 [133]. Akt1 is mainly involved in suppressing the production of inflammatory cytokines, while Akt2 has been reported to increase inflammatory functions in macrophages [134]. Activated Akt1 phosphorylates substrates including the forkhead box protein O 1 (FOXO1) [135] and glycogen synthase kinase 3 (GSK3α/β) [136], which result in inactivation of these molecules. Production of inflammatory cytokines is suppressed due to the inhibition of FOXO1 activity, while the production of anti-inflammatory cytokines, such as IL-10, is increased by enhanced cAMP response element-binding protein (CREB) function as a result of GSK3α/β inhibition [135, 136].

1.3 GUT IMMUNITY

The gastrointestinal (GI) tract, which is the largest organ in the body, has a dual function: nutrition uptake and maintaining immune homeostasis. The GI tract is constantly in contact with microorganisms and food antigens, and the presence of food, moisture and appropriate temperature makes the GI tract a favorable habitat for a diverse array of microorganisms. Therefore, mounting proper immune responses that prevent microbial invasion and maintain immune tolerance is crucial. Both the
innate and adaptive immune systems contribute to the development of a balanced and regulated host immune system, and any disturbance to this balance may negatively impact on the host’s health [137].

The GI tract microbiota, itself, plays also a central role in the host’s protection. They out-compete pathogenic bacteria via competition for binding sites, and production of antimicrobial compounds and volatile fatty acids [138, 139]. Also, their effects on intestinal structure and contributions to the development of a balanced and regulated host immune system have been reported [140, 141]. In addition, they provide the host with essential nutrients, including short chain fatty acids, amino acids, and vitamins [138].

1.3.1 The Structure of the Gut Immune System

The GI tract is a dynamic ecosystem that is a habitat for approximately $10^{14}$ microbes, which outnumber the total number of body tissue cells by a factor of ten [142]. The GI tract microbiota is separated from the host’s gut associated lymphoid tissue (GALT), the primary tissue involved in GI tract immunity, by intestinal epithelial cells (IECs). IECs consist of absorptive columnar epithelial cells with physical barrier function and microfold cells (M cells; possessing a unique antigen uptake function), as well as secretory cells, such as goblet cells (producing mucin, trefoil etc.), Paneth cells (producing antimicrobial factors such as α-defensin) and enteroendocrine cells (producing hormones and neuropeptides such as serotonin and secretin). The IECs act as a highly selective barrier, providing a degree of permeability for optimal nutrient and fluid uptake, while preventing translocation of microorganisms from the gut lumen [137]. The mucus layer above the IECs provides a higher degree of protection by inhibiting overgrowth of microbiota and potential pathogens using acidic pH, enzymes, such as lysozyme, secretory anti-microbial peptides, such as mucins and trefoil and immunoglobulins, such as IgA and IgM [137, 143, 144].

The lamina propria is a layer of connective tissue that lies beneath the epithelial monolayer, and contains diffused mucosa-associated lymphoid tissue (MALT) that is densely populated by macrophages, DCs, granulocytes such as neutrophils, mast cells
and lymphocytes. This layer also contains organized GALT and mucosal lymphoid follicles, including the Payer’s Patches (PPs) and the mesenteric lymph nodes (MLNs), which are very rich in immune cells including B cells, T cells, DCs and macrophages [137, 145].

1.3.2 Gut Macrophages

The gut is the largest reservoir of macrophages in the body [30]. Gut macrophages are mainly populated in the lamina propria and other parts of the gut such as mucosa, submucosa and muscularis mucosa [146]. Gut resident macrophages exhibit granular cytoplasm and active phagocytic behavior [146] and are identified by different cell surface markers such as CD11b, CD11c, F4/80, Fc-gamma receptor 1 (FcyRI; CD64), macrosialin (CD68), CD115, MHCII, CD206, hemoglobin scavenger receptor (CD163), Siglec F and αEβ7 receptor (CD103) [147]. In fact, distinguishing gut macrophages from other cells has to be performed by a combination of these markers, as CD11b⁺ and F4/80⁺ eosinophils [146] or CD11b⁺, CD11c⁺ and MHCII⁺ DCs [148] have been previously reported. Recently, CX3CR1⁺ or the fractalkine receptor has been shown to be associated with gut macrophages rather than gut DCs [148, 149]. On these grounds, gut macrophages in a steady state can be characterized by CD11b⁺, CD11c⁺, F4/80⁺, CX3CR1⁺ MHCII⁺, Siglec F⁻, and CD103⁻.

Studies have demonstrated that gut resident macrophages mainly originate from bone marrow-derived Ly6C⁺ high monocytes [150, 151]. It was further shown that these recruited Ly6C⁺ high monocytes differentiate to F4/80⁺ CD64⁺ CX3CR1⁺ Ly6C⁻ resident macrophages at least in part in a CCR2/CCL2-dependent manner [147]. Other chemokines such as TGF-β and IL-8, which are constantly produced by mucosal IECs and mast cells, have also been shown to be involved in the recruitment of blood monocytes [152, 153].

1.3.2.1 Gut Macrophage Roles

Gut macrophages are key players in maintaining gut immunity and homeostasis. In normal conditions, gut macrophages are tolerant to microbial products [147]. However, they retain their housekeeping functions, such as clearance of apoptotic
cells [154], as well as tissue remodeling [155]. Additionally, gut macrophages are highly phagocytic and bactericidal [156]. They lack respiratory burst activity [157] and do not produce NO [158] or inflammatory cytokines such as IL-1 and IL-6 [147, 156]. They, however, produce high levels of the anti-inflammatory cytokine IL-10 [147]. IL-10 plays a pivotal role in gut homeostasis. It induces production of the scavenger receptors such as CD206 and CD163. Additionally, IL-10 suppresses activation of gut macrophages to inflammatory stimuli [156]. Gut microbiota plays an important role in the production of IL-10 by gut macrophages. In fact, gut macrophages from germ free mice produce less IL-10 [159, 160]. Gut macrophages, however, produce low levels of TNF-α. Such effects have also been shown to be beneficial in regulating enterocyte growth, altering the permeability of the IEC barrier and inducing of matrix metalloproteinases and other tissue remodeling enzymes [161, 162]. Furthermore, gut macrophages produce prostaglandin E2 that stimulates proliferation of epithelial progenitors [163]. Overall, in the gut it appears that macrophages display a partial activation state with inflammatory anergy [156].

Gut macrophages also serve as APCs as they express high levels of MHCII [149]. It has been speculated that gut macrophages can differentiate naïve CD4+ T cells to forkhead box P3 (FoxP3+) T regulatory cells in the gut [164]. Such effects of gut macrophages are still debatable, as gut macrophages cannot migrate to MLNs in a steady state [148]. In addition, it has been shown that gut macrophages do not efficiently activate naïve CD4+ T cells in vitro [148]. Thus, other mediators may also be involved indirectly in this process. For instance, IL-10 is a substantial cytokine produced by gut macrophages and has been shown to regulate T regulatory cells that have migrated to the gut after initial priming in the MLNs [165, 166]. Gut macrophages may also facilitate the maintenance of other T cell populations. Indeed, microbiota-dependent IL-1β production in gut macrophages have been shown to contribute to the development of IL-17 producing T (Th17) cells in non-inflamed conditions [167].

1.3.2.2 Gut Macrophage Roles in IBD

The GI tract is one of the sites in the body that the immune system establishes a
profound presence due to heavily localized bacteria. Any disturbance to this balance may result in gut disorders, such as IBD [168]. IBD mainly comprises Crohn’s disease (CD) and ulcerative colitis (UC). CD is characterized by transmural inflammation in any part of the GI tract, mostly in the ileocecal region and terminal ileum, bowel obstruction, the presence of granulomas and the development of skip lesions. In contrast, UC inflammation is limited to the mucosa of the colon and rectum, and is characterized by bloody stool and severe diarrhea [169, 170]. Both CD and UC are caused by abnormal immune activation in the intestine [171]. It is postulated that the loss of immune tolerance to endogenous flora is responsible for initiating abnormal immune responses in genetically defective hosts [169]. In addition, the microbiome has also been found as a source of inflammation in IBD, as transferring the microbiome from IBD established T-bet-deficient mice (lacking Th1 immune response) to a wild-type mice caused intestinal inflammation [172]. Other theories such as genetic background and environmental factors have also been suggested to play a role in IBD [169, 173-176]. To date no cure has been found for IBD and most patients receive only antibiotics or anti-inflammatory drugs with negative consequences to reduce the symptoms and complications of the disease, which leads to less hospitalization and surgery. Therefore, new therapeutic treatments are needed to overcome these diseases [170, 177].

Macrophages are an abundant immune cells in the gut and are of major importance in the regulation of the immunological and inflammatory responses [30]. Dysregulation of cytokine production in macrophages has also been reported to associate with IBD. Macrophages from CD patients secrete high levels of pro-inflammatory IL-23 but low levels of anti-inflammatory IL-10. However, macrophages from UC patients mainly secrete high levels of pro-inflammatory IL-12 [178]. Macrophage polarization has also been shown to affect outcome of colitis. M1 macrophages contribute to pathogenesis of colitis; where M2 macrophages reduced the severity of the disease [134, 179, 180]. G-CSF was also produced less in IBD patients [181]. Less efficient bacterial clearance resulting from decreased ROS production [182] and phagocytosis [183] in macrophages have also been linked to IBD. Taken together, these findings indicate that an imbalance in any of the physiological functions of gut macrophages,
including inadequate response to GI tract flora, inappropriate clearing of microbes and ineffective switch from pro-inflammatory response to anti-inflammatory response, may tip the balance between GI bacteria and host’s immune system toward gut disorders such as IBD [171]. Indeed, recent genome-wide association studies revealed several single-nucleotide polymorphisms that contribute to the development of IBD: genes for microbial sensing (NOD2, IRF5, NFKB1, RELA, REL, RIPK2, CARD9 and PTPN22), clearance (ATG16L1, IRGM and NCF4) and integrating antimicrobial adaptive immune responses (IL-23R, IL-10, IL-12, IL-18RAP/IL-1R1, IFNGR/IFNAR1, JAK2, STAT3 and TYK2) [171, 184]. These analyses again suggest that defects in macrophage and DC function may contribute to the pathogenesis of IBD.

1.3.2.3 Mouse models of IBD

Animal models have been extensively used to increase our understanding of IBD. Mice can be used as an IBD model due to their inexpensive cost and small size compared to their counterparts such as monkeys. However, no ideal model that mimics all aspects of human IBD currently exists. To date, there are different models of IBD that can be grouped in three major categories based on the nature of inflammation and method of generation; the erosive self-limiting models of acute colitis, spontaneous models induced by targeted gene deletion and models induced by disruption of T-cell homeostasis [185]. As each animal model serves for different purposes, finding an appropriate model to study role of gut macrophages in colitis is an asset.

The erosive, self-limiting model of colitis can be used to resemble acute colitis with similar histopathological characteristics of UC such as neutrophil infiltration, mucosal erosions and ulcerations. This model can be produced by administration of dextran sulfate sodium (DSS) [186, 187]. DSS is a water-soluble heparin-like sulfated polysaccharide that is widely used to investigate the pathogenesis of UC [188]. Supplementing the drinking water of mice with DSS induces colonic mucosal inflammation with ulceration, body weight loss, bloody stools and diarrhea for several days [189]. Its mechanism of action is thought to be mediated by intestinal epithelium
damage through killing IECs and does not induce T cell responses, at least in the acute model [190, 191]. To investigate chronic inflammation, DSS can be administrated in low dose for several cycles [192]. Indeed, DSS-treatment of C.B17 SCID (sever combined immunodeficiency) mice resulted in colitis in these mice [189], indicating that innate immune cells such as macrophages and DCs are responsible for pathogenesis in this model.

In contrast to the DSS model, the trinitrobenzene sulfonic acid (TNBS) model induces colonic inflammation when administrated intrarectally in only susceptible strains of mice (but not C57BL/6j mice) and depends on the gut microbiota [186, 187, 193, 194]. This model closely resembles CD and has been shown to induce a CD4⁺ T cell-mediated-Th1 response [193, 194]. In addition, depletion of phagocytes in a TNBS model also showed to be effective in reducing colitis conditions in this model, confirming the role of macrophages and DCs in pathogenesis of IBD [195].

Spontaneous models induced by targeted gene deletion such as IL-10-deficient mice (IL-10⁻⁻), resembling CD, have also been used [196]. The IL-10⁻⁻ mice produce Th1-mediated immune responses, mucosal hyperplasia, a transmural mononuclear cell infiltration, occasional crypt abscesses and focal erosions [185]. This model has been used for studies of various immunologic agents (anti-TNF-α and anti-IFN-γ antibodies), antibiotics and probiotics. Similar to TNBS model, elimination of phagocytes in IL-10⁻⁻ mice also ameliorates the disease conditions in these mice, again indicating the importance of innate immune cells in this model [197].

Models induced by disruption of T-cell homeostasis have also been shown to be effective in resembling of IBD. This model is produced by adoptive transfer of CD4⁺ CD45RBhigh T cells (naive T-cells) from healthy wild-type mice into syngeneic recipients that lack T and B cells (such as SCID or RAG-deficient mice). This model produces transmural inflammation, epithelial cell hyperplasia, polymorphonuclear neutrophil and mononuclear leukocyte infiltration, crypt abscesses and epithelial cell erosions [185].
1.4 COLONY STIMULATING FACTORS

The colony stimulating factors (CSFs) are glycoproteins that include M-CSF, GM-CSF and G-CSF, each with differing colony stimulatory activity [198]. CSFs were initially considered as in vitro hematopoietic growth factors involved in the generation of myeloid cell populations from bone marrow precursor cells [199]. Additional studies, however, have identified other functions of CSFs, including differentiation and immunomodulatory effects [200, 201].

1.4.1 M-CSF

M-CSF, also known as CSF-1, is a hematopoietic growth factor required for the generation of the major macrophage populations [198, 201-203]. M-CSF also serves other functions, such as bone metabolism and macrophage chemotaxis [204, 205]. In addition, M-CSF has immune-related functions, including promoting resistance to inflammatory signals [206], modulating cytokine production and phagocytic activity of macrophages [207-209]. M-CSF is a steady state growth factor [201, 210] produced by variety of cells, including bone marrow stromal cells [211], activated monocytes/macrophages [212], activated granulocytes [213], endothelial cells [214] and fibroblasts [215]. Since M-CSF alone supports the survival and generation of macrophage-like cells, it is commonly used in vitro to prepare BMCs [216].

M-CSF deficient mice (M-CSF<sup>−/−</sup>; Csf1<sup>op/op</sup>/Csf1<sup>op/op</sup>) display deficiency in major macrophage populations [217, 218], demonstrating an important role of M-CSF in macrophage generation. In contrast to M-CSF<sup>−/−</sup> mice, M-CSF receptor deficient mice (M-CSFR<sup>−/−</sup>, CSF1R<sup>−/−</sup> or CD115<sup>−/−</sup>) have more severe defects in macrophage populations, as well as a more severe osteoporosis, than M-CSF<sup>−/−</sup>, [219]. Differences in phenotypes between M-CSF<sup>−/−</sup> and M-CSFR<sup>−/−</sup> can be explained by the identification of IL-34 as another ligand for M-CSFR [220]. Interestingly, IL-34 deficient mice (IL-34<sup>−/−</sup>) showed loss of microglia and Langerhans cells with minimal impact on other tissue macrophages [221].
1.4.1.1 M-CSFR Structure and Signal Transduction

M-CSFR is a tyrosine kinase transmembrane receptor which is encoded by the proto-oncogene *c-fms* [222]. M-CSFR is mainly expressed on progenitor and mature monocyte/macrophage cells [223]; however, its expression is also detected on decidual and placental trophoblast cells, indicating its additional role outside the hematopoietic system [224].

M-CSFR contains an extracellular ligand binding domain with five immunoglobulin-like loops, a single membrane-spanning helix and an intracellular protein tyrosine kinase domain [202, 206]. Ligand binding to M-CSFR results in the dimerization of receptor and subsequent phosphorylations at tyrosine residues of M-CSFR initiating several downstream signaling pathways, including Ras/ERKs and PI3K/Akt [225]. These signaling events lead to the activation and nuclear translocation of transcription factors, such as PU.1, and expression of many genes involved in cell survival (e.g. Bcl-2-related protein A1 and Myeloid cell leukemia 1), proliferation (e.g. cyclins A2, B1/2, D1/3, and E2) and differentiation (e.g. COX 1/2) [23, 71, 226, 227].

1.4.2 GM-CSF

GM-CSF, also known as CSF-2, is another hematopoietic growth factor which is able to promote development of granulocytes including neutrophils and eosinophils [228], monocytes/macrophages [216] and DCs [229]. GM-CSF, in contrast to M-CSF, is normally present in very low concentrations in the serum, but its production is rapidly elevated in response to inflammation and infections [198]. GM-CSF is produced by different cell types, including stromal cells [230], activated monocytes/macrophages [231], natural killer cells [232], endothelial cells [233], epithelial cells [234], CD4⁺ T cells [235] and Paneth cells, [236]. *In vitro*, GM-CSF is used to differentiate certain types of macrophages from BMCs [208, 237]. GM-CSF alone or in combination with IL-4 has also been used to induce the *in vitro* generation of DCs [238]; however, these DCs display a transcriptome pattern more closer to macrophages rather than DCs [239] and also express macrophage markers [70, 240].
GM-CSF deficient (GM-CSF−/−) or GM-CSF receptor (GM-CSFR or CSF2R) deficient (GM-CSFR−/−) mice generally display normal myelopoiesis except for abnormalities of alveolar proteinosis in the lung, indicating a role of GM-CSF in the maintenance of alveolar macrophages responsible for phagocytosis of surfactant protein [241-245]. Other studies have also shown that these mice are more susceptible to pulmonary infections, including *Mycobacterium tuberculosis* [246], *Pseudomonas aeruginosa* [247] and pulmonary group B streptococcal [248] infections. GM-CSF−/− mice also showed an increased neutrophilic infiltration in lungs upon bacterial infections, suggesting a defect in macrophage clearance of bacteria in the absence of GM-CSF [248]. Mice lacking both M-CSF and GM-CSF are still viable with similar characteristics related to M-CSF−/− and GM-CSF−/− mice. These double knockout mice still have a similar number of circulating monocytes to that of M-CSF−/− mice and functional phagocytic macrophages in the diseased lung. This suggests involvement of other compensatory mechanisms for macrophage production and function in vivo [249].

1.4.2.1 GM-CSFR Structure and Signal Transduction

GM-CSFR is a heterodimer of type I transmembrane glycoproteins comprised of a ligand-binding α subunit (CSF2Rα) and a signal-transduction β subunit (CSF2Rβ). GM-CSFR is expressed in myeloid cells but not B and T cells [250]. Other studies have also shown that GM-CSF is also active on non-hematopoietic cells such as fibroblasts [251] and endothelial cells [252]. Activation of GM-CSFR induces activation of several signaling cascades, including the Janus Kinase 2 (JAK2)/STATs (1/3/5), PI3K/AKT, Ras/MAPK and NF-κB pathways [253-256]. These events induce expression of specific genes related to cell survival, proliferation, differentiation and activation in monocytes and granulocytes.

1.4.3 G-CSF

G-CSF, also known as CSF3, is a 25 kDa-secreted glycoprotein and has a wide range of actions. G-CSF, as a hematopoietic factor, plays an important role in the survival, proliferation, differentiation and function of neutrophil progenitor cells and mature
neutrophils [257-259]. G-CSF is produced in response to inflammatory stimuli such as LPS, TNF-α, IL-17 and IL-1β by a variety of cell types, including monocytes/macrophages [260, 261], bone marrow stromal cells [262] and endothelial cells [263]. Similar to GM-CSF, serum concentrations of G-CSF remain low in the steady state; however, its production markedly increases upon inflammation or infection [264, 265]. Administration of G-CSF into non-human primates resulted in an increase in neutrophil numbers in the peripheral blood [266]. A study in mice has demonstrated that G-CSF significantly reduces the transit time through bone marrow stem cells to circulating neutrophils [267]. Likewise, G-CSF has been shown to increase mobilization of neutrophils into tissue from vascular compartments in humans [268]. A series of studies have also demonstrated that administration of G-CSF increases the levels of HSCs and progenitor cells in the peripheral blood, indicating a role of G-CSF in mobilization of multipotential hematopoietic progenitor cells from bone marrow to circulation [269, 270]. Overall, G-CSF is required for myeloid progenitor cells mobilization and increases survival and maturation of neutrophils. These effects make G-CSF a good candidate to compensate neutropenia in patients undergoing chemotherapy. Several clinical studies have proven the beneficial effects of G-CSF in this context, showing that administration of recombinant human G-CSF in febrile neutropenia and small-cell lung cancer patients reduces periods of neutropenia, infection episode and mortality [271-273].

Mice lacking G-CSF (G-CSF−/−) remain healthy, fertile and display normal long-term survival rates. However, these mice have a 70-80% reduction in peripheral neutrophil counts compared to wild-type mice, and a 50% reduction in bone marrow granulocyte/macrophage progenitor cells (indicated by frequency of colony-forming cells in response to different hematopoietic stimuli) [259]. Surprisingly, G-CSF−/− mice infected with Candida albicans had a normal neutrophilic response [274]. Likewise, Mycobacterium avium infection in these mice showed no depletion in myeloid precursor cells in the bone marrow without exacerbation of infection. However, subjecting these mice to Listeria monocytogenes infection resulted in a decrease and a delay in the production of peripheral neutrophils [259]. These findings suggest that emergency granulopoiesis in response to infections can still occur in the
absence of G-CSF with yet unknown compensatory mechanism.

G-CSF receptor (G-CSFR or CSF3R) deficient mice (G-CSFR\(^{-/-}\)) are also viable and fertile, but they develop more severe neutropenia than G-CSF\(^{+/+}\) mice, harboring 88% reduction in peripheral neutrophil counts compared to wild-type mice. However, G-CSFR\(^{+/+}\) mice are not neutropenic. Additionally, these mice display decreased hematopoietic progenitors in the bone marrow and deficiency in differentiation of the progenitor cells into granulocytes. Interestingly, these mice had normal neutrophil migration to the thioglycollate-inflamed peritoneum with normal myeloperoxidase activity, showing that G-CSF signaling is necessary for increasing the production of neutrophils in inflammatory conditions, but it is not an absolute necessity for granulocytopoiesis. This group also demonstrated that an addition of G-CSF to IL-6-stimulated cultures of G-CSFR\(^{-/-}\) progenitor cells promotes macrophage populations, indicating that G-CSF may also have a role in monocyte/macrophage production in a G-CSFR-independent manner [257].

1.4.3.1 G-CSFR Structure and Signal Transduction

G-CSFR is a type 1 transmembrane protein expressed mainly in neutrophils, monocytes/macrophages [260], lymphocytes [275], and hematopoietic stem cells and progenitor cells [257, 259]. It is also expressed in endothelial cells [276], placenta [277] and non-hematopoietic tumor cell lines [278]. Extracellular domain of G-CSFR contains an immunoglobulin-like domain, a cytokine recognition domain with four cysteine residues and a WSXWS motif required for ligand binding, and three fibronectin type III domains (Fig. 1.1). The cytoplasmic portion consists of three conserved regions with sequence homology to other cytokine receptors; Box1, Box2, and Box3 [279]. Box1 and Box2 have been linked in the transduction of proliferation signals, while Box3 has been shown to be involved in the differentiation process [280].

