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# **Role of CSF1/CSF1R signalling in avian macrophage biology**

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## Abstract

The mononuclear phagocyte system (MPS), which is a heterogenous family of functionally related cells, includes myeloid progenitors, blood monocytes, resident tissue macrophages, bone osteoclasts and conventional dendritic cells. In mammals, macrophage colony stimulating factor (M-CSF or CSF1) promote differentiation, proliferation and survival of myeloid progenitor cells into mononuclear phagocyte lineage cells by binding and signalling activity through a surface receptor (CSF1R). Interleukin-34 or IL34 is alternative growth factor which also signals via CSF1R.

CSF1, IL34 and the shared receptor CSF1R was shown to be conserved in birds, but their functions have not been studied in detail. The primary aim of this project is to study the role of CSF1R signalling in avian macrophage biology using three different approaches. The first approach involved the identification of chicken CSF1R specific kinase inhibitors, from a set of candidate mammalian CSF1R. Candidate CSF1R inhibitors were screened based on cell viability assay using IL-3 dependent pro B cell line Ba/F3 ectopically expressing chicken CSF1R and chicken bone marrow-derived macrophages (BMDM). To support these studies, biologically active, endotoxin-free recombinant chicken CSF1 protein was produced and re-folded from inclusion bodies using a bacterial system. Out of 10 potential CSF1R inhibitors screened, 6 inhibitors TIA086, TIA02-052, TIA02-054, TIA02-076, KUL01-123 and KUL02-016 were potent and selective for chicken CSF1R, whilst having no effect on growth in IL-3. Two inhibitors TIA02-054 and TIA02-076 were specific for the chicken CSF1R kinase compared to their actions on human CSF1R expressed in the same cells. The chicken CSF1R kinase inhibitors also effectively blocked CSF1-induced survival of primary BMDMs. BMDM survival was reduced even in the absence of exogenous CSF1 indicating a growth factor independent, autocrine CSF1/CSF1R signalling function in chicken macrophages.

The second approach to study CSF1 biology in the development of chicken MPS involved use of a novel neutralising monoclonal antibody to chicken CSF1 (ROS-AV183) that targets and blocks chicken CSF1R signalling activity. In order to test the activity of anti-ChCSF1 mAb on chicken macrophages both *in vitro* and *in vivo*, both anti-ChCSF1 mAb and Isotype control mAb reagents were purified from hybridoma culture by affinity chromatography and characterized further for purity, size by SDS PAGE and CSF1R signaling blocking activity by BaF3/ChCSF1R cell viability assay. Anti-ChCSF1 mAb completely inhibited survival of



primary chicken macrophages, irrespective of the presence or absence of CSF1, supporting the earlier finding regarding the autocrine CSF1 signalling behaviour of chicken macrophages. To determine the impact of anti-ChCSF1 mAb on postnatal birds *in vivo*, transgenic *CSF1R*-eGFP reporter birds were injected with antibody for four consecutive days. Anti-ChCSF1 mAb had no effect on the average growth rate, the relative weight gain or the normal development of hatchling birds. Anti-ChCSF1 mAb had no detectable effect on circulating CSF1 levels on the day of hatch or a week after treatment. Anti-ChCSF1 mAb significantly reduced *CSF1R*-eGFP transgene positive macrophages in bursa of Fabricius and caecal tonsil tissue, but not in spleen tissue. In bursa of Fabricius tissue, follicle associated epithelium (FAE) cell's proliferation and survival was altered post treatment. In caecal tonsil anti-ChCSF1 mAb substantially reduced B lymphocytes; this depletion was also evident in the circulation and spleen tissue. Tissue resident MHC-II<sup>+</sup> macrophages in spleen were effectively depleted, validating CSF1 dependency of tissue resident macrophages. In liver tissue, anti-ChCSF1 mAb treatment completely ablated Kupffer cell population. In bones anti-ChCSF1 mAb treatment depleted osteoclasts number. MicroCT scan analysis of bone femur architecture revealed significant reduction in the % bone volume and trabecular number, with a corresponding increase in the trabecular separation post anti-ChCSF1 mAb treatment of hatchling birds. In overview, the analysis indicated that CSF1 is required for post-hatch development of the MPS in birds and suggest trophic roles for CSF1-dependent macrophages in B cell development.

The third approach involved deletion of *CSF1R* in the chicken genome using CRISPR Cas9 editing in chicken primordial germ cells (PGCs). Out of the several guide RNAs (gRNAs) designed targeting different regions of *CSF1R* loci, gRNAs targeting exon 1 and 10 (encoding transmembrane domain of the receptor) were functionally validated for mutation. Guide RNAs targeting exon 1 and transmembrane domain region were effective in mutating receptor *CSF1R* in cultured PGCs with targeting efficiency of around 35% and 100% respectively. Transplantation of PGCs with biallelic deleted transmembrane domain region of *CSF1R* into germ cell deficient chicken embryos gave rise to one founder female G0 bird containing edited donor PGCs. Breeding of this chicken upon sexual maturation with transgenic *CSF1R*-eGFP male established 30 *CSF1R* heterozygous G1 birds containing *CSF1R* edited donor PGCs (39% germline efficiency). *CSF1R* heterozygous G1 birds had no obvious phenotypes compared to wild type hatch mates throughout the development of embryos and in adults. Furthermore, *CSF1R* homozygous mutant embryos (G2) were generated by breeding *CSF1R* heterozygous G1 chickens (26% germline efficiency). Analysis of 8-day old *CSF1R* homozygous mutant

embryos revealed deficiency in the expression of CSF1R protein in mononuclear phagocyte population. Hence, there was successful transmission of *CSF1R* knockout allele in G1 and G2 progeny. Analysis of the phenotype of the homozygous CSF1R mutant birds is ongoing.

The novel tools characterized in this project, anti-ChCSF1 antibody, chicken CSF1R kinase domain inhibitors and CSF1R-deficient transgenic chicken line will enable further detailed studies of the role of macrophages in chicken immunity and development.

## Lay summary

All living organisms contain an immune cell called macrophage that represent a heterogenous group of cells, found in every tissues and bones in body. Macrophages are important cells that display wide variety of fundamental roles. These include destruction of invading foreign pathogenic micro-organisms or body's dying cells, regulating immune system, wound healing, tissue homeostasis and overall development of the organism. Macrophages have key function in tissue morphogenesis and organ development. They are involved in vascular growth and bone resorption. To achieve this multi-functional role, macrophages respond to environmental factors by signalling via receptors or molecular markers expressed on the cell surface and by secreting effector molecules. Accordingly, macrophages survival, differentiation and expansion are dependent upon the colony stimulating factor (CSF1) and interleukin 34 (IL34) molecules signalling via cell surface receptor CSF1R.

Abnormalities in macrophage functions affects viability, resulting in inefficient immune response that cause various health problems. Mutation or absence of *Csf1* results in various developmental abnormalities like impaired growth, reproductive, skeletal, mammary gland and neurological defects in mammalian models, predominantly from deficiency in tissue macrophages. Developmental phenotypes are more severe in *Csf1r* mutated rodent models. CSF1 and/or CSF1R mutations is also implicated in a range of diseases like cancer and inflammatory diseases (autoimmune disorders, arthritis, atherosclerosis and obesity). Much about the macrophage biology including their origin and development have been studied in mammalian species but there is limited knowledge about the avian macrophage's biology. Chicken specific molecular reagents and antibodies characterising subsets of macrophages are also limited. Chickens are economically important livestock in poultry industries and therefore gaining knowledge about its immune responses becomes important. This project explores the significance of CSF1/CSF1R signalling in the development of chicken macrophages and immune cells.

Chicken embryo is a widely utilised model organism for studying avian immunology and blood haematopoiesis. Due to easy *in ovo* access and precise stages of development, fluorescent macrophage labelled chicken embryos, along with postnatal chickens were utilised in this project. For chicken macrophages functional studies, this thesis designed three approaches each aiming to reduce macrophage numbers. The first approach led to the identification of chicken

specific compounds that led to the inhibition of chicken macrophages survival. The second approach led to the depletion of subset of macrophages *in vivo*, highlighting the importance of CSF1 for postnatal macrophages growth and development in chickens. The third approach involved CSF1R gene editing, which led to the modification of genomic DNA and production of macrophage deficient chickens.

In summary, in this project, novel tools and chimeric chicken line were generated with the potential to study macrophages and immune system cells; and macrophage associated developmental phenotypes for future studies with chickens.



# Declaration

The thesis presented is the work of the author except where stated otherwise by reference and/or acknowledgement. Any work presented, which has been conducted by (or in collaboration with) others is explicitly acknowledged. No part of this work has been submitted for any other degree or professional qualification.

Name: Rakhi Dipak Harne

Date: 15.8.2019

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## Publications

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Zhiguang Wu, **Rakhi Harne**, Cosmin Chintoan-Uta, Tuan-Jun Hu, Robert Wallace, Amanda Maccallum, Mark Stevens, Pete Kaiser, Adam Balic, David A. Hume. Regulation and function of macrophage colony-stimulating factor (CSF1) in the chicken immune system, *Developmental and Comparative Immunology* (accepted manuscript)

## Oral presentations

Gene targeting in chicken primordial germ cells using CRISPRs. Talk presented at Gene Editing and Gene Regulation with CRISPR symposium, London, November 2016

**Rakhi D Harne**, Adam Balic, Mike McGrew, Alewo Idoko Akoh, Zhiguang Wu, David Hume. Role of CSF1/CSF1R signaling in avian macrophage biology. Abstract presented at, the Macrophage Satellite Symposium hosted by Macrophages @EdUni, QMRI, Little France campus, University of Edinburgh, May 2018

## Poster presentations

**Rakhi Harne**, Lucy Freem, Jenny Geddes, Mike McGrew, Adam Balic, David Hume. Role of CSF1/CSF1R signaling in avian immune response. Poster abstract presented at The Roslin Institute & R (D) SVS student research day, April 2016

**Rakhi Harne**, Lucy Freem, Jenny Geddes, Mike McGrew, Adam Balic, David Hume. Role of CSF1/CSF1R signaling in avian immune response. Poster presented at Coming of Age: The Legacy of Dolly at 20 symposium, September 2016

**Rakhi Harne**, Zhiguang WU, Lucy Freem, Jenny Geddes, Adam Balic, David Hume. Role of CSF1/CSF1R signaling in avian immune response. Poster presented at the XIV Avian Immunology Research Group (AIRG) meeting, Herrsching, Germany, September 2016

Lucy Freem, Almas Gheyas, Adam Balic, Jenny Geddes, **Rakhi Harne**, Choon-Kiat Khoo, Dave Burt, David Hume. Genetic variants influencing macrophage biology and disease resistance in poultry. Poster presented by Lucy Freem at the XIV Avian Immunology Research Group (AIRG) meeting, Herrsching, Germany, September 2016

Jenny Geddes, Lucy Freem, **Rakhi Harne**, Zhiguang Wu, David Hume. Bone marrow-derived macrophage culture to assess disease susceptibility in poultry. Poster presented by Jenny Geddes at the XIV Avian Immunology Research Group (AIRG) meeting, Herrsching, Germany, September 2016

**Rakhi Harne**, Mike McGrew, Adam Balic, Zhiguang Wu, Idoko-Akoh Alewo, David Hume. Role of CSF1/CSF1R signaling in the avian immune response. Poster abstract presented at The Roslin Institute & R(D)SVS student research day, May 2017

**Rakhi D Harne**, Mike McGrew, Adam Balic, Zhiguang Wu, Alewo Idoko Akoh, David Hume. Role of CSF1/CSF1R signaling in the avian immune response. Poster presented at Edinburgh Immunology group summer symposium, Edinburgh, June 2017

**Rakhi D Harne**, Mike McGrew, Adam Balic, Zhiguang Wu, Alewo Idoko Akoh, David Hume. Role of CSF1/CSF1R signaling in the avian immune response. Poster presented at Avian Genetics and Immunity symposium, Surrey, June 2017

**Rakhi D Harne**, Zhiguang Wu, Mike McGrew, Adam Balic, Alewo Idoko Akoh, David Hume. Role of CSF1/CSF1R signaling in the avian macrophage biology. Poster presented at European Macrophage and Dendritic Cell Society (EMDS) annual meeting, Madrid, Spain, September 2017

Amanda MacCallum, Lucy Freem, Jenny O'Dell, Zhiguang Wu, Kay Boulton, **Rakhi Harne**, Adam Balic and David Hume. Inbred chickens with naturally occurring genetic variation in immune response genes demonstrate differences in related phenotypes. Poster presented by Amanda MacCallum at the Avian Immunology Research Group (AIRG) meeting, Oxford, United Kingdom, September 2018



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# Abbreviations

Ab: Antibody

ADGRE1: Adhesion G protein coupled receptors

AF647: Alexa Fluor 647

AGM: Aorta-gonad-mesonephros

AMP: Anti-microbial proteins

APCs: Antigen presenting cells

AWERB: Animal Welfare and Ethical Review Board

BCA: Bicinchoninic acid colorimetric assay

BCR: B cell receptor

BF: Bursa of Fabricius

BMDMs: Bone marrow derived macrophages

BSA: Bovine serum albumin

BSDCs: Bursal secretory dendritic cells

CAM: Chorioallantoic membrane

Cat. no: Catalogue number

CBB: Coomassie Brilliant Blue

CHO: Chinese hamster ovary

CMP: Common myeloid progenitor

CNS: Central nervous system

CSF: Colony stimulating factor

CSF1: Colony stimulating factor 1

CSF1R: Colony stimulating factor receptor

CTL: Cytotoxic T lymphocytes

DAPI: 4',6-diamidino-2-phenylindole

DCs: Dendritic cells

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

EGF: Epidermal growth factor

eGFP: Enhanced green fluorescent protein

ELISA: Enzyme-linked immunosorbent assay

EMPs: Erythro-myeloid progenitors

ESCs: Embryonic stem cells

FACS: Fluorescence activated cell sorting

FAE: Follicle associated epithelium

FBS: Fetal bovine serum

FDC-P1: Factor dependent cell-Paterson 1

FDCs: Follicular dendritic cells

FIRE: *Fms* intronic regulatory element

Flt3: *fms* like tyrosine kinase 3

G-CFU: Granulocyte colony forming unit

G-CSF: Granulocyte colony stimulating factor

GM-CFU: Granulocyte/macrophage colony forming unit

GM-CSF: Granulocyte macrophage colony stimulating factor

HETs: Heterophil extracellular traps

HH: Hamburger and Hamilton (1951)

HRP: Horseradish peroxidase

HSCs: Hematopoietic stem cells

IFN- $\gamma$ : Interferon- $\gamma$

Ig: Immunoglobulin

IGF-1: Insulin growth factor-1

IL-10: Interleukin 10

IL-18: Interleukin 18

IL-3: Interleukin 3

IL-6: Interleukin 6

IL12: Interleukin 12

IL34: Interleukin 34

IL4: Interleukin 4

IPTG: Isopropyl  $\beta$ -D-thiogalactopyranoside

JM: Juxta membrane

KI: Kinase insert

LB: Luria-Bertani media

LPS: Lipopolysaccharide

M-CFU: Macrophage colony forming unit

M-CSF: Macrophage colony stimulating factor

mAb: Monoclonal antibody

MEM NEAAs: Minimum essential medium, non-essential amino acids solution

MFI: Mean fluorescence intensity

MHCII: Major histocompatibility complex

MPO: Myeloperoxidase

MPS: Mononuclear phagocyte system

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NETs: Neutrophil extracellular traps

O.D: Optical density

pAb: Polyclonal antibody

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffer saline

PBST: 1x PBS containing 0.1% (v/v) Tween 20

PCR: Polymerase chain reaction

PDGFR: Platelet-derived growth factor receptor

PEG: Polyethylene glycol

PELS: Peri ellipsoid lymphocyte sheath

PGCs: Primordial germ cells

RES: Reticuloendothelial system

RT: Room temperature

RTKs: Receptor tyrosine kinases

SCF: Stem cell factor

SEM: Standard error of the mean

TAMs: Tumour associated macrophages

TCR: T cell receptor

TK: Tyrosine kinase

TKIs: Tyrosine kinase inhibitors

TMB: 3,3',5,5'-Tetramethylbenzidine

TNF-  $\alpha$  : Tumour necrosis factor

Tregs: Regulatory T cells





# Chapter 1 Introduction and overview

## 1.1 General introduction

The domestic chicken, *Gallus gallus domesticus*, derived from the red jungle fowl is a major livestock species as well as a widely-used experimental research model organism. Chicken meat is the most consumed meat in the world and eggs are also a major source of protein nutrition. Infectious disease is a major challenge for poultry production at every level from small-holders to intensive producers. In order to manage infectious diseases, it is vital to understand the biology of the immune response components. This also contributes to improved management practices in poultry, for example effective vaccination strategy to acquire resistance to prevalent diseases such as highly pathogenic influenza virus, Marek's disease virus (MDV), infectious bursal disease virus, infectious bronchitis virus; alongside the other zoonotic diseases caused by bacteria *Salmonella* and *Campylobacter*. Compared to mammals, many aspects of innate and acquired immunity remain to be defined and progress has been limited by availability of reagents and characterization of many immune related genes and protein repertoire (Kaiser 2012, Stewart et al., 2013). For example, the chicken ortholog of the archetypal pro-inflammatory cytokine tumour necrosis factor (TNF- $\alpha$ ) and its receptor TNFR1 and TNFR2 genes, that have been characterized in mammals and lower vertebrates were only recently identified in chickens (Rohde et al., 2018). TNF- $\alpha$  play an important role in host defence, inflammation and lymphocyte homeostasis.

Additionally, mRNA expression atlas for the domestic chicken was published ([http://biogps.org/dataset/BDS\\_00031/chicken-atlas/](http://biogps.org/dataset/BDS_00031/chicken-atlas/)) giving access to genes expressed across a wide range of tissues and primary cells at different developmental stages, enabling comparison to similar assets for mammalian species (Bush et al., 2018). Nevertheless the availability of whole genome sequence of chicken (International Chicken Genome Sequencing 2004) and advances in functional genomics and targeted gene disruption (Sang 2006, Macdonald et al., 2012), have improved our understanding of disease resistance genes and myeloid/lymphoid cells associated biology in chickens.

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Studying immunology and developmental biology using chicken as an experimental model has an additional advantage with the straightforward access to the embryo *in ovo*, with well-defined stages described in the Hamburger-Hamilton (HH) system (Hamburger and Hamilton, 1992). There are many fundamental similarities in the immune responses between mammals, biomedical model species and chickens, but they differ in many ways with respect to gene expression, molecular markers, cells in various tissues, and combating pathogens and infectious diseases (Kaiser 2012). This project aims to gain knowledge about the biology of a crucial immune cell type, macrophages, in chickens. Previous studies revealed that, as in mammals, chicken macrophages depend upon two growth factors, macrophage colony stimulating factor (CSF1) and IL34, which signal through a common receptor CSF1R (Garceau et al., 2010). The specific focus of the study is to further dissect the role of CSF1R in avian macrophage biology. The introduction Chapter will cover some of the unique features of the avian immunity followed by a more specific consideration of macrophage biology.

### 1.2 Unique features of immune response in chickens

Early studies in chickens helped to decipher several aspects of immune responses that are shared across vertebrates. These include the response to allografts, the separate lineages of B and T lymphocytes, mechanism of gene conversion by some mammals, the close association of major histocompatibility complex (MHC) and disease resistance in chickens; and vaccinology (Kaiser 2012, Davison 2014). There are two broad types of immune responses known in vertebrates; innate, which is immediate, constitutive and relatively non-specific against broad classes of pathogenic infections and adaptive, which is pathogen-specific and provides life long and specific immune response. Although birds partly possess similar general immune components to mammals, they have also developed peculiar and diverse ways in which they react to microbial pathogens or lifelong challenges (Kaiser 2012) as discussed below.

Primary lymphoid organs provide a microenvironment for the lymphocytes or the effector cells of adaptive immune response to express receptors for antigens and mature progenitor or stem cells. Chickens have evolved with the most significant lymphoid organ structural difference, which also provided the first evidence for the

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clear separation of delineation of the two major arms of the adaptive immune system B and T lymphocytes (Cooper et al., 1965). B cell processing, selection and proliferation in chickens occurs within a follicular micro-environment of a unique and primary lymphoid organ known as Bursa of Fabricius (BF). The bursa is functionally equivalent to B cell deriving bone marrow in mammals (Ratcliffe 2006, Olah and Nagy 2013, Fellah et al., 2014). T lymphocytes on the other hand, mature and proliferate into another primary lymphoid organ known as thymus, in both mammals and birds. In mammals it consists of two lobes (Pearse 2006) whereas there are six to seven lobes around each side of the neck in chickens (Oláh et al. 2014).

The secondary lymphoid organs which are the major site for antigen presentation and encounter by immune effector cells are different in chickens. Chickens lack in lymph nodes, one of the highly structured secondary organs in mammals. The other secondary lymphoid organ spleen is present in both. With the absence of lymph nodes the chicken lymphatic system is poorly developed and most antigen encounter is thought to initiate locally at the site of infection or in widely-distributed lymphoid follicles or nodules filled with germinal centres found throughout the body (Balic et al., 2014, Oláh et al., 2014). Newly hatched chicks emerge with an incomplete functional immune system. Maternal or passive immunity is passed during embryogenesis via amniotic fluid and by absorption of yolk sack after hatch, providing transient protection against pathogens. This maternal immunity lasts up until one month post hatch (Hamal et al. 2006). Mammals also have transient passive maternal transmission of immunity through antibodies passed in colostrum milk (Hurley and Theil, 2011).

Another unique feature of chickens is in the way immunoglobulin (Ig) antibody repertoire diversity is generated in developing B lymphocytes. Diversity is associated with the variable (V) region of the heavy and light chain loci that is also the antigen binding site, which eventually translates into a complete functional Ig molecule. To generate a complete functional Ig molecule by B cells, primates and rodents rely exclusively on the process known as gene rearrangement that involves random assortment of Ig genes encoding V region of the heavy and light chain loci Diversity is further increased through somatic mutation upon B cell- antigen interaction. In birds, immunoglobulin gene rearrangement occurs in bursal follicles by a process known as

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“somatic intra-chromosomal gene conversion” that involve donor gene sequences known as pseudogenes (Tizard 2002, Ratcliffe 2006). Chickens have only one Ig locus containing one heavy chain and one light chain gene. The heavy chain locus contains one V region, one J region and around 15D segments in the heavy chain, and the light chain a single V and J region. Some diversity is generated by random joint of these regions and single base insertion. However, the main mechanism relies on insertion of V pseudogenes (V $\Psi$ ) located upstream of IgV<sub>H</sub> and IgV<sub>L</sub> region by gene conversion. As in mammals, the variable region after antigen encounter by B cells in birds is further diversified by somatic point mutations. Reviewed in (Tizard 2002, Ratcliffe 2006).

Interestingly in chicken's thymus, the T cell repertoire is developed by the process of gene rearrangement as in mammals (Davison 2014). Mechanism of gene conversion to encode Ig loci has been shown to be shared also with other farm animals including rabbits (Becker and Knight, 1990), swine (Butler et al., 1996), sheep, cattle and horses (Butler 1998, Ratcliffe 2006, Kurosawa and Ohta, 2011), although none of them have been observed to rely exclusively on this mechanism. In mammals, B cells gene rearrangement occurs continuously throughout life in bone marrow. In contrast, in chickens this process occurs in B cell progenitors during a brief period of embryonic development around E10-E15, and somatic gene conversion continues to take place in bursa until bursal involution around 4 to 6 months age at sexual maturity (McCormack et al., 1991; Fellah et al., 2014).

In mammals, the activation of T lymphocytes requires antigen presentation by another cell. Intracellular pathogenic peptide fragments are presented on the cell surface by MHC antigens encoded by a highly polymorphic region in mammals. The chicken MHC region is known as the B locus. Like the mammalian MHC, it is polymorphic and contains genes contributing to immune defence. It contains highly immunogenic BF-BL region encompassing only two copies of dominantly expressed class I (BF) and class II $\beta$  (B/L $\beta$ ) genes (reviewed in (Kaufman 2015)). Interestingly the chicken MHC region is much smaller, simpler and compact comprising of 92kB and 19 genes in total, in contrast to humans and mice models comprising of around 4 million base pairs and 300 genes (Davison 2014).

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In addition to the above unique features, avian immune system also differs in terms of various other repertoires. The first draft genome sequence (International Chicken Genome Sequencing 2004) identified distinct repertoires of immune-associated elements in the chicken. They include a distinct set of chemokines, small molecules that control the recruitment of immune cells to sites of inflammation and cytokines, known to mediate immune and inflammatory signals (Kaiser et al., 2005). In his review article (Kaiser 2012), Kaiser described this repertoire in chickens as “minimal essential cytokine, chemokine repertoire”, supporting a proposed theory of redundancy of mammalian cytokines. The availability of genomic sequence has facilitated identification of antibodies and reagents specific to chickens to understand their functions. As noted above, the first drafts of the chicken genome were incomplete, and many key immune-associated genes were absent. Macrophage growth factor CSF1 was cloned as a cDNA (Garceau et al., 2010) but no equivalent genomic locus was evident until the most recent genome assembly (GalGal5.0).

### 1.3 Immune response in chickens

Innate or native immunity provides the first line of defence against infections. The response is rapid but with less diverse specificity towards antigens. Pathogen recognition is by receptors encoded by the elements present in germ line DNA as opposed to gene rearrangements of B and T cell receptors. The first line of innate immunity involves multiple barrier functions such as epithelia, anti-microbial proteins and peptides (AMP) including lysozyme, proteins of the complement system and defensins (Juul-Madsen et al., 2014). AMPs work by forming pores on membranes of bacteria and fungi leading to direct cell death.  $\beta$  defensins are constitutively or inducible expressed at mucosal surfaces of respiratory, skin, digestive and urinary tract in chickens. Their role is briefly reviewed in (Van et al., 2008). Ovodefensins, a subset of  $\beta$  defensins, have a specific role in egg defence in birds and reptiles. Their expression in chickens is restricted to oviduct predicting a role in maintaining egg sterility (Whenham et al., 2015).

Foreign pathogens that penetrate epithelium and soluble anti-microbial molecules encounter a second line of defence that is cell-mediated. Classical effector innate cell types that mediate important immune functions in mammals include macrophages,

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polymorphonuclear granulocytes (neutrophils, eosinophils, basophils) and natural killer cells (Paul 2013). Chickens lack functional eosinophils in blood but have functional heterophils and thrombocytes, mammalian equivalent to neutrophils and platelets respectively. Thrombocytes in chickens are small nucleated blood cells by contrast to enucleated mammalian platelets. Their primary function in blood coagulation and wound healing is similar to mammals. In recent years thrombocytes were also revealed as an innate effector cell, which responds to TLR agonists as bacterial and viral products, followed by expressions and release of various mediators of inflammation, anti-microbial compounds and overall immune defence (Ferdous et al., 2017).

Heterophils provide potent and immediate anti-microbial immune response. They are known to provide quite effective and enhanced phagocytic activity towards killing of bacteria especially *Salmonella* (Genovese et al., 1999). Their biological function in inflammation and anti-microbial activities is reviewed in (Harmon 1998). Effective killing of micro-organisms by mammalian neutrophils usually involves generation of oxygen radicals, degranulation, phagocytosis and release of extracellular traps contain nuclear DNA. In mammals, myeloperoxidase (MPO) potentiates oxidative burst response via release of toxic reactive oxygen intermediates on pathogens. Interestingly in chickens, the heterophil granules do not contain MPO. Chicken heterophils, unlike mammalian neutrophils, rely mainly on non-oxidative or weak oxidative microbicidal activity response (Stabler et al., 1994). Degranulation leads to the release of various proteolytic enzymes shared with mammals (e.g. lysozyme) as well as  $\beta$  defensins, which are a major anti-microbial granular component in heterophils (Harmon 1998). Neutrophils can release some nuclear-granular material known as neutrophil extracellular traps (NETs) that induce extracellular bactericidal activity (Vorobjeva and Pinegin 2014). Similar trap-like molecules, heterophil extracellular traps (HETs) may contribute to bacterial defence in birds (Chuammitri et al., 2009).

Macrophages are the key effector motile immune cells. They are required for the normal development of organisms. When activated, macrophages secrete cytokines and thereby can regulate both innate and acquired immune response to infection. They comprise around 10% - 15% of cellular content in almost all tissue compartments,

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including peripheral blood (Hume 2008). In birds, as in mammals, the phagocytic and microbicidal activity of macrophages is enhanced further by the cytokine interferon- $\gamma$  (IFN- $\gamma$ ), which is released by activated CD4<sup>+</sup> T helper cells (Kaspers and Kaiser 2014).

Dendritic cells (DCs) in mammals are specialised antigen presenting cells (APCs) that express high levels of MHCII and co-stimulatory molecules required to activate naïve T cells (Macri et al. 2017). Chicken “DCs” have been generated *in vitro* from bone marrow precursors upon stimulation with recombinant GM-CSF (see section 1.7) and interleukin 4 (IL4) for 7 days (Wu et al., 2010; Wu and Kaiser 2011). However, these culture-derived cells in mammals are considered more macrophage-like (Jenkins and Hume 2014). In mammals, classical DC depend upon signals from a separate ligand, fms like tyrosine kinase 3 (FLT3) ligand (Kingston et al., 2009). Recent studies in our laboratory have focussed on identification of FLT3 ligand responsive DC in multiple tissues in the chicken (Hu et al., 2016; Hu et al., 2019).

Adaptive or acquired immunity provides slow but more specialized and effective immune response. Immunity depends upon specific antigen recognition by B and T cell receptors expressed on B (BCR) and T (TCR) lymphocytes respectively. Immune response by B-lymphocytes repertoire produces secreted effectors, antibodies. It is called humoral immunity because it can be transferred through cell-free plasma. T lymphocytes also produce secreted effectors (cytokines) that amplify the innate immune response and can also act directly to kill virus-infected cells. T cell immunity is referred to as cell-mediated because it can be transferred to another animal by cellular transplantation.

In chicken development, precursor B cells, emerging from yolk sac and bone marrow after their rudiment development at an early embryonic stage, enter the BF from mesenchyme through blood circulation between E10-15 and differentiate into mature B lymphocytes. These are observed as early as E12 with their numbers increasing to 90% in BF follicles at hatch (Fellah et al., 2014). Mature B cells migrate to the peripheral lymphoid organs. The function of the bursa was elucidated through the analysis of surgically bursectomized chickens. Such birds did not produce any antibody after exposure to *Salmonella typhimurium* antigen (St. Pierre and Ackerman



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1965). On the other hand surgical thymectomy in birds produced a striking reduction in the peripheral blood lymphocytes and spleen lymphoid follicles (Warner and Szenberg 1964).

Upon antigenic stimulation, chicken B cells differentiate to become plasma cells and release three classes of immunoglobulins in body serum and mucosal secretions identified as homologues to the mammalian IgM, IgA and IgG (Tizard 2002). There is no evidence for the other two mammalian isotypes, IgD and IgE (Tizard 2002, Ratcliffe 2006). IgM is mainly expressed on the cell surface of B cells and easily detected in serum and egg white, whereas IgY, (the functional ortholog of mammalian IgG) is found in abundant in the blood serum and egg yolk. IgA along with bile and intestinal secretions is predominantly produced in oviduct and secreted with albumin of egg white as the fertilized ovum passes down the oviduct while laying egg. IgM and IgA maternal antibody transfer occurs through secretions in oviduct from hen serum and release with egg white albumin, whereas IgY is found in yolk sac which comes into the serum and IgM, IgA into intestine of hatched chicks.

Based on TCRs chains expressed, T cells in mammals differentiate into functionally-distinct  $\alpha\beta$  TCR and  $\gamma\delta$  TCR T cells. Most known components relating to the biology of antigen presentation and the development of T lymphocytes accessory molecules are conserved in chickens (Chen et al., 1994 ). Additional co-receptor molecules further divide  $\alpha\beta$  T cells into sub lineages,  $CD4^+$  T helper population and  $CD8^+$  cytotoxic T cell population, after clonal selection and maturation (Smith and Göbel 2014). Mature T lymphocytes from thymus are released into the periphery to recognise foreign pathogenic antigens presented by MHC molecules. In mammals, a separate MHC region contains multiple class I and II genes. Class I molecules are expressed by almost all host cells and class II are expressed specifically by APCs including macrophages, B cells and dendritic cells. Class I MHC present peptide fragments derived from proteins found in cytoplasm or tumour and virus affected cells (endogenous antigen processing), whereas class II MHC present peptides derived from extracellular spaces into intracellular vesicles (exogenous antigen processing). Upon antigen presentation, MHC molecules signal TCRs expressed by T lymphocytes for the antigen specific immune response. MHC I molecules recognises or interacts with

TCR CD8<sup>+</sup> T cells that proliferate to become activated cytotoxic T lymphocytes (CTL) inducing direct killing of infected cells and MHCII molecules on APCs recognises TCR CD4<sup>+</sup> T helper cells to induce B cell mediated antibodies release as well as activation of macrophages harbouring intra-vesicular parasites or bacteria, also known as Th1 response (Kaufman 2014).

$\gamma\delta$  TCR T lymphocytes are mainly found in epithelial rich tissues and interestingly they represent 20-60% of the T cell population in chickens, compared to 5-10% peripheral lymphocytes in mammals (Kubota et al., 1999). Regulatory T cells (Tregs) in mammals which are known for its regulatory immunosuppression properties to protect host post inflammatory reaction or tolerance towards self-antigen have also been characterised in chickens and identified as CD4<sup>+</sup>CD25<sup>+</sup> cells in comparison to CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in mammals. At present, there is no functional ortholog of the Treg-associated transcription factor FoxP3 identified in birds (Selvaraj 2013).

### 1.4 Mononuclear phagocyte system

The process of internalisation of particles is called phagocytosis and this activity is intrinsic to antimicrobial defence by the innate immune system. The phagocytic activity of a cell leading to inflammation and immunity was first recognised and introduced by Elie Metchnikoff in the nineteenth century. He identified host cells as macrophages or “large eaters” (Tauber 2003). Macrophages were first considered part of a reticuloendothelial system (RES) or network of particulate phagocytic cells assumed to be emerging from vascular endothelium. Later it was recognised that they are mainly derived from precursors in the blood (monocytes) and bone marrow in the steady state and they were classified as part of a mononuclear phagocyte system (MPS) to distinguish them from polymorphonuclear leukocytes (Yona and Gordon 2015).

MPS is a heterogenous family of functionally related myeloid cells that are known to share with each other cell morphology, endocytic or phagocytic activity, specific expression of lytic enzymes and endocytic receptors, gene expression and ontology (Hume 2006, Hume 2008, Hume et al., 2019). They appear early in embryonic development from progenitors in the yolk sac, fetal liver or from pluripotent stem cells in the bone marrow and in the adult occupy many anatomical locations, expressing

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tissue specific microenvironment phenotypes (Okabe and Medzhitov 2016; Hoeffel and Ginhoux 2018). MPS family consists of myeloid progenitor cells, blood monocytes, tissue resident macrophages, bone resorbing multinucleated osteoclasts and conventional dendritic cells (cDC), (Hettinger et al., 2013; Jenkins and Hume 2014). Tissue resident macrophages are cells that are found in most organs: microglia in the brain; Kupffer cells in the liver; Langerhans cells in the epidermis; alveolar macrophages in the lung; red pulp macrophages of spleen; peritoneal macrophages that are adapted to tissue specific functional roles. Antigen presenting DCs and macrophages are differentiated from a common committed macrophage-DC progenitor cells in bone marrow and primarily function to induce and regulate immune responses throughout the lifespan in vertebrates (Geissmann et al., 2010). The ontogeny of avian DC subtypes describing their phenotype and distribution in primary and secondary lymphoid organs in chickens has been reviewed before (Nagy et al., 2016). These include bursal secretory dendritic cells (BSDCs), thymic DCs, Langerhans DCs, interdigitating DCs and follicular dendritic cells (FDCs) present in peripheral lymphoid organs (Nagy et al., 2016). In chicken liver, amongst the MPS cells an abundant non-phagocytic DC population (homologous to mammalian conventional DCs) was identified recently (Hu et al., 2019).

Monocytes can function as both precursor and effector cells. Mononuclear phagocytes differentiation and proliferation from a progenitor cell depends on specific growth factors and regulatory transcriptional factors. Their biology and differentiation has been studied extensively in mice and to some extent in humans and pigs, as reviewed in (Ginhoux and Guilliams 2016; Okabe and Medzhitov 2016; Rojo et al., 2017; Hume et al., 2019; Shrivastava and Shukla 2019).

### 1.5 Development origins of macrophages

The original view of MPS was that proliferating bone marrow promonocytes and progenitor cells differentiate into blood monocytes and then these blood monocytes continuously replenish tissue macrophages describing monocytes as immediate tissue resident macrophages precursor cells (Van et al., 1972). Based on lineage tracing the development of macrophages in the embryo in mice, the view of MPS has been redefined to recognise the embryonic origin of some adult resident tissue macrophages

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and their capacity for self-renewal throughout the life span in adults in the steady state (Hashimoto et al., 2013; Gomez et al., 2015; Hoeffel and Ginhoux 2018). According to this view the precursor cells of resident macrophages arise during early development in three sequential waves.

Erythro-myeloid progenitor cells (EMPs) originate from extra-embryonic yolk sac blood islands, giving rise to two cell populations. The first wave will produce yolk sac macrophages or primitive macrophages from EMPs, which do not go through a monocyte stage. The second wave generates definitive monocytes from fetal liver after EMPs migration to the liver. The third wave involves generation of definitive monocytes derived from multi-potential hematopoietic stem cells (HSCs) emerging from fetal liver and bone marrow that contributes to the pool of adult resident tissue macrophages (Ginhoux and Guilliams 2016). Primitive haematopoiesis origin in the yolk sac and long term definitive haematopoiesis is conserved between mice and chickens (Jaffredo et al., 2005). While EMPs arise from yolk sac in mouse embryo, definitive HSCs arise from aorta-gonad-mesonephros (AGM) region (Hoeffel et al., 2015). The formation of definitive HSCs from the AGM region is also conserved in chicken embryos (Jaffredo et al., 2005). In chickens the hematopoietic cell clusters emerge from the ventral wall of the dorsal aorta in the day 3 to 4 incubated embryos (Dieterlen-Lièvre and Martin 1981).

The contribution of embryonic precursor cells to adult self-renewing specific macrophages adapted to specific tissues remains a topic of debate (Ginhoux and Guilliams 2016). The general consensus is that EMP produced in the yolk sac are the main precursor cells for brain microglia, whereas other macrophage populations are mixtures of cells derived from fetal and adult monocytes, derived from EMP and HSC. The relative contribution depends upon how rapidly macrophages turn over; the macrophages of the dermis and skin for example are replaced relatively rapidly by blood monocytes (Hoeffel et al., 2015; Sheng et al., 2015). Regardless of their origin, several recent studies indicate that monocytes can replace resident macrophages and acquire the specific local adaptation when those cells are depleted (Guilliams and Scott 2017; Hume et al., 2019).

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To trace the origin of adult tissue macrophages in chickens, embryos were transplanted with yolk sac blood cells or bone marrow macrophages derived from ubiquitously eGFP expressing donors (Garceau et al., 2015). Yolk sac derived embryonic chicken macrophages were short lived, gave rise to macrophages throughout embryo development but were not retained in post hatch birds. On the other hand, self-renewing macrophage progenitors of bone marrow derived embryonic chicken macrophages gave rise to long term adult tissue macrophages in all organs. The study demonstrated that adult tissue macrophages can be generated from definitive HSCs arising from bone marrow or fetal liver derived monocyte progenitors rather than yolk sac derived EMPs (Garceau et al., 2015).