Upon binding to G-CSF, G-CSFR forms homodimers and activates multiple signaling pathways. G-CSFR lacks intrinsic kinase activity; therefore, it mainly relies on various non-receptor kinases such as the JAK family (JAKs), mainly JAK1, JAK2,
Figure 1.1. G-CSFR signaling pathways. Ligand binding to G-CSFR induces phosphorylation of JAKs and subsequent phosphorylation of four tyrosine residues located on the cytoplasmic portion of the G-CSFR. These phosphorylated tyrosine residues serve as docking sites for different proteins characterized by src homology 2 domains leading to activation of different signaling pathways that regulate various cell processes.
Figure 1.1

Plasma membrane

- Ig domain
- C-C
- WSXWS
- Fibronectin type III domains

Box1:
- Y703
- Stat3
- JAK1/2
- STAT5
- STAT1

Box2:
- Y729
- SOCS3
- MEK
- ERK1/2
- PI3K/Akt
- Survival
- Proliferation
- Differentiation
- Negative regulation

Box3:
- Y743
- Stat3
- Gab2
- Grb2
- Ras
- Raf
- Lyn

Negative regulation

Proliferation

Differentiation
and tyrosine kinase 2 (TYK2). Upon activation, the G-CSFR dimerizes and JAKs, which bind to a tryptophan residue localized between Box1 and Box2, are readily phosphorylated, leading to phosphorylation of four tyrosine (Y) residues (Y703, Y728, Y743, and Y763) in the murine receptor. Phosphorylation of Y703 and Y743 occurs at low levels of G-CSF, resulting in activation of STAT3 and subsequent proliferation and differentiation of neutrophils. At high levels of G-CSF, however, STAT3 activation is largely tyrosine-independent. It is also apparent that G-CSFR activation induces tyrosine-independent STAT1 and 5 activation, resulting in cell proliferation. Likewise, phosphorylation of Y763 mediates activation of Ras/ERK1/2 pathway through several adaptor proteins, leading to cell proliferation. Phosphorylation of Y728, however, involves in suppression of G-CSFR signaling through suppressor of cytokine signaling (SOCS) 3. Other pathways that are activated by G-CSFR, include PI3K/Akt and Src-like kinases Lyn and Hck pathways that are tyrosine-independent and crucial for cell survival, proliferation and differentiation [279].

1.4.3.2 Immunomodulatory Roles of G-CSF

Besides being a hematopoietic growth factor, G-CSF also exerts profound immunoregulatory effects in both innate and adaptive immunity [260, 281, 282]. In vitro studies revealed that G-CSF is able to down-regulate the production of inflammatory cytokines such as TNF-α and IL-12 in monocytes/macrophages, DCs and neutrophils induced by TLR agonists [260, 283]. Administration of anti-G-CSF antibody in pneumococcal pneumonia-infected mice showed elevated production of TNF-α, IL-1β and cytokine-induced neutrophil chemoattractant, indicating a role of G-CSF in host defense [284]. The mechanism underlying the inhibitory effect of G-CSF was shown to be mediated by G-CSF-dependent activation of JAK2/STAT3 pathway [285, 286]. JAK2/STAT3 activation inhibits activation of JNKs, known to be involved in pro-inflammatory cytokine production, and subsequent suppression of TNF-α production in LPS- or Escherichia coli-activated macrophages [285].

G-CSF may also directly or indirectly modulate adaptive immune effector cells, through promoting tolerogenic DCs in the peripheral blood [287, 288]. Follow up
studies have shown that those regulatory DCs are CD14+ monocyte derivatives and are capable of generating regulatory T cells with the ability to produce immunosuppressive cytokines such as TGF-β and IL-10 [289]. Rossetti et al., reported that CD14+ monocyte-derived DCs spontaneously secreted IL-10, but very little IL-12 in response to LPS and induced anergy in T cells. They also reported that G-CSF-treated DCs expressed higher co-stimulatory molecules, such as CD80 and CD86 [290]. Interestingly, in vivo administration of G-CSF reduced the expression of these co-stimulatory molecules on monocytes, B cells, and T cells [291]. Furthermore, monocytes collected from peripheral blood stem cells of G-CSF-treated breast cancer patients induced apoptosis in CD4+ T cells through Fas–Fas ligand interaction [292]. Other studies also reported that G-CSF-mobilized human donor monocytes suppressed T cell alloreactivity by different mechanisms, including production of IL-10, and inhibition of IL-12 and TNF-α secretions [291, 293, 294]. The other biological effects of G-CSF in relation to T cells is associated with the ability of G-CSF to promote the generation of IL-10 producing regulatory T cells [281, 295]. Indeed, G-CSF pretreatment of mice treated with LPS reduced IFN-γ (Th1 cytokine) production [296]. G-CSF may also directly modulate adaptive immune cells. A recent in vitro study has shown that G-CSF suppressed the production of IFN-γ and increased secretion of IL-4 in activated CD4+ T cells, suggesting the G-CSF immunomodulatory role on T cell polarization [297]. Overall, G-CSF seems to play an important role in shaping innate and adaptive immunity by inducing regulatory monocytes/macrophages and DCs, as well as changing the function of T cells, such as Th2 polarization, reflected by higher production of IL-10 and IL-4 rather than IFN-γ.

A growing body of experimental evidence suggests that G-CSF has also other functions in non-hematopoietic cells. G-CSF has been found to promote cardiac cell generation and repair after myocardial infarction in animal models through JAK/STAT pathway [298]. G-CSF has also been shown to have a neuroprotective role in a cerebral ischemia mouse model [299]. Overall, these finding suggest that G-CSF effects are not only restricted to myeloid lineage but also extend to other non-hematopoietic cells.
1.4.3.3 G-CSF Roles in the Gut

Several studies have previously highlighted the importance of G-CSF in gut immune regulation. Previously published work from our laboratory has documented that colon tissue explants from G-CSFR−/−, but not wild-type, mice had high levels of spontaneous inflammatory cytokine production such as TNF-α, IL-12 and IL-23, which have been shown to play important roles in the pathogenesis of IBD [181]. However, no apparent features of spontaneous colitis were detected in G-CSF−/− or G-CSFR−/− mice. Indeed, these observations were in agreement with the ability of G-CSF in down-regulating inflammatory cytokines in macrophages, DCs and neutrophils [283, 285, 300], promoting maturation of tolerogenic DCs [289] and differentiation of T lymphocytes to regulatory T cells [301]. In addition, it was demonstrated that exogenously applied recombinant G-CSF is beneficial in both murine DSS- and hapten-induced colitis models [302, 303], as well as in CD patients [304-306]. Interestingly, cells isolated from non-inflamed sections of IBD patients undergoing gut surgery for colon cancer showed significant reduction in the G-CSF production [181]. In contrast, inflamed sections of the gut from those patients have been shown to express substantial levels of G-CSF [307]. Differences in G-CSF production in the inflamed and non-inflamed sections could be due to the massive ongoing inflammation in the inflamed region of the gut that has been shown to be associated with production of both inflammatory (TNF-α and IL-1) and non-inflammatory (IL-10) cytokines, which implies that the balance of cytokines is more crucial rather than the presence or absence of a particular cytokine [308]. Moreover, G-CSF has been shown to have protective effects on IECs. Administration of G-CSF to rats treated with the chemotherapeutic agent reduced villus atrophy, enterocyte apoptosis, and gut bacterial translocation [309]. Overall, it appears that G-CSF plays a crucial role in gut immune regulation, where both avid immune surveillance and tolerance against infiltrating commensal microbes are required.

1.5 PROBIOTICS

Probiotics are defined as “living microorganisms, which, when administered in adequate amounts, confer a health benefit to the host” [310]. Although people have
consumed probiotics for many years, their health effects have only recently been addressed scientifically. To date, microorganisms with probiotic properties mainly include members of the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Bacillus*. Other bacteria such as avirulent *E. coli*, *Enterococcus faecium* and yeasts have also been used infrequently as probiotics [311, 312].

Probiotic microorganisms are believed to out-compete pathogens for nutrients or binding sites in the gastrointestinal tract, while some secrete antimicrobial substances, such as short chain fatty acids (SCFA), hydrogen peroxide and bacteriocins to adjacent microorganisms [139, 313]. Probiotics also enhance the host intestinal barrier functions by increasing the production of mucins, antibacterial peptides such as defensins, and maintaining or restoring tight junctions [314, 315]. Another strategies by which probiotics elicit their effect is by increasing anti-inflammatory cytokines such as IL-10 and G-CSF, while decreasing pro-inflammatory cytokines such as TNF-α [285, 300]. In addition, probiotics elicit immunomodulatory effects on the host T cells through modulation of DCs [316]. Furthermore, the immunomodulating effects are not limited to the level of intestinal mucosa, but also reach distant sites [317].

Several strains of probiotic bacteria have been implicated as therapeutic reagents for preventing and/or treating various gastrointestinal diseases such as traveler’s diarrhea and IBD, as well as atopic diseases such as arthritis and allergies [318-320]. However, these generalized probiotic effects are required to be itemized for specific strains, and in-depth understanding of the mechanisms of probiotics effects should be sought for their efficient and effective use [321]. For instance, *L. casei* subsp. *alactus* alone induces pro-inflammatory cytokines in murine DCs; however, the stimulatory effect is diminished when it is used in combination with *L. reuteri* [322]. It was also observed that the administration of mixtures of *L. casei*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* increases the production of IgG1 (a Th2 response) in a mouse model, but *L. acidophilus* alone results in the production of IgG2a (a Th1 response) [323]. Further studies will greatly increase our understanding on specific
biological features of each probiotic strain and their modes of action, leading to proper applications of probiotics.

1.5.1 Probiotics and Immune Sensing Mechanisms

All microbes harbor MAMPs and induce inflammatory responses through activating PRRs. Although probiotics harbor MAMPs, they rarely induce inflammatory diseases, but instead effectively modulate host immune responses [324, 325]. During the last 10 years, several mechanisms modulating the host cell signaling pathways by bacterial probiotics have been unraveled, which includes modulation of NF-κB [326], MAPKs [285, 327] and Akt [314] pathways.

In macrophages, probiotics were shown to affect signaling pathways altering cytokine production profiles. For instance, NF-κB, as an important signaling molecule in pro-inflammatory cytokine production, was shown to be inhibited by *E. coli* M17, resulting in decreased production of pro-inflammatory TNF-α, IL-1β and IL-6 [328]. LTA from *L. plantarum* was found to inhibit LPS-induced TNF-α in macrophages through NF-κB and MAPKs signaling pathways [329]. Supernatant of *L. casei* strain Shirota was also shown to have TNF-α-inhibitor activity in LPS-treated macrophages through suppression of NF-κB signaling pathway [327]. Similar findings were also observed in secreted factors from *B. bifidum* B536 and *Ruminococcus gnavus* FRE1 [330]. However, the probiotic factors responsible for the signaling modulation are still unknown.

1.5.2 Probiotic Characteristics of *L. rhamnosus*

*Lactobacillus*, a Gram-positive aerotolerant anaerobic bacteria, is a common constituent of the indigenous microbiota in the human intestinal [331] and urogenital [332, 333] tracts. Several strains of this genus have been used as probiotics and their effects on inflammatory diseases, such as IBD, in human and animal disease models have been well documented [324, 334, 335].

*L. rhamnosus* GG (LGG) is a well-studied strain of this species and has been shown to have disease-ameliorating effects on various animal inflammatory disease models
and human patients [324, 336, 337]. LGG produces soluble proteins that inhibit TNF-α production in LPS- or LTA-activated murine macrophages with no effect on IL-10 production [325]. LGG also produces p75 (75 kDa) and p40 (40 kDa) proteins that have a cytoprotective effect on IECs through activation of the epidermal growth factor receptor (EGFR) and its down-stream signaling molecule Akt [314, 338, 339]. Activation of EGFR by p40 is mediated by the release of the heparin binding (HB)-EGF which is released by activated ADAM17 (a disintegrin and metalloproteinase domain-containing protein 17) [340]. Delivery of p40 to DSS-treated mouse colon reduced intestinal epithelial apoptosis and disruption of barrier function [341]. Also, a recent study has indicated that LGG-fermented milk, containing p40 and p75, significantly reduced severity of the DSS-induced murine model of colitis [342].

_ L. rhamnosus_ GR-1 (GR-1) is closely related to LGG and renders a number of properties considered important for probiotics [285, 320, 343]. Both LGG and GR-1 induce miniscule amounts of pro-inflammatory cytokines such as TNF-α, but potently induces anti-inflammatory cytokines such as G-CSF and IL-10. G-CSF produced by LGG- or GR-1-exposed macrophages inhibits production of pro-inflammatory cytokines in _E. coli_ or LPS-activated macrophages [285]. Administration of yogurt supplemented with probiotic strains including GR-1 has also been shown to increase T regulatory cells in the peripheral blood of IBD patients. In addition, the treatment resulted in a decreased percentage of TNF-α and IL-12-producing monocytes and DCs [320]. Overall, these findings indicate that LGG and GR-1 induce anti-inflammatory effects by directly releasing a soluble factor(s) or indirectly by inducing production of immunomodulatory cytokines, such as G-CSF and IL-10. To date, the signaling mechanisms and GR-1 factor(s) responsible for the G-CSF preferential production in macrophages are unknown.
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Chapter 2

HYPOTHESIS AND SPECIFIC AIMS
2.1 RATIONALE AND HYPOTHESIS

Granulocyte-colony stimulating factor (G-CSF) is a pleiotropic cytokine with various functions in the immune system. It is primarily involved in neutrophil generation [1], but its role in macrophage generation has also been documented [2, 3]. An anti-inflammatory role of G-CSF in down-regulating inflammatory cytokine production has also been reported in immune cells, including macrophages and DCs [4-8]. It is also apparent that G-CSF has potential therapeutic properties in gut inflammatory diseases such as inflammatory bowel diseases (IBD) [9-13]. To date, little is known about the role of G-CSF in macrophage generation and gut immunity. Additionally, LGG and GR-1 have been shown to render immunomodulatory effects through preferentially and potently inducing G-CSF in macrophages. However, how GR-1 preferentially induces G-CSF in macrophages still remain to be unraveled. This thesis aims to elucidate the effects of G-CSF on macrophages and gut immunity, and examine the signaling mechanisms involved in the preferential production of G-CSF in macrophages. According to previous findings, I hypothesize that G-CSF plays an important role in the generation of gut-homing immune regulatory macrophages and in the maintenance of gut immune homeostasis.
2.2 SPECIAL AIMS

2.2.1 Aim 1: Examine the role of G-CSF in the generation and characteristics of macrophages.

Macrophages represent the most abundant mononuclear phagocytes in the gut lamina propria and play a critical role in immune responses, inflammation and tissue homeostasis [14-17]. Gut macrophages originate from bone marrow under the influence of IL-3, and growth factors such as macrophage (M)-CSF and granulocyte/macrophage (GM)-CSF [18-21]. G-CSF primarily influences the generation and differentiation of granulocytes, mainly neutrophils [1]. However, studies on G-CSF<sup>−/−</sup> or G-CSFR<sup>−/−</sup> mice indicated that G-CSF is also involved in macrophage generation [2, 3]. To date, the influence of G-CSF on macrophage development is poorly understood. In Chapter 3, I will be evaluating effects of G-CSF on the generation of macrophages using in vitro bone marrow cell culture models. Then, I will be addressing the characteristics of G-CSF-responsive macrophages in regards to morphology, cytokine secretion and cell-surface marker profiles. Finally, I will be examining the in vivo localization of adoptively transferred in vitro G-CSF-treated bone marrow derived macrophages (G-BMDM) in mice. Collectively, this study demonstrates that G-CSF plays an important role in the generation of gut-homing, M2-like macrophages.
2.2.2 Aim 2: Examine the role of endogenous G-CSF in DSS-induced acute colitis.

Several studies have shown that exogenous administration of G-CSF is effective in ameliorating disease severity in murine colitis models and IBD patients [9-13]. However, the role and mechanism of endogenous G-CSF in gut immunity is still unknown. As illustrated in Aim 1, G-CSF involves in the generation of gut-homing M2-like macrophages [22]. Therefore, I will be using oral administration of dextran sulfate sodium (DSS) to induce self-limiting acute colitis in mice and further characterize macrophages using hallmarks of M1 and M2 macrophages, and determine if adoptive transfer of G-CSF-treated macrophages reduce severity of colitis in DSS-treated G-CSFR^{-/-} mice. Taken together, this study indicates that G-CSF plays a protective role in DSS-induced acute colitis through generating gut-homing macrophages.
2.2.3 Aim 3: Examine the signaling mechanisms involved in the preferential G-CSF production in macrophages by GR-1.

Previously, our laboratory showed that G-CSF plays a key role in suppressing production of TNF-α or IL-12/23 p40 in macrophages and DCs, respectively [6, 23]. G-CSF is potently and preferentially produced by LGG- or GR-1-exposed macrophages; however, the microbial factor(s) and its mechanism by which *L. rhamnosus* elicits such effects on macrophages are largely unknown. In Chapter 5, I will be evaluating the cytokine profile of GR-1-treated macrophages and will be comparing it to the profile of macrophages stimulated with other stimuli. Additionally, I will be determining the responsible receptor for GR-1 recognition in macrophages and subsequent downstream signaling pathways initiated by GR-1 in these cells. Moreover, I will be addressing the signaling pathway responsible for preferential G-CSF production in GR-1-exposed macrophages and will be partially characterizing the GR-1 factor(s) responsible for such preferential G-CSF production in macrophages. Collectively, this study demonstrates that a protein-like factor(s) secreted from GR-1 preferentially induces G-CSF production in macrophages through selectively activating ERKs, NF-κB and Akt in a TLR2-dependent manner.
2.3 REFERENCES


Chapter 3

G-CSF PREFERENTIALLY SUPPORTS THE GENERATION OF GUT-HOMING GR-1\textsuperscript{HIGH} MACROPHAGES IN M-CSF-TREATED BONE MARROW CELLS.

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3.1 INTRODUCTION

Macrophages are distributed throughout the body and play key roles in metabolic, immunological and inflammatory processes [1]. In adults, tissue macrophage populations are maintained by self-renewal of local macrophages, which initially arise from the yolk sac in development, and recruitment of circulating monocytes, which originate from common myeloid progenitors in the bone marrow [2-4]. Macrophages also show remarkable plasticity and functional diversity determined by environmental cues. In general, macrophages are classified into two different types: interferon-γ (IFN-γ)- and tumor necrosis factor (TNF)-α-exposed ‘classically activated’ or ‘M1’ macrophages, which release high amounts of pro-inflammatory cytokines, such as interleukin (IL)-1β, -6, -12 and -23, in response to microbial infections, and IL-4-, -10- and -13-exposed ‘alternatively activated’ or ‘M2’ macrophages, which are involved in anti-inflammatory responses [5]. The repertoire of M2 macrophages has expanded and now encompasses at least two main subtypes, namely wound-healing and regulatory macrophages, based on their function and cytokine release profiles [6, 7]. Of the more than 20 soluble hematopoietic factors, the colony-stimulating factors (CSFs), including macrophage (M)-CSF and granulocyte/macrophage-CSF (GM-CSF), are crucial for the generation of myeloid cells in the bone marrow and differentially affect the type of macrophages that can be produced from hematopoetic bone marrow precursors and monocytes [8, 9]. In general, GM-CSF is known to generate dendritic cells (DCs), granulocytes, alveolar macrophages and invariant natural killer T cells, whereas M-CSF is involved in the generation and maintenance of monocytes/macrophages [10]. Therefore, M-CSF-treated bone marrow cells (M-BMCs) have frequently been used as a source for primary macrophages in vitro and were shown to have anti-inflammatory/regulatory macrophage “M2-like” characteristics in cytokine production upon activation [11]. Unlike M-CSF, GM-CSF-BMCs (GM-BMCs) give rise to inflammatory DCs and have often been used as a model for generating DCs. However, further studies demonstrated that GM-BMCs resemble macrophages rather than DCs, based on transcriptomic analysis [12] and cytokine profiles [11]. In line with these notions, GM-CSF, which is rapidly induced during infections, is particularly implicated in inflammatory processes and generation
of inflammatory “M1-like” macrophages [11]. M-CSF, which is constitutively and ubiquitously expressed, is involved in generating the steady state tissue macrophages, counteracting GM-CSF effects, suppressing pro-inflammatory stimuli and restoring tissue homeostasis from inflammatory damage [11, 13-17].

Distinct from M-CSF and GM-CSF, granulocyte-CSF (G-CSF) is intimately involved in the generation, mobilization and activation of granulocytes, namely neutrophils [18-22]. However, recent studies have revealed that G-CSF also plays a role in immunomodulation and maintenance of gut homeostasis [10, 23-25]. In the bone marrow, G-CSF is produced by stromal cells, which supports proliferation and survival of G-CSF-dependent myeloid precursor cells. G-CSF is also produced by fibroblasts, endothelial cells and macrophages in response to various inflammatory cytokines, including IL-1, IL-17 and TNF-α, as well as pathogen-associated or danger-associated molecular pattern molecules such as lipopolysaccharide (LPS) and the acute-phase protein serum amyloid A, respectively [22, 26, 27]. Normal serum levels of G-CSF are low, but the levels greatly increase during infection or chronic inflammatory diseases such as rheumatoid arthritis. Peak levels of G-CSF in the serum or tissues are expected to recruit and activate neutrophils, which is key for restricting infection and inducing local inflammation. Consistent with these effects, administration of G-CSF can exacerbate pathophysiology in animal models of arthritis and graft-versus-host disease [28], whereas G-CSF deficiency or neutralizing antibodies can alleviate symptoms. In contrast, a high constitutive level of G-CSF is detected in the colon [29], and administration of G-CSF confers protective effects on colitis, pulmonary inflammation [30], neuronal inflammation, cell death [31, 32], and cardiac ischemia/reperfusion injury [33]. Furthermore, G-CSF reduces TNF-α and IL-12 production in myeloid cells [29, 34], and promotes the generation of tolerogenic DCs and T lymphocytes [23, 35-37]. These seemingly opposing effects suggest that G-CSF has tissue- and disease-specific roles.

In early in vitro studies, G-CSF was shown to support the survival of both M-CSF- and GM-CSF-responsive progenitor cells in bone marrow, suggesting a role in macrophage development [38]. Consistent with these observations, mice deficient in
G-CSF or G-CSFR harbour ~85-90 % and ~50% reduced circulating neutrophils and monocytes, respectively, compared to wild-type mice [20]. G-CSFR<sup>−/−</sup> mononuclear cells also showed higher levels of apoptosis in bone marrow cell cultures [20], suggesting a role of G-CSFR in supporting the survival of monocytes/macrophages. Therefore, lower numbers of circulating monocytes in G-CSFR<sup>−/−</sup> mice could be due to their defects in maintaining myeloid precursor cells necessary to sustain continual differentiation of monocytes/macrophages in different tissue compartments. To date, the role of G-CSF in the development of tissue-specific macrophages has not yet been explored. Here, using in vitro bone marrow cell culture models, we found that G-CSF plays an important role in the generation of Gr-1<sup>high</sup>/F4/80<sup>+</sup> “M2-like” macrophages, which are localized mainly in the intestine after adoptive transfer.
3.2 MATERIALS AND METHODS

3.2.1 Mice

C57BL/6j wild-type mice were obtained from Jackson Laboratories (Bar Harbour, ME, USA). Homozygote G-CSFR\(^{-/-}\) mice (C57BL/6 background) were originally obtained from Dr. D.C. Link (Washington University Medical School, St Louis, MO, USA) and maintained in the animal care and veterinary facility at Western University under conventional conditions. C57BL/6 mice were crossed to G-CSFR\(^{-/-}\) mice, and G-CSFR\(^{+/+}\) offspring were then crossed with each other to generate G-CSFR\(^{+/+}\) and \(^{-/-}\) genotypes. Mice were genotyped for G-CSFR\(^{+/+}\) by PCR using the following primers; forward: AGCCCTCTCCATCGGAAAGTTTGA, reverse: AGCGAAGTTCACACTCCTGTTCCA. Mice were genotyped for G-CSFR\(^{-/-}\) by PCR using the following primers; forward: AGAGGCTATTCGGCTATGACTG, reverse: CCTGATCGACAAGACCGGCTTC. Matched pairs of littermates were used whenever possible. Male and female mice, aged 4-12 weeks, were used in all experimental procedures. The \(ly5\)-EGFP-\(ki\) mice were originally obtained from Dr. Thomas Graf (Albert Einstein University, NY, USA) and have been maintained in a barrier facility as homozygous mice on a C57BL/6 background. These transgenic mice express EGFP under the control of the lysozyme M promoter in mature myeloid and granulocytic populations [39]. All experimental protocols were approved by the Western University Animal Use Subcommittee, which follows the regulation of the Animals for Research Act (Ontario, Canada) and the Canadian Council on Animal Care.

3.2.2 Reagents

The antimitotic agent nocodazole was purchased from Sigma-Aldrich (St. Louise, MO, USA). M-CSF, GM-CSF and G-CSF, FITC-anti-mouse F4/80, PE-anti-mouse Ly6C, PE-Cy5-anti-mouse CD11c, APC-anti-mouse CD11c, PE-Cy7-anti-mouse Gr-1 (Ly6G), PE-anti-mouse CD11b, PE-anti-mouse CD71, PE-anti-mouse PDL2, APC-anti-mouse CD206 antibodies, the Fixable Viability Dye eFluor® 660 (FVD eFluor® 660), and the enzyme-linked immunosorbent assay (ELISA) kits of TNF-\(\alpha\), IL-10,
IL-23 and chemokine (C-C motif) ligand (CCL) 2 were purchased from eBioscience (San Diego, CA, USA). LPS from *E. coli* O111:B4 was obtained from List Biological Laboratories (Campbell, CA, USA). IL-4 and IL-13 cytokines were obtained from PeproTech (Rocky Hill, NJ, USA). CellTrace™ carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation kit was purchased from Invitrogen/Molecular Probes (Eugene, OR, USA).