Transgenic chickens with macrophage restricted fluorescent (red and green) reporter gene have enabled the examination of the growth and development of both embryonic and adult tissue macrophages (Balic et al., 2014). *In vivo* imaging of transgenic embryos identified the first appearance of primitive macrophages in yolk sac at stage HH13. The fluorescent reporter gene was not expressed in erythrocytes and definitive HSCs arising in HH21 stage chicken embryos. It expressed in macrophages appearing in speckled pattern that co-localised with CD45<sup>+</sup> HSCs arising from mesenchyme but not with HSCs of the epithelial layer of dorsal aorta (Garceau et al., 2010; Balic et al., 2014). The pattern of macrophages appearing from the yolk sac, their distribution in mesenchyme and tissues were consistent with the previous studies of the development of chicken embryonic phagocytes (Cuadros et al., 1992). The fluorescent reporter gene was not expressed on thrombocytes which appeared at stage HH29 embryos. Further behavioural studies revealed that these macrophages rapidly proliferated locally in tissues, exhibited phagocytic potential with their ability to recognize microbial antigens and entered into blood vessels lumen. Chicken embryonic macrophages are accumulated in regions of apoptotic cells and do not respond to wounding (Balic et al., 2014).

### 1.6 Mononuclear phagocyte surface markers

As mentioned above the number of markers and reagents characterised for studying macrophages and monocytes in birds is limited. In mammals, antigenic markers recognising cells of the MPS lineage *in situ* have been identified by use of monoclonal

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antibodies against surface markers (Hume et al., 1983; Waddell et al., 2018) and macrophage restricted transgenic reporters (Pridans et al., 2014) that contributed to their biological and functional studies. Expression of several surface markers is shared between monocytes and macrophages. F4/80 is one such antigen marker that is expressed on major cell population of the MPS in mice. Morphology, tissue location and function of MPS cells *in vivo* using F4/80 antibody has been studied extensively (Hume et al., 2002; Hume 2006; Hume 2008). F4/80 is a cell surface glycoprotein that is part of a family of genes EGF TM7 homologous to human epidermal growth factor (EGF) module containing mucin like hormone receptor 1 (EMR1) and CD97 (Kwakkenbos et al., 2004). There is no apparent ortholog of any of the EGF-TM7 family in the chicken genome. The property of F4/80 antibody binding to an epitope that is resistant to glutaraldehyde fixation and paraffin embedding of mouse tissues enabled the recognition and localization of macrophages in several anatomical niches separating them from other cell types. In mice, macrophage specific F4/80 antigen is expressed mainly in the basement membrane of epithelial and endothelial surfaces along with lymphoid and hematopoietic tissues. These include bone marrow stroma and mature resident tissue macrophages as Kupffer cells, Langerhans cells of the skin epidermis, microglia, thymus, macrophages in the red pulp of the spleen, pancreas, kidney, peritoneal macrophages and specific regions of lymph node and gut associated lymphoid areas (Hume et al., 1983; Hume 2008). Recently, a monoclonal antibody against pig myeloid differentiation marker ADGRE1 was characterized (Waddell et al., 2018). Adhesion G protein coupled receptors family or ADGRE1 loci also encodes for F4/80 antigen and is highly expressed in the bone marrow, monocyte derived, or alveolar macrophages as examined in large mammalian species human, pig, rat, sheep, goat, ruminants, and horse but with variation in their protein coding sequences. In pigs, ADGRE1 antibody recognized monocytes, liver and lung tissue macrophages and granulocytes of bone marrow and peripheral blood. ADGRE1 expression was inducible during differentiation into monocytes and macrophage from progenitors in the bone marrow (Waddell et al., 2018).

Although F4/80 antigen recognizes well defined cells of MPS, its expression is not detected on other MPS cells as osteoclasts, marginal zone macrophages, alveolar

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macrophages and classical dendritic cells. Macrophages differentiation, proliferation and survival depends upon its growth factor CSF1 signaling via its receptor (CSF1R). CSF1R is therefore another MPS cell surface marker expressed in mononuclear phagocyte lineage cells of embryo as well as in adult tissues whose functional role and defined key transcriptional regulatory elements in mammals have been studied thoroughly as reviewed in (Hume et al., 2017; Rojo et al., 2017). Transgenic reporter animals based upon expression of CSF1R, have been described, previously in mice *Csf1r*-eGFP, *Csf1r*-mAPPLE (Sasmono et al., 2003; Ovchinnikov et al., 2010; Hawley et al., 2018), sheep *Csf1r*-eGFP (Pridans et al., 2016) and chickens *CSF1R*-eGFP (MacGreen) *CSF1R*-mAPPLE (MacApple) (Balic et al., 2014). The key enhancer within the *CSF1R* locus the intron sequence *Fms* intronic regulatory element (FIRE) was shown to be conserved in amniotes and absolutely necessary for the maximal expression of *CSF1R* reporter gene in macrophages *in vivo* (Himes et al., 2001; Balic et al., 2014; Hume et al., 2017; Rojo et al., 2017).

In MacGreen transgenic mice (*Csf1r*-eGFP), the reporter gene marked all cells of the myeloid lineage throughout development of embryo and trophoblast placenta (which is also recognized as site for CSF1R gene expression from separate promoter). In tissues the reporter gene marked splenic red pulp macrophages, Kupffer cells in liver, alveolar and interstitial lung macrophages; lamina propria of the intestine, brain microglia, renal and testis interstitial macrophages; osteoclasts, thymic macrophages, Langerhans cells of the epidermis and mesenteric lymph nodes (Sasmono et al., 2003). In chickens, an orthologous *CSF1R* reporter gene identified the phenotype of blood monocytes (Balic et al., 2014). It was co-expressed with the chicken macrophage/monocyte marker KULO1<sup>+</sup> cells (Mast et al., 1998), MHCII<sup>+</sup> (Kaufman 2014). As in mammals, it was also detectable at lower levels on other myeloid cells. In lymphoid tissues, *CSF1R* reporter gene was expressed in ellipsoid and in peri-ellipsoid lymphocyte sheath (PELS) in spleen; BSDCs and inter-follicular region in bursa; FDCs in caecal tonsils; brain microglia; sinusoid located Kupffer cells; interstitial tissue of the parabronchial wall in lung; breast skeletal muscle and in epidermis of feather pulp (skin). As chickens lack lymph nodes, macrophages are present in lymphoid follicles (Oláh et al., 2014). Imaging with *CSF1R* reporter gene

chickens identified these lymphoid follicle aggregates in the gut tissue as scattered aggregates present in single follicles or hundreds of follicles. They are present in various organised lymphoid organs. In the germinal centres, lymphoid aggregates are present as B cell dominated region in medulla surrounded by rich T cell area, and transgene expressing cells form dense network of cells within the B cell region (Balic et al., 2014).

The use of *CSF1R* reporter transgene as a definitive marker for mononuclear phagocytes in chicken transgenic lines was taken advantage of in this project to evaluate the *in vivo* biology of macrophage development. Macrophage restricted transgenic reporter animals highlighted the diversity and trophic functions of macrophages in development, homeostasis as well as inflammatory states (Pollard 2009; Gautier and Yvan-Charvet 2014).

### 1.7 Colony forming cells and stem cells

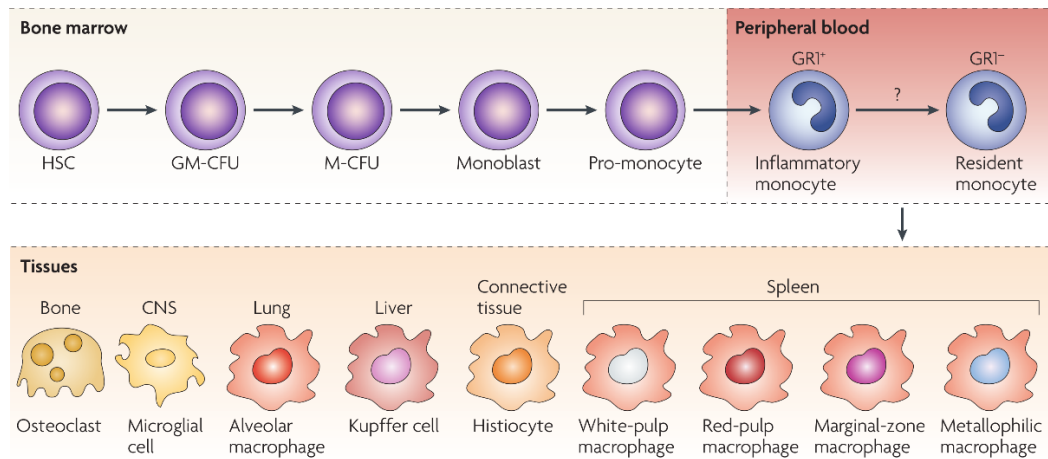
Monocytes originate through differentiation steps from stem cell progenitors and colony forming cells in the bone marrow and fetal liver upon exposure to stimuli that commit them to myeloid and then monocyte lineage. Multipotential HSC is differentiated into a common myeloid progenitor stem cell (CMP) that is a precursor of many different cell types, polymorphonuclear granulocytes, macrophages, dendritic cells and mast cells. CMP is also referred to as the granulocyte/macrophage colony forming unit (GM-CFU), a colony forming cell that commits to two lineages macrophage colony forming unit (M-CFU) and granulocyte colony forming unit (G-CFU). These lineages in mice and humans can be separated with the use of cell surface markers. G-CFU differentiates into polymorphonuclear granulocytes and M-CFU divide and sequentially differentiate into other colony forming cells monoblasts, pro-monocytes before becoming monocytes, that exit bone marrow and migrate to bloodstream. Osteoclasts also develop from monocyte/macrophages cell lineage. In peripheral blood, monocytes differentiate into inflammatory monocytes to resident tissue macrophages to localize in specific tissues (Gordon and Taylor 2005).

Proliferation and differentiation of HSCs into GM-CFU is due to synergy between several cytokines or colony stimulating growth factors or hematopoietic cell growth



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factors giving rise to macrophages with distinct phenotypes. These include CSF1, also known as macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF, or CSF2), multi-CSF (IL3), IL6 and stem cell factor (SCF, also known as kit ligand). Granulocyte colony stimulating factor (G-CSF, or CSF3) differentiates G-CFU into granulocytes. Orthologs of all three mammalian cytokines CSF1, CSF2 and CSF3 has been identified in the chicken genome (Avery et al., 2004; Gibson et al., 2009; Garceau et al., 2010). Proliferation and differentiation of M-CFU into monocytes and mature macrophages is primarily regulated by CSF1. IL34, a recently identified growth factor can also drive macrophage differentiation and signals through a common cell surface receptor, CSF1R. To commit M-CFU into macrophage survival and differentiation, stimulation of CSF1R is vital. All cells of the monocyte/macrophage cell lineage express CSF1R. All biological actions of CSF1 are by signaling through CSF1R (Jeannin et al., 2018). Significance of CSF1/IL34/CSF1R signaling in macrophage differentiation became evident from studies in IL34 deficient and *csfl* or *csflr* deficient *op/op* mice and rats that exhibit severe deficiency in tissue macrophages leading to post-natal growth retardation (Ryan et al., 2001; Dai et al., 2002; Wang et al., 2012; Pridans et al., 2018).



**Figure-1-1: Differentiation and heterogeneity of cells of the MPS**

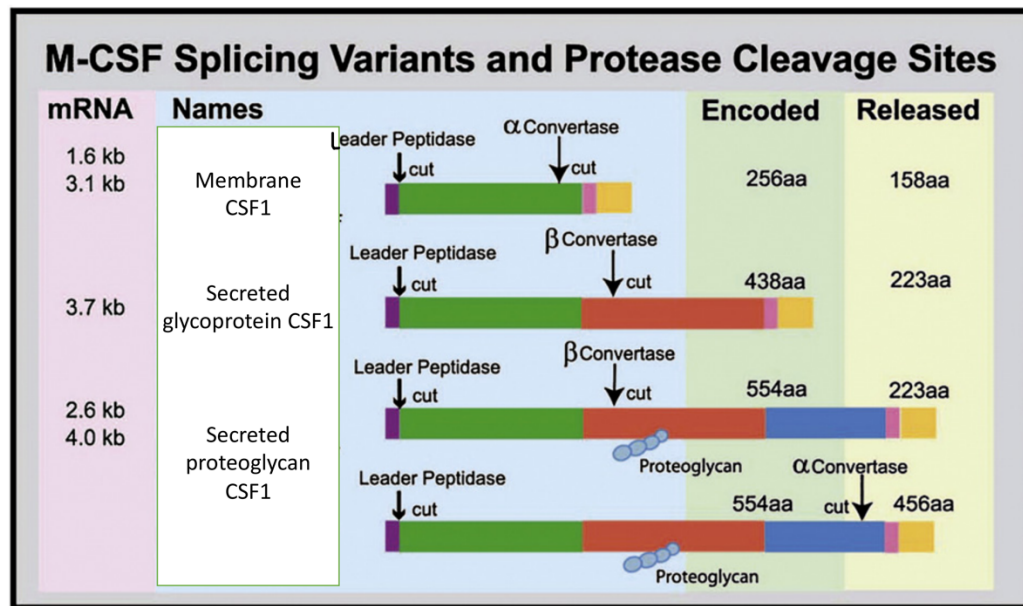
Developmental stages of monocyte and macrophage differentiation depicting precursor multipotential HSC origin in bone marrow. Stimulation of M-CFU by growth factor CSF1 commits precursor cells to monocyte/macrophage lineage cells. Monocytes exit bone marrow and enter into peripheral blood in the steady state or in response to inflammation. The two monocyte subsets known in mice GR1<sup>+</sup> or inflammatory monocytes and GR1<sup>-</sup> or resident monocytes is shown. GR1<sup>+</sup> monocytes enter inflamed tissues. Blood monocytes differentiate into tissue macrophages contributing to specific tissue micro-environment. CNS, central nervous system; GM-CFU, granulocyte/macrophage colony-forming unit; M-CFU, macrophage colony-forming unit. Adapted from (Mosser and Edwards 2008).

### 1.7.1 Colony stimulating factor-1 (CSF1)

Homodimeric glycoprotein CSF1 was the first CSF discovered for its ability to stimulate progenitor cells into pure macrophage colonies *in vitro* (Stanley et al., 1978). The *Csf1* gene is located at chromosome 1 in humans and chromosome 3 in mice. Primary mRNA is transcribed into varying lengths, 4.0, 3.7, 3.1, 2.6 or 1.6 kb mRNA forms via alternative splicing and post translational modification. In humans, the 1.6 and 3.1 kb transcripts encode 256-amino-acid proteins that give rise to a shorter, membrane-bound CSF1 protein. The 3.7 kb transcript encode a 438-amino-acid protein and 4 and 2.6 kb transcripts encode a 554-amino-acid long peptide. The three isoforms correspond to the membrane bound, secreted glycoprotein and secreted proteoglycan isoforms of CSF1 respectively (depicted in figure: 1-2) (Pixley and Stanley 2004; Douglass et al., 2008; Chockalingam and Ghosh 2014).

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The mature biologically active form of CSF1 which stimulates macrophage proliferation is a disulfide linked homodimer and stays attached to the cell surface or processed by proteolytic cleavage to form secreted isoforms that circulates in peripheral blood (Pixley and Stanley 2004). Proteases including a signal peptidase,  $\alpha$ -convertase and  $\beta$ -convertase process the nascent protein into isoforms. The majority of the CSF1 protein (95–99%) is processed to soluble form from membrane bound precursors and released into circulation, while (1–5%) remain bound to the extracellular surface membrane (Douglass et al., 2008; Chockalingam and Ghosh 2014). Based upon detection of multiple *CSF1* mRNA transcripts, the formation of three functional membrane bound, secreted glycoprotein and secreted proteoglycan CSF1 isoforms and alternative splicing is likely conserved from mammals to birds (Garceau et al., 2010).



**Figure-1-2: Schematic representation of CSF1 mRNA transcripts and various isoforms of CSF1 protein obtained**

CSF1 in general consists of five sections, an N-terminal signal peptide composed of 32 amino acids, a receptor binding domain of 149 amino acids (required for biological activity), a spacer region with varying length, a C-terminal transmembrane domain with 24 amino acids and a cytoplasmic tail composed of 35 amino acids with variable inserts between these two parts. From left to right, purple: leader sequence, green: common 149-amino-acid M-CSF core, red: a spacer region, dark pink: a 25-amino-acid transmembrane region, and orange: a 35-amino-acid intracellular region. The first black arrow indicates where the leader sequence is cleaved quickly during processing by a signal peptidase. The larger arrow represents where other protease activity subsequently cleaves CSF1 to yield various soluble species. Picture adapted from (Douglass et al., 2008)

The three isoforms of CSF1 gene can be functionally separated. Transgenes encoding each of these isoforms have been constructed earlier using CSF1 promoter and first intron and validated for their functional roles in *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice in a normal tissue-specific and developmental pattern. These studies confirmed different but overlapping phenotype roles for all CSF1 isoforms and identified several cellular sites of CSF1 expression (Ryan et al., 2001; Dai et al., 2004). CSF1 is produced by a range of cell types in mice. Endothelial cells synthesize secreted proteoglycan and glycoprotein isoforms that function on CSF1 target cells at remote locations (humoral regulation), whereas both cell-surface glycoprotein and the proteoglycan isoforms is involved in

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local regulation, but not much is known about their differential regulation (Pixley and Stanley 2004).

Previous studies from our lab demonstrated that the *CSF1* gene exists in chicken as well as zebra finch genomes (Garceau et al., 2010). The identified chicken *CSF1* gene was genetically mapped to chromosome 26. As noted above, two transcripts for chicken CSF1 were identified. The large transcript comprising of eight coding exons, of size 2698 bps and translating to protein of 490 amino acids and a small transcript that encodes a protein of 270 amino acids and has a large portion of exon 6 missing, that encodes for proteolytic cleavage site in mammals, releasing CSF1 from membrane anchored precursor. It was therefore proposed that the smaller transcript might be the chicken ortholog of mammalian cell surface membrane binding CSF1 protein. Molecular modelling study of avian CSF1 (and IL34, see below) depicted four helix bundle structure and formation of a homodimer, similar to mammalian CSF1 (and IL34) that confirmed the overall structural conservation in birds and mammals for the two ligands. Conserved intrachain disulfides stabilize the structure in both mammals and birds. In contrast to mammals and fish, wherein cysteine residues contribute to an interchain disulfide-bond at the dimer interface of CSF1 (Pandit et al., 1992), avian CSF1 protein forms noncovalent dimers through a large hydrophobic interface. The amino acid alignment studies highlighted the divergence in the contact residues for CSF1 bound to CSF1R from mouse CSF1 protein. There was also substantial divergence in these contact residues between chicken and zebra finch (Garceau et al., 2010). Subsequent studies in the laboratory revealed rapid evolution of the extracellular domain of CSF1R and CSF1 binding in avian evolution (DA Hume, In press)

In contrast to other CSFs, CSF1 is constitutively and ubiquitously expressed by many different cell types like monocytes, smooth muscle, fibroblasts, osteoblasts, stromal cells, endothelial cells, and tumor cells (Stanley and Chitu 2014). CSF1 also regulates trophoblast lineage cells of pregnant uterus via CSF1R signaling as CSF1R is exceptionally expressed on these cells outside of mononuclear phagocyte lineage cells (Arceci et al., 1989; Guleria and Pollard 2000). Elevation in the circulating CSF1

levels has been detected in numerous disease states including cancer, inflammation, and autoimmune diseases (Chitu and Stanley 2006).

CSF1 induced signalling is a major regulator of the proliferation and differentiation of mononuclear phagocytes in mammals as well as chickens. *In vivo* administration of CSF1 expands mononuclear phagocyte system. In mice, recombinant human CSF1 treatment promoted liver and kidney growth repair along with increased blood circulating mature monocytes and macrophages of peritoneal cavity, liver and spleen (Hume et al., 1988; Alikhan et al., 2011). In rats, human CSF1 injection induced monocytosis (Ulich et al., 1990). In chickens, injections of novel form of CSF1 protein, chicken CSF1-Fc in embryos and post hatch CSF1R reporter transgenic birds caused leucocytosis and increased macrophage numbers in spleen, bursa, liver, lung, kidney and muscles as well as increase in bone density (Garceau et al., 2015).

### 1.7.2 Interleukin 34 (IL34)

Differences in phenotype in *Csf1r<sup>-/-</sup>* mice compared to the *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice first indicated the possible existence of a second ligand for CSF1R. Specifically, Langerhans cells are present in *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice, but ablated in *Csf1r<sup>-/-</sup>* mice (Dai et al., 2002). Subsequently, through comprehensive proteomic analysis, a new cytokine IL34 that stimulated monocyte viability was identified and purified (Lin et al., 2008). In humans, the *IL34* gene is located on chromosome 16 and encodes a full-length mature protein of 222 amino acid. It is secreted as a glycoprotein and the biologically active form is a four-helix bundle, noncovalently linked homodimer structure, different from the disulfide linked homodimer structure of mammalian CSF1 (Jeannin et al., 2018). The four-helix bundle structure of CSF1 and IL34 is shared by many other cytokines and growth factors.

Examination of IL34 deficient reporter mice, confirmed that keratinocytes of the epidermis and brain microglia were selectively depleted in these mice and that IL34, an alternate ligand for receptor CSF1R has a non-redundant developmental and maintenance function by promoting differentiation, specifically of the myeloid cells in the skin epidermis and CNS. IL34 is mainly expressed in epidermis and CNS in mice and humans both and the knockout has limited impact on the development of other

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monocyte cells, tissue macrophages and dendritic cell subsets (Chihara et al., 2010; Greter et al., 2012; Wang et al., 2012; Stanley and Chitu 2014).

The chicken ortholog of human IL34 gene was identified previously and mapped to chromosome 11 (Garceau et al., 2010). Avian IL34 is made of six coding exons, with a transcript length of 1,416 bps, translating to protein of 178 amino acids. Subsequently Zebra finch orthologous IL34 gene was identified and mapped to chromosome 11. It encodes a protein of 180 amino acids. Structural comparison of chicken and mammalian IL34 protein form revealed similar four helix bundle homologous to CSF1. Amino acid sequences comparison revealed more conservation of chicken IL34 with mammalian form IL34 than chicken CSF1. Chicken IL34 lacks cysteines forming intrachain and interchain disulphide bonds. However, the dimer interface did contain exposed hydrophobic residues, suggesting non-covalent homodimer structure. Chicken IL34 protein sequences amino acid residues at CSF1R binding site are totally different than those in CSF1 at the same position. This study confirmed an alternate binding site of IL34 on chicken CSF1R, different from CSF1 binding site (Garceau et al., 2010).

### 1.7.3 Colony stimulating factor-1 receptor (CSF1R)

CSF1R (also known as FMS, c-FMS, CD115, or M-CSFR) is a 150 kDa glycoprotein encoded by the *c-fms* proto-oncogene (Sherr et al., 1985). CSF1R belongs to the type III tyrosine kinase subfamily of the platelet-derived growth factor receptor (PDGFR) family, which also includes KIT, PDGFR $\alpha$ , PDGFR $\beta$  and FLT3/FLK2 proteins. CSF1R consists of extracellular ligand-binding five Ig-like (D1–D5) domains, a transmembrane domain, and an intracellular tyrosine domain comprising of juxta membrane (JM), tyrosine kinase (TK) and kinase insert (KI) regions. The first three N-terminal domains D1-D3 are site for ligand recognition and D4–D5 domains are required for dimerization of receptor. CSF1R in inactivated state exists as auto-inhibited monomer form that is mediated by JM domain (Walter et al., 2007).

CSF1 and IL34 binding to the extracellular domain of CSF1R stabilizes receptor dimerization and brings about autophosphorylation of seven conserved tyrosine residues in intra-cellular cytoplasmic domain leading to overall activation of

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macrophages which triggers key pathways associated with phagocytosis, survival and proliferation (Stanley and Chitu 2014; Rojo et al., 2017). CSF1/IL34 activation of CSF1R receptor leads to gene transcription, protein translation and cytoskeletal remodeling.

CSF1R gene orthologues have been identified in all vertebrates. Protein alignment homology studies between mammalian CSF1R and chicken CSF1R revealed extreme conservation of the intracellular tyrosine kinase domain (AAs 540–977) amongst all species including birds (Garceau et al., 2010). CSF1/IL34/CSF1R system is conserved across evolution in birds and fish, however there are species specific cross reactivities due to variations in the interaction sites of the ligand (CSF1/IL34) receptor CSF1R complexes (Garceau et al., 2010; Hume et al., 2017). For example, comparison of CSF1 binding sites to CSF1R amongst mammals and avian species revealed many divergent residues at the CSF1 binding sites depicting non conservation of CSF1 ligand as well as the extra-cellular CSF1 binding domain of CSF1R. The ligand CSF1 as well as the extra cellular domain of CSF1R is divergent amongst various species (Garceau et al., 2010)

CSF1R elements and factors required for macrophage specific gene expression are conserved in birds (Hume et al., 2017). Transcriptional studies of avian CSF1R revealed that the CSF1R promoter in birds is purine rich and TATA less similar to mammalian CSF1R promoter. Chicken CSF1R and enhancer FIRE region contains certain conserved multiple consensus repeats of the mammalian CSF1R and FIRE region. These include binding sites for transcription factors PU.1, C/EBP and AML1. These studies showed that transcription of avian CSF1R is controlled in the same way as in mammals (Garceau et al., 2010).

*CSF1R* gene is expressed at low levels on monocytes and increases as cells differentiate toward mature macrophages (Hawley et al., 2018). CSF1R expression is mainly restricted to the cell surface of monocytes and macrophages. During embryonic development in mammals, CSF1R is expressed in placental trophoblasts. There have been reports that CSF1R is expressed by various neuronal and epithelial cell populations, but a critical review of these studies indicates that the evidence is not



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compelling (Hume et al., 2019). The authors conclude that *CSF1R* mRNA and protein is exclusively expressed on cells of the MPS and their progenitors and accordingly, the impacts of *CSF1R* mutations in human patients and animals (see below) are exclusively attributable to the loss of MPS functions. As noted above, macrophages and blood monocytes can therefore be localised using monoclonal antibody against *CSF1R* or with reporter genes driven by elements of the *CSF1R* promoter in birds and mammals alike (Sasmono et al., 2003; Garcia-Morales et al., 2013; Balic et al., 2014; Pridans et al., 2016).

The functional activity of *CSF1R* can be assayed conveniently by expressing the receptor in a factor-dependent cell line. One such study used *in vitro* bioassay with BaF3 cells ectopically expressing feline *CSF1R*, human and pig *CSF1* to study the species cross-reactivity of various *CSF1* and *IL34* molecules (Gow et al., 2013). BaF3 cells transfected with porcine *CSF1R* showed low level expression of macrophage differentiation markers F4/80 and CD11b. On the other hand, BaF3 cells transfected with chicken *CSF1R* and selected for growth in chicken *CSF1* for a long duration differentiated and underwent growth arrest exhibiting high level expression of macrophage differentiation markers F4/80 and CD11b (Garceau 2014). These results suggested that BaF3 cells are myeloid rather than lymphoid in origin as described initially (Palacios and Steinmetz 1985; Gow et al., 2012). BaF3 cells had also been used to dissect various *CSF1R* mutant alleles in the autosomal dominant neurodegenerative disease HDLS (Pridans et al., 2013) and to assay candidate *CSF1R* kinase inhibitors (Irvine et al., 2006). Chicken *CSF1R* has been expressed in BaF3 cells to demonstrate the biological activity of cloned *CSF1* and *IL34* (Garceau et al., 2010).

### 1.8 Pleiotropic effects of macrophage deficiency

Mice and rats homozygous for the natural or targeted null mutation of the ligand *CSF1* *Csf1<sup>op</sup>/Csf1<sup>op</sup>* in mouse; *Csf1<sup>tl/tl</sup>* in rat and the receptor *Csf1r* gene (*Csf1r<sup>-/-</sup>*) have been invaluable for determining their pleiotropic functions on tissue macrophages and osteoclasts *in vivo* and their importance in postnatal growth and development in mammals (Wiktor-Jedrzejczak et al., 1990; Ryan et al., 2001; Dai et al., 2002; Van et al., 2002; Li et al., 2006; Harris et al., 2012; Pridans et al., 2018). *Csf1* or *Csf1r*

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deficient *op/op* mice exhibit neuronal dysfunction, infertility, mammary gland and insulin secreting pancreatic cells developmental abnormalities. *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mouse and *Csf1<sup>u/n</sup>* rats with a natural mutation in *Csf1* locus exhibit severe macrophage numbers and effector function defects associated with *Csf1* deficiency, which includes postnatal growth retardation, deficiency in *Csf1* dependent bone resorbing osteoclasts, leading to osteopetrosis and reduced fertility (Chitu and Stanley 2006; Pollard 2009). Mice with a homozygous recessive mutation in the coding region of *Csf1* gene on chromosome 3, have severely deficient mature macrophages and low number of osteoclasts leading to osteopetrosis (Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990). In mice, phenotype can be partly compensated with age indicating partial redundancy of *Csf1* (Begg et al., 1993).

Detailed phenotypic analysis of rats with targeted disruption of *Csf1r* gene has been carried out recently (Pridans et al., 2018). *Csf1r<sup>-/-</sup>* rats survived longer than *Csf1r<sup>-/-</sup>* mice, at least on an inbred background (Hume et al., 2019). Many of the macrophage phenotypes mediated by *Csf1r* mutation is shared between mice and rats. These include reduced circulating monocyte numbers, lack of tooth eruption, reproductive abnormalities, deficiency in liver and peritoneal macrophages, loss of Langerhans cells and post-natal growth retardation (Dai et al., 2002; Pridans et al., 2018). However, the *Csf1r<sup>-/-</sup>* rats lack the severe brain phenotype and effects on the sensory nervous system, insulin/pancreatic islet development, and gastrointestinal tract observed in mice (Pridans et al., 2018). In a very recent study, the *Csf1r* enhancer FIRE region which is highly conserved in mammals as well as birds and is present in the intron sequence was deleted in mice to examine physiological impacts and tissue macrophages (Rojo et al., 2019). Compared to *Csf1* and *Csf1r* mutated mice, FIRE deleted mice were not osteoclast deficient. The study revealed selective functional requirement of FIRE for *Csf1r* transcription in mononuclear phagocytes. FIRE was required for *Csf1r* dependent tissues such as those of the embryo, microglia, Langerhans cells, peritoneum, heart and kidney, but not required for the development of other *Csf1r* dependent tissues as intestine, lung, dermis, spleen, liver, and bone. FIRE deleted mice had normal circulating monocytes but abolished *Csf1r* expression in bone marrow

monocytes and progenitor cells (Rojo et al., 2019). Impacts of macrophage deficiency due to modulation in CSF1/CSF1R signaling are discussed more in Chapter 4.

Inhibitors that target macrophages in mammals are of high interest due to their active role in inflammation as well as development and maintaining homeostasis in embryonic and post-natal stage. CSF1/CSF1R signalling activation promotes differentiation, survival and proliferation of monocyte and macrophage lineage cells, but overexpression of either of these molecules has been linked to malignant disorders (Hamilton et al., 2016; El-Gamal et al., 2018). Therefore, molecules that inhibit the function of the receptor or its ligands have significant therapeutic potential (discussed more in Chapter 3).

### 1.9 Chicken model for genome editing

Chickens are recognised as a valuable research model in developmental studies involving embryogenesis or specific gene functions (Woodcock et al., 2017; Davey et al., 2018; Sid and Schusser 2018). Technologies enabling successful targeted defined mutations of chicken genome have accelerated the use of the chicken model (Davey et al., 2018). Effective use of genome editing technologies facilitate molecular research in development, immunology or industry applications contributing to our understanding of avian species. The easy *in ovo* access to chicken embryos and convenient use of microsurgical procedures provide novel opportunities that are limited in mammalian models. Recent advances include fate mapping the origin of cells (Le Douarin and Dieterlen-Lièvre 2013), stable fluorescent proteins expressed ubiquitously under specific enhancers (McGrew et al., 2008) and development of lineage-specific reporter transgenics (Balic et al., 2014). Translational opportunities for poultry industry applications include potential generation of infectious disease resistance avian lines and production of economically important proteins in eggs (Lillico et al., 2005; Lee et al., 2017; Herron et al., 2018).

The first genetically modified chicken was generated successfully using retroviral vector injected into the yolk sac near to the developing blastoderm, that integrated foreign DNA into the germline G1 and G2 (Salter et al, 1987). Alternative approach avoiding potential hazards of retroviruses replication in poultry included use of

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plasmid DNA injection into chicken zygote (germinal disk) that led to stable transmission of foreign DNA into progeny transgenic chickens (Love et al., 1994). The first transgenic chickens with high frequency of stable germline transmission for many generations were generated using a lentivirus vector derived from avian viruses and blastoderm in developing embryo (McGrew et al., 2004). Although a decent germline transmission efficiency was obtained there were challenges involved with the limiting size of the transgene, precise edits or silencing of virus vectors.

Primordial germ cells (PGCs) are germ cells that originate from embryonic stem cells in the oocyte and carry genetic information from one generation to another. They differentiate into functional sperm or oocyte into developing embryonic gonads. Availability of PGCs culture medium and their subsequent transplantation into embryonic vasculature enabled generation of genome-edited chicken models (Van de Lavoie et al., 2006; Whyte et al., 2015). Besides retrovirus vectors (McGrew et al., 2004; Lillico et al., 2007; Motono et al., 2010) other approaches for the precise deletions and integration into the chicken genome using PGCs include transposons (Macdonald et al., 2012; Tyack et al., 2013), homologous recombination (Schusser et al., 2013) and programmable engineered nucleases (discussed in Chapter 5). Genetically modified chickens with targeted gene knockout have been produced in PGCs, although there have been limited studies in avian species at present which reported successful generation of genome modified knockouts in birds including chicken and quail.

### 1.10 Rationale of study

Chickens provide a tractable model to study the development and function of the mononuclear phagocyte system. The expression of the fluorescent *CSF1R* reporter gene in transgenic chickens co-incident with the endogenous *CSF1R* protein expression in macrophage lineage cells has permitted us to view and examine the chicken macrophages in embryos as well as post hatch birds lymphoid tissues and blood monocytes (Balic et al., 2014). This model is a great asset for further studies on MPS in chickens, allowing us to quantify or visualise the lymphoid follicles or different subset of macrophage population. Studies that preceded the current project (Garceau et al., 2015), showed that *CSF1* regulates macrophage proliferation and

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differentiation in embryo and postnatal birds and addressed the relative longevity of yolk sac and bone marrow derived macrophages (BMDMs). The novel form of recombinant CSF1-Fc protein injections in embryo and post hatch birds showed that there is a macrophage restricted CSF1R signalling in tissues, blood monocytes and bone osteoclasts. This finding in turn opened up several questions about the role of CSF1/CSF1R signalling in the development of chickens. Is CSF1 required for normal postnatal development in birds as it is in mammals? Given the severe pleiotropic effects of CSF1R mutations in rats, mice and humans (Hume et al. 2019) are CSF1R-dependent macrophages also critical for development in birds?

Prior to the start of this project, several tools that enable study of CSF1R-dependent chicken mononuclear phagocyte system were developed and characterised previously by Hume lab. For the purpose of this project, these reagents were utilized, or stock generated for various objectives to enable studying CSF1- dependent macrophages in chickens and to explore similarities and differences with mammalian systems. These include MacReporter transgenic chicken lines (*CSF1R*-mAPPLE and *CSF1R*-eGFP) (Balic et al., 2014) and other reagents as recombinant chicken CSF1 vector plasmid for bacterial isolation of CSF1 protein, hybridoma for chicken CSF1 monoclonal antibody (mAb) purification (Wu *et al*, manuscript in progress), alexa fluor (AF) 647 labelled novel CSF1-Fc protein for flow cytometry and an antibody that recognises chicken CSF1R expression *in vivo*. (Garceau et al., 2010; Garcia-Morales et al., 2013; Balic et al., 2014; Garceau et al., 2015). The antibody that recognises a subset of chicken MPS cells involved in the elimination of apoptotic cells anti-TIM4 mAb was only recently characterised (Hu et al., 2019) and has been used in this project for immunohistochemistry. Moreover, growth factor dependent pro-B cell line Ba/F3 expressing chicken CSF1R; and bone marrow derived chicken macrophages grown in recombinant CSF1 for up to 7 days (Garceau et al., 2010) were utilised for *in vitro* cell based assays.

## 1.11 Aim, objectives and hypothesis

Studies from this project examine CSF1R signalling functions in chickens *in vivo* and *in vitro* interpreting the biology of monocytes and other cell types in which CSF1R is expressed or affected, specifically the phenotypes and morphological changes observed post macrophage functional changes. The principal aim was to study CSF1/CSF1R biology in chickens and its impact upon the avian macrophage development. To address this question, three complementary approaches were examined, each aiming to reduce the level of CSF1R signal.

1. To identify potential chicken CSF1R kinase inhibitor compounds that can affect the viability of chicken macrophages *in vitro* and *in vivo* (Chapter 3).
2. To characterise a novel chicken CSF1 specific neutralising mAb ROS-AV183, that targets and blocks chicken CSF1R signalling activity (Chapter 4).
3. To generate CSF1R knockout transgenic chickens by targeted gene disruption of *CSF1R* loci and analyse the impact of mutation on mononuclear phagocytes and developmental phenotype (Chapter 5).

Accordingly, objectives of the study were as follows,

- Characterisation of mass purified recombinant chicken CSF1 protein, anti-chicken CSF1 mAb (ROS-AV183) and isotype control mAb utilised for *in vivo* subcutaneous injection experiments.
- To identify candidate chicken CSF1R kinase inhibitors via a cell viability assay using a factor dependent pro B cell line Ba/F3 expressing chicken form of CSF1R protein.
- To test the impact of chicken CSF1R kinase inhibitors *in vitro* on the survival of CSF1-dependent primary chicken macrophages.
- To study the impact of anti-chicken CSF1 mAb *in vitro* on the survival of CSF1-dependent primary chicken macrophages.

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- To determine the impact of neutralising anti-chicken CSF1 mAb against chicken CSF1 on blood monocytes, tissue macrophage numbers, bone density and overall development.
- To localise CSF1 protein in lymphoid tissues via immuno-fluorescence
- To target *CSF1R* receptor gene located on chromosome 13 in chicken PGCs using CRISPR/CAS9 genome editing tools
- To produce CSF1R knockout transgenic chicken model via germline transmission.
- Analyse the phenotypes associated with *CSF1R* knockout allele in G1 heterozygous and G2 homozygous embryos at different days of incubation.