3.2.3 Generation of Bone Marrow-Derived Macrophages

BMCs were flushed out from femurs and tibia of G-CSFR^{+/+} and/or ^{−/−} mice using a 25.5 gauge needle and phosphate buffered saline (PBS). Isolated cells were cultured in complete RPMI 1640 (c-RPMI; Sigma-Aldrich, St. Louise, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louise, MO, USA), 100 U ml^{-1} of penicillin, 0.1 mg ml^{-1} of streptomycin (Sigma-Aldrich, St. Louise, MO, USA), 5 mM sodium pyruvate (Sigma-Aldrich, St. Louise, MO, USA), 5 mM MEM non-essential amino acids (Sigma-Aldrich, St. Louise, MO, USA), and M-CSF or GM-CSF at 37°C in a humidified atmosphere with 5% CO₂. The culture media was replaced with fresh media every 2 days.

3.2.4 Cell Proliferation Assay

BMCs were harvested from G-CSFR^{+/+} and ^{−/−} mice on the day of the experiment and cells were distributed evenly onto 24 well plates. Duplicate wells were created for M-CSF (20 ng/ml) ± G-CSF (10 ng/ml), GM-CSF (20 ng/ml) ± G-CSF (10 ng/ml) and G-CSF (10 ng/ml). Cells were quantified using a haemocytometer every day for 5-7 days.

3.2.5 Flow Cytometry and Magnetic-Activated Cell Sorting

For the analysis of F4/80, Gr-1, Ly6C, CD11c, CD11b, PDL2, CD206 and CD71 expression, cells were harvested, washed twice, stained for cell surface markers and then read on the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry analysis was done using the Flowjo software (version 7.6.5; Treestar, Inc., OR, USA). Gr-1^{low}/F4/80^{+} (P1), Gr-1^{high}/F4/80^{+} (P2) and Gr-
$1^+/F4/80^- (P3)$, or $F4/80^+$ cells were sorted using the FACS Aria III cell sorter or the Magnetic-Activated Cell Sorting (MACS; Miltenyi Biotec, CA, USA), respectively, following the manufacturer’s protocol.

### 3.2.6 CSFE Proliferation Assay

Cell proliferation was analyzed by a CFSE dilution assay according to the manufacturer's instructions. Briefly, MACS-sorted P1 cells were counted, stained with 1 µL of 5 mM CFSE per mL and incubated at 37 °C for 15 min in the dark. Cells were then washed twice with 5 volumes of ice-cold FBS. Intracellular CFSE intensities were measured by flow cytometry immediately after washing or after culturing cells 2 days with M-CSF or M-CSF + G-CSF.

### 3.2.7 Transmission Electron Microscopy

Isolated BMCs from wild-type mice were cultured in the presence of M-CSF (20 ng/ml) ± G-CSF (10 ng/ml). On day 5, cells were sorted for $F4/80^+$, Gr-1<sub>low</sub> (P1) and $F4/80^+$, Gr-1<sub>high</sub> (P2) cells using the FACS Aria III cell sorter (BD Biosciences, CA, USA). The sorted cells were then washed with PBS twice and fixed with 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer for 2 h at room temperature. Grids with specimens were prepared according to the Transmission Electron Microscope Facility Protocol at Western University (Canada), and micrographs were taken with a transmission electron microscope. Briefly, after fixing with 2.5% glutaraldehyde, cells were washed with 0.1 M cacodylate buffer three times, and cells were further fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, and then rinsed with 0.1 M cacodylate buffer. Cells were enrobed in 5% Noble Agar and washed with distilled water 5 times, further fixing with 2% uranyl acetate for 2 h in the dark, followed by dehydration in 50% (15 min), 70% (16 h), 85% (15 min), 95% (15 min), and two changes of 100% ethanol (15 min each). They were then cleared by two changes of propylene oxide each 15 min and infiltrated with epon resin:propylene oxide (1:1) for 3 h, epon resin:propylene oxide (3:1) for 16 h at room temperature, and two changes with 100% epon resin for a total of 6 h. Following that, samples were baked for two days at 60°C. Thin sections were mounted on grids, and examined
under the electron microscope (Philips CM-10 80 Kv and AMT Digital Camera, OR, USA).

3.2.8 Enzyme-Linked Immunosorbant Assays

Isolated BMCs were cultured in the presence of M-CSF (20 ng/ml) + G-CSF (10 ng/ml). On day 5, cells were sorted using FACSaria III cell sorter, washed with PBS twice and cultured in a 96-well plate for 2 h. Subsequently, cells were treated with 100 ng/ml of LPS and cytokine levels were determined in cell culture supernatants using commercially available quantitative ELISA kits from eBioscience (San Diego, CA, USA). The same procedure was performed for unsorted bone marrow cells that were cultured in the presence of M-CSF (20 ng/ml) or GM-CSF (10 ng/ml) ± G-CSF (10 ng/ml) for 5 days.

3.2.9 Cell Death Assay

A 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay or FVD eFluor® 660 staining was used to assess cell death. For MTT assay, G-CSFR<sup>+/+</sup> BMCs were cultured with c-RPMI supplemented with either M-CSF (20 ng/ml) or GM-CSF (10 ng/ml). On day three, the cultures were split into four 96-well plates for analysis on day 4, 5 and 6. Triplicate wells were created for M-CSF (5 and 10 ng/ml) or GM-CSF (1 and 5 ng/ml) ± G-CSF (10 ng/ml). Analysis on days 4 through 6 were done the same and conducted as follows. MTT was added at a final concentration of 0.5 mg/ml and plates were then incubated for 2 h at 37°C. The plates were then centrifuged at 2900 rpm for 10 min to pellet cells and the media was carefully aspirated out. Dimethyl sulfoxide was then added to each well and the plates were incubated at room temperature in the dark while shaking gently to dissolve formed crystals. Optical densities of the wells were analyzed using an automatic ELISA plate reader (Bio-Rad Laboratories, Inc., CA, USA) at a wavelength of 590 nm. For FVD eFluor® 660 staining, cells were washed twice with PBS and re-suspended in PBS containing 1 µL/ml eFlour 660 dye at a density of 1-10 × 10<sup>6</sup> cells. After incubation on ice in the dark for 30 min, cells were washed twice, stained for surface markers as described above and analyzed by flow cytometry.
3.2.10 Splenocyte and Intestinal Lamina Propria Cell Isolation

G-CSFR$^{+/+}$ and $^{-/-}$ mice were euthanized by CO$_2$ inhalation and were dissected immediately after sacrifice. Splenocyte Isolation: An incision was made along the right side of the mouse and the spleen was removed, mechanically disrupted and passed through a 70 µm filter. The suspension was centrifuged and re-suspended in 4 ml Ammonium-Chloride-Potassium cell lysis buffer and left for 4 min at room temperature with gentle shaking. Then, 10 ml of c-RPMI was added to the suspension and was centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cells were re-suspended in c-RPMI to $1 \times 10^6$ cells/ml and samples were prepared for flow cytometry analysis as described above. Intestinal Cell Isolation: Intestinal lamina propria cells were isolated as previously described [40] with some modifications. Briefly, intestines were flushed out with 1x PBS supplemented with 5% FBS and the Peyer's patches excised. The intestines were opened longitudinally, washed with ice-cold 1x PBS twice and cut into 0.5-cm segments. These segments were incubated twice in Hank's balanced salt solution with 2 mM EDTA and 1mM dithiothreitol at 37°C with shaking for 20 min. Remaining pieces of intestine were passed through a 100 µm cell strainer and washed with 1x PBS. Intestinal tissues were then digested twice with 1x PBS including 0.13 wünsch units/ml of Liberase-TM (Roche Applied Science, CA, USA), 0.04 mg/ml of DNase I (Sigma-Aldrich, St. Louise, MO, USA) and 3 mg/ml of dispase II (Roche Applied Science, CA, USA) at 37°C with shaking for 30 min. Cells were then passed through a 40 µm cell strainer and washed twice with 1x PBS with 5% FBS. The cells were then re-suspended in 10 ml of the 40% percoll and the mixture was then overlaid on top of 5 ml of 80% percoll. Following the centrifugation at 1000 g for 20 min without breaking, lamina propria cells were collected from the interphase of two different percoll solutions and washed immediately with 1x PBS with 5% FBS. The cells were then stained for flow cytometry as described above.

3.2.11 Adoptive Cell Transfer Experiment

BMCs from lys-EGFP-ki transgenic mice [41] were harvested as described earlier and cultured in the presence of M-CSF (20 ng/ml) ± G-CSF (10 ng/ml). On day 5, F4/80$^+$
cells were sorted using MACS and the expression of EGFP was confirmed by flow cytometry (Appendix A.1). F4/80$^+$ cells (1.0 × 10$^6$, unless otherwise indicated) were then intravenously injected to wild-type recipient mice and various tissues (intestine, spleen, liver, kidney, lung, heart and bone marrow) were obtained after 2, 5, 15 and 30 days after adoptive transfer. These tissues were fixed in 10% neutral-buffered formalin for 48 h, paraffin-embedded and placed on poly-L-lysine coated slides after being sliced in 50 µm thickness. The tissue slides were then stained by hematoxylin and eosin, and EGFP$^+$ cells were examined by the Olympus 1X51 fluorescent microscope (Olympus America Inc., NY, USA) attached to the QImaging Retiga 2000R digital camera (QImaging Co., BC, Canada). Images were processed using the QCapture Pro image analyzing software (version 6.0; QImaging Co., BC, Canada).

3.2.12 Statistical Analysis

Statistical analysis was carried out using Prism 5.0c for Mac OS X (GraphPad Software, La Jolla, CA, USA). Student’s $t$ tests and one-way ANOVA, followed by Tukey’s multiple comparison post-hoc test, were performed as described in the figure legends.
3.3 RESULTS

3.3.1 G-CSF preferentially increases numbers of M-BMCs.

To examine the role of G-CSF in macrophage proliferation, BMCs from G-CSFR\(^{+/+}\) and \(^{-/-}\) mice were cultured in the presence of M-CSF or GM-CSF, and viable cell numbers were counted each day for 7 days. In both M-CSF and GM-CSF treated cell cultures, G-CSFR\(^{-/-}\) bone marrow cell numbers were growing significantly slower than G-CSFR\(^{+/+}\) cells (Fig. 3.1.A-B). These results are consistent with a previous study showing that G-CSF supports survival of both M-CSF and GM-CSF responsive progenitor cells in bone marrow [38]. However, when G-CSF was exogenously added to cell culture media, cell numbers of M-BMCs were significantly increased after 3 days of cell culture, but not in GM-BMCs (Fig. 3.1C-D). G-CSF alone was not able to sustain continued survival/proliferation of BMCs. These results suggest that G-CSF had a growth promoting effect on M-BMCs, but not GM-BMCs.
Figure 3.1. G-CSF preferentially increases cell number of M-BMCs. (A-B) BMCs from wild-type and G-CSFR<sup>−/−</sup> mice were treated with either M-CSF (20 ng/ml) (A) or GM-CSF (20 ng/ml) (B), and cell numbers were counted each day for 7 days. (C-D) Similarly, BMCs from wild-type mice were treated with either M-CSF (20 ng/ml) ± G-CSF (10 ng/ml) (C) or GM-CSF (20 ng/ml) ± G-CSF (10 ng/ml) (D). Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s t-test).
Figure 3.1

A

C

G-CSFR+/+ - M-CSF
G-CSFR−/− - M-CSF

% increase in cell number

0 1 2 3 4 5 6 7

Day

B

G-CSFR+/+ - GM-CSF
G-CSFR−/− - GM-CSF

% increase in cell number

0 1 2 3 4 5 6 7

Day

D

G-CSFR+/+ - M-CSF
G-CSFR+/+ - M-CSF + G-CSF
G-CSFR−/− - G-CSF

% increase in cell number

0 1 2 3 4 5

Day

G-CSFR+/+ - GM-CSF
G-CSFR+/+ - GM-CSF + G-CSF

% increase in cell number

0 1 2 3 4 5

Day
3.3.2 G-CSF induces proliferation of Gr-1<sup>high</sup>/Ly6C<sup>+</sup> macrophage-like and neutrophil-like cells in M-BMCs.

To further examine myeloid cell types that arise from G-CSF-, M-CSF- and GM-CSF-treated BMCs, cells were stained with various macrophage surface markers including F4/80, Gr-1, Ly6C, CD11b and CD11c. As shown in Fig. 3.2A, M-BMCs showed 3 distinct cell populations, based on F4/80 and Gr-1 staining intensities: P1, Gr-1<sub>low</sub>/F4/80<sup>+</sup>; P2, Gr-1<sub>high</sub>/F4/80<sup>+</sup>; P3, Gr-1<sup>+</sup>/F4/80<sup>+</sup>. Further examination of other surface markers showed that both P1 and P2 were positive for CD11b and CD11c; however, levels of Ly6C staining were high in P2 but much lower in P1 (Fig. 3.2B, top lane). P3 was Ly6C<sup>high</sup>, CD11b<sup>low</sup> and CD11c<sup>-</sup>. These results suggest that P1 and P2 cells were macrophage-like and P3 were neutrophil-like cells. G-CSF alone generated a very small number of cells, which were mainly P3 (Fig. 3.2A left panel and 3.2C). M-CSF generated about 1.5 × 10<sup>6</sup> P1 cells (~38% in proportion), 0.3 × 10<sup>6</sup> P2 (~7%) and 0.25 × 10<sup>6</sup> P3 (~5%) cells from 1 × 10<sup>6</sup> BMCs seeded. M-CSF together with G-CSF significantly increased cell numbers of all populations but much greater extents in P2 and P3 cells, which resulted in proportional increases of P2 and P3 with a slight, but non-significant decrease of P1 (Fig. 3.2C bottom panel). These results suggest that G-CSF support the generation of P2 and P3 cells, as well as survival of M-CSF responsive progenitor cells. Although G-CSF increased cell numbers, overall intensities for Ly6C and CD11c of these cells did not change (data not shown).

GM-CSF induced generation of at least four distinct populations based on F4/80 and Gr-1 staining (Fig. 3.3A). Similar to M-BMCs, P3 of GM-BMCs were Ly6C<sup>high</sup>/CD11b<sup>+</sup>/CD11c<sup>-</sup>; P1 was Ly6C<sup>low</sup>/CD11b<sup>-</sup>/CD11c<sup>+</sup>; P2 was Ly6C<sup>high</sup>/CD11b<sup>-</sup>/CD11c<sup>-</sup>; P4 was Ly6C<sup>low</sup>/CD11b<sup>-</sup>/CD11c<sup>-</sup> (Fig. 3.3B). Unlike M-BMCs, G-CSF had no effects on cell numbers and composition of populations in GM-BMCs (Fig. 3.3C). The differences in the cell populations between M-BMCs and GM-BMCs were not likely due to differences in granulocyte/macrophage progenitors, since BMCs from both wild-type and G-CSFR<sup>+</sup> mice generated the same cell population compositions in the presence of M-CSF or GM-CSF (Appendix A.2).
Collectively, these results suggest that G-CSF supported generation of P2 and P3 cells from M-BMCs.
Figure 3.2. G-CSF increases Gr-1\textsuperscript{high}/F4/80\textsuperscript{+}/Ly6C\textsuperscript{+}/CD11b\textsuperscript{+}/CD11c\textsuperscript{+} (P2) and Gr-1\textsuperscript{+}/F4/80\textsuperscript{+}/Ly6C\textsuperscript{+}/CD11b\textsuperscript{+}/CD11c\textsuperscript{+} (P3) cell populations in M-BMCs. BMCs from wild-type mice were treated with M-CSF (20 ng/ml), M-CSF + G-CSF (10 ng/ml) or G-CSF (10 ng/ml) alone for 5 days. (A) Based on the Gr-1 and F4/80 expression characteristics, cells were divided into 3 populations: P1, Gr-1\textsuperscript{low}/F4/80\textsuperscript{+}; P2, Gr-1\textsuperscript{high}/F4/80\textsuperscript{+}; P3, Gr-1\textsuperscript{+}/F4/80\textsuperscript{+}. (B) Characteristics in expression of Ly6C, CD11c and CD11b markers were further examined in each cell population. (C) Number and percentage of each cell population was analyzed and plotted. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s t-test or one-way ANOVA with Tukey's multiple comparison post-hoc test; columns accompanied by the same letter are not significantly different from each other).
Figure 3.2

A  G-CSF  M-CSF  M-CSF + G-CSF

F4/80  Gr-1

B  M-CSF + G-CSF

P1  P2  P3

Ly6C  Count

CD11b  CD11c

C

Cell number (per 1 x 10^6 BMCs)

2.5 x 10^6
2.0 x 10^6
1.5 x 10^6
1.0 x 10^6
0.5 x 10^6
0

P1  P2  P3

*  *  *

Cell population (%)

50
40
30
20
10
0

P1  P2  P3

a  a  a

b  b  b

G-CSF  M-CSF  M-CSF + G-CSF
Figure 3.3. **G-CSF had no effect on cell numbers and surface marker expressions on GM-BMCs.** BMCs from wild-type mice were treated with GM-CSF (10 ng/ml), GM-CSF + G-CSF (10 ng/ml) for 5 days. (A) Based on the Gr-1 and F4/80 expression characteristics, cells were divided into 4 populations: P1, Gr-1^{low}/F4/80^{+}; P2, Gr-1^{high}/F4/80^{+}; P3, Gr-1^{+}/F4/80^{-}; P4, Gr-1^{-}/F4/80^{-}. (B) Characterization of Ly6C, CD11c and CD11b marker surface expression was examined on each of the four cell populations. (C) Number and percentage of each cell population was quantified and plotted. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by one-way ANOVA with Tukey's multiple comparison post-hoc test).
Figure 3.3

A

Isotype  
GM-CSF  
GM-CSF + G-CSF

B

GM-CSF + G-CSF

Ly6C

CD11b

CD11c

C

Cell number (per 1 x 10^6 BMCs)  
Cell population (%)
3.3.3 G-CSF supports cell survival/proliferation in M-CSF-deprived cell culture conditions.

Since G-CSF had a growth supporting effect on M-BMCs, we further examined whether G-CSF can support survival/proliferation of M-BMCs at low M-CSF concentrations. BMCs were cultured at a high dose of M-CSF (20 ng/ml) or GM-CSF (10 ng/ml) for 3 days, and then the doses of M-CSF and GM-CSF were lowered to suppress cell proliferation or induce cell death with or without G-CSF (10 ng/ml) supplements for an additional 3 days. With 10 ng/ml of M-CSF cell culture, cell survival/proliferation of M-CSF-derived cells, examined by MTT assays, remain stable (Fig. 3.4A, left panel); however, at 5 ng/ml of M-CSF, cell death was increased after 2 days (Fig. 3.4A, right panel). Adding G-CSF in these M-CSF culture conditions improved cell survival/proliferation. With 5 ng/ml of GM-CSF cell culture, overall cell survival/proliferation remained unchanged (Fig. 3.4B, left panel), but cell death started to increase after 2 days of 1 ng/ml GM-CSF cell culture (Fig. 3.4B, right panel). Unlike M-BMCs, adding G-CSF in these GM-CSF culture conditions failed to support cell survival/proliferation. These results suggest that G-CSF could specifically compensate the function of M-CSF but not GM-CSF in bone marrow cell culture.
Figure 3.4. G-CSF supports growth of M-BMCs but not GM-BMCs in CSF-deprived cultures. Wild-type BMCs treated with M-CSF (20 ng/ml) or GM-CSF (10 ng/ml) for 4 days. These cells were sub-cultured with 2 different doses of M-CSF (10 ng/ml or 5 ng/ml; A) or GM-CSF (5 ng/ml or 1 ng/ml; B) with or without G-CSF (10 ng/ml) for the next 3 days. Cell survival was measured by MTT assays each day. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 between samples with and without G-CSF by Student’s t-test).
Figure 3.4

A

- G-CSF
+ G-CSF

B

- G-CSF
+ G-CSF
3.3.4 G-CSF protects cell death of M-CSF-derived P2 and P3 cells.

G-CSF specifically promoted growth of P2 and P3 of M-BMCs (Fig. 3.2). We further examined whether G-CSF also specifically support continued growth or prevent cell death of these cells at low M-CSF concentrations. Culturing M-BMCs at a low dose of M-CSF (5 ng/ml) significantly reduced P1, but not P2 and P3, which were low even at optimal M-CSF concentrations. Adding G-CSF (10 ng/ml) in these culture conditions significantly increased P2 and P3 without affecting P1 (Fig. 3.5A and B). Further staining of these cells with the cell death dye FVD eFluor® 660 showed that basal cell death levels of P1 were relatively low (~22%), which became significantly increased (~80%) after 3 days of cell culture at the low M-CSF concentration (Fig. 3.5C). Treatments of G-CSF had no effect on the extent of P1 cell death. Unlike P1, P2 cells showed high basal levels of cell death (~65%) at both concentrations of M-CSF, which was significantly reduced by G-CSF (~45%). Similarly, P3 cells also showed ~22% of basal cell death levels at both M-CSF concentrations, which was reduced by G-CSF (Fig. 3.5C). These results, together with Fig. 3.4, suggest that G-CSF selectively protected cell death of M-CSF-derived P2 and P3 BMCs cultured at sub-optimal and optimal doses of M-CSF.
Figure 3.5. G-CSF increases survival of P2 and P3 cells in M-BMCs. Wild-type BMCs were treated with M-CSF (20 ng/ml) for 4 days and then sub-cultured with 20 ng/ml or 5 ng/ml of M-CSF, or M-CSF (5 ng/ml) + G-CSF (10 ng/ml) for an additional 2 days. (A) Based on the Gr-1 and F4/80 expression characteristics, cells were divided into 3 populations: P1, Gr-1\textsuperscript{low}/F4/80\textsuperscript{+}; P2, Gr-1\textsuperscript{high}/F4/80\textsuperscript{+}; P3, Gr-1\textsuperscript{+}/F4/80\textsuperscript{−}. (B) Percentage of each cell population was quantified and plotted. (C) Percentage of dead cells (FVD eFluor\textsuperscript{®} 660\textsuperscript{−} cells) from P1, P2 and P3 populations was quantified by flow cytometry and plotted. (B-C) Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by one-way ANOVA with Tukey's multiple comparison post-hoc test; columns accompanied by the same letter are not significantly different from each other).
Figure 3.5

A

M-CSF (20 ng/mL)  M-CSF (5 ng/mL)  M-CSF (5 ng/mL) + G-CSF

B

Cell population (%)  

C

Cell death (%)  

Legend:
- 20 ng/ml M-CSF
- 5 ng/ml M-CSF
- 5 ng/ml M-CSF + G-CSF
3.3.5 G-CSF enhanced the generation of P2 cells from P1 M-BMCs.