### Hypothesis:

CSF1/CSF1R signalling controls macrophage differentiation in birds, and indirectly controls development and immunity in ways that are relevant to production traits

## Chapter 2 Materials and methods

Unless otherwise specified, all procedures were carried out at The Roslin Institute, University of Edinburgh. Information on the general reagents mentioned in this Chapter including catalogue number and stock/working concentrations used is given in Chapter 8, appendix A

### 2.1 Purification of endotoxin free chicken CSF1 protein

#### 2.1.1 Transformation of chemically competent bacterial cells

pTLW54 is a bacterial expression plasmid for chicken CSF1 (N31- P189), and was prepared by Dr Tom Wilson of Zoetis (formerly Pfizer Animal Health (PAH), Kalamazoo, MI, USA) as part of a collaboration to develop CSF1 as a therapeutic agent in birds (Garceau et al., 2015). Optimized for expression in bacteria, chicken CSF1 insert was cloned into pET-28(b) vector or plasmid pTLW54. An aliquot of this plasmid was transformed into chemically competent One Shot® BL21 Star cells by standard methods and transformants were selected on agar plates containing antibiotic kanamycin (50 µg/ml). Single cell colonies were picked up for inoculation in Luria-Bertani (LB) growth media and culture preparation. Colonies were expanded in LB medium containing kanamycin at concentration of 50 µg/ml, in small (5 ml) to large volumes (100 ml, 1 litre and 2 litre). The culture was incubated at either 25°C or 37°C at 220 rpm in a shaking incubator.

#### 2.1.2 Plasmid purification and analytical digestion

5 ml culture of pTLW54 transformed BL21 star cells was harvested by centrifugation at 8000 rpm for 3 mins at room temperature (RT). Plasmids was isolated from cell pellets using Qiagen's mini prep kit (cat. no. 27104) and according to the manufacturer's instructions. To isolate and purify plasmids in large volume stock, Qiagen's endofree plasmid maxi prep kit (cat. no. 12363) was used as per manufacturer's instructions. Plasmids were isolated from 100 ml of starter culture. Plasmid DNA pellets were resuspended in 200 µl TE buffer in 1.5 ml Eppendorf tubes. Concentration of isolated plasmid pTLW54 DNA in ng/µl was determined using the Nanodrop ND-1000 spectrophotometer. Tubes were kept at RT overnight for solubilization of DNA pellet and stored at 4°C for short term use or -20°C for long term use.

To confirm the presence of the CSF1 cassette in pTLW54, a double restriction digestion reaction was setup as per the engineered restriction sites on plasmid pTLW54 and accordingly reaction was setup using 500 ng of plasmid and restriction enzymes *EcoRI*-HF and *NlaIII*. The reaction was incubated at 37°C for one hour to enable complete digestion, and DNA fragments



were visualized by 1.5% agarose gel electrophoresis. The results confirmed release of the expected 567 bp fragment encoding chicken CSF1.

### 2.1.3 Bacterial culture induction

Expression of CSF1 in pTLW54 is controlled by the lac operon and inducible by Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG). A 100 ml starter culture of plasmid pTLW54/One Shot BL21 star cells was grown in media as described in 2.1.1 at 37°C, 220 rpm. When the optical density (O.D) of the culture reached approximately 1, as measured by mass spectrophotometry at a wavelength of 600 nm, the cells were harvested by centrifugation and the pellet stored overnight at 4°C. The following day, fresh 100 ml LB/kanamycin media was added to the pellet and the starter culture was refreshed in 1 litre LB/kanamycin broth (1:10 dilution) using a 2 litre vented flask. The culture was allowed to grow at 37°C in a shaking incubator at 220 rpm to mid log phase (O. D<sub>600nm</sub> measurement of 0.8 (approximately 3 to 4 hrs). At this stage, CSF1 protein expression was induced by adding 0.5 mM IPTG and additional kanamycin at 50  $\mu$ g/ml. The induced cultures were kept in a shaking incubator at 220 rpm at either 37°C for 3 hrs or 25°C overnight to test the optimal CSF1 expression post induction. After induction the cultures were harvested from the different flasks for protein solubilization by centrifugation at 4000 rpm, for 20 mins at 4°C. The pellet was stored at -80°C until column purification. 1 ml aliquot samples of pre (control) and post induced *E.coli* culture were used to detect CSF1 protein expression by immuno-blotting.

### 2.1.4 Detection of expressed protein by Immuno-blotting

Presence and purity of recombinant CSF1 protein was analyzed by SDS PAGE. Accordingly, sample pellets from control and induced cultures were suspended in 100  $\mu$ l of sterile buffer, phosphate buffer saline (PBS). 12.5  $\mu$ l of 2x Laemmli sample buffer, containing 5% 2-mercaptoethanol was added to 12.5  $\mu$ l of each sample. The mixture was vortexed and heated at 95°C for 10 mins, spun briefly and then loaded on 4–15% Mini-PROTEAN® TGX™ precast protein gels. Finally, the mixture was separated in running buffer (25 mM tris-base, 192 mM glycine and 0.1% SDS, pH 8.3) at 160 V for an hour. A dual color standards ladder (BioRad, cat. no. 1610374) was loaded in one lane. SDS gels were stained with Coomassie Brilliant Blue (CBB) buffer (0.1% CBB-250, 10% acetic acid and 40% methanol) by gently rocking on a shaker for one hour, followed by washing with distilled water to remove excess stain residues. Gels were then placed in de-stain solution (20% methanol, 10% acetic acid), rocking gently with continuous monitoring of bands until optimal visualization with naked eye.

For immuno-blotting of expressed chicken CSF1 protein, samples were transferred onto 0.25  $\mu$ m pore size nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Transfer was performed for 2 hrs at constant voltage of 100 V. The membrane was blocked with 5% non-fat skimmed milk in PBST (PBST (0.1% (v/v) Tween 20 in 1x PBS) buffer overnight at 4°C in tubes on a roller shaker. The following day, the membrane was incubated in a primary antibody, anti-chicken CSF1 IgG1 (5 mg/ml) (1:5000 dilution) dissolved in PBST, for 1 hr at RT, while rocking on a roller shaker. The membrane was washed with PBST buffer three times for 10 mins each then probed with secondary antibody goat anti-mouse pAb to M<sub>s</sub> IgG+IgM+IgA HRP (1:2000 dilution) (Abcam, cat. no. AB6006, 1mg/ml) for 1 hr at RT followed by further PBST washes. CSF1 protein expression was detected by chemiluminescence, performed using Pierce™ ECL Plus substrate reagents kit (Life Technologies, cat. no. 32132) following the manufacturer's protocol. The lot was developed in an X-ray film cassette.

### 2.1.5 Isolation, refolding and column purification

The IPTG induced *E.coli* cell pellet from 1 litre of culture stored at -80°C was utilized for bacterial CSF1 protein isolation. This procedure was performed with the assistance of Dr. Andy Gill using the protein purification facilities in his lab at The Roslin Institute. The pellet was resuspended in five volumes of lysis buffer (50 mM tris pH 8.5 10 mM EDTA) on ice, and vortexed. Cells were lysed by passing the suspension two times through a high pressure (15K PSI) microfluidizer cell disruptor (Constant Systems Ltd). The inclusion bodies were pelleted by low speed centrifugation (12,000 x g for 20 mins) at 15°C. The pellet was washed two times with lysis buffer to remove the *E.coli* membrane and cell wall material, then stored at -20°C. Inclusion bodies were resuspended in solubilization buffer (50 mM tris pH 8.0, 7 M guanidine HCl, 5 mM EDTA, 5 mM DTT) and incubated for 1 hr at RT in a circular rotator. The protein solution was clarified by centrifugation at 15000 x g for 20 mins. Refolding of the denatured protein was initiated by slowly adding the protein solution (clear supernatant) to 200 ml of refold buffer (55 mM tris pH 8.2, 21 mM NaCl, 0.8 mM KCl, 1.1 M guanidine, 1 mM EDTA, 1 mM reduced glutathione, 1 mM oxidized glutathione), using a peristaltic pump, at a rate of 1 ml/min, with continuous stirring overnight at 4°C. The clear refolded protein solution was then poured into a dialysis membrane and dialyzed against 2 litre dialysis buffer (pre-Q-Sepharose) (25 mM tris pH 8.5, 30 mM NaCl) stirring continuously at 4°C overnight. The following day, dialysis buffer was replaced, and overnight dialysis was repeated. The dialyzed

protein solution inside the membrane was clarified for chromatography by centrifugation at 12000 x g for 20 mins at 4°C. Supernatants from several batches were combined.

An AKTA chromatography system (GE Healthcare Life Sciences) was used to perform an ion exchange chromatography of CSF1 protein solution. An XK26 chromatography column containing washed Q sepharose resins was equilibrated with 5BV (buffer valve) Q-sepharose A (QA) loading buffer (25 mM tris pH 8.5, 20 mM NaCl). Clarified protein sample was loaded onto the column at 3 ml/min. The column was washed with approximately 5CVs (column volumes) of buffer QA. Elution of the protein was accomplished with the following gradient: 10 BV 0-25% Q-sepharose B (QB) buffer (25 mM tris pH 8.5, 0.8 M NaCl), 2 BV 25-100% QB buffer, 2 BV 100% QB hold, 1 BV 0% QB (re-equilibration). Protein containing fractions were pooled together to dialyze against PBS pH 7.2, then sterile filtered, and stored in aliquots at -80°C after quantification.

### 2.1.6 Endotoxin assay

To test bacteria-sourced endotoxin contamination in the purified CSF1 protein solution, an endotoxin assay was performed using murine macrophage RAW264-ELAM cell line, which upon stimulation, like LPS, induces expression of detectable reporter gene eGFP (Himes et al., 2001).

RAW264-ELAM cells were cultured in RPMI-1640 medium supplemented with heat inactivated 10% fetal bovine serum (FBS), penicillin (10,000 units/ml) streptomycin (10,000 µg/ml) antibiotic, and 2 mM glutamax (hereby referred to as complete RPMI media) in 10 cm<sup>2</sup> sterilin bacteriological plates at 37°C incubator with 5% CO<sub>2</sub>, until confluent (2 to 3 days). Adherent RAW ELAM cells were harvested by scraping the plate with a syringe, an 18 g blunt needle, and media containing 0.03 M EDTA.

For the endotoxin assay, harvested RAW ELAM cells were seeded in a 96-well U bottom plate, with each well containing 5x10<sup>5</sup> cells, in a volume of 190 µl and 10 µl of purified recombinant CSF1 protein, equating 200 µl per well. CSF1 protein was tested for endotoxin contamination at a dilution range of 700, 500, 350, 35, 3.5, 0.35 and 0.035 ng/ml. As a negative control, endotoxin free chicken CSF1 protein (0.239 mg/ml stock, supplied by Zoetis) and as a positive control LPS from *Salmonella Minnesota* strain Re 595 was used. The 96-well plate was then incubated for 4 hrs in a 37°C incubator at 5% CO<sub>2</sub>. Cells were analyzed by flow cytometry. Briefly, the plate was centrifuged for 3 mins at 300 g, gently inverted and the cell pellet was

washed two times by repeat centrifugation, each time re-suspending in 200  $\mu$ l FACS buffer (PBS with 0.5% bovine serum albumin and 0.05% sodium azide). Cells were transferred to FACS tubes in 200  $\mu$ l FACS buffer, with the tubes kept on ice for the entire duration. Stain that detects dead cells, sytox blue 1.0  $\mu$ M was added just prior to analysis, to exclude dead cells. eGFP expression was measured as fluorescence intensity using BD LSR Fortessa (BD Biosciences, UK). Doublets were separated out based on SSC-A/H or FSC-A/H profiles and data was analyzed using FlowJo software (FlowJo, Ashlan, USA).

### 2.1.6.1 Endotoxin removal from recombinant protein

Endotoxin contamination from the batch produced chicken CSF1 protein was removed using Pierce High-Capacity Endotoxin removal spin columns 0.50 ml (Thermo Fisher Scientific, cat. no. 88274). CSF1 protein after final dialysis was in PBS buffer with pH 7.2. To optimize endotoxin binding to the poly-L-lysine resins in the column, CSF1 was dialyzed against the column equilibration buffer (20 mM sodium phosphate buffer and 100 mM NaCl, pH 6.5). Subsequent steps were followed as per manufacturer's instructions for the batch method with spin columns. The entire final-pooled CSF1 protein solution was tested for endotoxin contamination by RAW ELAM endotoxin assay.

### 2.1.7 Quantification of purified protein

Purified CSF1 protein was quantified using a Pierce bicinchoninic acid BCA protein assay kit (Thermo Fischer Scientific, cat no. 23227) following manufacturer's instructions, and by Nanodrop, by calculating UV absorbance at 280 nm. For standard curve, a series of dilutions of known concentration of bovine serum albumin (BSA) 0-2000  $\mu$ g/ml, was prepared and assayed alongside unknown CSF1 protein solution. For accurate measurement of CSF1 protein concentration, average of values obtained from BCA assay and nanodrop reading was considered. Concentration from nanodrop absorbance reading at 280 nm was calculated using the following formula.

$$\text{Concentration (Moles/L)} = (\text{Abs } 280 \text{ nm} \div \epsilon) \times M \times \text{dilution factor}$$

Where,  $\epsilon$  is the extinction co-efficient of protein, and M is the molecular weight.

## 2.2 Cell culture

### 2.2.1 Chicken bone marrow harvest and primary macrophage culture

Chicken bone marrow derived macrophage culture from bone marrow progenitor cells was produced as described previously (Garceau et al., 2010; Garceau et al., 2015; Garcia-Morales et al., 2015). Briefly,  $2 \times 10^7$  cells harvested from the leg bones of three- or five-week-old birds were plated per 10 cm<sup>2</sup> sterilin bacteriological plate in complete RPMI media supplemented with 350 ng/ml or 200 ng/ml of recombinant chicken CSF1. The plate was incubated in a 37°C incubator with 5% CO<sub>2</sub> levels up until 7 days, when they become confluent and a population of macrophages is visible. Chicken BMDMs were then harvested for *in vitro* cell viability assay (see below). Medium was replaced with fresh complete RPMI media with CSF1 on day 4.

### 2.2.2 BaF3/ChCSF1R cells

BaF3 cells stably transfected with chicken CSF1R (Garceau et al., 2010) were maintained in complete RPMI media supplemented with IL3 (5%) from X63 supernatant (Karasuyama and Melchers 1988, Tagoh et al., 2002 ). For the inhibitor experiments where BaF3/ChCSF1R cells were selected in CSF1, endotoxin free recombinant purified chicken CSF1 100 ng/ml was added instead of IL3. Cells were cultured in 75 cm tissue-culture treated Nunclon Delta flasks in 20 ml media and allowed to proliferate in a 37°C incubator with 5% CO<sub>2</sub>. The two-day suspension cell line yield from a single 75 cm flask was approximately  $1.5 \times 10^7$  cells.

### 2.2.3 Cryo-preservation

Harvested and sub-cultured cell lines of RAW ELAM, Ba/F3 cells transfected with chicken CSF1R (selected in purified chicken CSF1), and chicken BMDMs from layer lines were cryo-preserved in vials for long term storage of stocks. Cells were harvested, and then slowly resuspended drop by drop in freezing medium (FBS containing 10% dimethyl sulfoxide (DMSO)). Cells were frozen at approximately  $1-2 \times 10^7$  cells/ml/vial. For cryo-preservation of the anti-chicken CSF1 mAb and isotype control IgG1 mAb hybridoma cell lines, cells were frozen at a density of approximately  $2.5 \times 10^5$ /ml. Vials were gradually frozen by storing for two days at -80°C, and then transferred to a -155°C freezer for longer term storage. When plating for the experiments, each vial was thawed for approximately two minutes in a water bath at temperature 37°C and the cells were re-suspended gently by dropwise addition of complete RPMI culture medium.

### 2.3 Tyrosine kinase inhibitors preparation

Candidate mammalian CSF1R tyrosine kinase inhibitors were kindly gifted from our collaborators at Norway (Prof. Eirik Sundby, Norwegian University of Science and Technology) details of which are summarised in the table 2-1. All inhibitors were suspended in sterile DMSO to make a stock concentration of 10 mM and stored at -20°C until required. The final concentrations of DMSO in the well were <0.5%. For the cell viability assay, appropriate inhibitors concentrations were prepared using the cell culture medium. Viability of BaF3/ChCSF1R cells and chicken BMDMs was then measured by MTT assay (see below). Percentage viability of cells was calculated as (Absorbance at 570 nm/Absorbance at 570 nm of untreated cells) \*100

*Table 2-1: Potential CSF1R kinase inhibitors to test cell viability*

Inhibitor ID	Compound ID	Molecular Weight	Amount (mg)
1	TIA070	345.41	9.9
2	TIA086	345.41	9.2
3	TIA02-052	345.41	9.8
4	TIA02-054	372.47	9.4
5	TIA02-056	358.45	7.7
6	TIA02-076	345.41	9.3
7	KUL01-123	344.42	9.6
8	KUL02-016	362.41	9.7
9	KUL02-028	432.55	9.3
10	KUL02-056	432.55	9.7

### 2.4 Cell viability test or MTT assay

The activity of purified CSF1 and candidate CSF1R inhibitors on CSF1 induced chicken CSF1R signaling was tested in two assays. Both involve the indirect measurement of live cell numbers based upon cell metabolism of MTT. The NAD(P)H-dependent cellular oxidoreductase enzymes reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan which can be measured. For this assay, 96-well plate was seeded with  $2 \times 10^4$  BaF3/ChCSF1R cells (for purified chicken CSF1 concentration optimization),  $4 \times 10^4$  BaF3/ChCSF1R cells (for screening candidate CSF1R kinase inhibitors) or 7-day cultured chicken BMDMs (for inhibitors and anti-chicken CSF1 mAb activity validation) in a total volume of 50  $\mu$ l media. To assay CSF1-induced proliferation, 50  $\mu$ l of endotoxin free purified recombinant chicken CSF1 protein in two-fold serial dilution from 1  $\mu$ M was added to BaF3/ChCSF1R cells. To test the metabolic activity of cells two or three-fold serially diluted CSF1R inhibitors and anti-chicken CSF1 mAb (25  $\mu$ l each) was added to BaF3 cells and chicken BMDMs. Additionally, 25  $\mu$ l of appropriate growth factors, IL3 (5% X63 supernatant) or chicken CSF1 solubilized in cell culture media were added. All dilutions were plated in triplicates. Plate was then incubated for 72 hrs in 37°C incubator with 5% CO<sub>2</sub>, following which 10  $\mu$ l of MTT solution from 5 mg/ml stock was added to BaF3/ChCSF1R cells (final concentration 0.5 mg/ml). For the adherent chicken BMDM cell plate, 50  $\mu$ l of 1 mg/ml MTT solution was added. Plates were incubated for 3 hrs in a 37°C incubator with 5% CO<sub>2</sub>. After 3 hrs, any visible insoluble formazan particles were solubilized by adding 100  $\mu$ l per well of a solubilization buffer (50% isopropanol, 10% SDS and 0.01 M HCl) following which BaF3/ChCSF1R cell plates were incubated overnight at 37°C incubator. Chicken BMDM plates were incubated at 37°C incubator for 10 mins. Plate absorbance was read at 570 nm in a spectrophotometer. The test wells were blanked with blank media only wells and measurements were analyzed in excel 2013.

### 2.5 EC<sub>50</sub> and IC<sub>50</sub> value calculation

Absorbance data from the MTT assay was analyzed by drawing dose response curves using GraphPad Prism 7 software (San Diego, CA, USA). Log values of inhibitor doses or antibody serial dilutions were plotted against normalized viability of cells using nonlinear regression curve fit. The EC<sub>50</sub> value of bacterially purified recombinant chicken CSF1 and IC<sub>50</sub> concentration values of inhibitors and anti-chicken CSF1 mAb at half metabolic activity of cells were then determined from the nonlinear regression curve.

## 2.6 Batch purification of anti-chicken CSF1 and control antibodies

### 2.6.1 Hybridoma culture

Chicken CSF1-Fc fusion protein was used as an immunogen in mice and hybridoma cells that recognize chicken CSF1 were characterized and cryo-preserved (Wu *et al*, manuscript in preparation). Cryo-preserved mouse anti-chicken CSF1 mAb (ROS-AV183) and isotype control IgG1 mAb (mouse anti-ovine CD335 (GR13.1)) hybridoma cell vials were provided by colleague Dr. Zhiguang Wu. Anti-CSF1 mAb and isotype control mAb were then purified from large preparations of cell culture supernatant from these hybridoma cells. Briefly, vials were defrosted for 2 mins in a 37°C water bath. Growth media (Dulbecco's modified eagle's medium (DMEM), FBS (10%), penicillin (10,000 units/ml) streptomycin (10,000 µg/ml) antibiotics, sodium pyruvate 1x, minimum essential medium, non-essential amino acids (MEM NEAA) solution 1x, glutamax 1x) was added gently drop by drop to the cell suspension and centrifuged for 5 mins at 400 x g at RT. The pellet was re-suspended in growth media by pipetting up and down. Hybridoma cells were cultured in T25 flask at 37°C in an incubator with 5% CO<sub>2</sub> overnight. Hybridoma cells were monitored regularly for cell density to reach 2.5 x 10<sup>5</sup> cells/ml, following which they were further expanded in T75 flasks for optimum growth and passaged in growth media containing 10% Ig depleted serum. Subsequently cells were passaged in media containing reduced 5% Ig depleted serum until they became confluent (normally 2-3 days) at which point hybridoma cells were harvest. Supernatant from mass produced hybridoma culture flasks was filtered using the filter unit (500 ml) from Nalgene (Thermo Fisher Scientific) and stored at 4°C until further purification by Protein G column chromatography.

### 2.6.2 HiTrap Protein G column purification

Monoclonal antibodies from large preparations of anti-CSF1 mAb and isotype control mAb hybridoma culture supernatants were purified with Protein G column (1 ml) by affinity chromatography. (<https://www.gelifesciences.com/en/us/shop/chromatography/prepacked-columns/affinity-specific-groups/hitrap-protein-g-hp-antibody-purification-columns-p-03524>). Setup includes a peristaltic pump, syringe tubing, a column holder stand and filters (0.45 µm, 0.2 µm). The column was washed with binding buffer (PBS, pH 7.0-7.2) for 5-7 mins at a flow rate of 1 ml/min. The flow rate was kept constant throughout the purification procedure. Precaution was taken to avoid air bubbles inside the tubing. Sterile filtered anti-



CSF1 mAb and isotype control mAb hybridoma cells supernatant was loaded on the Protein G columns. After complete binding with the supernatant, the column was washed with binding buffer for 15 to 20 mins. At pH 7.0, there is a strong binding affinity between Protein G and IgG Abs, therefore elution was achieved by changing the medium to lower pH elution buffer (0.1 M glycine-HCL, pH 2.7). Approximately 1 ml of eluate fractions, were collected into neutral buffer (30 µl of 1 M tris-HCL, pH 9.0), to balance low pH in the elution buffer and preserve antibody activity. After washing in PBS, the Protein G column was stored in 20% ethanol at 4°C until required for further batches of supernatant mAbs purification. Protein concentration of fractions eluted from the chromatography column was determined using Nanodrop. Fractions with readings above 0.1 mg/ml and a sharp normal curve were selected for further purification. For desalting, concentrating antibodies and to remove low molecular weight contaminants, Slide-A-lyzer 20K, 0.5-3 ml capacity dialysis cassette from Thermo Fisher scientific (cat. no. 66003) was used as per manufacturer's instructions. Anti-CSF1 mAb and isotype control mAb was dialysed against PBS buffer for 24 hrs, changing the buffer three times post every 4 to 5 hrs. They were filtered using a 0.22 µm Millex-GV filter unit (cat. no. SLGV0135L) and stored as 1 ml aliquots at 4°C.

### 2.6.3 Antibodies stock preparation and quantification

Concentrations of purified anti-CSF1 mAb and isotype control mAb from several batches were measured with Nanodrop using the following formula,

$$\text{Concentration (mg/ml)} = \text{Average absorbance } 280 \text{ nm} \div (\epsilon L)$$

where,  $\epsilon$  is the extinction co-efficient of protein, and L is the path length. Most antibodies have a 1% extinction coefficient of 13-15. A 1 mg/ml solution of IgG is expected to give an absorbance of about 1.36 in a 1 cm light path cuvette or with the nanodrop equipment. Thus, antibodies concentration was determined as

$$\text{Concentration (mg/ml)} = \text{Average absorbance at } 280 \text{ nm}/1.36$$

Antibodies were concentrated further to make a stock concentration of 5 mg/ml to inject 500µg/100µl dose in transgenic hatchling MacGreen birds for *in vivo* studies. This was performed using Millipore's Amicon ultra-0.5 centrifugal 10K filter devices following manufacturer's instructions. From an initial sample volume of 500 µl utilized, concentrate volume of 15-20 µl was typically obtained with high protein recovery.

### 2.6.4 SDS PAGE analysis

Different batch aliquots of purified anti-CSF1 mAb and isotype control mAb were characterised by SDS PAGE to assess their purity and quality, before pooling them all together to make a common stock of 5 mg/ml. Antibodies were diluted in PBS to obtain a concentration of 100 µg/ml, of which 10 µg/ml/well batch samples were loaded on SDS PAGE gels. To visualise antibody integrity under reducing and non-reducing conditions, samples were prepared in 2x Laemmli buffer, with or without 2-mercaptoethanol respectively. Electrophoresis and CBB staining of SDS gels were performed as described in section 2.1.4.

## 2.7 Methods for *In vivo* anti-chicken CSF1 mAb injection experiment

### 2.7.1 Experimental design

Transgenic chickens expressing *CSF1R*-eGFP or *CSF1R*-mAPPLE reporter birds (Balic et al., 2014) were bred and obtained from the National Avian Research Facility (NARF) at The Roslin Institute, University of Edinburgh. All birds were maintained in accordance with the UK Home Office Code of Practice regulations for Housing and Care of Animals Bred, supplied or used for Scientific Purposes. The chicken anti-CSF1 mAb injections experiment was approved by Roslin Institute Animal Welfare and Ethical Review Board (AWERB). The study was carried out under Home Office project licence PPL 60/4420 19b/1 (Prof. Mark Stevens), as per the regulations of the Animal (Scientific Procedures) Act 1986. All birds were humanely culled by dislocation of the neck and death confirmed by decapitation (schedule 1 method of the Animals (Scientific Procedures) Act 1986).

Seventeen freshly hatched *CSF1R*-eGFP chicks were housed without vaccination and identified with wing tags. Their weights were measured and as per matching body weights divided into two groups. On day 1 of age one group of hatchlings (treatment group-9) received 500 µg per bird (c. 10 mg/kg = 10 µg/g) of purified anti-CSF1 mAb (ROS-AV183) in a total volume of 100 µl, injected subcutaneously at 2 two sites (50 µl each) in the breast muscle region, while the other group of birds (control group-8), received 100 µl of isotype control mAb in two 50 µl doses at the same sites. 1 ml Hamilton syringes and 75 g fine needles were used for this purpose. Antibody injections were repeated at 2, 3 and 4 days of age, such that a total of four daily equivalent doses are given on consecutive days. On day 7 all birds from both the groups were killed by schedule 1 method for collection of blood and tissue samples. On day 1 a separate group of 8 birds were culled to define baseline parameters. Body weights for

the treatment and control groups were measured throughout all 7 days, prior to injections or sacrifice to monitor growth rate. All birds were closely monitored for their health, behaviour and overall viability post every 2 hrs of injections.

### 2.7.2 Blood collection

Post decapitation of hatchlings, blood was immediately collected in heparinized tubes immediately to prevent blood clotting. It was utilised for flow cytometry analysis and for obtaining sera for enzyme-linked immunosorbent assay (ELISA).

### 2.7.3 Tissue collection

Lymphoid tissues (spleen, caecal tonsils, bursa, liver, lung and bones (tibias and femurs)) were dissected and collected in ice cold PBS for whole mount imaging, immuno-cyto-fluorescence or immuno-histo-fluorescence. A small section of above lymphoid tissues, gut, brain, feathered skin and breast muscle were put in RNA later (Qiagen) and stored at -80°C.

### 2.7.4 Circulating chicken CSF1 quantification by ELISA

Serum was separated from the heparinised blood samples via centrifugation at 10,000 rpm for 20 mins at 4°C and stored in -20°C until ELISA assay. To measure CSF1 levels in serum samples, a sandwich capture ELISA was developed using optimised concentration of anti-chicken CSF1 mAb, coating ROS-AV181 and detecting ROS-AV183, as described previously (Balu et al., 2011; Wu et al., 2016). An ELISA plate was coated with ROS-AV181 diluted to 2.0 µg/ml (50 µl/well) in a coating buffer (0.5 M carbonate buffer, pH 9.6) and incubated at 4°C overnight. The plate was washed three times in washing PBST buffer and free sites were blocked with 100 µl/well of blocking buffer (0.5% casein in PBS) by incubating at RT for 1 hr. After washing with wash buffer three times 50 µl of treatment and control group (serum) or purified recombinant chicken CSF1 (stock 0.239 mg/ml) as standard was added. Plate was washed three times in wash buffer and 50 µl/well biotinylated capturing antibody ROS-AV183 in PBS to 0.25 µg/ml was added. It was then kept for incubation for 1 hr on a rocker at RT and then washed three times with wash buffer. Streptavidin-HRP (Thermo Fischer Scientific, cat no. 21130) was diluted to 1/10,000 and 50 µl/well was added. The plate was kept for 1 hr on the rocker at RT, washed three times in wash buffer and 50 µl/well of substrate buffer TMB (Thermo Fischer Scientific, cat. no. 34029) was added at RT for 2-10 min. Reaction stopped with 50 µl/well 2 N H<sub>2</sub>SO<sub>4</sub> and plate read at 450 nm on a spectrophotometer. Analysis was performed using MiniTab.

### 2.7.5 Whole mount Imaging

*CSFIR*-eGFP transgene expression in the lymphoid tissues (spleen, bursa and caecal tonsil) was visualised by whole mount imaging by placing the tissues in PBS in a petri dish. Images were captured with Zeiss Axio Cam HR microscope under UV light using a green fluorescent filter channel for the appropriate constant amount of time. Surface area of the lymphoid tissues and mean fluorescent intensities of *CSFIR* transgene were measured using Zen blue 2012 edition. Average values with standard deviation graphs were plotted using GraphPad Prism 7 software.

### 2.7.6 Isolation of peripheral blood mononuclear cells (PBMCs) from blood

Blood from transgenic *CSFIR*-eGFP hatchling birds was collected into a BD Vacutainer collection tubes with spray-coated K<sub>2</sub>EDTA. Blood from 8-week old non-transgenic hyline chicken was also collected as a negative control. Whole blood was diluted with PBS (1:2 ratio) and gently overlaid onto density gradient medium lymphoprep (1:1 ratio) with a density 1.077 g/mL (STEM cell Technologies, cat. no. 1114547) in a 50 ml falcon tube. PBMCs were then isolated by centrifugation at 400 g for 20 mins without break. Cells accumulated in the buffy coat at the interface were carefully collected and washed two times in fluorescence-activated cell sorting or FACS buffer (PBS, 0.5% BSA, 0.05% sodium azide) by centrifuging at 400 g for 5 mins at 4°C for immuno-staining with FACS. Cell count was performed with trypan blue.

### 2.7.7 Isolation of spleen tissue single cell suspension

Spleen tissues from all 17 birds were dissected and collected into cold PBS in petri dishes. After whole mount imaging they were cut in half for flow cytometric analysis and immuno-fluorescence imaging. To prepare single cell suspension for FACS analysis, half cut spleen sections were meshed through a 100 µm cell strainer (BD Falcon) and diluted in PBS (1:1 ratio). Cells were centrifuged at 400 g for 5 mins at 4°C and pellets washed two times in FACS buffer (as for blood PBMCs for immuno-staining with FACS). Cell count was performed with trypan blue.

### 2.7.8 Immunocyto-fluorescence and flow cytometric analysis

Blood PBMCs and spleen single cell suspension were harvested as described above in sections 2.7.6 and 2.7.7. Cells were prepared at a density of 0.5-1.0 x 10<sup>6</sup> per 50 µl volume and distributed in wells of 96-U-bottom plate. Cells were subsequently blocked in FACS buffer by

making volume made up to 200  $\mu$ l/well with FACS buffer. The plate was centrifuged at 300 g for 3 mins at 4°C and supernatant flicked off in the sink. The pellets were then re-suspended on the plate shaker, following which cells were incubated with appropriate fluorochrome labelled primary Ab, isotype control Ab, viability control sytox blue stain (1.0  $\mu$ M) or FACS buffer alone for unstained controls (50  $\mu$ l/well) for 30 mins on ice at 4°C in dark conditions, since transgenic birds and fluorochromes were used. Dilution concentrations were determined with separate titration assays prior to animal experiment. The plate was centrifuged at 300 g for 3 mins at 4°C and supernatant flicked off in the sink. The pellets were then re-suspended on the plate shaker and cells washed two times with 200  $\mu$ l FACS buffer, to remove any unbound primary antibodies. The pellets were re-suspended in 150  $\mu$ l/well of FACS buffer and cells transferred to FACS tubes for profiling on BD LSR Fortessa (BD Biosciences, UK). The tubes were kept on ice until analysis. Sytox blue stain was added just prior to analysis to exclude dead cells. Doublets were separated out based on SSC-A/H or FSC-A/H profiles and data analyzed using FlowJo software (FlowJo, Ashlan, USA).

### 2.7.9 Immunohisto-fluorescence and confocal imaging

After whole mount imaging, lymphoid tissues (spleen, caecal tonsils, bursa, liver) from anti-CSF1 mAb treated *CSF1R*-eGFP birds were fixed in 4% paraformaldehyde in PBS 4°C overnight, washed in PBS and placed in 30% sucrose solution in PBS at 4°C. These were then cryo-embedded in Tissue-TEK OCT compound (Sakura Finetechnical, Tokyo, Japan) at appropriate orientations in a weighing boat and stored in -80°C until sectioning. OCT blocks were sectioned at 10  $\mu$ m onto Superfrost Plus slides (Menzel-Gläser, Germany) using Leica CM1900 cryostat and left to dry for 1 hr at RT. Sections were bordered with a dako pen and blocked for 1 hr in blocking solution (10% skimmed milk powder, 10% normal horse serum, 0.1% triton X-100 in PBS (MST-PBS)). Bursa and caecal tonsil sections were stained with primary Ab anti-chicken BU-1. Spleen and liver sections were stained with primary Ab anti-chicken Tim4. All antibodies were diluted in MST-PBS and sections kept for overnight incubation at 4°C. They were washed in PBS on a rocker three times (10 mins each) and re-incubated with secondary antibodies donkey anti-mouse AF647, rabbit anti-GFP AF488, along with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei for 1 hr at RT in dark. Sections were washed in PBS on the rocker three times (10 mins each) and mounted in Prolong gold anti-fade mounting medium (Invitrogen, cat. no. P36930) for downstream analysis. Slides were kept for drying 24 hrs at RT in dark conditions before imaging. To visualise the distribution of

macrophages, sections were imaged using LSM 710 inverted microscope and images analysed by Zen Black software.

### **2.7.9.1 Localization of chicken CSF1 in immune tissues**

Freshly dissected lymphoid organs (caecal tonsils, liver, lung) from a 5-week-old hyline chicken were washed in PBS and immediately cryo-embedded in OCT compound in weighing boat. Tissue blocks were sectioned at 10  $\mu\text{m}$  as described above and slides selected for Immuno-staining after visualising on bright field microscope. Sections were fixed in ice cold methanol for 10 mins on ice and washed three times in PBS (5 mins each) on a rocker. These were blocked and stained with primary antibodies, anti-chicken CSF1 AF647 mAb or isotype control AF647 mAb overnight at 4°C. After washing, sections were stained with DAPI. Slides were then washed, mounted and images captured as described in section 2.7.9.

### **2.7.10 Image processing**

Immunohisto-fluorescence liver section images at 40X magnification were processed using ImageJ. Briefly, average cell count and mean fluorescence intensity (MFI) of TIM4<sup>+</sup> cells were calculated from images taken from five different regions of liver per bird and graphs plotted as mean  $\pm$  SEM in GraphPad Prism software.

### **2.7.11 Analysis of bone architecture**

Anti-CSF1 mAb treated hatchling femur bones were analysed by micro-CT scan to evaluate bone density. Hatchling bones are fragile therefore they were dissected with caution to avoid breakage. Femur bones were fixed in 4% paraformaldehyde overnight and stored in 70% ethanol at 4°C for micro-CT scan imaging and for subsequent sectioning. Bone marrow cells from tibias were flushed, washed in PBS and utilised for: CSF1R transgene expression by FACS and cryo-preservation.

Post-paraformaldehyde fixation, femurs were embedded into 0.5% agarose gel in a proper orientation and sent to the micro-CT facility at Chancellors building, Royal Infirmary campus of University of Edinburgh. Bones were scanned with a Skyscan 1172 scanner (Kontich, Belgium) at 60 kV and 150 mA at a resolution of 5  $\mu\text{m}$ . Images were reconstructed using the SkyScan NRecon program. 3D images were analyzed using SkyScan CTAn software. A volume of 100 slices was measured from the distal condyle surface and average readings from four different regions calculating parameters as bone volume, trabecular thickness, trabecular number and trabecular separation were plotted using GraphPad Prism 7 software.

Post-micro-CT scan, femurs were sent to the Clinical Pathology Laboratory at R(D)SVS for sectioning to stain for osteoclasts. Bones were decalcified in 14% EDTA, pH 7.0, for 3 days at RT, and embedded in paraffin wax. Sections were cut and dried overnight at 37°C followed by drying at 60°C for 25 min. Femur slides were dewaxed in xylene, rehydrated in ethanol and washed for immuno-histochemistry.

### 2.7.12 Statistical analysis

Data of all graphs were plotted using GraphPad Prism 7 (San Diego, CA, USA) or MiniTab software. Data was analyzed using Mann-Whitney nonparametric test. Results are presented as the mean +/- standard error of the mean (SEM). Significance was indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

## 2.8 Guide RNA and primer design

The chicken CSF1R gene sequence was downloaded from <http://ensembl.org/>. Guide RNAs (gRNAs) were designed targeting four different regions of interest for receptor CSF1R. DNA sequences of the regions of interest were initially aligned to the chicken genome using the BLAT tool (<https://genome.ucsc.edu>) to confirm 100% identity to the chicken genome. These were then used as input sequences to design gRNAs using the web tools CHOPCHOP (<http://chopchop.cbu.uib.no/>) and gRNA design service from MIT (<http://crispr.mit.edu/>). Suitable gRNAs were ranked computationally and the guides closer to the cleavage sites were selected as forward guide oligo. A reverse guide oligo was obtained using seqbuilder (Lasergene 13, DNASTAR) by reverse complement. A total of eight gRNAs were designed (two for each target region) and cloned separately into vector encoding Cas9 nuclease (see below).

Primer sequences for amplification of appropriate gRNA target sites were designed using primer3 software (<http://primer3.ut.ee/>). Annealing temperatures were calculated using Tm calculator (<https://tmcaculator.neb.com>) from NEB. All gRNA oligonucleotides and primer sequences designed were synthesized by Sigma Aldrich. Primers were diluted to 10  $\mu$ M in distilled water from 100  $\mu$ M primer stock. Target CSF1R regions of interest gRNA sequences and respective primers are listed in table 2-2.