To examine whether P2 cells originated from P1 or bone marrow precursor cells, F4/80$^+$ cells were isolated using the MACS column after culturing BMCs with M-CSF for 4 and 7 days. Cells were further cultured for the next 2 days in the presence of M-CSF or M-CSF + G-CSF and expression of Gr-1 and F4/80 was examined. As expected, no P3 or residual BMCs were detected (Fig. 3.6A), suggesting complete removal of residual BMCs and P3 cells by the column. M-CSF alone was able to generate P2 cells, which was apparent after a total of 9 (7 + 2) days of cell culture (middle panel). G-CSF further enhanced P2 generation, resulting in corresponding decreases of P1, in both day 4 and day 7 M-BMCs (Fig. 3.6B, left panels). However, when cell proliferation was inhibited by the antimitotic agent nocodazole (10 µg/ml), an increase in the P2 ratio over P1 by G-CSF was not detected (Fig. 3.6B, right panel). We then examined whether P2 cells originated from P1 by culturing only P1 cells. As shown in Fig. 3.6C, G-CSF enhanced the generation of P2 cells from isolated P1 M-BMCs. Also, both P1 and P2 cells completed one cell division after 2 days (Fig. 3.6D). These results, together with nocodazole data, suggest that P2 cells were derived from P1 M-BMCs, which was enhanced by G-CSF.
Figure 3.6. Increase of Gr-1$^{\text{high}}$/F4/80$^+$ (P2) cells by G-CSF is due to proliferation of P1 M-BMCs. BMCs were treated with M-CSF (20 ng/ml) for 4 and 7 days, F4/80$^+$ cells sorted by MACS columns, and then further cultured with M-CSF or M-CSF + G-CSF (10 ng/ml) in the presence or absence of the antimitotic agent nocodazole (Nocoda) for an additional 2 days. (A) Based on Gr-1 and F4/80 expression, 2 populations of cells were detected: P1, Gr-1$^{\text{low}}$/F4/80$^+$; P2, Gr-1$^{\text{high}}$/F4/80$^+$. (B) Percentages of P1 and P2 populations (left panels) and the ratio of P2 to P1 with or without nocodazole in day 4 M-BMCs (right panel) were plotted. (C-D) P1 cells were isolated from M-BMCs and stained with CFSE. These cells were further cultured with M-CSF or M-CSF + G-CSF for an additional 2 days. G-CSF enhanced the generation of P2 cells from P1 (C), and both P1 and P2 cells went through cell cycle at a similar rate (D). (B-C) Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 and N.S., not significant by Student’s t-test).
Figure 3.6

A

B

C

D

M-CSF
M-CSF + G-CSF

F4/80
Gr-1

Day 4

Day 7

Day 4

Day 7

<math>\text{Cell population (%)}</math>

<math>\text{Day 4}</math>

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3.3.6 P2 cells are “M2-like” macrophages as defined by cytokine and surface marker expression.

GM-CSF and M-CSF induce inflammatory and regulatory macrophages, respectively, from BMCs. Consistent with previous studies, M-BMCs produced higher levels of IL-10 and CCL2 and lower levels of TNF-α than GM-BMCs, and undetectable levels of IL-23, whereas GM-BMCs produced high levels of TNF-α and IL-23, and lower levels of IL-10 and CCL2 than M-BMCs in response to LPS (Fig. 3.7A). G-CSF had no effects on overall production of TNF-α and IL-23 in M- and GM-BMCs; however, slightly reduced levels of IL-10 and CCL2 production in M-BMCs. G-CSF had no effects on cytokine production in GM-BMCs. These results suggest that G-CSF had only minor or no effects on overall “M1-” and “M2-like” phenotypes of GM- and M-BMCs, respectively, in response to LPS. To further examine contributions of P1, P2 and P3 cells in these cytokine productions, each subpopulation of M-BMCs treated with G-CSF were isolated by FACSaria III cell sorter and treated with LPS as before. P1 and P2 cells produced similar levels of TNF-α; however, the levels of IL-10 and CCL2 were slightly but not significantly reduced in P2 cells when compared to those of P1 cells (Fig. 3.7B). P3 cells also produced similar levels of IL-10 as P1 and P2 cells, but the levels of TNF-α and CCL2 were low and unchanged by LPS. As expected, production of IL-23 was not detected in any of the cell populations. To examine responses to M2 stimuli, M-BMCs treated with G-CSF were treated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) and expression of M2 surface markers PDL2 (programmed cell death 1 ligand 2) [42], CD206 (mannose receptor, C type 1) and CD71 (transferrin receptor) [43] were examined. As shown in Fig. 3.7C, expression of PDL2, CD71 and CD206 were induced in all P1, P2 and P3 cells in response to IL-4/IL-13, but the highest amounts of these markers were induced in P2 cells. LPS or LPS + IFN-γ did not induce these markers in any of the cell populations (data not shown). Wright-Giemsa staining of P1, P2 and P3 cells also suggested that P1 and P2 cells were similar to each other in their overall characteristics including size, mononuclear morphology and adherence to culture dishes, whereas P3 cells were about half the size of P1 and P2 cells and non-adherent (data not shown). Ultra
structures of P1 and P2 cells, resolved by transmission electron microscopy, showed
similar macrophage-like spherical morphologies [44] with limited membrane ruffles
and developed vacuoles of about 12 microns in diameter (Fig. 3.7D). Collectively,
these data suggest that both P1 and P2 cells are M2-like macrophages but P2 cells are
more potent in expressing M2-specific surface markers in response to IL-4/IL-13,
compared to P1 cells.
Figure 3.7. Gr-1\textsuperscript{high}/F4/80\textsuperscript{+} (P2) cells are M2-like cells in cytokine production by LPS and cell surface marker expression by IL-4 and IL-13. (A) Wild-type BMCs were treated with either M-CSF (20 ng/ml) ± G-CSF (10 ng/ml) or GM-CSF (10 ng/ml) ± G-CSF (10 ng/ml) for 5 days. Cells (1.0 x 10\textsuperscript{5}) were then plated on a 96-well plate and treated with LPS (100 ng/ml) for 6 h. Concentrations of cytokines in the cell culture media were measured by ELISA. (B) Wild-type BMCs were treated with M-CSF (20 ng/ml) + G-CSF (10 ng/ml) for 5 days, and P1 (Gr-1\textsuperscript{low}/F4/80\textsuperscript{−}), P2 (Gr-1\textsuperscript{high}/F4/80\textsuperscript{+}) and P3 (Gr-1\textsuperscript{−}/F4/80\textsuperscript{−}) cells were isolated using the FACS\textsuperscript{Aria} III cell sorter. Each sorted population of cells (1.0 x 10\textsuperscript{5}) was treated with LPS (100 ng/ml) for 6 h and cytokine concentrations in the cell culture media were measured by ELISA. (C) Mean fluorescent intensity (MFI) of PDL2, CD71 and CD206 markers were further examined in each cell population. (A-C) Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by one-way ANOVA with Tukey's multiple comparison post-hoc test; columns accompanied by the same letter are not significantly different from each other). (D) Images shown are transmission electron micrographs of representative cells found in the sorted P1 and P2 populations. N.D, not detectable.
Figure 3.7

A

B

C

D

P1

P2
3.3.7  G-CSF enhanced gut-homing phenotype of M-BMCs.

Since monocytes migrate to local compartments/tissues during normal homeostasis, we examined where M-BMCs treated with G-CSF migrated. To this end, BMCs from lys-EGFP knock-in mice [41] were treated with M-CSF or M-CSF + G-CSF for 5 days and F4/80$^+$ cells were injected into tail veins of recipient mice. Injected M-BMCs were detected in the lamina propria of the small intestine after 2 days of injections, but not in other tissues including the spleen, liver, kidney, lung, heart and bone marrow (Fig. 3.8A, shown in arrows; Appendix A.3). Interestingly, G-CSF greatly enhanced the numbers of gut-homing M-BMCs in both small and large intestines (Fig. 3.8A-B). These transplanted cells could be detected in the intestine throughout the time frame examined (>30 days; Fig. 3.8C).
Figure 3.8. G-CSF enhances the localization of adoptively transferred F4/80⁺ M-BMCs to the lamina propria of intestines. (A) BMCs from lys-EGFP-ki transgenic mice were treated with either M-CSF (20 ng/ml) or M-CSF (20 ng/ml) + G-CSF (10 ng/ml) for 5 days. F4/80⁺ cells were isolated by MACS and 1.0 × 10⁶ cells were injected into recipient mice through tail vein. Two days after injection, sections from the large and small intestines of the recipient mice (total of 4 mice in 2 different times) were viewed through a fluorescent microscope as described in Methods. Images in the bottom row show representative fluorescent microscopy results. Images in the top row are representative overlays of phase contrast microscopic digital images with their corresponding fluorescent images from the same tissue section area. Arrows indicate EGFP⁺ cells. (B) Average numbers of EGFP⁺ cells per 40× field of view (>20) from the small and large intestine tissue sections were counted and plotted. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s t-test). (C) Similarly, the small and large intestine tissue sections after indicated days of adoptive transfer (2.0 × 10⁵ cells) were prepared and viewed as (A).
Figure 3.8

A

Small Intestine

Large Intestine

B

Day 2  Day 5  Day 15  Day 30

C

Day 2  Day 5  Day 15  Day 30
3.3.8  Gr-1\textsuperscript{high} and Gr-1\textsuperscript{low} intestinal resident macrophages (Ly6C\textsuperscript{low}/CD11c\textsuperscript{+}/F4/80\textsuperscript{+}) are reduced in G-CSFR\textsuperscript{−/−} mice.

Since G-CSF enhanced tissue localization of M-BMCs, we examined whether G-CSFR\textsuperscript{+/−} mice are defective in populating mononuclear phagocytes in the intestine during steady state. Lamina propria leukocytes of the intestine were isolated from G-CSFR\textsuperscript{+/−} and \textsuperscript{−/−} mice and examined for the surface expression of different markers. Based on the same flow cytometry analysis scheme used for \textit{in vitro} BMCs, intestinal lamina propria leukocytes isolated from wild-type mice contained F4/80\textsuperscript{+}/Gr-1\textsuperscript{low}/Ly6C\textsuperscript{low}/CD11c\textsuperscript{+} (~22%, P1), F4/80\textsuperscript{+}/Gr-1\textsuperscript{high}/Ly6C\textsuperscript{low}/CD11c\textsuperscript{+} (~10%, P2) and F4/80\textsuperscript{−}/Gr-1\textsuperscript{+}/Ly6C\textsuperscript{low}/CD11c\textsuperscript{−} (~20%, P3) cells (Fig. 3.9A-B). Cells from G-CSFR\textsuperscript{−/−} mice contained significantly lower numbers of P1 (~10%) and P2 (~4%) cells than those from G-CSFR\textsuperscript{+/−} mice, whereas no differences were detected in the numbers of P3 cells (Fig. 3.9C). These data suggest that G-CSFR plays a key role in populating both P1 and P2 mononuclear phagocytes in the intestine \textit{in vivo}.
Figure 3.9. G-CSFR<sup>−/−</sup> mice harbour reduced numbers of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> macrophage-like (Ly6C<sup>low</sup>/CD11c<sup>+</sup>/F4/80<sup>+</sup>) cells in the intestinal lamina propria compared to those of wild-type mice. The intestinal lamina propria cells were isolated from wild-type and G-CSFR<sup>−/−</sup> mice and hematogenous cells enriched on a percoll step gradient. (A) Based on the Gr-1 and F4/80 expression characteristics, cells were divided into 3 populations: P1, Gr-1<sup>low</sup>/F4/80<sup>+</sup>; P2, Gr-1<sup>high</sup>/F4/80<sup>+</sup>; P3, Gr-1<sup>+</sup>/F4/80<sup>+</sup>. (B) Each cell population was further characterized based on the expression of Ly6C and CD11c. (C) Percentage of each cell population was quantified and plotted. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s t-test).
Figure 3.9

A

B

C

G-CSFR<sup>+/+</sup>  G-CSFR<sup>−/−</sup>

Cell population (%)

P1  P2  P3

Cell population (%)

P1  P2  P3

Cell population (%)

P1  P2  P3

*
3.4 DISCUSSION

Although G-CSF has been dubbed the “generator and activator of granulocytes” due to its best known activities toward neutrophils, early in vitro bone marrow cell cultures and G-CSFR−/− mouse studies suggested that G-CSF is also involved in the generation of monocytes/macrophages. Here, we show that in a single CSF bone marrow cell culture model, G-CSF specifically supported persistent growth of M-BMCs but not GM-BMCs (Fig. 3.1C & D). GM-CSF alone was able to support generation of at least four heterogeneous cell populations, including Gr-1+/F4/80− neutrophil-like cells (Fig. 3.3), whereas M-CSF gave rise to a more homogeneous cell population than GM-CSF, mainly Gr-1low/Ly6Clow/F4/80+/CD11b+/CD11c+ macrophage-like (P1) cells (Fig. 3.2). Enhanced growth of M-BMCs by G-CSF mainly attributes to the generation of Gr-1high/F4/80+/Ly6C+/CD11b+/CD11c+ (P2) macrophage-like and Gr-1+/Ly6C+/F4/80+/CD11blow/CD11c− (P3) neutrophil-like cell populations (Fig. 3.2). These results were consistent with in vivo roles of GM-CSF and M-CSF in supporting granulocyte/macrophage progenitors and macrophage/DC precursor cells, respectively, in bone marrow [10]. The macrophage-DC precursor cells, derived from granulocyte-macrophage progenitors, lose their granulocyte potential and give rise to monocytes and common DC precursors [2, 45-48]. Subsequently, certain populations of monocytes migrate to tissues and differentiate into tissue macrophages, such as CD103+/CD11b+/F4/80+ cells in the gut [49, 50]. DCs can further differentiate to either classical or plasmacytoid DCs, which can constitute CD103+ or CD103− DCs in the gut [45, 51]. M-CSF is also involved in the transition of Ly6C+ monocytes to Ly6Cmonocytes [52-54]. Consistent with these studies, we found that BMCs cultured with M-CSF for more than 8 days reduced expression of Ly6C, regardless of the presence of G-CSF (data not shown). Therefore, M-CSF may support growth of macrophage-DC precursors and maturation of Ly6C+ cells to Ly6Cmonocytes, which are detected in the circulation at steady state [10] and the intestine (Fig. 3.9B). Unexpectedly, M-CSF together with G-CSF was able to generate both Gr-1+/F4/80− neutrophil-like (P3) and Gr-1high/F4/80+ macrophage-like (P2) cells, when G-CSF alone supported neither P2 nor P1 cells (Fig. 3.2A). These results are consistent with the fact that G-CSF induces cell
differentiation commitment [55], and suppresses apoptosis of progenitor and mature cells [56]. Furthermore, addition of G-CSF to BMCs after culturing 4 days with M-CSF suppressed apoptosis of P2 and P3 cells induced by sub-optimal concentrations of M-CSF (Fig. 3.5C). Although previous studies suggested that G-CSF has only apoptosis suppressive effects without proliferation-inducing activities [55, 56], signalling via the G-CSFR was shown to induce cell differentiation and proliferation through two distinct domains of the receptor [57]. Therefore, it is possible that synergistic effects of two separate signalling cascades induced by the M-CSF-receptor (M-CSFR) and G-CSFR may be required for the generation of P2 and P3 cells in in vitro bone marrow cell cultures. Particularly, G-CSF enhanced generation of P2 cells from P1 cells in M-BMCs, which was abolished by nocodazole (Fig. 3.6). Therefore, both cell-specific suppression of apoptosis and promotion of proliferation could contribute to the expansion of P2 cells in M-BMCs.

Previous studies showed that BMCs treated with GM-CSF or M-CSF produce distinct M1- and M2-like cytokine profiles, respectively, in response to microbial components such as LPS [11, 58, 59]. Consistent with their reports, GM-BMCs produced high levels of IL-23 and TNF-α, and low levels of CCL2 in response to LPS (Fig. 3.7A). Addition of G-CSF in the culture media had no effects on their production profiles. In contrast, M-BMCs produced high levels of IL-10 and CCL2, and low levels of IL-23 in response to LPS. Addition of G-CSF in the culture media slightly reduced production of IL-10 and CCL2. The decrease of CCL2 production was likely due to an increase of P3 cells in the culture, since P3 cells fail to produce CCL2 (Fig. 3.7B). P1 and P2 cells were equipotent in producing IL-10 and CCL2, suggesting that both P1 and P2 cells were M2-like macrophages. However, when M-BMCs were activated by IL-4/IL-13, expression levels of M2-specific surface markers were greater in P2 than P1 cells (Fig. 3.7C). Morphologies of both P1 and P2 cells were similar, showing typical macrophage-like ultra-structures with large vacuoles and limited membrane ruffles (Fig. 3.7D). Based on these results, we believe that G-CSF enhanced the generation of M2-like macrophages in conjunction with M-CSF. Further studies are required to unravel the distinctive characteristics and function of P2 cells in comparison to P1 cells.
M-CSF was shown to control macrophage populations in peritoneal macrophages and Kupffer cells [10, 53, 60], and differentiation of specific DC subsets such as epidermal Langerhans cells, kidney, lung and intestinal macrophages/DCs [51, 61, 62]. Considering its known biological functions, we expected that M-BMCs would be distributed to these tissues after adoptive transfer of M-BMCs. However, M-CSF-treated \textit{lys}-EGFP-\textit{ki} F4/80$^+$ BMCs were mainly localized in the small intestinal lamina propria of recipient mice (Fig. 3.8A). When these cells were treated with G-CSF, significantly more numbers of cells were localized in both the small and large intestines, and survived more than 30 days (Fig. 3.8). G-CSF also enhanced numbers of gut-homing M-BMCs even after 6 h of adoptive transfer (Appendix A.3B-C), suggesting that G-CSF enhanced gut-homing tropism of M-BMCs. In line with the effect of G-CSF, the lamina propria of the intestine from G-CSFR$^{-/-}$ mice were also shown to harbor greatly reduced numbers of Gr-1$^\text{high}$ and Gr-1$^\text{low}$ mononuclear cells (Ly6C$^\text{low}$/CD11c$^-$/F4/80$^+$), very similar to those of P2 and P1 macrophage-like cells, respectively, based on the four surface marker expression profiles (Fig. 3.9). However, no differences were detected in the numbers of intestinal Gr-1$^+/F4/80^+$ neutrophil-like (P3) cells between G-CSFR$^{+/+}$ and $^{-/-}$ mice (Fig. 3.9). These results are quite surprising, considering the substantial decrease in the proportion of neutrophils in the spleen and bone marrow of G-CSFR$^{-/-}$ mice (Appendix A.4). At this moment, it is unknown whether these transferred cells differentiated to typical resident macrophages after they arrived in the intestine. Previously, adoptive transfer of CCR2$^+/CX3CR1^\text{lo}$ inflammatory monocytes were short-lived, but CCR2$^-/CX3CR1^\text{hi}$ regulatory monocytes persisted longer and cells that could be recovered had a CD11c$^+/MHC-II^+$ DC-like phenotype [63]. Also Ly6C$^\text{hi}$ monocytes gave rise to CX3CR1$^+$ small intestinal lamina propria mononuclear phagocytes and failed to differentiate into classical splenic DCs [46]. Therefore, it will be interesting to examine whether M-BMCs treated with G-CSF, particularly P2 cells, share similar surface marker expression and function of CCR2$^-/CX3CR1^+$ mononuclear cells in the intestinal lamina propria. In addition, Gr-1$^+/CD11b^+$ cells are found during early lineage-committed precursors of myeloid cells [64] and comprise 20-30\% of BMCs [65]. Gr-1$^+/CD11b^+$ cells are also known as myeloid-derived suppressor cells [66, 67]
and adoptive transfer of these cells protects allergen-induced airway inflammation [68] and colitis [69]. Therefore, it will be interesting to see if G-CSF-induced P2 cells exhibit similar functions as these myeloid-derived suppressor cells.

G-CSF was shown to have anti-inflammatory and anti-apoptotic roles in immune cells, including macrophages, DCs and T/B lymphocytes [70-72]. In the intestine, G-CSF may be important for guarding against invading pathogens, maintaining tolerance to indigenous microbes and supporting cell survival in the presence of various gut luminal stresses [25]. Supporting this notion, the administration of recombinant G-CSF was shown to have beneficial effects in both animal colitis models [73, 74] and open-label pilot studies in inflammatory bowel disease patients [75, 76]. The in vitro bone marrow cell culture model used here will not represent the much more complex and heterogeneous monocytes/macrophage cell populations in vivo; however, simplistic cell cultures using a single CSF will provide valuable information on the role of specific cytokines under examination. Using the M-CSF or GM-CSF bone marrow cell culture models, we found that G-CSF also appeared to specifically support the survival of M-BMCs, which showed M2-like cytokine production profiles and gut-homing characteristics after adoptive transfer. These results suggest a novel function of G-CSF in the differentiation of macrophages to M2-like characteristics and in the homing of these cells to the intestine.
3.5 REFERENCES


Chapter 4

PROTECTIVE ROLE OF ENDOGENOUS G-CSF IN DEXTRAN SULFATE SODIUM-INDUCED ACUTE COLITIS THROUGH GENERATING GUT-HOMING MACROPHAGES

4.1 INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is primarily known for its role in the generation, mobilization and function of neutrophils [1-3] which are key innate immune cells fighting against invading microbes [4-7]. G-CSF also plays an important role in regulating both innate and adaptive immune responses through modulating activation of macrophages and dendritic cells (DCs) [8-10], and promoting generation of tolerogenic DCs [11] and regulatory T cells [12]. These dual modalities of G-CSF in immune regulation could be important in maintaining intestinal immune homeostasis, where both avid immune surveillance and tolerance against infiltrating commensal microbes are required. Previous studies have demonstrated that administration of recombinant G-CSF ameliorates colitis in acute and chronic experimental colitis [13, 14] and Crohn’s disease patients [15-17], suggesting its beneficial role in immune homeostasis of the intestine. However, the role of endogenous G-CSF in intestinal immune homeostasis is largely unknown.

Intestinal macrophages are highly populated in the intestinal lamina propria, residing underneath the single epithelial layer, and Peyer’s patches [18, 19]. These cells form an intestinal immune barrier for invading microbes, and also orchestrate immune and wound repair responses that prevent inadvertent development of colitis [20-22]. However, in pathologic conditions, monocytes/macrophages, rapidly recruited into the inflamed intestines, can mediate inflammatory responses through releasing pro-inflammatory cytokines or promoting inflammatory T cell differentiation [23, 24].

Unlike most tissue macrophages derived from local progenitor cells [25, 26], intestinal macrophages are mainly refurnished by circulating monocytes originated from bone marrow cells (BMCs) [19, 27]. Circulating monocytes released from the bone marrow migrate to the intestine where they differentiate into resident macrophages under the influence of local cytokine milieu [24]. Recruited monocytes or resident macrophages can then be polarized to either classically activated (M1) or alternatively activated (M2) macrophages in response to lipopolysaccharide (LPS)/interferon (IFN)-γ or interleukin (IL)-4/IL-13, respectively [28-31]. M1 macrophages are characterized as inflammatory macrophages by their high capacity
to produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-12, IL-23, and inducible nitric oxide synthase (iNOS) [29, 32]. In contrast, M2 macrophages highly express regulatory cytokines, such as transforming growth factor (TGF)-β and IL-10 [32], and various markers, including Arginase I [33], chitinase-like 3 (Ym1) [34], found in inflammatory zone 1 (FIZZ1) [34, 35], cluster of differentiation (CD)71, CD206 [36] and programmed death ligand 2 (PDL2) [37], which are involved in anti-inflammatory, wound-healing or anti-parasitic responses [28, 29, 31].

In addition to the generation of neutrophils, G-CSF was also shown to be involved in the generation of macrophages. It was shown to support the survival of macrophage progenitor cells in in vitro bone marrow cell cultures [1, 38] and mice deficient in G-CSF harbor ~50% reduced circulating monocytes, compared to wild-type mice [39]. Recently, we showed that G-CSF plays an important role in the generation of regulatory Gr-1<sup>high</sup>/F4/80<sup>+</sup> macrophages, which are localized mainly in the intestine after adoptive transfer [40]. Here, we further examined the role of endogenous G-CSF in intestinal immune homeostasis using a dextran sulfate sodium (DSS)-induced acute colitis model in G-CSFR<sup>−/−</sup> mice. We found that G-CSFR<sup>−/−</sup> mice were more susceptible to DSS-induced colitis than G-CSFR<sup>+/−</sup> or G-CSFR<sup>+/+</sup> mice. Macrophages derived in the presence of G-CSF enhanced expression of several M-like markers, such as PLD2, CD71 and CD206, and suppressed LPS/IFN-γ-induced expression of iNOS, CD80 and CD86. Adoptive transfer of these macrophages significantly ameliorated the severity of colitis in G-CSFR<sup>−/−</sup> mice. These results suggest that endogenous G-CSF plays a protective role from developing inadvertent colitis, likely through generating gut-homing macrophages.
4.2 MATERIALS AND METHODS

4.2.1 Mice

C57BL/6j wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Homozygote G-CSFR\(^{-/-}\) mice with C57BL/6j background were obtained originally from Dr. D. C. Link (Washington University Medical School, St. Louis, MO, USA) and maintained in the animal care and veterinary facility at the University of Western Ontario (London, ON, Canada) under conventional conditions. C57BL/6j and G-CSFR\(^{-/-}\) were crossed, and G-CSFR\(^{+/+}\) offspring were then crossed to generate G-CSFR\(^{+/+}\), \(^{+/+}\) and \(^{-/-}\) littermates for use in experimental procedures. G-CSFR\(^{+/+}\), \(^{+/+}\), and \(^{-/-}\) were housed in conventional conditions, and were intermixed randomly during experiments such that each would be co-housed with other mice of different genotypes. Aged-matched mice between 6 to 12 weeks of age were used in all experimental procedures. All experimental protocols were approved by the University of Western Ontario Animal Use Subcommittee, which follows the regulations of the Animals for Research Act (Ontario) and the Canadian Council on Animal Care.