*Table 2-2: Oligo sequences designed targeting four different regions of interest of receptor CSF1R gene and the corresponding primers for amplification of these target sites*

Target region	Oligos	Sequence	Primer s	Sequence
Exon 1	Exon 1 FO1 (gRNA1)	caccgTGCTGACCACACCTGCTGTC	Ex 1 for 1	AGGCCTGAGGTAGTGCTTATC
	Exon 1 RO1	aaacGACAGCAGGTGTGGTCAGCAc	Ex 1 rev 1	CTTTCTGTCCCCTGTCTCT
	Exon 1 FO2 (gRNA2)	caccGACCTACCGTGCCAGACAGC	Ex 1 for 2	CCCACCCTGAATTCTCTCTGT
	Exon 1 RO2	aaacGCTGTCTGGCACGGTAGGTCg	Ex 1 rev 2	CACACTTTCTGTCCCACTGTC
Trans-membrane domain	TM FO3 (gRNA3)	caccgATGGGACTGAAGAGTTGGTT	TM for 3	TGGTGCTTGCTACTCC TACAA
	TM RO3	aaacAACCAACTCTTCAGTCCCATc	TM rev 3	AGGAGATTTTCACCCACCCAT
	TM FO4 (gRNA4)	caccgTGACCTGGTTGTACTTGTAG	TM for 4	TTGCTGCTCTTGGTCTGTCTA
	TM RO4	aaacCTACAAGTACAACCAGGTCAc	TM rev 4	TGCACTTACACATCCCAGAGA
			TM for 11	GAAGCCTGAACGAACAGTCA
			TM rev 11	ATTTTGGTTTTGGTGCATGGGC
ATP Binding site	ATP FO5 (gRNA5)	caccgAGAAGATTCAGTCTCAAAG	ATP for 5	AGTTCCCATAGATGCTCACC
	ATP RO5	aaacCTTTGAGGACTGAACTTTCTc	ATP rev 5	GTGCCCAAATGACTCATGAT
	ATP FO6 (gRNA6)	caccgAGATGTTGAAATGTAAGCCT	ATP for 6	ATCTGTCATCTTGTCTGGGCT
	ATP RO6	aaacAGGCTTACATTTCAACATCTc	ATP rev 6	TCCATAGGTACATGCTCCCAG
Catalytic domain	Cat FO7 (gRNA7)	caccgTGACTTAGTTCTATGGAAC	Cat for 7	GGTATGCTGGGGAGAGATGAA
	Cat RO7	aaacAGTTCCATAGAACTAAGTCAc	Cat rev 7	TGCACCACTCACATTTCCAAG



Target region	Oligos	Sequence	Primer s	Sequence
	Cat FO8 (gRNA8)	caccGTTCCCTCTTCCTTA CCAA	Cat for 8	GACTCAGTGACAGTG GCTTTG
	Cat RO8	aaacTTGGTAAGGAAGAG GGAAC	Cat rev 8	CATGAAGCACCTCCA GTTTCC
			Cat for 9	ACTCAGTGGACATGG AGAAGG
			Cat rev 9	TACCCCATCTGAAAA CCTCCC
			Cat for 10	GAGGACTGGGGAAGG TACAAG
			Cat rev 10	TCAGATCAGAGGCAG TTGGA

## 2.9 Preparation of gRNA CRISPR constructs

To generate gRNA expressing construct, vector pSpCas9(BB)-2A-Puro (PX459) V2.0 was used. pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988) (Ran et al., 2013). To generate designed gRNA expressing CRISPR plasmids, all oligonucleotides were cloned into vector PX459, using methods described in (Ran et al., 2013). Brief steps in the procedure are described from sections 2.9.1 to 2.9.3.

### 2.9.1 Cloning gRNA oligonucleotides and sequence validation

The first step involved, preparation of gRNA oligo inserts. Accordingly, the forward and reverse synthesized oligos were re-suspended to make stock concentration of 100  $\mu$ M. To phosphorylate and ligate gRNA oligos, the following reaction mixture was prepared.

Forward strand gRNA	1 $\mu$ l
Reverse strand gRNA	1 $\mu$ l
T4 ligation buffer, 10X (NEB)	1 $\mu$ l
T4 polynucleotide kinase (NEB)	1 $\mu$ l
ddH <sub>2</sub> O (Ambion)	6 $\mu$ l

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Total 10  $\mu$ l

Phosphorylation and annealing of gRNA oligos was carried out in a thermocycler under following conditions: 37°C for 30 min; 95°C for 5 min; 25°C for 15 mins, ramp down to 0.1°C/sec and held at 4°C.

All gRNA oligo inserts reaction mixture were diluted to 1:200 in ddH<sub>2</sub>O. Guide RNA oligo inserts were cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 vector using BbsI sites. Accordingly, the following ligation reaction mixture was prepared, along with a separate negative control (vector PX459 V2 only and no insert).

PX459, 100 ng	11.4 $\mu$ l (stock 880 ng/ml) + 88.6 $\mu$ l ddH <sub>2</sub> O
(1:200) diluted gRNA oligo insert	2 $\mu$ l
Tango buffer, 10X (Thermo Scientific)	2 $\mu$ l
DTT, 10 mM (Promega)	1 $\mu$ l
ATP, 10 mM (Promega)	1 $\mu$ l
FastDigest <i>BbsI</i> (Thermo Scientific)	1 $\mu$ l
T7 ligase (NEB)	0.5 $\mu$ l
ddH <sub>2</sub> O	1.1 $\mu$ l
Total	20 $\mu$ l

Reaction was ligated by incubating mixture for a total of 1 hr at a constant lid temperature of 100°C. Cycle number 1-6, 37°C for 5 min. To digest leftover residual linearized DNA the ligation reaction was treated with PlasmidSafe exonuclease. Accordingly following reaction mixture was prepared,

Ligated gRNA/ PX459 reaction	11 $\mu$ l
PlasmidSafe buffer, 10X (Epicentre)	1.5 $\mu$ l

ATP, 10 mM (Promega)	1.5 $\mu$ l
PlasmidSafe exonuclease (Epicentre)	1 $\mu$ l
Total	15 $\mu$ l

The reaction mixture was incubated at 37°C for 30 mins followed by 70°C for 30 mins. Generated CRISPR plasmids were stored at -20°C until sequence validation. To prepare these plasmids for sequencing, they were transformed into XL10-Gold ultracompetent cells from Agilent (cat. no. 200315), along with a negative control plasmid, following manufacturer's instructions. Transformed cells were plated onto a LB plate containing 100  $\mu$ g/ml of ampicillin and incubated overnight at 37°C for colony growth. Early the following day, single cell colonies were inspected for growth. From each plate, three single cell colonies were selected using individual sterile pipette tips and inoculated into 5 ml of LB growth media containing 100  $\mu$ g/ml of ampicillin. The CRISPR plasmids transformed culture was then grown by incubating the tubes at 37°C for 12 to 16 hrs. To verify correct insertion of gRNA oligos into PX459 V2 vector, CRISPR plasmids were isolated from cultures using a plasmid miniprep kit from Promega (cat. no. A1330), following manufacturer's protocol. Plasmid purity and concentration was determined using a Nanodrop ND-1000 spectrophotometer and plasmid samples were sent for sequencing.

### 2.9.2 Sanger sequencing and gRNAs orientation validation

Vector PX459 V2 contains U6 promoter for transcription of the U6 transcript (Ran et al., 2013) in conjunction to which designed gRNA oligos were cloned. To prepare CRISPR plasmids for sequencing, 0.2 ml tubes containing 500 ng/ $\mu$ l of plasmid DNA and 1  $\mu$ l of 3.2 pmole/ $\mu$ l of U6 forward primer (as well as U6 reverse primer in separate tube to sequence from both the ends) were prepared. All the 8-gRNA cloned CRISPR plasmids (three different single cell colonies purified per plasmid), targeting various regions of chicken receptor *CSF1R* were sent to the Edinburgh Genomics facility at The Roslin Institute, in replicates for Sanger sequencing.

To verify the orientation of cloned gRNA oligos in CRISPR plasmids, they were referenced against PX459 V2 vector sequence in MegAlign software. 20 nucleotide gRNA sequences perfectly aligned with cloned site of CRISPR plasmid constructs were processed further to prepare MaxiPrep stock of plasmids.

### 2.9.3 Sequence validated CRISPR constructs stock preparation

To generate the stock of 8 CRISPR plasmids, Qiagen's endotoxin free Maxi Prep plasmid kit was used as described in section 2.1.2 of this chapter. The pellet was resuspended in 200 µl of buffer TE overnight at 4°C and plasmids stored in aliquots of 50 µl, to be used during transfection experiment.

### 2.10 Handling of chickens

All commercial and transgenic chicken lines both, generated from this project and previously produced, were bred and maintained at the National Avian Research Facility (NARF) at The Roslin Institute in accordance with the UK Home Office Code of Practice regulations for Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. All chicken related experiments for the production of CSF1R knockout birds were approved by The Roslin Institute's AWERB committee. The study to generate the transgenic G0 founder line was carried out under Home Office project licence PPL 70/8940, protocol 1 (Prof. Helen Sang). The study to breed and maintain G0, G1 (heterozygous for *CSF1R*) and G2 (homozygous for *CSF1R*) transgenic lines was carried out under Home Office project licence PPL 70/8940, protocol 2 (Prof. Helen Sang).

### 2.11 Chicken primordial germ cells (PGCs) media and culture

A defined serum free culture condition medium for the derivation, expansion, self-renewal and clonal growth of germline competent chicken PGCs has been described before (Whyte et al., 2015). This study markedly facilitated the propagation of germline competent PGCs *in vitro* for extended periods of time. Chicken PGCs were cultured in defined avian knockout (KO) DMEM FAOT medium as per described in (Whyte et al., 2015). Composition of avian KO DMEM FAOT medium used to culture chicken PGCs is given in Chapter 8, in appendix A (section 8.1).

To sub-culture PGCs, cells were resuspended into PGCs media to avoid clumping and centrifuged for 4 mins at 1600 rpm. The cell pellet was re-suspended into fresh PGCs media and plated in 500 µl media in a 24 well plate followed by incubation at 37°C in a 5% CO<sub>2</sub> incubator. The media was changed every three days. When the cell numbers reached  $1 \times 10^5$ /ml (in around 3 to 4 days), they were split and maintained at a density no more than  $2-4 \times 10^5$  cells/ml per well. Genome edited PGCs non clonal and single cell clone PGCs were cryo-

preserved for long term storage. They were frozen in avian KO DMEM basal media containing 8% DMSO, 10% chicken serum (Biosera, cat. no. CH-515/500) and 20 mM calcium chloride using standard procedures. PGCs were cryo-preserved at a cell density of  $2 \times 10^5$  cell/vial in 0.5 ml volume and stored at  $-150^\circ\text{C}$ .

### 2.11.1 Lipofectamine transfection

CRISPR plasmids encoding for guides targeting the chicken *CSF1R* exon 1 (gRNA 1 and 2) and transmembrane domain (gRNA 3 and 4) region were delivered to PGCs by lipofectamine transfection. Accordingly, PGCs were transfected with CRISPR plasmids in combinations as gRNA 1, gRNA 2, gRNA 1+2 and gRNA 3+4 using lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, cat. no. 11668030). When PGCs attain 80% confluency in a 24 well plate, around  $2 \times 10^5$  cells per well, cells were utilized for transfections. 2  $\mu\text{l}$  of lipofectamine was added to 148  $\mu\text{l}$  of Optimem I reduced serum medium (Gibco, cat. no. 31985-0620) and separately 2  $\mu\text{g}$  of CRISPR plasmid was added to Optimem I to make a final volume of 150  $\mu\text{l}$  in a screw top Eppendorf tube. Tubes were incubated at RT for 20 mins. Both the tubes were mixed gently and flicked 5 times to mix them together. Tube was further incubated for 25 mins to allow complexes to form. To this transfection mixture, PGCs suspension washed and resuspended in 50  $\mu\text{l}$  of Optimem I, was gently added and pipetted up and down followed by incubation for 6 hrs in  $37^\circ\text{C}$  incubator set at 5%  $\text{CO}_2$ . Tube at this stage was loosely capped for the entry of  $\text{O}_2$  and  $\text{CO}_2$  to transfected PGCs. Cells were centrifuged at 2200 rpm for 10 mins and pellet re-suspended in PGCs media to seed in 24 well plate. Control PGCs culture was also maintained in the same plate. PGCs media was changed every 3 days gently.

To select for puromycin bearing CRISPR plasmids with stable integration of gRNAs, 0.6  $\mu\text{g}/\text{ml}$  of antibiotic puromycin was added to PGCs at 24 hrs post transfection for 48 hrs, followed by media change. Clumps of living PGCs were expanded for two to three weeks following same procedure as described in section 2.11 and utilised for genomic DNA isolation to assess Cas9 cleavage, determine targeting efficiencies and/or single cell clonal expansion.

### 2.11.2 Genotype validation by PCR and sequencing

PGCs transfected with each of the individual CRISPR constructs were cultured in large amounts (3 to 4 wells of 24 well plate), were then harvested by centrifugation and the pellet was utilised for genomic DNA extraction. Genomic DNA was isolated using QiaAmp DNA micro kit (Qiagen, cat. no. 56304) and QiaAmp MinElute columns (Qiagen, cat. no. 1020909)

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following manufacturer's instructions. Genomic fragments containing guide RNA target sites in *CSF1R* (exon 1 and transmembrane domain) along with wild type PGCs DNA were PCR amplified using Q5® Hot Start High-Fidelity DNA polymerase (NEB, cat. no. M0493L) and appropriate specific primers as listed in table 2-2. Genomic DNA was diluted to 100 ng/μl and PCR performed under following conditions:

Initial denaturation 98°C 30 seconds, 35 Cycles of 98°C for 10 seconds, 67°C for 30 seconds and 72°C for 30 seconds/kb; final extension 72°C 2 mins, held at 4°C.

All PCR amplified samples were run on 1% agarose gel using SYBR safe DNA gel stain (Thermo Fisher Scientific, cat. no. S33102), to check for the presence, absence (in case of inversions) or variation in sizes of DNA products (in case of deletion). Confirmation of CRISPR constructs Cas9 cleavage INDEL mutations at target sites in the transfected mixed cell population was carried out by Sanger sequencing. To sequence PCR amplified products, they were cloned into pGEM-T Easy vector (Promega, cat. no. A137A) for transfected mixed cell population, while the single cell clonal population was sequenced at Edinburgh Genomics post PCR amplification (see section below). To clone into pGEM-T Easy vector, products were purified using QIAquick PCR purification kit (Qiagen) following manufacturer's instructions and A tailed prior to ligation into vector. Accordingly following reaction mixtures were prepared and incubated at 70°C for 20 mins.

Amplified PCR product	5 μl
10x reaction buffer	1 μl
25 mM MgCl <sub>2</sub>	0.6 μl
0.1 mM dATP	2 μl
Taq polymerase (Thermo Scientific)	1 μl
ddH <sub>2</sub> O	0.4 μl
Total	10 μl

A-tailed PCR product	2 $\mu$ l
Rapid ligation buffer 2x	5 $\mu$ l
pGEMT vector 50 ng/ $\mu$ l (Promega)	1 $\mu$ l
T4 DNA ligase (3 Weiss units/ $\mu$ l)	1 $\mu$ l
ddH <sub>2</sub> O	1 $\mu$ l
Total	10 $\mu$ l

Ligation reaction controls (Promega supplied insert + vector, positive control and no insert vector only as a negative control) were also included. Reaction mixture was incubated overnight at 4°C and utilised next day for transformation into XL10 gold ultra-competent cells followed by purification of cloned plasmids as described in section 2.9.1. For blue/white screening of colonies, 20  $\mu$ l of X-gal (Promega, 50 mg/ml, cat. no. V394A) and 20  $\mu$ l of IPTG (Sigma Aldrich, 200 mM, cat no. 1284) was added to the ampicillin plates (100  $\mu$ g/ml) just before spreading the transformed culture. To confirm cloning of Cas9 cleaved target site inserts (which were amplified by PCR) into the pGEMT vector, single enzyme restriction digestion with EcoRI HF (NEB, cat no. R3101S) was performed in cut smart buffer and digested products were visualised by agarose gel electrophoresis. To validate the mutated insert plasmid sequence, cloned vector pGEMT was sequenced using T7 promoters (<https://www.promega.co.uk/products/pcr/pcr-cloning/pgem-t-easy-vector-systems/?catNum=A1360>) as per the steps described in section 2.9.2 at Edinburgh genomics. Sanger sequencing data was viewed and analysed using tools SeqBuilder and MegAlign Pro 14 in Lasergene 14 DNASTAR software.

### 2.11.3 Transfected PGCs T7 endonuclease I mismatch assay

To analyze mutation in PGCs targeted with CRISPR Cas9 plasmids encoding gRNA 1 and 2, a T7 endonuclease I (NEB, cat no. IM0302S) assay was performed according to manufacturer's instructions. T7 endonuclease I identify single base mismatches. Briefly, PCR amplified products from genomic DNA Cas9 targeted guide 1 and 2 were purified using QIAquick PCR purification kit (Qiagen). The product (200 ng/ $\mu$ l) was further denatured and annealed in a thermocycler to form heteroduplexes with and without mutations, which was further digested

with T7 endonuclease I in 10x NEBuffer 2. Accordingly, the reaction mixture was prepared as a purified PCR product (10 µl), ddH<sub>2</sub>O (8 µl) and 10x NEBuffer 2 (2 µl). The conditions for denaturing and annealing used are as follows: Initial denaturation 95°C for 5 mins, annealing 85°C for 0.1 ramp to 85°C at 2°C/sec and 25°C for 0.1 ramp to 25°C at 0.1°C/sec. Held at 4°C. The reaction mixture for T7 digestion consisted of denatured annealed PCR product (20 µl), T7 endonuclease I (1 µl), 10x NEBuffer 2 (1 µl) and ddH<sub>2</sub>O (8 µl) was incubated at 37°C for 15 mins. T7 digested and non-digested products were resolved on 1% agarose gel stained with Gelred (Biotium, cat no. 41002).

### 2.11.4 Estimating genome editing efficiency

To analyze on-target genome editing efficiency, the above generated DNA fragments were quantified using ImageJ software (<https://imagej.net>). Quantification was based on relative band intensities. Indel percentage was determined by the formula (Ran et al., 2013),

$$100x (1 - (1 - (b + c)/(a + b + c))^{1/2})$$

Wherein a, is the integrated intensity of the undigested PCR product and b and c are the integrated intensities of each cleavage product.

### 2.11.5 Single cell clonal expansion and validation

When post transfection PGCs reach 70% confluency, clumps of cells were separated by pipetting and single cell per well was manually seeded into 96-well plate by serial dilution and using a bright field microscope. A minimum of 10 single cell clones per gRNA transfected were cultured and expanded in 250 µl of PGC media (added gradually in small volumes to single cell as they double) per well for two weeks with the media replenishment every three days. When the cell density reached 30-50% single PGCs clonal population were transferred to 48-well plate for expansion. When the cell density reached 75% into 48-well plate, cells were transferred to 24-well plate for expansion (2 to 3 weeks). Single cell clones were further genotyped by genomic DNA isolation and mutation identification by Sanger sequencing.

## 2.12 Targeted PGCs injections into surrogate embryos

For PGCs injections into embryos, a PGCs suspension was collected, centrifuged at 1600 rpm for 4 mins and pellet resuspended in appropriate volume to obtain cell density of 8000 PGCs/µl



for injections. To trace PGCs migration 1 µl of fast green dye (Sigma Aldrich) was added to the cell suspension immediately prior to injection.

To inject genome modified donor female PGCs, *DDX4* germ cell deficient female host embryos were utilised (Taylor et al., 2017). Fertile eggs obtained from the breeding of Novagen brown *DDX4* negative hemizygous males and wild type females were incubated for 2.5 days. Donor PGCs were then injected into the dorsal aorta of stage 16HH recipient embryos (Hamburger and Hamilton, 1992) through a window in the shell method as described in (McGrew et al., 2004; Taylor et al., 2017). Window was sealed with chorioallantoic membrane (CAM) from inside the shell as well as parafilm with medium amount of heat. Sealed eggs were kept for incubation at 38°C until hatch (around 21 days). At this point, embryos that failed to survive (detected by candling) were discarded as per Home office license procedures. Hatched G0 progeny (founder birds' line) were maintained in appropriate conditions.