4.2.2 Reagents, Antibodies and Cytokines

ELISA for TNF-\(\alpha\), IL-1\(\beta\) and IL-10 were obtained from eBioscience (San Diego, CA, USA). LPS from *Escherichia coli* O111:B4 was obtained from List Biological Laboratories (Campbell, CA, USA). FITC anti-mouse F4/80, PE-Cy7 anti-mouse Gr-1, PE anti-mouse CD71, PE anti-mouse PDL2, APC anti-mouse CD206, APC anti-mouse CD80 and PE anti-mouse CD86 antibodies were purchased from eBioscience (San Diego, CA, USA). IL-4, IL-13 and IFN-\(\gamma\) cytokines were obtained from PeproTech (Rocky Hill, NJ, USA). The murine recombinant M-CSF and G-CSF were obtained from eBioscience (San Diego, CA, USA).

4.2.3 DSS Colitis Mouse Model

1.5% weight/volume DSS (molecular weight: 40,000-50,000 Da, USB Corporation, Cleveland, Ohio) was dissolved in autoclaved drinking water and administered *ad libitum* to the mice for 5 days. Control mice were given normal drinking water. On
day 5, the DSS solution was replaced with water without DSS for an additional 4-5 days. Mice were monitored daily for weight loss, diarrhea and rectal bleeding. Disease activity scoring was performed as follows: weight loss (percentage of starting weight): < 10% = 1, < 15% = 2, < 20% = 3; diarrhea: slightly loose feces and/or slight rectal prolapse = 1, loose feces and/or obvious prolapse = 2, watery diarrhea and/or severe prolapse = 3; bleeding: spotty blood in feces or around anus = 1, slightly bloody feces or moderate blood around anus = 2, bloody stool or severe bleeding around anus = 3; activity: still moving spontaneously but reduced = 1, little spontaneous movement = 2, moves only with gentle touch = 3. The sum of the all values constitutes the colitis score. Mice were euthanized if the total score reached higher than 9 or if more than 20% body weight loss was observed.

4.2.4 Histology and Immunohistochemistry

After the mice were sacrificed, the caecum was removed and the remaining colon tissue was cut into 0.5 cm pieces. Tissues were then fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned (5 µm), and stained with hematoxylin and eosin (H&E) for visualization of intestinal tissue damage, or used for immunohistochemical staining for myeloperoxidase (MPO). H&E stained sections were evaluated by a pathologist in a blinded fashion. Histological scoring was performed as follows (modified from [41]): 0 = normal; 1 = lesions that are not transmucosal; 2 = 0-25% of mucosa affected; 3 = 25-50% of mucosa affected; 4 = >75% of mucosa affected. Immunohistochemical staining was performed after heat-induced epitope retrieval by heating slides to 95°C in 10 mM Tris buffer (pH 10) containing 0.05% Tween-20. Staining was performed using the Santa Cruz ABC staining kit (Santa Cruz Biotechnology, Santa Cruz, California) according to the manufacturer’s instructions. Briefly, slides were incubated in 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 minutes to quench endogenous peroxidase activity. Slides were then incubated in 1.5% blocking serum in PBS for 1 hour, followed by overnight incubation at 4°C with 10 µg/mL anti-MPO antibody (R&D Systems, Minneapolis, Minnesota). Staining was developed the next day and slides were counterstained with hematoxylin. For counting of the number of MPO positive
cells per field of view, a single tissue section containing the highest degree of staining on the slide was chosen, and densely stained cells in each field of view at 100× magnification were counted.

4.2.5 Generation of BMDM and G-BMDM

Macrophage-CSF (M-CSF)-treated bone marrow-derived macrophages (BMDM) and G-CSF- and M-CSF-treated bone marrow-derived macrophages (G-BMDM) were generated as previously described [40]. Briefly, BMCs were harvested from femurs and tibia of G-CSFR<sup>+/+</sup> and/or G-CSFR<sup>−/−</sup> mice using a 25.5-gauge needle and PBS. Red blood cells were lysed using ammonium chloride and EDTA. BMCs were then cultured in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 U ml<sup>−1</sup> penicillin, 0.1 mg ml<sup>−1</sup> streptomycin (Sigma-Aldrich), 5 mM sodium pyruvate (Sigma-Aldrich), 5 mM MEM non-essential amino acids (Sigma-Aldrich), and M-CSF (20 ng/ml) or M-CSF and G-CSF (10 ng/ml). Cells were then maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 7 days. The culture media was replaced with fresh media every 2 days.

4.2.6 Flow cytometry

BMDM and G-BMDM were harvested, washed twice, surface labeled with fluorescently conjugated antibodies against F4/80, Gr-1, PDL2, CD206, CD71, CD80 and CD86. Labeled cells were washed and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry analysis was done using FlowJo software (version 7.6.5; TreeStar, Ashland, OR, USA).

4.2.7 Adoptive transfer of BMDM/G-BMDM and estimation of the number of cells localized in the intestine

5 × 10<sup>6</sup> BMDM or G-BMDM were injected intraperitoneally into G-CSFR<sup>−/−</sup> recipient mice 1 day before and 2 days after DSS treatment. Blood samples were obtained immediately after sacrifice from each mouse in a 1.5 ml tube via cardiac puncture and allowed to clot at room temperature for 30 min. The blood samples were then
centrifuged (2000 g, 4°C, 10 min) and the serum was collected and stored at −80°C until analyzed for serum cytokine levels. In addition, colon tissue was excised (feces was gently forced out), washed extensively with PBS, and measured for weight and length. The entire colon tissues were then opened longitudinally, cut into 0.5 cm pieces and then 150 mg of the tissues were weighed and kept in TRIzol reagent (Life Technologies) for further quantitative real-time PCR (qRT-PCR) screening. The number of BMDM and G-BMDM were calculated by extrapolating from a standard curve generated by series of dilutions of G-CSFR transcripts obtained from $1 \times 10^6$ BMDM or G-BMDM injected, using qRT-PCR. Estimation of % of recovered BMDM and G-BMDM in intestinal tissues were then calculated from the total numbers of cells present in random intestinal tissue samples (~150 mg) using the same qRT-PCR procedures.

4.2.8 Quantitative Real-Time PCR

Total cellular RNA was isolated from the colon tissues using TRIzol according to manufacturer’s instructions. 1 µg of total RNA was then reverse-transcribed with M-MuLV reverse transcriptase (New England BioLabs) according to the manufacturer’s instruction in the presence of oligo (dT) primer. cDNA was then used for qRT-PCR analysis using the Brilliant SYBR Green PCR Master Mix (Applied Biosystems) on the Rotor-Gene RG3000 quantitative multiplex PCR instrument. Mouse oligonucleotide primers used in qRT-PCR analysis are listed in Table 4.1.
Table 4.1. List of primers used for qRT-PCR with corresponding amplicon sizes
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-GCATTGTGGAAGGGCTCATG-3'</td>
<td>361</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TTGCTGTTGAAGTCGAGGAG-3'</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5'-CTTGAAATAGCTCCCAGAA-3'</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CATTGGGAACCTCTCATCC-3'</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward: 5'-TGCTATGCTGCTTGCTCTTA-3'</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCATTCCGATAAGGCTTGG-3'</td>
<td></td>
</tr>
<tr>
<td>Arginase I</td>
<td>Forward: 5'-CAGAAGAATGGAAGAGTCAG-3'</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAGATATGCACGGAGTCACC-3'</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward: 5'-GGAGAGCCCTGGATACCAAC-3'</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAGGGTCAGCAGACAGAAGTT-3'</td>
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</tr>
<tr>
<td>Ym1</td>
<td>Forward: 5'-CATGATGACAGATGCCTGTGGA-3'</td>
<td>100</td>
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<td></td>
<td>Reverse: 5'-AGGGCCCTTATTGAGAGGACCTTT-3'</td>
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<tr>
<td>FIZZ1</td>
<td>Forward: 5'-ACTGCCCTGCTGCTACTGACTGACT-3'</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AAAGCTGCTTCTGCTACACC-3'</td>
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</tr>
<tr>
<td>IL-1β</td>
<td>Forward: 5'-GCTTCAGGCAGGCACTACAC-3'</td>
<td>239</td>
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<tr>
<td></td>
<td>Reverse: 5'-CGACAGCAGACGGTTTTC-3'</td>
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<tr>
<td>iNOS</td>
<td>Forward: 5'-CAGCTGGCTGTACAAACCTT-3'</td>
<td>95</td>
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<tr>
<td></td>
<td>Reverse: 5'-CATTGGAAAGCTGGCATCCTT-3'</td>
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<tr>
<td>G-CSF receptor WT (exon 4 and 5)</td>
<td>Forward: 5'-CATCCAACCTGGGGAGACGAC-3'</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTGGCAGGGGAGATGCCT-3'</td>
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</table>
4.2.9 Statistical Analysis

Statistical analysis was carried out using Prism 5.0c for Mac OS X (GraphPad Software, La Jolla, CA, USA). Student’s $t$-tests, one-way ANOVA with Tukey’s multiple comparison post hoc test and two-way ANOVA with Bonferroni post-test were performed as described in the figure legends.
4.3 RESULTS

4.3.1 G-CSFR deficiency exacerbates DSS-induced colitis in mice.

To examine the role of endogenous G-CSF in colitis, we first examined susceptibility of G-CSFR\(^{-/-}\) and G-CSFR\(^{+/+}\) mice to DSS-induced acute colitis in comparison to G-CSFR\(^{+/+}\) mice. As shown in Fig. 4.1A, non-DSS-treated G-CSFR\(^{+/+}\) mice (dotted lines) showed no difference in body weights relative to G-CSFR\(^{+/+}\) mice. Treatments of DSS through drinking water (ad libitum) for 5 days started causing severe body weight loss in Day 6 in all mice; however, G-CSFR\(^{-/-}\) mice showed more pronounced and rapid body weight loss than G-CSFR\(^{+/+}\) and \(^{+/+}\) mice by Day 7, and some G-CSFR\(^{-/-}\) mice lost more than 20% of their starting body weight by Day 8 (Fig. 4.1A, upper panel). Consistent with these results, DSS-treated G-CSFR\(^{-/-}\) mice exhibited higher diarrhea, rectal bleeding and lethargy scores during the course of DSS treatment (Day 7), which then decreased to the levels of G-CSFR\(^{+/+}\) mice on Day 9 (Fig. 4.1A, bottom left panel). The reason for the decrease observed on Day 9 was due to early termination of these mice because of high scores on Day 8, rather than recovery from colitis. In agreement with these results, overall colitis scores including body weight, diarrhea, rectal bleeding and lethargy were significantly higher in G-CSFR\(^{-/-}\) than G-CSFR\(^{+/+}\) or G-CSFR\(^{+/+}\) mice toward to end of DSS-treatment (Fig. 4.1A, bottom right panel).

Colons from mice euthanized on Day 9 were stained with H&E for histological evaluation. Non-DSS-treated G-CSFR\(^{+/+}\) and \(^{-/-}\) mice retained intact colonic structure (Fig. 4.1B, upper panels), whereas all DSS-treated mice had extensive epithelial cell destruction, crypt loss and tissue infiltration (Fig. 4.1B, lower panels). However, colons from DSS-treated G-CSFR\(^{-/-}\) mice showed more extensive tissue damage than those from DSS-treated G-CSFR\(^{+/+}\) and \(^{+/+}\) mice. In agreement with these results, DSS treatment led to a significant increase in histopathological scores (0-4, 4 being the most tissue damage) of G-CSFR\(^{-/-}\) colons compared to their G-CSFR\(^{+/+}\) counterparts (Fig. 4.1C). Indeed, all examined G-CSFR\(^{-/-}\) colons scored 2 to 4, while G-CSFR\(^{+/+}\) tissue scores ranged from 0-4. Additionally, 3 out of the 5 G-CSFR\(^{-/-}\) mice examined
histologically also revealed the formation of a pseudomembranous structure (Fig. 4.1B, lower right panel), which appeared to be admixed with a large population of bacteria and pockets of bacterial clusters surrounded by infiltrating cells (arrow). These results suggest the presence of a secondary bacterial infection in the DSS-treated G-CSFR−/− mice. In contrast, no pseudomembranous structures or bacterial infections were detected in any of the colon segments from G-CSFR+/+ or +/- mice.
Figure 4.1. G-CSFR deficiency exacerbates DSS-induced colitis in mice. (A) G-CSFR*/*, +/- or -/- mice were administered 1.5% dextran sulfate sodium (DSS) in the drinking water for 5 days, and fresh water thereafter. Body weight loss, diarrhea/rectal bleeding/lethargy scores, and overall colitis scores (encompassing weight loss, stool consistency, rectal bleeding and loss of activity) were plotted from Day 0 to 9. Figures show pooled data of more than 3 independent experiments (n ≥ 10 per group, except Day 9 (n = 5 for G-CSFR +/- and -/-, n = 2 for G-CSFR*/*)). *, p < 0.05 by two-way ANOVA with a Bonferroni post-test. Symbols represent mean ± SEM. (B) Colon sections stained with H&E. Non DSS-treated G-CSFR*/* and +/- colon tissue showed healthy and normal appearance. Nine days after DSS treatment, colon tissue of all mice groups showed intestinal tissue damage; however, G-CSFR*/* and +/- tended to show a lesser degree of tissue damage than G-CSFR -/. DSS-treated G-CSFR-/- mice showed the presence of a pseudomembranous structure with clusters of bacteria, digitally magnified inset of DSS-treated G-CSFR-/-; bacteria indicated by arrow. Original images were obtained at 400× magnification. (C) Histological damage scores (0-4, 4 being the most tissue damage) from 4-6 tissue sections of each group were plotted. All G-CSFR-/- tissues examined scored 2 to 4, whereas G-CSFR*/*+ scores ranged from 0-4. *, p < 0.05 by Student’s t-test.
Figure 4.1

A

Body Weight (% of starting body weight) over time for different genotypes:
- **G-CSFR**+
- **G-CSFR** homozygous
- **G-CSFR** heterozygous
- **G-CSFR** heterozygous
- **G-CSFR** homozygous
- **G-CSFR** homozygous

B

Diarrhea/rectal bleeding score over time for different genotypes:
- **G-CSFR**+
- **G-CSFR** homozygous
- **G-CSFR** heterozygous
- **G-CSFR** homozygous

C

Overall colitis score over time for different genotypes:
- **G-CSFR**+
- **G-CSFR** homozygous
- **G-CSFR** heterozygous
- **G-CSFR** heterozygous

Histological Score:
- **G-CSFR**+
- **G-CSFR** homozygous
- **G-CSFR** heterozygous

DSS-treated G-CSFR+
DSS-treated G-CSFR−
4.3.2 G-CSFR<sup>−/−</sup> mice harbor lower basal levels of F4/80<sup>+</sup> macrophage-like cells without alterations in F4/80<sup>+</sup>/Gr-1<sup>+</sup> neutrophil-like cells in basal and DSS-exposed intestines.

We then examined whether F4/80<sup>+</sup> macrophage-like and F4/80<sup>+</sup>/Gr-1<sup>+</sup> neutrophil-like cells are similarly populated in the intestine between G-CSFR<sup>+/+</sup> and G-CSFR<sup>−/−</sup> mice. As shown in Fig. 4.2A, G-CSFR<sup>−/−</sup> cells were similarly populated in neutrophil-like cells (~20% of all gut lamina propria cells), but significantly lower in macrophage-like cells (~10%), when compared to those (~20% in both cell types) of G-CSFR<sup>+/+</sup> mice. To determine whether circulating neutrophil deficiency participated in the exacerbation of inflammatory conditions in the DSS-treated G-CSFR<sup>−/−</sup> mice, we evaluated neutrophil infiltration into the intestine of DSS-treated mice using immunohistochemical staining for MPO. Non-DSS-treated mice showed on average less than one positive cell per entire tissue section (data not shown). However, the average number of MPO<sup>+</sup> cells in colon segments from DSS-treated G-CSFR<sup>−/−</sup> mice (Fig. 4.2B; right panels shows a digitally magnified inset of left column, arrows indicate example MPO<sup>+</sup> cells) showed a trend for slightly higher numbers of MPO<sup>+</sup> cells relative to DSS-treated G-CSFR<sup>+/+</sup> or <sup>−/−</sup> mice (Fig. 4.2C), although this did not reach statistical significance. Taken together, these data imply that reduced macrophage, but not neutrophil, infiltration may be responsible for the increased DSS-induced colitis susceptibility in G-CSFR<sup>−/−</sup> mice.
Figure 4.2. G-CSFR$^+$ mice harbor lower basal levels of F4/80$^+$ macrophage-like cells without alterations in Gr-1$^+$/F4/80$^-$ neutrophil-like cells in basal and DSS-exposed intestines. (A) The intestinal lamina propria cells were isolated from G-CSFR$^{++}$ and G-CSFR$^{--}$ mice and hematogenous cells were enriched on a Percoll step gradient. Percentage of F4/80$^+$ macrophage-like and F4/80$^-$/Gr-1$^+$ neutrophil-like cells were quantified and plotted. Data shown as mean ± SEM ($n \geq 3$; *, $p < 0.05$ by Student’s $t$-test). (B and C) G-CSFR$^{++}$ and $^{--}$ mice colon tissue sections prepared 9 days after the initiation of 1.5% dextran sulfate sodium (DSS) treatment were stained for myeloperoxidase (MPO). (B) While G-CSFR$^{++}$ tissue sections showed a few number of cells but with strong MPO staining (digitally magnified inset of DSS-treated G-CSFR$^{++}$; MPO staining = brown color; arrow indicates example MPO$^+$ cell), G-CSFR$^{--}$ showed high number of MPO$^+$ cells but with weak staining (digitally magnified inset of DSS-treated G-CSFR$^{--}$; arrow indicates example MPO$^+$ cell). Original images were obtained at 400× magnification. (C) Quantification of MPO$^+$ cells per field of view from more than 4 tissue sections from each group was plotted. Line represents the mean. No significant changes in between groups were detected.
Figure 4.2

A

![Graph showing intestinal macrophages and intestinal neutrophils percentage for G-CSFR+/+ and G-CSFR-/- mice.]

B

![Images of DSS-treated G-CSFR+/- and DSS-treated G-CSFR-/- mice showing intestine macrophage population.]

C

![Graph showing number of MPO+ cells per field of view for G-CSFR+/-, G-CSFR+/-, and G-CSFR-/- mice with and without DSS treatment.]

4.3.3 G-CSF enhances expression of several M2-like cell surface markers in BMDM in response to IL-4/IL-13.

To examine the effects G-CSF on macrophage activation, primary macrophages were generated by culturing BMCs with M-CSF (BMDM) or G-CSF and M-CSF (G-BMDM) 5 days and treated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for an additional 2 days. On day 7, expression of M2 cell surface markers including PDL2, CD71 (transferrin receptor) and CD206 (mannose receptor, C type 1) were examined. As shown in Fig. 4.3A, IL-4/IL-13 significantly induced expression of PDL2, CD206 and CD71 in both BMDM and G-BMDM. However, expression levels of these markers were significantly higher in G-BMDM than BMDM. Similar experiments using G-CSFR<sup>−/−</sup> mice showed no such G-CSF effects on these markers in G-CSFR<sup>−/−</sup> BMDM, suggesting that G-CSFR was the receptor responsible for exogenous G-CSF responses in BMDM. We further quantified mRNA expression levels of intracellular M2 markers in these cells, including Arginase I, TGF-β, Ym1 and FIZZ1. However, unlike surface markers, expression levels of these intracellular markers were not significantly different between G-BMDM and BMDM (Fig. 4.3B). These results suggest that G-CSF generated a distinct macrophage population with different capabilities in responding to M2-stimuli.
Figure 4.3. G-CSF enhances expression of several M2-like cell surface markers in BMDM in response to IL-4/IL-13. G-CSFR\(^{+/+}\) (WT) and G-CSFR\(^{-/-}\) (KO) BMCs were treated with M-CSF (20 ng/ml) (BMDM) or M-CSF + G-CSF (10 ng/ml) (G-BMDM) for 5 days. Then, cells were treated with IL-4 (20 ng/ml) + IL-13 (20 ng/ml) for an additional 2 days. (A) Mean fluorescence intensity (MFI) of PDL2, CD71 and CD206 cell surface markers of WT and KO M- and G-BMDM were examined. (B) mRNA expression of Arginase I, TGF-β, Ym1, and FIZZ1 was analyzed in IL-4/IL-13-treated WT M- or G-BMDM. Data shown as mean ± SEM \([n \geq 3]; *, p < 0.05\) by one-way ANOVA with Tukey’s multiple comparison post hoc test; columns accompanied by the same letter (a, b or c) are not significantly different from each other.
Figure 4.3

A


B

4.3.4 G-BMDM are less responsive to M1-stimuli in expressing iNOS, CD80 and CD86 than BMDM.

To examine the effects of G-CSF on the response of BMDM to M1-stimuli, BMDM were similarly cultured as above, except with IFN-γ (20 ng/ml) and LPS (50 ng/ml) for an additional 2 days, and examined for the expression of M1 markers including IL-1β, iNOS, CD80 and CD86. G-BMDM expressed significantly lower levels of iNOS and CD86, but not IL-1β, when compared with those of BMDM (Fig. 4.4). Although the expression levels of CD80 in G-BMDM were slightly but not significantly higher than those of BMDM, treatments of IFN-γ and LPS induced CD80 expression in BMDM, but not in G-BMDM.
Figure 4.4. G-BMDM are less responsive to M1-stimuli in expressing iNOS, CD80 and CD86 than BMDM. G-CSFR+/+ BMCs were treated with M-CSF (20 ng/ml) (BMDM) or M-CSF + G-CSF (10 ng/ml) (G-BMDM) for 5 days. Then, cells were treated with IFN-γ (20 ng/ml) + lipopolysaccharide (LPS; 50 ng/ml) for an additional 2 days. mRNA expression of IL-1β and iNOS and mean fluorescence intensity (MFI) of CD80 and CD86 cell surface markers were examined. Data shown as mean ± SEM [n ≥ 3; *, p < 0.05 by one-way ANOVA with Tukey’s multiple comparison post hoc test; columns accompanied by the same letter (a, b or c) are not significantly different from each other].
Figure 4.4

**INOS mRNA fold change**

- BMDM
- M-BMDM
- M+G-BMDM

**IL-1β mRNA fold change**

- BMDM
- M-BMDM
- M+G-BMDM

**iNOS mRNA fold change**

- BMDM
- M-BMDM
- M+G-BMDM

**MFI of PDL2**

- BMDM
- M-BMDM
- M+G-BMDM

**MFI of CD80**

- BMDM
- M-BMDM
- M+G-BMDM

**MFI of CD86**

- BMDM
- M-BMDM
- M+G-BMDM

Legend:

- None
- IFN-γ + LPS
4.3.5 Adoptive transfer of G-BMDM exert a protective effect on DSS-induced mouse colitis in G-CSFR⁻/⁻ mice.

Next, we examined the role of G-BMDM in murine DSS-induced colitis in mice. To this end, $5 \times 10^6$ G-BMDM or BMDM were adoptively transferred into G-CSFR⁻/⁻ mice twice, 1 day before and 2 days after starting DSS treatments. Adoptive transfer of G-BMDM significantly prevented body weight loss, which was comparable to that of G-CSFR⁺/+ mice, whereas BMDM had no effects on body weight loss (Fig. 4.5A, upper panel). Consistent with these results, adoptive transfer of G-BMDM, but not BMDM, into DSS-treated G-CSFR⁻/⁻ mice also showed significantly lower diarrhea and rectal bleeding, and overall colitis scores which were similar to those of DSS-treated G-CSFR⁺/+ mice (Fig. 4.5A, bottom panels). In addition, DSS caused more extensive colon shortening, which is an additional disease severity indicator in colitis, in G-CSFR⁻/⁻ than G-CSFR⁺/+ mice. However, adoptive transfer of G-BMDM into G-CSFR⁻/⁻ mice prevented the colon shortening to a similar extent of G-CSFR⁺/+ mice (Fig. 4.5B). We estimated that about 28% of total adoptively transferred G-BMDM ($2.8 \times 10^6$ G-BMDM/mouse) were localized in the intestine of these mice, based on qRT-PCR-based estimation as described in the Materials and Methods (Fig. 4.5C).
Figure 4.5. Adoptive transfer of G-BMDM exert a protective effect on DSS-induced mouse colitis in G-CSFR<sup>−/−</sup> mice. G-CSFR<sup>+/+</sup> and <sup>−/−</sup> mice were given 1.5% dextran sulfate sodium (DSS) in the drinking water for 5 days, and fresh water thereafter. G-CSFR<sup>−/−</sup> mice were injected with 5 × 10⁶ M-CSF (20 ng/ml)-treated (BMDM) or M-CSF + G-CSF (10 ng/ml)-treated (G-BMDM) cells 1 day before and 2 days after DSS treatment. (A) Body weight loss, diarrhea/rectal bleeding and overall colitis scores (encompassing weight loss, stool consistency, rectal bleeding, and loss of activity) were plotted from Day 0 to 10. *, p < 0.05 by one-way ANOVA with Tukey’s multiple comparison post hoc test. Symbols represent mean ± SEM. (B) Colon length of mice after 10 days of DSS treatment was measured and plotted. (C) Gut-homing phenotype of the adoptive transferred-M- and G-BMDM in DSS-treated G-CSFR<sup>−/−</sup> mice was evaluated by investigating the presence of mRNA expression of G-CSF receptor wild-type (G-CSFR exon 4 and 5) in colon tissues. Data shown as mean ± SEM [n ≥ 3; *, p < 0.05 by one-way ANOVA with Tukey’s multiple comparison post hoc test; columns accompanied by the same letter (a, b or c) are not significantly different from each other].
4.3.6 Adoptive transfer of G-BMDM suppresses pro-inflammatory cytokines and iNOS in DSS-treated G-CSFR−/− mice.

Since adoptive transfer of G-BMDM protected DSS-induced colitis, we further examined the expression of cytokines and iNOS in serum and/or colon tissues from day 10 samples of DSS- and non-treated G-CSFR+/+ and G-CSFR−/− mice. As shown in Fig. 4.6A, DSS treatment significantly induced TNF-α, IL-1β and iNOS mRNA in colon tissues, which was more pronounced in G-CSFR−/− than G-CSFR+/+ mice. G-CSFR−/− colon tissues adoptively transferred with G-BMDM showed lower expression of these inflammatory markers, which were comparative to those of G-CSFR+/+ colons. Similarly, production of the pro-inflammatory cytokines TNF-α and IL-1β were enhanced in G-CSFR−/− mice, when compared with those in G-CSFR+/+ mice, and adoptive transfer of G-BMDM to G-CSFR−/− mice suppressed expression of these cytokines to the levels of G-CSFR+/+ (Fig. 4.6B). However, no significant changes were detected in the levels of IL-10 production regardless of DSS treatment (Fig 6B; right panel). Overall, these results indicate that endogenous G-CSF plays a protective role in DSS-induced colitis in mice, which is at least in part mediated by generating gut-homing macrophages.
Figure 4.6. Adoptive transfer of G-BMDM suppresses pro-inflammatory cytokines and iNOS in DSS-treated G-CSFR−/− mice. G-CSFR+/+ and −/− mice were given 1.5% dextran sulfate sodium (DSS) in the drinking water for 5 days, and fresh water thereafter. G-CSFR−/− mice were injected with \(5 \times 10^6\) M-CSF (20 ng/ml) + G-CSF (10 ng/ml)-treated (G-BMDM) cells 1 day before and 2 days after DSS treatment. (A) mRNA expression of TNF-α, IL-1β and iNOS was measured using qRT-PCR from colon tissues of mice with or without adoptive transfer of G-BMDM. Data shown as mean ± SEM \([n \geq 3;\] by one-way ANOVA with Tukey’s multiple comparison post hoc test; columns accompanied by the same letter (a, b, or c) are not significantly different from each other]. (B) Cytokine levels of TNF-α, IL-1β and IL-10 were measured from serum of mice using ELISA. *, \(p < 0.05\) by Student’s \(t\)-test. Each point represents an individual mouse, line shows the mean. Pooled data of two independent experiments.
**Figure 4.6**

A

![Graph showing TNF-α mRNA fold change, IL-1β mRNA fold change, and iNOS mRNA fold change](image)

B

![Graph showing TNF-α (pg/ml), IL-1β (pg/ml), and IL-10 (pg/ml)](image)
4.4 DISCUSSION

Systemic administration of recombinant G-CSF induces immune suppressive and wound healing responses [41-43], which can render beneficial effects on animal and human colitis [16, 44-47]. However, the role of endogenous G-CSF in immune homeostasis of the intestine is not well understood. Here, we show that G-CSFR⁻/⁻ mice did not develop spontaneous colitis, but these mice developed pseudomembranous structures and more severe colitis than G-CSFR⁺/⁺ or G-CSFR⁺⁻ mice when 1.5 % DSS was administered (Fig. 4.1), suggesting a protective role of endogenous G-CSF in acute colitis. In fact, it is often the case for mice genetically mutated in key innate immune factors only when phenotypes are manifested after certain challenges such as DSS or microbial infections [48-50]. Different intestinal microflora and/or the presence of unique pathogenic microbes could have contributed to the phenotypes of G-CSFR⁻/⁻ mice [51, 52]. However, G-CSFR⁻/⁻ mice were housed intermixed with G-CSFR⁺/⁺ and ⁺/⁻ mice during the course of the experiment; therefore, G-CSF deficiency, rather than differences in microbial exposure, was likely responsible for the development of pseudomembrane structures and severe colitis.

G-CSFR⁻/⁻ mice did not show apparent defects in the numbers of resident (Fig. 4.2A) and recruited neutrophils (MPO⁺ cells; Fig. 4.2B & C). These results are consistent with previous studies shown that, although circulating neutrophil counts are decreased up to 85%, emergency generation neutrophils and their recruitments to local infection/inflammatory sites during infection or inflammation are not compromised in G-CSF⁻/⁻ or G-CSFR⁻/⁻ mice [53-55]. However, G-CSF also enhances bactericidal function of neutrophils, development of severe colitis and formation of pseudomembranous structures in G-CSFR⁻/⁻ mice could be due to functional defects of intestinal neutrophils. Indeed, low intensities of MPO staining in G-CSFR⁻/⁻ mice may indicate a functional defect in G-CSFR⁻/⁻ neutrophils, as MPO is important for the bactericidal activity of neutrophils [56] and their ability to produce neutrophil extracellular traps [57]. Further studies are required to explore whether and how these defects influence the outcome of severe colitis in G-CSFR⁻/⁻ mice. Unlike neutrophils,
we found lower numbers of intestinal macrophages in G-CSFR<sup>−/−</sup> mice than wild-type mice (Fig. 4.2A). This observation is consistent with the facts that G-CSF<sup>−/−</sup> mice harbors ~50% lower circulating monocytes than wild-type mice [39], resident intestinal macrophages are mainly originated from circulating monocytes [19, 27], and G-CSF promotes generation of gut-homing Gr-1<sup>high</sup>/F4/80<sup>+</sup> monocytes [40]. Therefore, we further examined the characteristics of macrophages cultured in the presence of G-CSF and their role in colitis.

Of more than 20 soluble hematopoietic factors, M-CSF and granulocyte/macrophage-CSF (GM-CSF) play crucial roles in the generation and characteristic differentiation of myeloid cells in the bone marrow [58, 59]. In general, M-CSF is involved in the generation and maintenance of “M2-like” macrophages, which potently produce anti-inflammatory/regulatory cytokines upon activation; whereas, GM-CSF generates “M1-like” DCs or macrophages skewed toward pro-inflammatory responses [59, 60]. Previously, we showed that G-CSF enhances the generation of M-CSF-derived, but not GM-CSF-derived, macrophages which are mainly localized in the colon after the adoptive transfer [40]. In line with this observation, macrophages cultured together with G-CSF expressed higher levels of M2 markers (PDL2, CD71 and CD206; Fig. 4.3), but lower levels of M1 markers (iNOS and CD80/86; Fig. 4.4), in response to IL-4/-13 and IFN-γ/LPS, respectively. However, it had no effects on the expression of several other M1 and M2 markers, such as Arginase I, Ym1, FIZZ1, TGF-β and IL-1β, suggesting an effect of G-CSF on macrophages distinct from a generalized dichotomic view of macrophage activation/differentiation. At this moment, it is not clear whether G-CSF directly induced expression or suppression of these markers, and/or contributed to the proliferation of specific subsets of macrophages. Since, G-CSF did not induce such effects in fully differentiated macrophages (data not shown), we suspect that G-CSF promoted generation of macrophages with unique characteristics. Further experiments using cell proliferation inhibitors or mutant G-CSFR specifically defective in the cell proliferation or differentiation signaling cascade [61, 62] will likely provide information on these questions.
Regardless the mechanism of G-CSF in inducing M2-like responses, we examined the role of macrophages in colitis and found that G-BMDM, but not BMDM, protected G-CSFR−/− mice from DSS-induced acute colitis (Fig. 4.5). DSS treatments induced higher levels of TNF-α, IL-1β and iNOS in the colon, and TNF-α and IL-1β in the serum in G-CSFR−/− mice than wild-type mice, which were prevented by adoptively transferring G-BMDM (Fig. 4.6). Since these pro-inflammatory cytokines are main mediators of colitis in DSS-induced colitis in animals and inflammatory bowel disease patients [63-66], we tempt to speculate that G-CSF at least in part positively contribute to immune homeostasis of the intestine through enhancing intestinal localization of immune regulatory macrophages and suppressing production of pro-inflammatory cytokines. However, it is still unknown how and whether directly or indirectly G-BMDM rendered the colitis ameliorating effects. Since resident macrophages, which are tolerant to microbes in inducing pro-inflammatory responses, actively involved in phagocytosis of invading microbes, and immune modulating and wound healing processes [67-70], we suspect that adoptively transferred G-BMDM likely conferred beneficial effects during DSS-induced colitis through enhancing immune barrier function (eg. phagocytosis and killing microbes) without overt inflammation and/or wound healing processes. Consist with the beneficial effects of G-CSF, we previously showed that G-BMDM comprise mainly with Gr-1+/CD11b+ cells [40], which are known as myeloid-derived suppressor cells [71, 72] and adoptive transfer of these cells protects allergen-induced airway inflammation [73] and colitis [74].

In summary, this study demonstrates that G-CSFR−/− mice were more susceptible to DSS-induced acute colitis than wild-type mice and adoptive transfer of G-BMDM protected G-CSFR−/− mice from the colitis. These results suggest that endogenous G-CSF plays an important role in maintaining intestinal immune homeostasis and preventing colitis, likely through enhancing population of immune regulatory macrophages in the intestine.
4.5 REFERENCES


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Chapter 5

PREFERENTIAL PRODUCTION OF G-CSF BY A PROTEIN-LIKE LACTOBACILLUS RHAMNOSUS GR-1 SECRETORY FACTOR THROUGH ACTIVATING TLR2-DEPENDENT SIGNALING EVENTS WITHOUT ACTIVATION OF JNKS

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5.1 INTRODUCTION

Microorganisms induce diverse immune responses, which can be either harmful or beneficial to the host. Unlike pathogenic microbes, probiotics are microorganisms usually isolated from fermented food or healthy individuals, and confer a health benefit on the host when administered in adequate amounts [1, 2]. Among various probiotic bacteria, Lactobacillus, a Gram-positive facultative anaerobic bacterium, is a common constituent of the indigenous microbiota in the human intestinal and urogenital tracts [3, 4], and has been used as a probiotic for preventing or treating infectious and inflammatory diseases [5, 6]. However, different Lactobacillus species and strains elicit strikingly different immune responses from a variety of immune cells and experimental systems [7-10]. L. rhamnosus GG (LGG) is a well-studied strain that can induce inflammatory responses in dendritic cells (DCs) and aggravate dextran sulfate sodium (DSS)-induced acute colitis in mice [11, 12]. LGG also has anti-inflammatory effects on mouse and human macrophage cell lines [7, 13], and renders beneficial effects on chronic DSS-induced mouse colitis and pouchitis in human [14, 15]. Two soluble factors from LGG, referred to as p75 and p40, prevent apoptotic cell death of intestinal epithelial cells through activating the epithelial growth factor receptor [16, 17] and are beneficial in ameliorating DSS-induced acute colitis, as well as oxazolone and trinitrobenzenesulfonic acid-induced chronic colitis in mice [18]. L. rhamnosus GR-1 (GR-1), which is closely related to LGG, colonize both the intestinal and urogenital tracts after oral supplements [19-21]. Previously, GR-1 was shown to increase IL-10 and granulocyte-colony stimulating factor (G-CSF) production, and suppress tumor necrosis factor (TNF)-α production in human placental trophoblast cells [22, 23]. GR-1 also renders anti-inflammatory effects on macrophages [24, 25] and DCs [26], and promotes the generation of regulatory T cells in humans [27]. However, detailed immune responses and signaling mechanisms elicited by GR-1 remain largely unknown.

Macrophages are key innate immune cells, orchestrating immune responses through releasing various pro- and anti-inflammatory cytokines. These cells are highly populated in the gut lamina propria, interacting directly with microorganisms that
have crossed the gut barrier [28]. Macrophages detect microbe-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Although probiotics harbor MAMPs, they rarely cause infections and inflammatory diseases, and discretely modulate host immune responses through inducing distinct cytokine and chemokine profiles [7-10, 29]. Although details remain to be elucidated, several strains of probiotic bacteria have been shown to have immunomodulatory activities through selectively activating PRRs or regulating signaling cascades initiated by PRRs. For example, cell wall components such as lipoteichoic acid (LTA) [30-32], cell wall associated polysaccharide [33], S layer protein A [34], bacteriocins [35, 36], pilus [37] and histamine [38, 39] have each been shown to modulate pro-inflammatory responses in macrophages and DCs through activating specific or unidentified receptors. Also, several probiotic bacteria were shown to suppress pro-inflammatory cytokine expression or promote anti-inflammatory cytokines by inhibiting activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [31, 40, 41], c-Jun N-terminal kinases (JNKs) [42] and extracellular regulated kinases (ERKs) [31, 41]. The present study examined cytokines produced, signaling cascades activated, and bacterial factor(s) released by GR-1 in macrophages. We found that a protein-like factor released by GR-1 specifically and potently induced immunomodulatory G-CSF through activating NF-κB, ERKs and protein kinase B (also known as Akt), but not JNKs, in a TLR2-dependent manner.
5.2 MATERIALS AND METHODS

5.2.1 Mice

C57BL/6j wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained in the animal care and veterinary facility at the University of Western Ontario (London, ON, Canada) under conventional conditions. Male and female mice, aged 4-10 weeks, were used in all experimental procedures. All experimental protocols were approved by the University of Western Ontario Animal Use Subcommittee, which follows the regulations of the Animals for Research Act (Ontario) and the Canadian Council on Animal Care.

5.2.2 Cell cultures, bacteria and reagents

Immortalized mouse bone marrow-derived macrophages (BMDM) with C57BL/6j background were originally obtained from Dr. Bharat Aggarwal (MD Anderson Cancer Center, University of Texas, Houston, TX) and cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin (Sigma-Aldrich), 5 mM sodium pyruvate (Sigma-Aldrich), 5 mM MEM non-essential amino acids (Sigma-Aldrich) (referred to as complete (c)-RPMI). Cells were then maintained at 37°C in a humidified atmosphere with 5% CO₂. L. rhamnosus GR-1, obtained from Dr. Gregor Reid (The Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, London, ON, Canada), was grown anaerobically in De Man, Rogosa and Sharpe (MRS) agar (Becton Dickinson) using anaerobic packs (Becton Dickinson) at 37°C for 24 h. For cell culture challenge, L. rhamnosus GR-1 were grown from a single colony in MRS broth (Becton Dickinson) at 37°C for 48 h. Uropathogenic E. coli GR-12, originally isolated from the urine of a patient with pyelonephritis [43], and Staphylococcus aureus str. Newman were grown aerobically overnight in Luria-Bertani (LB) medium (Becton Dickinson) and brain-heart infusion (BHI) broth (Becton Dickinson), respectively, with agitation at 37°C. All bacteria were harvested by centrifugation at 6000 g for 10 min, washed three times with phosphate-buffered saline (PBS) (pH 7.4), and diluted to obtain a final optical density
of 1.0 at 600 nm (representing approximately $10^9$ CFU/ml) in PBS. Lipopolysaccharide (LPS) from *E. coli* O111:B4 was purchased from List Biological Laboratories (Campbell). PAM$_2$CSK$_4$ (PAM2) and PAM$_3$CSK$_4$ (PAM3) were purchased from Invivogen. LTA from *S. aureus*, lipase (from Candida rugosa) and RNase A were obtained from Sigma-Aldrich. DNase was purchased from Roche. LY294002, Akt inhibitor II, NF-κB activation inhibitor, wortmannin, SB202190, U0126, and JNK inhibitor II were purchased from Calbiochem (EMD Biosciences). Antibodies for phospho-p38 (p-p38), p-ERKs, p-SAPK/JNKs, p-Akt, p-inhibitory κB (IκB) and β-actin were purchased from Cell Signaling Technology (NEB Biosciences).

5.2.3 Generation of primary BMDM

Bone marrow cells (BMCs) were harvested from femurs and tibia of mice using a 25.5-gauge needle and PBS. Isolated cells were cultured in c-RPMI supplemented with murine recombinant macrophage (M)-CSF (20 ng/ml; eBioscience). Cells were then maintained at 37°C in a humidified atmosphere with 5% CO$_2$ for 7 days. The culture media was replaced with fresh media every two days after culture initiation.

5.2.4 Crude LTA purification

Crude LTA was extracted from LGG and *S. aureus* using the butanol extraction method as previously described [44]. Briefly, bacterial cells were sonicated for 15 min, re-suspended in n-butanol/water (1:1, v/v) and stirred for 30 min at room temperature. The suspension was then centrifuged at 8000 g for 30 min, resulting in a two-phase system. The aqueous phase was lyophilized to give crude LTA. Subsequently, the lyophilized sample was re-suspended in PBS and used for further experiments.

5.2.5 Macrophage cell challenge and cytokine determination

Macrophages were challenged for 4 h and 24 h in a 96-well plate format with live bacteria (if not indicated otherwise, 20 colony forming units (CFU)/cell), bacterial spent culture supernatant (SCS; 1/25 dilution), LTA (10 μg/ml), and LPS (100
ng/ml). Samples for TNF-α and G-CSF analysis by enzyme-linked immunosorbent assays (ELISA) were obtained from cell culture supernatant after 4 and 24 h challenge, respectively, and stored at -20°C until use.

5.2.6 ELISA

To measure the TNF-α and G-CSF levels in cell culture supernatant, ELISA kits were purchased from eBioscience (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively. Standard curves were generated using recombinant proteins provided by the manufacturer.

5.2.7 Western blot

Total cell lysate extraction and Western blot analysis were performed as previously described [45]. Briefly, total cell lysates were extracted using ice-cold lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na₃VO₄, 1 mM DTT, 75 mM β-glycerophosphate, 0.1 mM PMSF, 1 μg/mL aprotinin, 10 μg/mL pepstatin A, 1 μg/mL leupeptin, and 1% Triton X-100. Following the incubation of cells with lysis buffer on ice for 5 min, cell lysates were extracted by centrifuging the homogenate at 18000 g for 15 min. Extracts were then mixed with SDS-PAGE loading buffer, heated to 100°C for 5 min, resolved on 11% SDS-PAGE polyacrylamide gels (Bio-Rad), and transferred onto nitrocellulose membranes. Following that, membranes were blocked with 5% w/v skim milk for 30 min, immunoblotted with antibodies, and visualized using an enhanced chemiluminescence detection system (ECL; Pierce Bioscience). Band intensity quantification was performed using ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, 1997–2009, http://rsb.info.nih.gov/ij/) [46].

5.2.8 Cytokines and Chemokines PCR Array

Total RNA was isolated using RNeasy Mini kit (QIAGEN Canada) according to the manufacture’s recommendations. Reverse transcription was conducted using the RT² First Strand Kit (QIAGEN) according to the manufacture’s protocol. The Q-PCR was performed using a RT² Profiler PCR Array System Kit-mouse cytokines and
chemokines PCR array (QIAGEN) on an Applied Biosystems StepOnPlus instrument according to the manufacturer guidelines.

5.2.9 Statistical analysis

Pooled results of several independent experiments were used in all analyses. Results are reported as mean ± SEM. Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post hoc test were used to determine significance at \( p < 0.05 \) among experimental groups using Prism 5.0c for Mac OS X (GraphPad Software, La Jolla, CA).
5.3 RESULTS

5.3.1 Production of G-CSF is the most prominent among 84 cytokines and chemokines examined in GR-1-treated macrophages.

We previously showed that the probiotic GR-1 potently induces G-CSF but poorly induces TNF-α production in macrophages [47]. To further examine production of other cytokines and chemokines induced by GR-1 and their levels in comparison to other stimuli, immortalized BMDM were treated with live GR-1 (20 CFU/cell), the TLR4 ligand LPS (1 µg/ml), TLR2/6 ligand PAM2 (1 µg/ml) and TLR1/2 ligand PAM3 (1 µg/ml) for 5 h, and the expression of 84 cytokines and chemokines were examined using the RT² Profiler PCR Array System Kit. As shown in Fig. 5.1 (upper left panel) and Appendix A.5, GR-1 potently induced expression of G-CSF mRNAs (>60-fold of those induced in non-treated cells), whereas all others were induced less than 5-fold. LPS also induced G-CSF mRNA to ~80-fold; however, it also potently induced other cytokines and chemokines, including C-C motif ligand (CCL)5, CCL12, C-X-C motif ligand (CXCL)1, CXCL3, CXCL9, CXCL10, CXCL11, IL-1α/β, IL-6, IL-12, TNF-α and TNF superfamily member 10 more than 50-fold. PAM2 and PAM3 also potently induced G-CSF more than 100-fold; however, they also induced IL-1α/β, IL-16 and TNF-α more than 50-fold. These results demonstrate that GR-1 induced a distinct cytokine expression profile from LPS, PAM2 and PAM3.
Figure 5.1. Production of G-CSF is the most prominent among 84 cytokines and chemokines examined in GR-1-treated macrophages. Cytokine mRNA levels were measured in immortalized BMDM treated with GR-1 (20 colony forming units (CFU)/cell), lipopolysaccharide (LPS; 1 µg/ml), PAM2CSK4 (PAM2; 1 µg/ml), and PAM3CSK4 (PAM3; 1 µg/ml) using PCR array. The graph plots the log10 of normalized gene expression levels in a control condition (x-axis) versus an experimental condition (y-axis).
Figure 5.1

[Graph showing the relationship between GR-1, G-CSF, TNF-α, LPS, PAM2, PAM3, and Control with logarithmic scales for the x and y axes.]
5.3.2 Preferential G-CSF production by GR-1 is unique among tested bacteria in primary BMDM.

We further examined whether preferential production of G-CSF was also observed in other bacteria and also in non-transformed cells. Macrophages derived from BMCs of C57BL/6j mice in the presence of M-CSF were treated with live GR-1, *E. coli* GR-12 and *S. aureus* with 1-200 CFU/cell, and production of TNF-α (in 4 h) and G-CSF (in 24 h) were measured using ELISA. Both *E. coli* and *S. aureus* induced TNF-α as low as 1 CFU/cell, whereas GR-1 did not induce TNF-α even at 10 CFU/cell (Fig. 5.2A). GR-1 with higher than 20 CFU/cell induced TNF-α, but the levels was less than one-half of those induced by *E. coli* and *S. aureus*. Unlike TNF-α, G-CSF was significantly produced by GR-1 as low as 10 CFU/cell and maximally at 50 CFU/cell (Fig. 5.2B). *E. coli* maximally produced G-CSF even at 1 CFU/cell, whereas *S. aureus* gradually induced more G-CSF production starting at 1 CFU/cell and maximally at 20 CFU/cell. Further examination on the kinetics of the production of these cytokines showed that GR-1 induced G-CSF 12 h after treatment at similar levels induced by LTA (Fig. 5.2C; right panel). Unlike G-CSF, production of TNF-α was induced as early as 4 h of LTA treatment. However, GR-1 did not induce TNF-α until 12 h post-treatment and the levels were significantly lower than those induced by LTA (Fig. 5.2C; left panel). Nonetheless, the levels of TNF-α were significantly lower than those induced by LTA. Similar results were also obtained in immortalized BMDM. These results suggest that live GR-1 preferentially produced G-CSF with similar kinetics as LTA, but induced TNF-α significantly less efficiently than LTA.
Figure 5.2. Preferential G-CSF production by GR-1 is unique among other bacteria in primary BMDM. (A-B) Primary BMDM treated with live *S. aureus*, live *E. coli* GR-12 or live GR-1 at the indicated bacteria:macrophage (colony forming units (CFU/cell) ratios. Production of TNF-α (A; in 4 h) and G-CSF (B; in 24 h) was measured from spent cell culture media using ELISA. \[ n \geq 3; p < 0.05 \] by one-way ANOVA with Tukey’s multiple comparison post hoc test; columns accompanied by the same letter (a, b or c) are not significantly different from each other]. (C) Primary BMDM treated with live GR-1 (20 CFU/cell) or lipoteichoic acid (LTA; 10 µg/ml) for indicated time points. Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM \[ n \geq 3 \].
Figure 5.2

A

B

C

TNF-α (pg/ml)

G-CSF (pg/ml)

Live bacteria (CFU/cell)

TNF-α (pg/ml)

G-CSF (pg/ml)

Live bacteria (CFU/cell)

0
500
1000
1500
2000
2500
3000

0
2
4
12
24 (h)

0
2
4
12
24 (h)

ILTA

GR-1

LTA

GR-1
5.3.3 Activation of ERKs, NF-κB and Akt but not JNKs by GR-1 plays a key role in the preferential production of G-CSF over TNF-α.

To examine signaling pathways activated by GR-1, immortalized BMDM were treated with GR-1 at 20 CFU/cell for different time points and Western blots against phosphorylated IκB, Akt and MAPKs (ERKs, p38 and JNKs) were performed. Consistent with previous studies, LTA activated all MAPKs, Akt and NF-κB signaling cascades after 15-30 min of treatment (Fig. 5.3A). GR-1 also potently activated ERKs, but weakly p38, Akt and NF-κB. However, no activation of JNKs by GR-1 was detected. To further examine which of these signaling cascades were involved in G-CSF and TNF-α production, these cells were pretreated with various inhibitors, and then treated with GR-1 or LTA. As shown in Fig. 5.3B, production of G-CSF by GR-1 and LTA was inhibited by the NF-κB inhibitor (NF-κB activation inhibitor), ERKs inhibitor (U0126), phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmanin or LY94002) and Akt inhibitor (Akt inhibitor II), but not by the p38 inhibitor (SB203580) and JNKs inhibitor (JNK inhibitor II) (Fig. 5.3B, left panel). Unlike G-CSF, the production of TNF-α by LTA was inhibited by all the MAPK inhibitors (U0126, SB203580 and JNK inhibitor II), but not by the PI3K and Akt inhibitors (wortmanin, LY94002 and Akt inhibitor II) (Fig. 5.3B, right panel). These results suggested that GR-1 preferentially induces G-CSF production in macrophages through selectively activating ERKs, NF-κB and Akt, but not JNKs, in a TLR2-dependent manner.
Figure 5.3. Activation of ERKs, NF-κB and PI3K/Akt but not JNKs by GR-1 plays a key role in the preferential production of G-CSF over TNF-α. (A) Immortalized BMDM were treated with live GR-1 (20 colony forming units (CFU)/cell) or lipoteichoic acid (LTA; 10 µg/ml) for 15, 30 and 60 min. Phosphorylation of MAPKs (ERKs, p38 and JNKs), Akt and I-κB was analyzed by Western blot using phospho- or anti-peptide antibodies against indicated proteins. Bar graphs demonstrate band intensity quantification (ratio of protein phosphorylation to β-actin) using ImageJ. Data shown as mean ± SEM [n ≥ 3]. (B) Immortalized BMDM treated with GR-1 (20 CFU/cell) or LTA (10 µg/ml) in the presence or absence of various inhibitors (NF-κB-NF-κBi; 10 µM, p38-SB203580; 10 µM, JNKs-JNK inhibitor II; 250 nM, MEK/ERKs-U0126; 25 µM, Akt-Akt inhibitor II; 1 µM, PI3K-wortmannin; 10 µM and PI3K-LY94002; 10 µM). Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s t-test).
Figure 5.3

A

![Graph showing the ratio of p-MAPKs/β-actin in Immortalized BMDM](image)

B

![Graph showing cytokine production in GR-1 (20 CFU/cell) and LTA (10 µg/ml)](image)
5.3.4 TLR2 plays a key role in inducing G-CSF in GR-1-treated primary BMDM.

GR-1, as a Gram-positive bacterium, harbors several MAMPs including lipoproteins/LTA, which activate TLR2. To examine if this receptor is involved in GR-1-induced G-CSF production, primary BMDM derived from TLR2-deficient (TLR2<sup>-/-</sup>) mice were treated with GR-1 or PAM3. G-CSF production was undetectable in TLR2<sup>-/-</sup> BMDM in response to either GR-1 or PAM3 (Fig. 5.4A). TLR2<sup>-/-</sup> BMDM failed to produce TNF-α by either GR-1 or PAM3. Consistent with these results, TLR2<sup>-/-</sup> BMDM did not activate ERKs, p38, Akt and NF-κB in response to GR-1 (Fig. 5.4B). GR-1 activated JNKs in neither wild-type nor TLR2<sup>-/-</sup> BMDM (data not shown), as in immortalized BMDM (Fig. 5.3A). These data suggest that production of G-CSF and activation of ERKs, p38, Akt and NF-κB by GR-1 was mediated through TLR2.
Figure 5.4. TLR2 plays a key role in inducing G-CSF in GR-1-treated primary BMDM. (A) Primary BMDM from wild-type (WT) and TLR2−/− mice were treated with live GR-1 (20 colony forming units/cell (CFU/cell)) or Pam3CSK4 (PAM3; 1 μg/mL). Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM [n ≥ 3]. (B) Primary BMDM from WT and TLR2−/− mice were treated with live GR-1 (20 CFU/cell) for 15, 30 and 60 min. Phosphorylation of MAPKs (ERKs, p38 and JNKs), Akt and IκB was analyzed by Western blot using phospho- or anti-peptide antibodies against indicated proteins. Bar graphs demonstrate band intensity quantification (ratio of protein phosphorylation to β-actin) using ImageJ. Data shown as mean ± SEM [n ≥ 3].
Figure 5.4

A

Cytokine (pg/ml)

CNT  GR-1  PAM3

WT  TLR2⁻/⁻

B

Ratio of p-MAPKs/β-actin (arbitrary units × 10⁴)

p-ERKs  p-p38

GR-1  WT  TLR2⁻/⁻

BMDM  BMDM

Ratio of p-Akt/β-actin (arbitrary units × 10⁴)

GR-1  WT  TLR2⁻/⁻

BMDM  BMDM

Ratio of p-IκB/β-actin (arbitrary units × 10⁴)

GR-1  WT  TLR2⁻/⁻

BMDM  BMDM

Ratio of p-AKT/β-actin (arbitrary units × 10³)

GR-1  WT  TLR2⁻/⁻

BMDM  BMDM
5.3.5 A heat-labile protein-like molecule(s) secreted by GR-1 preferentially induces G-CSF production.

Since TLR2 activation was required for G-CSF production by GR-1, we first examined whether LTA/lipoproteins isolated from GR-1 induced a similar G-CSF preferential production effect in immortalized BMDM. LTA/lipoproteins from GR-1 were ~ 10-fold less effective in inducing both G-CSF and TNF-α than those from S. aureus (Fig. 5.5A). However, unlike live GR-1, LTA/lipoproteins from GR-1 induced both TNF-α and G-CSF at similar levels, suggesting that LTA/lipoproteins were not the factor responsible for the G-CSF preferential production effect. We then examined whether a factor(s) released from GR-1 had a similar effect on G-CSF production as in live GR-1. As shown in Fig. 5.5B, GR-1 cell free SCS, but not the media (MRS) alone, preferentially produced G-CSF over TNF-α, and the production of G-CSF was abolished in SCS treated with heat (95°C for 3 h), trypsin and proteinase K, but not with lipase, DNase and RNase (Fig. 5.5B). Treatments of trypsin or proteinase K had no effects on LPS-induced TNF-α and G-CSF production, indicating that inhibition of G-CSF production in GR-1-SCS was not due to residual effects of these proteases on macrophages. To estimate the molecular size(s) of the GR-1 molecule(s), GR-1-SCS was filtered through different sizes of membranes. As shown in Fig. 5.5C, the molecule(s) passed through 100 kDa membranes but not 30 kDa membranes, suggesting that the GR-1 molecule(s) was between 100 and 30 kDa in native size. Collectively, these results indicate that the GR-1 factor(s) responsible for preferential G-CSF production in macrophages is a heat-labile protein-like molecule(s) of 30-100 kDa in native size.
**Figure 5.5. A heat-labile protein-like molecule(s) secreted by GR-1 preferentially induces G-CSF production.** (A) Immortalized BMDM were treated with indicated concentrations of GR-1- or *S. aureus*-n-butanol-extracted crude LTA/lipoproteins. Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM (*n* ≥ 3; *, *p* < 0.05 by Student’s *t*-test). (B) Immortalized BMDM were treated with GR-1 spent culture supernatant (SCS; 1/25 dilution) or MRS culture media (1/25 dilution) which had been treated with proteinase K (200 μg/mL), lipase (200 μg/mL), trypsin (200 μg/mL), DNase (200 μg/mL), RNase (200 μg/mL), and heat (95°C for 3 h). Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM (*n* ≥ 3; *, *p* < 0.05 by Student’s *t*-test). (C) Immortalized BMDM were treated with MRS culture media (1/25 dilution), GR-1-SCS (1/25 dilution) or GR-1-SCS which had been fractioned using Centricon centrifugal filter devices with a 100 or 30 kDa molecular weight cutoffs. Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media using ELISA. Data shown as mean ± SEM (*n* ≥ 3).
Figure 5.5

A

Cytokine (pg/ml)

GR-1 LTA
μg/ml

S. aureus LTA
μg/ml

B

Cytokine (pg/ml)

GR-1 SCS

C

Cytokine (pg/ml)

> 30 kDa
< 30 kDa
> 100 kDa
< 100 kDa

MRS

Heat 3 h
Trypsin
Proteinase K
Lipase
DNase
Rnase
Trypsin + LPS
Proteinase K + LPS

CNT
GR-1
MRS
GR-1 SCS
> 30 kDa
< 30 kDa
> 100 kDa
< 100 kDa
5.4 DISCUSSION

Here, we showed that among 84 cytokines and chemokines examined, GR-1 potently induced G-CSF (Fig. 5.1 and Appendix A.5), and the production of G-CSF by GR-1 was as efficient as other known pro-inflammatory stimuli in both kinetics and amounts (Fig. 5.2). This observation is consistent with our previous studies in macrophages [24, 26] and placental trophoblast cells that showed high levels of G-CSF production by GR-1 [22, 23]. GR-1 at higher than 20 CFU/cell or after 12 h post-exposure was able to induce TNF-α, albeit at significantly lower levels than those induced by E. coli GR-12 or S. aureus (Fig. 5.2). Therefore, GR-1 could induce pro-inflammatory cytokines, but much less efficiently when compared to other bacteria or LTA.

Inefficient production of TNF-α by GR-1 was likely due to poor stimulatory effects of GR-1 cell wall components. LTA prepared from GR-1 was ~10-fold less efficient in inducing TNF-α when compared to LTA from S. aureus (Fig. 5.5A). Previous studies have shown that LTA purified from several strains of Lactobacillus, including L. plantarum str. WCFS1 [30], L. casei str. YIT9029 [31], L. fermentum str. YIT0159 [31] and LGG [48] are inefficient in producing pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. It is still unknown why LTA from several strains of Lactobacillus are inefficient in inducing pro-inflammatory cytokines. Interestingly, mutant L. plantarum WCFS1 and LGG that lacks D-alanylated LTA induces more anti-inflammatory but less pro-inflammatory cytokines in monocytes than wild-type LTA [30], and rendered protective effects on colitis [12, 30]. However, in another study, alanylation of LTA had no role in the production of pro-inflammatory cytokines in LGG-treated macrophages and peripheral mononuclear cells [48]. Regardless the levels of alanylation, GR-1 LTA is not likely involved in the G-CSF preferential effect of GR-1, as GR-1 LTA at high doses induced both TNF-α and G-CSF at similar extents (Fig. 5.5A).

At this moment, the identity of GR-1 factor(s) responsible for the preferential production of G-CSF is elusive. This study suggested that a protein-like factor, which
is sensitive to heat and proteases, such as trypsin and proteinase K, with a molecular weight between 30 and 100 kDa, is responsible for G-CSF preferential production by GR-1 (Fig. 5.5B-C). To date, probiotic proteins known to preferentially induce anti-inflammatory cytokines include surface layer proteins from *L. reuteri*, *L. casei*, [49] and *L. acidophilus* NCFM [34], and bacteriocins released from *L. plantarum* strains [36, 50]. Immunomodulation elicited by surface layer proteins likely acts through DC-specific ICAM3-grabbing non-integrin (DC-SIGN) [49, 51], which is distinct from the GR-1 factor(s) that required TLR2 (Fig. 5.4A). Various *Lactobacillus* species including *L. rhamnosus* release bacteriocins, which can directly modulate immune responses [50, 52, 53]. Therefore, bacteriocin-related proteins released by GR-1 may have been involved in the G-CSF production. Further studies on the possible involvement of bacteriocin-related proteins in G-CSF production are warranted.

GR-1 was shown to release factors that have immunomodulatory effects on non-immune cells. GR-1-SCS significantly induced G-CSF and macrophage inflammatory protein 1α/β, but suppressed various LPS-induced pro-inflammatory cytokines including TNF-α in human decidual cells [54] and in CD-1 pregnant mice [55]. However, in urinary bladder cells, GR-1-SCS enhanced activation of NF-κB [56] and production of TNF-α, but not IL-6 and IL-8, induced by *E. coli* [57]. In these studies, GR-1 or GR-1-SCS alone induced NF-κB but little TNF-α production, which is consistent with our results (Fig. 5.3A and 5.4B). Although it is unknown whether these responses are mediated by the same factor, molecular characteristics in a protein-like entity with native molecular size above 30 kDa appear to be common in both studies (Fig. 5.5) [56].

We further examined the signaling pathways required for G-CSF preferential production in GR-1-treated macrophages. GR-1 activated ERKs, p38, Akt and NF-κB, but not JNKs, in immortalized and primary BMDM in a TLR2-dependent manner (Fig. 5.3A and 5.4B). Based on experiments using specific inhibitors, activation of ERKs, Akt and NF-κB pathways were involved in G-CSF production, whereas activation of ERKs, p38, JNKs and NF-κB pathways were required for TNF-α
production (Fig. 5.3B). These results are consistent with previous reports showing that p38 and JNKs are required for production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 [58, 59]. Also, the p38 and JNKs, but not ERKs, pathways are involved in the signal transduction of LPS-induced TNF-α and IL-1β production by Kupffer cells [60] and RAW 264.7 macrophages [61]. Therefore, lack of JNKs activation in GR-1-treated macrophages likely contributed to the inefficient production of pro-inflammatory cytokines. The PI3K/Akt signaling axis can suppress TNF-α production through multiple pathways [62], particularly through inactivating NF-κB but activating cAMP response-binding protein (CREB), resulting in suppression of IL-12 and induction of IL-10 production [63]. We showed that the inhibitors specific for Akt or PI3K inhibited TNF-α, but not G-CSF, production (Fig. 5.3B). These results are consistent with previous studies showing that activation of ERKs, NF-κB and PI3K/Akt pathways are crucial for G-CSF expression [64, 65]. Recent studies also showed that activation of PI3K/Akt is required for TLR-mediated G-CSF production, through inducing expression of the transcription factor octamer-binding factor-2 (Oct-2) [66, 67]. Therefore, activation of PI3K/Akt and ERKs pathways by GR-1 likely contributed to G-CSF-specific production by GR-1-treated macrophages.

An intriguing fact is that GR-1-induced G-CSF production required TLR2 (Fig. 5.4A). At this moment, it is unknown how activation of TLR2 by GR-1 led to ERKs and p38 activation without JNKs activation, resulting in G-CSF production without inducing pro-inflammatory cytokines. However, the anti-inflammatory responses by activating TLR2 are not restricted to GR-1. *Bifidobacterium pseudocatenulatum* CECT7765 was recently shown to produce anti-inflammatory cytokines such as IL-10 and suppress TNF-α production through TLR2 [68]. Even purified LTA or whole cells from lactobacilli were previously shown to modulate TNF-α expression through a TLR2-dependent mechanism [30, 31, 69, 70]. Further experiments delineating mechanisms of TLR2 in activating immunomodulatory responses is of great interest.

In addition to the primary function of G-CSF in the generation and mobilization of neutrophils, G-CSF enhances anti-microbial function of mature neutrophils [71-73],
and regulates immune responses through suppressing production of various pro-inflammatory cytokines in myeloid cells [24, 25], generating regulatory gut-homing macrophages [74], promoting maturation of tolerogenic DCs [26, 75, 76], and generation of IL-10-secreting CD4+ type 1 T regulatory cells [77]. Therefore, G-CSF production by GR-1 may play an important role in enhancing anti-bacterial activities of neutrophils and preventing cell death of intestinal/urogenital epithelial cells induced by various stresses, without inducing inflammatory responses. Beneficial effects of probiotics are not limited in the intestine. Interestingly, a recent study showed that administration of *L. plantarum* 299v or GR-1 protected cardiac failure and hypertrophy in the rat heart [78, 79]. Since G-CSF also renders cytoprotective effects against various stresses in neuronal and cardiac cells [80-82], we are tempted to speculate that G-CSF may also contribute to the cardio-protective effects of *L. plantarum* 299v or GR-1.

In summary, this study demonstrated that a protein-like factor(s) from GR-1 preferentially and potently produced G-CSF in macrophages through selectively activating ERKs, NF-κB and Akt in a TLR2-dependent manner.
5.5 REFERENCES


Chapter 6

SUMMARY OF RESULTS, CONCLUSIONS AND FUTURE DIRECTIONS
6.1 SUMMARY OF RESULTS

Several *in vitro* and *in vivo* studies have shown that G-CSF has a role in the generation of monocytes/macrophages [1, 2]. Additionally, G-CSF induced by the probiotic strain of *L. rhamnosus* GR-1 down-regulates expression of inflammatory cytokines in activated macrophages [3]. However, details on the mechanisms of these observations had remained to be explored. The first part of this thesis focused on examining the immune regulatory effects of G-CSF in the gut through macrophages (Chapters 3 and 4). I have established that G-CSF induced the generation of gut-homing M2-like macrophages in BMDM. I also showed that G-CSF was an important factor in the regulation of the gut immune system, as demonstrated in the DSS-induced acute colitis mouse model. The second part of this thesis elucidated the signaling mechanisms involved in the preferential production of G-CSF in GR-1-exposed macrophages (Chapter 5). I have shown that GR-1 induced high levels of immunomodulatory G-CSF production with low levels of pro-inflammatory cytokines in macrophages through activating TLR2, and subsequent NF-κB, ERKs and PI3K/Akt signaling pathways. The GR-1 factor responsible for the G-CSF preferential effect was a heat-labile protein-like molecule(s) of 30-100 kDa in size.
6.1.1 Chapter 3: G-CSF preferentially supports the generation of gut-homing Gr-1^{high} macrophages in M-CSF-treated bone marrow cells

Chapter 3 describes the role of G-CSF in the generation of macrophages. Since G-CSF alone was not able to sustain continued survival/proliferation of BMCs (Fig. 3.1C and Fig. 3.2A; left panel), I investigated its impact on BMCs in the presence of other CSFs, M-CSF and GM-CSF. I found that the addition of G-CSF mainly promoted the growth of BMCs in the presence of M-CSF (Fig. 3.1C), but not GM-CSF (Fig. 3.1D). M-CSF alone generated a homogenous cell population, mainly Gr-1^{low}/F4/80^{+} macrophage-like cells, from BMCs (Fig. 3.2). G-CSF and M-CSF induced Gr-1^{high}/F4/80^{+} macrophage-like (Fig. 3.2 and Fig. 3.7D) and Gr-1^{+}/F4/80^{−} neutrophil-like cell populations (Fig. 3.2). G-CSF prevented cell death (Fig. 3.4 and Fig. 3.5) and enhanced cell proliferation of Gr-1^{high}/F4/80^{+} macrophage-like cells (Fig. 3.6). In addition, I also established that these Gr-1^{high}/F4/80^{+} macrophage-like cells have M2-like cytokine (3.7A-B) and cell surface marker expression profiles (3.7C). Adoptive transfer of F4/80^{+} BMCs that were treated with G-CSF resulted in higher localization of BMCs in the intestinal lamina propria of recipient mice (Fig. 3.8) but not in other tissues examined (Appendix A.3). Accordingly, the number of intestinal Gr-1^{high}/F4/80^{+} and Gr-1^{low}/F4/80^{+} cells was reduced in G-CSFR^{−} mice compared to their wild-type counterparts with no differences in the number of Gr-1^{+}/F4/80^{−} neutrophil-like cells. These results suggested that G-CSF can contribute to the survival and proliferation of M-CSF-exposed BMCs, which showed M2-like cytokine production and cell surface marker profiles and gut-homing characteristics following adoptive transfer.
6.1.2 Chapter 4: Protective role of endogenous G-CSF in dextran sulfate sodium-induced acute colitis through generating gut-homing macrophages

As shown in Chapter 3, G-CSF seemed to be involved in gut-homing of M-CSF derived macrophages. In Chapter 4, therefore, I first aimed to elucidate the endogenous role of G-CSF in gut immune regulation using the DSS-induced acute colitis model. Mice (G-CSFR$^{+/+}$, $^{+/−}$ and $^{−/−}$) received DSS (1.5% w/v) in their drinking water and were monitored for the severity of colitis. Following DSS treatments, all mice showed significant body weight loss, diarrhea, colitis scores (Fig. 4.1A) and tissue damage (Fig 4.1B-C), but these effects were more pronounced in G-CSFR$^{−/−}$ mice (Fig 4.1A-B), which also showed a decreased ability to clear infections after DSS treatment, reflected by formation of pseudomembranous structures (Fig 4.1B, lower right panel). Since no development of spontaneous colitis was observed in G-CSFR$^{−/−}$ mice (Fig. 4.1A), it can be postulated that the G-CSF signaling defect may not be a primary factor of colitis induction but rather a driving factor in colitis progression. Next, I tested whether G-CSFR$^{−/−}$ mice with low circulating neutrophil counts had similar deficiency in the gut neutrophil populations. Interestingly, I found no difference in the neutrophil-like cell population in the steady-state (Fig. 4.2A; left panel) or in the infiltration of these cells in inflammatory state after DSS treatment (Fig. 4.2B-C). These observations indicated that emergency generation of neutrophils during inflammation and recruitment to local inflammatory sites were not defective in these mice and neutrophils might not be involved in the G-CSFR$^{−/−}$ DSS-induced colitis phenotype.

As shown in Fig. 3.7, macrophages treated with G-CSF had M2-like characteristics, reflected by their cytokine and cell surface marker expression profiles. To investigate further the G-CSF-treated BMDM characteristics, I continued to analyze the M1- and M2- cell surface marker and mRNA expression of these cells in response to M1 and M2 stimuli. There appeared to be a significant increase in expression of M2-cell surface markers (Fig. 4.3A); however, there was no such effect in the mRNA expression of M2-specific genes in G-CSF-treated BMDM in response to M2 stimuli.
(Fig. 4.3B). In contrast, G-CSF significantly reduced M1 markers, iNOS mRNA and CD86 cell surface marker expressions on G-CSF-treated BMDM in response to M1 stimuli (Fig. 4.4). These results indicated that G-CSF-treated BMDM may not readily fall into all M2 macrophage characteristics, but are regulatory and modulatory in nature (the reason why we called them M2-like cells).

Since G-CSF induced the generation of gut-homing M2-like macrophages (Chapter 3), G-CSFR−/− mice had less F4/80+ macrophages, but not neutrophils (Fig. 3.9 and Fig 4.2) and G-CSFR−/− mice were more susceptible to DSS-induced acute colitis (Fig. 4.1), I explored the effects of adoptively transferred G-BMDM during DSS-induced acute colitis. I found that adoptive transfer of G-BMDM, but not BMDM, rendered disease-ameliorating effects in DSS-induced acute colitis in G-CSFR−/− mice (Fig. 4.5 and Fig. 4.6). Collectively, these findings pointed out that G-CSF plays an important role in the regulation of gut immune response in DSS-induced acute colitis model.
6.1.3 Chapter 5: Activation of TLR2-dependent signaling cascades and preferential production of G-CSF by a protein-like *Lactobacillus rhamnosus* GR-1 secretory factor.

In Chapter 5, I aimed to reveal the mechanism underlying the G-CSF production in GR-1-exposed macrophages. I found that GR-1 induced high levels of G-CSF mRNA expression in macrophages, whereas all other pro-inflammatory cytokines and chemokines mRNA were minimally expressed or absent (Fig. 5.1). Next, I showed that this cytokine profile was unique to GR-1 as other bacteria (Fig. 5.2) or other TLR stimuli (Fig. 5.1) potently induced G-CSF and pro-inflammatory cytokines. Accordingly, I showed that preferential production of G-CSF was mainly mediated through ERKs, NF-κB and PI3K/Akt but not JNKs signaling pathways (Fig 5.3). I further showed that activation of all ERKs, NF-κB and PI3K/Akt signaling pathways were mediated through TLR2 (Fig. 5.4). Given that GR-1-SCS induced preferential production of G-CSF in macrophages similar to live GR-1 (Fig. 5.4C), I sought to characterize the responsible factor(s). I found that GR-1 responsible factor(s) involved in preferential G-CSF production was a heat-labile protein like molecule(s) (Fig. 5.4B) of 30-100 kDa in size (Fig. 5.4C). Taken together, I showed that GR-1 induced preferential G-CSF production in a TLR2/ERKs/NF-κB/PI3K/Akt-dependent pathway by means of a protein like molecule(s) of 30-100 kDa in size.
6.2 CONCLUSION

The main findings and conclusion of this thesis are summarized as follows:

- G-CSF promoted the generation of gut-homing M2-like Gr-1$^{\text{high}}$/F4/80$^+$ macrophages in M-CSF-treated BMCs.

- G-CSF had a disease ameliorating effect in DSS-induced acute colitis.

- *L. rhamnosus* GR-1 preferentially induced G-CSF production through activation of TLR2-dependent NF-κB and PI3K/Akt signaling pathways.

- The *L. rhamnosus* GR-1-secreted factor(s) responsible for preferential G-CSF production in macrophages was a heat-labile protein-like molecule(s) of 30-100 kDa in size.
6.3 FUTURE DIRECTIONS

The work presented in Chapters 3 and 4 provide many new and exciting avenues of research for examining the role and mechanism of G-CSF in gut immune regulation through macrophages. Further investigating the role of G-CSF in macrophage development and gut immune regulation can be aims of future research.

In Chapter 3, I demonstrated that G-CSF promoted growth of Gr-1+ /F4/80+ macrophages in only M-CSF, but not GM-CSF-exposed macrophages (Fig. 3.2 and Fig. 3.3). This indicates that G-CSF could potentiate the macrophage generation function of M-CSF in BMC culture. Activation of both M-CSF receptor and GM-CSF receptor induce similar signaling pathways, including JAK/STAT, Ras/ERKs and PI3K/Akt pathways [4-8]. Whether G-CSF activates a specific signaling pathway(s), such as JAK2-STAT5 [9], that are absent in M-CSF signaling but present in GM-CSF signaling or potentiates a specific signaling pathway, such as Ras/ERKs, that are differently regulated by M-CSF or GM-CSF [10], would be a matter of interest to investigate. G-CSF enhanced homing of adoptively transferred macrophages to the gut (Fig. 3.8 and Appendix A.3); however, whether these transferred cells were differentiated to resident macrophages after arriving in the gut is still unknown. Gut resident macrophages marked by CX3CR1^{high}/Ly6C^{-} have been shown to be recruited from CX3CR1^{low} Ly6C^{high} blood monocytes [11]. Therefore, it would be interesting to see the effects of G-CSF on the expression of CX3CR1 in BMDM after adoptive transfer. Moreover, Gr-1+ /CD11b+ cells were shown to be myeloid-derived suppressor cells [12, 13] with colitis protective effects [14]. Therefore, it would be interesting to examine whether G-CSF-induced Gr-1+ /F4/80+ macrophages exhibit similar functions as these myeloid-derived suppressor cells. Their suppressive effects can be assessed by examining proliferation of T cells in the presence or absence of purified Gr-1+ /F4/80+ cells or their condition media.

Furthermore, G-CSF also increased Gr-1+ /F4/80+ cells in M-CSF-exposed BMCs (Fig. 3.2). Since Gr-1 is transiently expressed in the myeloid lineage [15], it is not clear whether this cell population represent neutrophils or macrophage precursor cells.
Further characterization of Gr-1⁺/F4/80⁻ cells in their morphology and neutrophil functions such as LPS/phorbol myristate acetate-induced formation of neutrophil extracellular traps (NETs) assay [16] will reveal whether these cells are *bona fide* neutrophils.

In Chapter 4, I showed that G-CSF played a protective role in DSS-induced acute colitis model (Fig. 4.1). As the DSS model mainly involves innate immunity, the role of G-CSF in other colitis models should also be examined. The TNBS model of acute colitis is also used due to its efficiency in inducing colitis and convenience in handling [17, 18]. In contrast to the DSS model, the TNBS model involves adaptive immunity for the development of colitis. Since the adaptive immune system also plays an important role the development of colitis, examining the role of G-CSF by using G-CSFR⁻/⁻ mice in the TNBS model will unravel the function of G-CSF in the context of gut adaptive immunity. Another suitable model to study the role of G-CSF in the gut inflammation is the CD4⁺CD45RB<sup>high</sup> T cell transfer model [19]. CD4⁺CD45RB<sup>high</sup> cells can be adoptively transferred into newly generated G-CSFR⁻/⁻ RAG⁻/⁻ (lacking adaptive immunity) mice for the induction of colitis. This model will further evaluate the role of G-CSF in non-adaptive immune cells in the growth of a T cell dependent colitis. I also determined in Chapter 4 that adoptive transfer of G-CSF-exposed macrophages reduced the severity of colitis in G-CSFR⁻/⁻ mice (Fig. 4.5 and Fig. 4.6). The CD4⁺CD45RB<sup>high</sup> T cell transfer model can be used for co-transfer of G-CSF-exposed macrophages and CD4⁺CD45RB<sup>high</sup> cells to assess the importance of G-CSF-exposed macrophages in gut immune regulation.

The IL-10⁻/⁻ mouse model is a well-characterized spontaneous colitis model that indicates the importance of the anti-inflammatory cytokine IL-10 in maintenance of gut homoeostasis [20]. In addition, depletion of phagocytes in IL-10⁻/⁻ mice ameliorates the colitis conditions in these mice [21]. Having observed the immunomodulatory role of G-CSF in down-regulating inflammatory cytokines [3, 22, 23] and robust production of pro-inflammatory cytokines by macrophages in IL-10⁻/⁻ mice [24], it would be best to evaluate the function of adoptively transferred G-CSF-
exposed macrophages with an M2-like cytokine profile in newly generated G-CSFR\(^{-/-}\) IL-10\(^{-/-}\) mice.

In addition, I found in Chapter 3 that G-CSF-exposed BMCs contained 3 different cell populations, Gr-1/F4/80\(^+\), Gr-1\(^+\)/F4/80\(^+\) and Gr-1\(^+\)/F4/80\(^-\) (Fig. 3.2A). Finding the responsible population in reducing severity of colitis in G-CSFR\(^{-/-}\) mice might provided more insight into the mechanisms underlying the beneficial effects of G-CSF.

In Chapter 4, only DSS-treated G-CSFR\(^{-/-}\) mice, but not G-CSFR\(^{+/+}\) and \(^{+/}\) mice, formed a pseudomembranous structure admixed with a large population of bacteria, suggesting that these mice were defective in controlling bacterial infection following DSS-induced tissue damage. It is still undetermined whether and how these features affect the outcome of severe colitis in G-CSFR\(^{-/-}\) mice. Since all mice were kept under conventional housing conditions, using specific-pathogen free conditions would offer unique opportunities to study the effects of these structures on the outcome of colitis in G-CSFR\(^{-/-}\) mice.

In Chapter 5, there were no differences in IκB phosphorylation and degradation in between GR-1 and LTA treatments, indicating that GR-1 induces IκB degradation. I and others [25-28] have shown that NF-κB signaling pathway is important for expression of TNF-α, IL-12 and G-CSF, but how GR-1-dependent NF-κB activation induces only G-CSF is still undetermined. It is, therefore, important to examine whether GR-1 can inhibit or reduce the nuclear translocation of NF-κB or NF-κB DNA binding activity, leading to preferential G-CSF production by other pathways. Given that PPAR-\(\gamma\) involves in nuclear export of RelA subunit of NF-κB, it would also be pertinent to investigate whether GR-1 inhibit NF-κB transcription activity. It is also interesting to investigate whether GR-1 can manipulate the non-canonical pathway of NF-κB in order to induce preferential G-CSF production. GR-1 also activated PI3K/Akt pathway with minimal effects on JNKs activation. Activation of PI3K/Akt pathway has been shown to negatively regulate MAPKs- and NF-κB-dependent inflammatory cytokine production in macrophages through inhibition of
key inflammatory transcription factors including NF-κB, AP-1 and Egr-1 [29]. However, we ruled out such possibilities due to the fact that inhibition of PI3K/Akt pathway did not result in increased production of TNF-α (Fig 5.3). It is possible that GR-1 preferentially produce G-CSF over TNF-α through directly inhibiting the activation of JNKs. To date, several molecules have been shown to be involved in the JNKs specific inhibition. The X-link inhibitor of apoptosis (XIAP) and growth arrest and DNA damage induced protein (Gadd45β) prevent JNKs activation by ubiquitin-mediated degradation of TAK1 [30] and directly binding and inhibiting MKK7 activity [31], respectively. Therefore, it will be interesting to investigate the activation of common upstream activator of JNKs, such as MKK7, or XIAP and Gadd45β, in GR-1-treated macrophages.

I have not yet identified the GR-1 factor responsible for the preference production of G-CSF. Given that the GR-1 factor(s) was a secreted protein-like molecule(s) of 30-100 kDa in size, using a size-exclusion and anion or cation column chromatography would further purify the GR-1 factor(s). In fact, I have tried to identify the GR-1 factor(s) through gradient fast protein liquid chromatography (FPLC) on the 80% ammonium sulfate fraction and the Centricon precipitated GR-1-SCS samples that had the preferential G-CSF production activity. However, the results were not promising as the factor would not bind to the Mono Q column and induces G-CSF and to some extent TNF-α in almost all fractions in BMDM (data not shown). However, I observed that released products from GR-1 cultured in PBS for 2 h would not be able to induce preferential G-CSF production (data not shown). Further mass spectrophotometry analysis on the GR-1-PBS incubated culture supernatant and GR-1-SCS revealed only one recognized protein with high homology score to fibrinogen binding protein (FBP) from L. rhamnosus. However, the recombinant FBP produced from E. coli BL21 with histidine-tagged fusion showed no preferential G-CSF activity in BMDM. Two dimensional gel electrophoresis and subsequent tandem mass spectrometry of the released proteins from GR-1 or GR-1 cultured in PBS would help us to find secreted candidate protein. Once complete sequences of candidate proteins are revealed, the recombinant protein can be expressed in E. coli and purified recombinant protein can be tested for further confirmation of its G-CSF stimulating
activity. Similar to GR-1-SCS, LGG-SCS has been shown to strongly induce G-CSF production in macrophages. LGG secreted proteins, p40 and p75, have been both shown to activate Akt [32-34]. By using GR-1 mutant in p75 SCS, we, however, ruled out that this protein is involved in preferential G-CSF production by GR-1 in macrophages (Appendix A.6).

One of the strategies by which probiotics elicit their effects is by increasing anti-inflammatory cytokines such as IL-10 and G-CSF, while decreasing pro-inflammatory cytokines such as TNF-α [3, 22]. Thus, the anti-inflammatory capacity of probiotics may be useful in treatment of IBD. I showed that G-CSFR−/− mice exhibited more severe inflammatory conditions in a DSS-induced acute colitis model than wild-type mice (Fig. 4.1), indicating an importance role of G-CSF in intestinal immune regulation. I also showed that the GR-1 or its SCS was able to induce G-CSF in macrophages. Given that G-CSF is able to down-regulate inflammatory cytokines, it is expected that the production of G-CSF by oral administration of GR-1 or intraperitoneal injection of GR-1-SCS would reduce inflammatory responses in colitis. This will extend our understanding on the mechanistic basis of the proposed probiotic GR-1 health effects. While results from previous studies on therapeutic implication of LGG for IBD remain controversial [35-37], further detailed and extensive experiments would reveal the role of GR-1 in colitis.
6.4 REFERENCES


dithiocarbamate inhibits NF-kappa B mobilization and TNF production in human monocytes. The Journal of Immunology 151, 6986-6993.


Appendix A
Supplementary Figures and Tables
Appendix A.1. BMCs from *lys-EGFP-ki* transgenic mice were expressing EGFP. BMCs from *lys-EGFP-ki* transgenic (right panel) and wild-type (left panel) mice were cultured in the presence of M-CSF (20 ng/ml) + G-CSF (10 ng/ml). On day 5, F4/80+ cells were sorted using MACS and were analyzed for EGFP expression before intravenously injecting to G-CSFR+/+ mice using flow cytometry.
Appendix A.1

Wild-type BMCs

lys-EGFP-ki BMCs
Appendix A.2. BMCs from wild-type and G-CSFR<sup>−/−</sup> mice show no differences in Gr-1 and F4/80 surface marker expression in the presence of M-CSF or GM-CSF. BMCs from wild-type and G-CSFR<sup>−/−</sup> mice were treated with GM-CSF (10 ng/ml) or M-CSF (20 ng/ml) for five days. (A and B) Based on the Gr-1 and F4/80 expression characteristics, cells were divided into 3 populations: P1, Gr-1<sup>low</sup>/F4/80<sup>+</sup>; P2, Gr-1<sup>high</sup>/F4/80<sup>+</sup>; P3, Gr-1<sup>+</sup>/F4/80<sup>−</sup>. (C and D) Percentage of each cell population was quantified and plotted. Data are average of two independent experiments.
Appendix A.2

A

G-CSFR\(^{+/+}\): GM-CSF

G-CSFR\(^{-/-}\): GM-CSF

B

Cell population (%)

\[\text{G-CSFR}^{+/+}: \text{GM-CSF}\]

\[\text{G-CSFR}^{-/-}: \text{GM-CSF}\]

C

G-CSFR\(^{+/+}\): M-CSF

G-CSFR\(^{-/-}\): M-CSF

D

Cell population (%)

\[\text{F4/80} \quad \text{Gr-1}\]

\[\text{G-CSFR}^{+/+}: \text{M-CSF}\]

\[\text{G-CSFR}^{-/-}: \text{M-CSF}\]
Appendix A.3. G-CSF increases localization of F4/80^+ M-BMCs in the intestine. BMCs from *lys-EGFP-ki* transgenic mice were treated with M-CSF (20 ng/ml) + G-CSF (10 ng/ml) for five days. F4/80^+ cells were isolated by MACS and 1.0 × 10^6 cells were injected to the recipient mice through tail veins. (A) After two days of adoptive transfer (1.0 × 10^6 cells), spleen, liver, kidney, lung, heart and bone marrow tissue sections were prepared and viewed through a fluorescent microscope. (B) Six hours after injection, sections from the large and small intestines of the recipient mice (total of four mice in two different times) were viewed through a fluorescent microscope. Images show representative fluorescent microscopy results. (C) Average numbers of EGFP^+ cells per 40× field of view (> 20) from the small and large intestine tissue sections were counted and plotted. Data shown as mean ± SEM (n ≥ 3; *, p < 0.05 by Student’s *t*-test).
Appendix A.3

A

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B

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C

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* Indicates significant difference.
Appendix A.4. Numbers of macrophages and neutrophils are significantly reduced in spleen and bone marrow in G-CSFR $^{-/-}$ mice compared to those of wild-type mice. Splenocytes (A-B) and BMCs (B-C) were prepared from wild-type and G-CSFR $^{-/-}$ mice. Based on the Gr-1 and F4/80 expression characteristics, cells were divided into different populations. (B and D). Percentage of each cell population was quantified and plotted. Data shown as mean ± SEM (n = 3; *, p < 0.05).
Appendix A.4

A

G-CSFR\textsuperscript{+/+}  

G-CSFR\textsuperscript{-/-}

B

\begin{align*}
\text{Cell population (\%)} & \text{ G-CSFR\textsuperscript{+/+}} & \text{ G-CSFR\textsuperscript{-/-}} \\
\text{P1} & \text{\textbullet} & \text{\textbullet} \\
\text{P2} & \text{\textbullet} & \text{\textbullet} \\
\text{P3} & \text{\textbullet} & \text{\textbullet} \\
\end{align*}

C

\begin{align*}
\text{Cell population (\%)} & \text{ Isotype} & \text{ G-CSFR\textsuperscript{+/+}} & \text{ G-CSFR\textsuperscript{-/-}} \\
\text{P1} & \text{\textbullet} & \text{\textbullet} & \text{\textbullet} \\
\text{P2} & \text{\textbullet} & \text{\textbullet} & \text{\textbullet} \\
\text{P3} & \text{\textbullet} & \text{\textbullet} & \text{\textbullet} \\
\end{align*}

D

\begin{align*}
\text{Cell population (\%)} & \text{ G-CSFR\textsuperscript{+/+}} & \text{ G-CSFR\textsuperscript{-/-}} \\
\text{PA} & \text{\textbullet} & \text{\textbullet} \\
\text{PB} & \text{\textbullet} & \text{\textbullet} \\
\end{align*}
Appendix A.5. Induction of expression of cytokine and chemokine genes in immortalized BMDM treated with GR-1 and TLR2/4 agonists.
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1 Genes that were not amplified or not detectable in the PCR array were marked as 'N.D' (not detectable).

* Genes that were not amplified or not detectable in the PCR array of all treatments were not reported. For the complete list of genes refer to http://www.sabiosciences.com/ArrayList.php?pline=PCRArray (cytokines and chemokines PCR Array).
Appendix A.6. GR-1 mutant in p75 (Msp1) has similar capability to GR-1 WT in inducing preferential G-CSF production over TNF-α. Production of TNF-α (in 4 h) and G-CSF (in 24 h) was measured from spent cell culture media of BMDM treated with GR-1-spent culture supernatants (SCS; 1/25 dilution) of wild-type (WT) and Δp75 GR-1 using ELISA. Data shown as mean ± SEM.
Appendix A.6

![Graph showing cytokine levels (TNF-α and G-CSF) under different conditions (CNT, WT, Δp75, GR-1 SCS). The graph includes error bars indicating variability.](image-url)
Appendix B: Ethics Approvals
Sung Kim - eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2010-222::2

From:  
To:  
Date:  26/11/2012 11:25 AM  
Subject:  eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2010-222::2  
CC:  

Western  

2010-222::2:  

AUP Number: 2010-222  
AUP Title: Evaluating the anti-inflammatory effects of G-CSF in enterocolitis mouse models.  
Approval Date: 04/15/2010  

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-222 has been approved.  

1. This AUP number must be indicated when ordering animals for this project.  
2. Animals for other projects may not be ordered under this AUP number.  
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.  

REQUIREMENTS/COMMENTS  
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.  

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.  

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee

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The original material was published in:

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Year, month, volume, issue, pages, and DOI:

Accepted: Jun 11, 2014/DOI: 10.1189/jlb.JA0814-1720

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Comments:
Dear [Cody Mooneyhan],

I am writing this letter in request of written permission to reproduce the publication entitled "G-CSF preferentially supports the generation of gut-homing Gr-1macrophages in M-CSF-treated bone marrow cells." Journal of Leukocyte Biology, Volume 96, DOI: 10.1189/jlb.1A0314-172R, June 2014, on which I am first author, as part of my Ph.D. thesis.

Thank you very much,

Shahab Meshkibaf

Permission granted.

[Signature]
Shahab Meshkibaf

EDUCATION

Ph.D. in Microbiology & Immunology 2011-2015
Western University, London, Ontario, Canada
Thesis research with Dr. Sung O. Kim entitled “The gut immune regulatory function of granulocyte colony-stimulating factor through macrophages and signaling mechanisms controlling its production by probiotics”
- Examined effects of probiotics/G-CSF on polarization/signalling of macrophages and dendritic cells.
- Investigated the protective role of polarized macrophages in DSS-induced colitis mouse model.

M.Sc. in Medical Microbiology & Infectious Diseases 2009-2011
University of Manitoba, Winnipeg, Manitoba, Canada
Thesis research with Dr. Denis O. Krause entitled “Gut microbiome analysis in piglet models infected with E. coli K88: The role of charcoal and dietary crude protein supplemented with probiotic E. coli UM2 and UM7.”
- Examined levels of crude protein in conjunction with probiotic E. coli strains in an E. coli K88 infectious diseases piglet model.
- Investigated effects of charcoals in an E. coli K88 infectious diseases piglet model.

B.Sc. in Molecular & Cellular Biology-Emphasized in Microbiology 2002-2006
Azad University, Karaj Branch, Karaj-Iran

SCHOLARLY AND PROFESSIONAL ACTIVITIES

Teaching Assistance 2013-2015
Western University, London, Ontario, Canada
- Laboratory techniques in microbiology and immunology (MICROIMM 3600G)

Student Mentoring 2012-2015
Western University, London, Ontario, Canada
- Supervised and guided seven students to understanding of scientific methods through immunology, microbiology and molecular biology experiments and case-based learning.

Graduate Representative to the Seminars 2012-2015
Western University, London, Ontario, Canada

Committee member at IIRF 2012-2015
Western University, London, Ontario, Canada

Research assistant with Dr. C. Creuzenet 2013
Western University, London, Ontario, Canada
- Joint project for analyzing the function and role of H. pylori protein (HcpE) on monocyte differentiation and activity.
Research assistant with Dr. Khafipour 2009-2011
University of Manitoba, Winnipeg, Manitoba, Canada
• Joint project for evaluating the effect of stress on gut microbiome in piglet models.

Teaching Assistance Summer 2008
University of Guelph, Guelph, Ontario, Canada
• Cellular and Molecular Biology

Bozorgmehr Laboratory 2004-2006
Karaj, Tehran, Iran
• Executed and analyzed tests in areas including microbiology, hematology, serology and genetics to aid diagnosing and treating diseases.
• Assisted in testing, reviewing, and reporting lab data on all performed tests.
• Assisted management in QC microbiology/immunology lab operating costs and negotiating equipment and service contracts.

Volunteered at Pooyesh Medical Laboratory 2004-2005
Karaj, Tehran, Iran
• Assisted in collecting and preparing specimens, performing laboratory tests in areas including biochemistry and serology to aid diagnosing and treating diseases.
• Verified data records and reported lab results on all performed tests.
• Assisted in selling the molecular biology kits, PCR-related products, protein markers, culture media and lab ware technologies to hospitals and private labs.

Teaching Assistant 2002-2004
Negarestan Institute, Tehran, Iran
• Biology

SCHOLARSHIPS AND AWARDS
• Graduate Thesis Research Award Fund, Western University 2013
• Western Graduate Research Scholarships (WGRS), Western University 2012-2015
• Travel Award, Western University 2012 & 2013

PUBLICATIONS
Abstracts and Poster Presentations:
• S. Meshkibaf and S. O. Kim. G-CSF preferentially supports the generation of gut-homing Gr-1<sup>high</sup> macrophages in M-CSF-treated bone marrow cells. Canadian Society of Immunology (CSI). Quebec city, Quebec, Canada. March, 2014.

Manuscripts:
• S. Ha, C. Reid, S. Meshkibaf and S. O. Kim. HDAC8 plays a role in lethal toxin-induced cytokine suppression in macrophages. (Under Review; Journal of Immunology)
• S. Meshkibaf, Marcelo Gottschalk and S. O. Kim. Preferential production of G-CSF by a protein-like lactobacillus rhamnosus GR-1 secretory factor through activating TLR2-dependent signaling events without activation of JNKs. (Under Review; Journal of BMC Microbiology)
• S. Meshkibaf and S. O. Kim. Granulocyte colony-stimulating factor. (In progress)