### **2.13 Genome edited PGCs germline transmission.**

Female G0 founder birds were maintained to reach sexual maturity (approximately six months). All the male G0 progeny were culled. G0 hens were bred to homozygous *CSFIR*-eGFP transgenic male (Balic et al., 2014) via artificial insemination to generate G1 *CSFIR* mutant heterozygotes. All the G1 off-springs were screened to identify *CSFIR* deleted progeny. *CSFIR* heterozygous G1 male and female birds were raised to sexual maturity and bred to obtain *CSFIR* homozygous G2 progeny. *CSFIR* deleted heterozygotes (G1) and homozygotes (G2) were analysed by whole mount imaging for embryo development (as described in section 2.7.5) and *CSFIR*-eGFP transgene expression *in ovo* at different time points. Adult heterozygotes were analysed by FACS.

### **2.14 Genotype and sex determination of chimeric chickens**

CAM samples at hatch or embryos *in ovo* and blood samples from older birds were obtained in 1.5 ml Eppendorf tubes from chimeric chickens. To determine genotype and sex of G0, G1 and G2 progeny, genomic DNA of all the birds was isolated from both CAM and blood samples. CAM samples were stored at -20°C and blood samples stored at RT until extraction. Genomic DNA from CAM samples was isolated using REDEExtract-N-Amp Tissue PCR Kit (Sigma Aldrich, cat no. XNAT) following manufacturer's instructions and diluted CAM lysate utilised for setting up PCR. Blood DNA was extracted using Puregene DNA purification kit (Qiagen) as per manufacturer's instructions.

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*CSF1R* targeted heterozygotes and homozygotes were identified by PCR amplification of genomic DNA as described in section 2.11.2. To screen germ cell deficient *DDX4* knockout sterile G0 progeny, PCR for the presence of GFP transgene was performed (Taylor et al., 2017). To determine sex of chicks in all three generations, PCR for the W-chromosome of both CAM and blood DNA for the confirmation was performed as described in (Clinton et al., 2001). Primers for these PCR were provided by Lorna Taylor. To identify *CSF1R*-eGFP transgene expressing reporter chickens (Balic et al., 2014) amongst the G2 birds, PCR using HIV primers was performed on CAM DNA samples. These primers were provided by Hazel Gilhooley.

*Table 2-3: List of primers used for genotyping and sexing of chickens*

Name	5' → 3'
GFP specific	Forward ACGTAAACGGCCACAAGTTC Reverse AAGTCGTGCTGCTTCATGTG
W	Forward CCCAAATATAACACGCTTCACT Reverse GAAATGAATTATTTTCTGGCGAC
GAPDH	Forward CAGATCAGTTTCTATCAGC Reverse TGTGACTTCAATGGTGACA
HIV	Forward GAGAGAGATGGGTGCGAGAG Reverse GCTGTGCGGTGGTCTTACTT

Reaction conditions followed were as follows:

**GFP:** Initial denaturation 95°C 5 mins, 30 Cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds/kb; final extension 72°C 5 mins, hold 4°C.

**W:** Initial denaturation 94°C 5 mins, 20 Cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds/kb; final extension 60°C 10 mins, hold 4°C.

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**GAPDH:** Initial denaturation 94°C 4 mins, 35 Cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds/kb; final extension 60°C 10 mins, hold 4°C.

## Chapter 3 CSF1R tyrosine kinase inhibitors targeting chicken macrophages

### 3.1 Introduction

The aim of this thesis is to gain knowledge of CSF1R-dependent macrophage biology in birds and to identify similarities and differences in CSF1R signalling compared to the mononuclear phagocyte system of mammals. Previous studies have shown that the basic function of CSF1R and its two ligands, CSF1 and IL34, as macrophage growth factors is conserved in birds (Garceau et al., 2010; Garceau et al., 2015). The overall objective of this project was to establish the tools to manipulate CSF1R signalling *in vivo* in order to understand the significance of CSF1R biology in the development of both innate and acquired immunity in chickens. Three alternative approaches were described in the introductory Chapter 1 (section 1.11). This Chapter will focus on the first approach, to identify candidate CSF1R kinase inhibitors that can affect the viability of chicken macrophages *in vitro*, before proceeding to manipulating their functions *in vivo* (Chapters 4 and 5).

Kinases are enzymes that catalyse phosphoryl transfer from donor molecule as ATP to a specific protein substrate, a process known as phosphorylation. Receptor tyrosine kinases (RTKs) are a large family of molecules essential for signal transduction pathways that leads to activation and mediation of cellular communications. They bind mainly to growth factors and hormones; and are key regulators of normal cellular processes such as cell cycle, metabolism, differentiation, survival and cell migration (Lemmon and Schlessinger 2010). Because of their active role in development and homeostasis, any kind of genetic mutation, abnormalities, or overexpression of the receptor/ligand leads to the onset or progression of various malignancies and cancer. As outlined in the main introduction, the differentiation, proliferation and survival of mononuclear phagocyte lineage cells is controlled by signals from the macrophage colony-stimulating factor receptor (CSF1R) initiated by binding of one of its two ligands, macrophage stimulating factor (CSF1) or IL-34 (Hume and MacDonald 2012; Jenkins and Hume 2014). Excessive activation via CSF1R mediated signalling has been implicated in inflammatory diseases, cancer, autoimmune and bone diseases (Chitu and Stanley 2006; El-Gamal et al., 2012; El-Gamal et al., 2018) and accordingly, CSF1R has been widely-recognised as a therapeutic target. Macrophages are also involved in immunosuppression and damage repair. They display various functional phenotypes depending upon polarization signals and tissue/organ specificity (Cannarile et al., 2017; Guerriero 2018). Tumour associated

macrophages (TAMs) are a major cellular component of tumours and are known to promote tumour progression, angiogenesis and resistance to various therapeutic agents (Priceman et al., 2010; DeNardo et al., 2011; Escamilla et al., 2015). Given the protective role of macrophages in innate and adaptive immune responses and contribution to tissue injury in inflammatory conditions in various disease states, there has been tremendous growth in development of drugs targeting macrophages in mammalian models via the CSF1R signalling pathway.

Therapeutic approaches to inhibition of CSF1R include monoclonal antibodies against the receptor or its ligands CSF1/IL34, thereby blocking their binding. Additionally, small molecule bioactive inhibitors or tyrosine kinase inhibitors (TKIs) that inhibit tyrosine kinase activity of the receptor, for example by occupying ATP binding site of the receptor. Many companies have developed or are developing small molecules that target the CSF1/CSF1R signalling axis (reviewed in (Hamilton et al., 2016; El-Gamal et al., 2018). Many of these candidate drugs have been tested in experimental disease animal models and clinically in human patients. Some are undergoing clinical trials to validate efficacy and safety. This Chapter will provide a brief overview of the various approaches used to target CSF1R signalling, with further emphasis on identifying potential candidate chicken CSF1R specific inhibitors.

### 3.1.1 Targeting CSF1R signalling with tyrosine kinase inhibitors

As described in introduction Chapter 1, CSF1R belongs to type III of the RTKs family. The kinase domain has substantial homology to other class III RTKs receptors: FLT3; KIT; and PDGFR  $\alpha$  and  $\beta$  (Qiu et al., 1988). Due to the structural similarity and high conservation of the kinase domain of class III RTKs, few inhibitors are truly specific for CSF1R (Murray et al., 2003; Dewar et al., 2005). Extended reviews of the specificity and efficacy of CSF1R kinase inhibitors have been published previously (El-Gamal et al., 2012; Hume and MacDonald 2012).

The most-studied inhibitor of CSF1R kinase, GW2580, is an orally available drug with relatively high specificity for CSF1R kinase. At 60 nM it completely inhibited human CSF1R kinase and was less potent to other structurally related human kinases such as KIT, FLT3, and PDGFR- $\beta$ . GW2580 inhibited proliferation of CSF1 induced human and rat monocytes at IC<sub>50</sub> values of 0.47  $\mu$ M and 0.2  $\mu$ M respectively (Conway et al., 2005; Conway et al., 2008). GW2580 has been widely-studied *in vitro* and *in vivo* in multiple preclinical animal disease models. Some key studies are summarised in table 3-1.

**Table 3-1: Pre-clinical animal studies with orally available CSF1R inhibitor GW2580**

Species	Disease models	Comments	References
Humans	Chronic lymphocytic leukaemia (CLL) patients.	Inhibits monocyte growth and bone degradation in osteoclasts.	(Conway et al., 2005) (Edwards et al., 2018)
Rats	Arthritis; Multiple sclerosis.	Inhibits monocyte growth and bone degradation in osteoclasts.	(Conway et al., 2005) (Conway et al., 2008) (Borjini et al., 2016)
Mice	Solid tumours; Prostate cancer; Pancreatic adenocarcinoma; Mammary adenocarcinoma; Prion disease; Ovarian cancer; Amyotrophic lateral sclerosis (ALS); Transgenic model of Alzheimer's-like pathology; Spinal cord injury.	Reduces infiltration and angiogenesis in various solid tumours;  Inhibits myeloid leukaemia cells and induced macrophage migration in peritoneal cavity;  Supresses tumour growth in combination with radiotherapy/ chemotherapy treatment;  Inhibits microglial proliferation.	(Conway et al., 2005) (Priceman et al., 2010) (Xu et al., 2013) (Weizman et al., 2013) (Gómez-Nicola et al., 2013) (Mitchem et al., 2013) (Moughon et al., 2015) (Martínez-Muriana et al., 2016) (Olmos-Alonso et al., 2016) (Gerber et al., 2018)

One important finding in relation to kinase inhibitors is that they do not prevent internalisation and degradation of the ligand. CSF1 is removed from the circulation by receptor-mediated endocytosis, mainly by macrophages of the liver and spleen. (Bartocci et al., 1987; Irvine et al., 2006). Irvine et al. (2006) described a novel inhibitor of CSF1R kinase (CYC10268). Whereas the inhibitor blocked CSF1R signalling and compromised the viability of CSF1-dependent mouse macrophages, it did not prevent CSF1-induced receptor internalisation. Antibodies that block CSF1R binding, or mutations in CSF1R, lead to increased circulating CSF1 (Dai et al., 2002; MacDonald et al., 2010; Pridans et al., 2018). In principle, blocking the receptor with anti-CSF1R could lead to a rebound monocytosis when the inhibitor is removed, however this is likely to be less of a problem with a kinase inhibitor.

Some key clinical and pre-clinical studies with several specific or non-specific CSF1R kinase inhibitors are summarised in Table 3.2

**Table 3-2: Clinical and pre-clinical studies with other CSF1R inhibitors targeting CSF1/CSF1R signalling activation**

Compound name	Target specificity	Comments	Disease models	References
PLX3397 or Pexidarnitib	CSF1R; KIT; FLT3; PDGFR.	Orally available; Crosses blood-brain barrier; Depletes microglia; Reduces macrophages growth and tumour development; Increases efficacy in combination with standard care treatments.	Mouse model of GIST; Prostate cancer patients; Mouse model of prostate cancer; Patients with recurrent glioblastoma; Patients with Dt-GCT; Mouse model of Alzheimer's disease; Mouse model of glioma.	(Kim et al., 2014) (Elmore et al., 2014) (Escamilla et al., 2015) (Butowski et al., 2016) (Tap et al., 2015) (Brahmi et al., 2016) (Spangenberg et al., 2016) (Yan et al., 2017) (Sosna et al., 2018)
BLZ945	CSF1R	A brain-penetrant; Depletes malignant cells in various tumors models.	Mouse model of mammary and cervical carcinogenesis; Mouse model of multiple sclerosis.	(Strachan et al., 2013) (Beckmann et al., 2018)
ARRY-382	CSF1R; KIT.		Chronic lymphocytic leukaemia CLL patients.	(Edwards et al., 2018)
PLX5622	CSF1R	Orally available; Brain penetrant; Dose dependent elimination of microglia.	Mouse model of Alzheimer's disease; Mouse model of multiple sclerosis; Mouse embryonic	(Dagher et al., 2015) (Spangenberg et al., 2016) (Acharya et al., 2016) (Nissen et al., 2018) (Rosin et al., 2018) (Seitz et al.,

			microglia study.	2018) (Huang et al., 2018)
JNJ-40346527	CSF1R; KIT.	Orally available	Patients with active rheumatoid arthritis;  Patients with Relapsed or Refractory Hodgkin Lymphoma.	(Genovese et al., 2015) (Von et al., 2015)
Ki20227	CSF1R; VEGFR; KIT; PDGFR.	Orally available;  Reduces osteoclasts differentiation and suppresses osteolysis or bone destruction;  Exhibit anti-inflammatory effect in mouse model of arthritis;  Reduces disease severity in animal model of multiple sclerosis.		(Ohno et al., 2006) (Ohno et al., 2008) (Uemura et al., 2008)
AZD7505	CSF1R	Reduces tumour mass.	Mouse model of pancreatic cancer.	(Scott et al., 2013) (Candido et al., 2018)
CYC10268	CSF1R	Regulates macrophages effector functions.		(Irvine et al., 2006)

### 3.1.2 Targeting CSF1R signalling with antibodies

CSF1R signalling in macrophages can be alternatively targeted with blocking monoclonal antibodies against the receptor CSF1R or the ligand CSF1. Accordingly, *in vitro* and *in vivo* studies have been performed using antibodies developed against the human or mice receptor CSF1R in various human or mice models. Some clinical or pre-clinical studies are summarised in Table 3.3. Importantly, having established chicken as a novel vertebrate model to study the development of mononuclear phagocyte system (Garceau et al., 2015), and the added



advantage of access to CSF1R fluorescent transgenic reporter lines (Balic et al., 2014), newly-generated mAb to chicken CSF1R (Garcia-Morales et al., 2013) and chicken CSF1 (Wu *et al*, manuscript in progress) provide useful biological tools to explore chicken macrophage biology

**Table 3-3: Clinical and pre-clinical studies with monoclonal antibodies targeting CSF1R signalling**

<b>Compound name</b>	<b>Target specificity</b>	<b>Comments</b>	<b>Disease models</b>	<b>References</b>
RG7155 or Emactuzumab  (humanized IgG1 anti-human CSF1R)	CSF1R	Blocks CSF1R receptor dimerization interface;  Blocks ligand binding in a competitive manner;  Apoptosis of CSF1 differentiated human macrophages.	Dt-GCT patients;  Patients with various solid malignancies.	(Ries et al., 2014) (Cassier et al., 2015) (Brahmi et al., 2016)
AMG820 (Human CSF1R antibody)	CSF1R	Blocks ligand binding and receptor activation.	Patients with advanced solid tumors.	(Papadopoulos et al., 2017)
AFS98 antibody  (Rat IgG2A anti-mouse CSF1R)	CSF1R	Binds CSF1R with greater affinity than M279;  Reduces infiltration, inflammation and tissue damage in renal injury models;  Reduces total murine monocytes and resident peritoneal macrophages;  Inhibits TAMs and osteoclasts in murine tumour model;  Depletes pancreatic islet macrophages.	Mouse model of renal damage;  Several mice tumour models;  Auto-immune diabetic mouse model.	(Jose et al., 2003) (Lim et al., 2009) (Lenzo et al., 2012) (Fend et al., 2013) (Carrero et al., 2017) (Hume and MacDonald 2012)
M279 antibody  (Rat IgG1 anti-mouse CSF1R)	CSF1R	Depletes murine tissue and tumour macrophages with exception of lung,		(MacDonald et al., 2010) (Hume and MacDonald 2012)

		brain, uterus and ovary.		
H27K15 antibody (Human CSF1R antibody)	CSF1R	Non-ligand competitive binding;  Does not block CSF1/CSF1R endocytosis;  Reduces osteoclast differentiation;  Prevents M2 polarised macrophages.		(Haegel et al., 2013) (Grellier et al., 2014)

At the beginning of this project, analysis of CSF1R evolution (Garceau et al., 2010; Gutowska 2015), comparison of homology between avian and mammalian forms of CSF1R highlighted the rapid evolution of the extracellular domain. Accordingly, there is no cross-reactivity between avian and mammalian ligands and CSF1R. Indeed, chicken CSF1 does not activate the receptor of zebrafish (Garceau 2014; Gutowska 2015). By contrast, the intra-cellular tyrosine kinase domain (aa 540 to 977) of the receptor is strongly conserved between birds and mammals. However, one key difference is that cysteine C665, conserved in all mammals, is changed to arginine (R665 in chicken) in all birds for which proteins sequences are available ((Garceau et al., 2010); DA Hume, personal communication). Further studies, comparing dose dependent cell viability activity of the inhibitor GW2580 on CSF1 induced bone marrow cells of mammals (mouse and pig) and chickens showed that GW2580 failed to block the CSF1 induced proliferation of chicken macrophages, but on the other hand completely inhibited mammalian macrophage growth (Garceau 2014). Molecular modelling studies between human/chicken CSF1R and inhibitor, highlighted that the non-conserved residue (cysteine 665 in humans and arginine 665 in birds) makes contact with the inhibitor and this sequence difference likely explains the lack of efficacy of GW2580 in chicken.

Previous studies in our laboratory group led to the isolation and characterization of anti-chicken CSF1R monoclonal antibody (Garcia-Morales et al., 2013). This provides a useful marker for chicken blood monocytes and tissue macrophages, however, unlike the anti-mouse and human CSF1R antibodies discussed above in the table 3.3, it did not block CSF1 binding. Apart from CSF1R specific kinase inhibitor GW2580, no other CSF1R-specific inhibitor has been tested

to validate the growth of chicken macrophages. To identify novel agents that could be used in future studies in chickens, the efficacy of a set of candidate mammalian CSF1R tyrosine kinase domain inhibitors developed as part of a collaborative project with a Norwegian drug development group was tested.

Chicken CSF1 was previously expressed as a recombinant protein in bacteria and in transfected mammalian cells, and as a CSF1-Fc fusion protein by industry partners Zoetis (formerly Pfizer Animal Health (PAH), Kalamazoo, MI, USA) (Garceau et al., 2010; Garceau et al., 2015). To enable the proposed studies, it was first necessary to establish the methods for generation and purification of endotoxin-free recombinant chicken CSF1 in the laboratory, and to test biological activity using the chicken CSF1R reporter cell line. That task forms the first part of this chapter. Using the purified recombinant chicken CSF1, the objectives of this Chapter are:

- To compare the activities of candidate mammalian CSF1R kinase inhibitors on chicken and human CSF1R
- To test the effect of active CSF1R kinase activities on CSF1-dependent primary chicken macrophages

## 3.2 Results

### 3.2.1 Recombinant chicken CSF1 protein purification

Large scale production of homogenous recombinant myeloid growth factor proteins have advanced the understanding of biological functions *in vivo*, in structural analysis and in potential clinical applications (Clark and Kamen 1987). Native human CSF1 was originally purified from human sources including urine and pancreatic carcinoma cell lines (Wu et al., 1979, Csejtey and Boosman 1986, Wong et al., 1987). However, much larger yields became available when cDNA clones of CSF1 from human, mouse, pig and chicken were transformed into mammalian cell systems with appropriate expression vectors (Kawasaki et al., 1985; Clark and Kamen 1987; Wong et al., 1987; Halenbeck et al., 1988; Garceau et al., 2010; Gow et al., 2012). Mammalian cells, including Chinese hamster ovary (CHO) COS1 or HEK293T, possess post translational modification and produce native proteins with appropriate glycosylation. Various isoforms of CSF1 could be cloned, detected and produced in culture supernatants via secretion in sufficient amounts to support analysis of biological functions *in vitro* and *in vivo*

(Clark and Kamen 1987; Garceau et al., 2010; Gow et al., 2012). Recombinant human CSF1 protein produced in cell culture had equivalent biological activity to the native form produced from natural human sources, as validated with a primary bone marrow macrophage proliferation assay (Wong et al., 1987; Halenbeck et al., 1988), and subsequently by injection of the recombinant protein into mice (Hume et al., 1988).

Glycosylation is not required for biological activity. Accordingly, the production of biologically active recombinant CSF1 protein from human, mouse, pig and chicken have also been described using various bacterial expression systems (Halenbeck et al., 1989; Krautwald and Baccarini 1993; Koths 1997; Garceau et al., 2010; Gow et al., 2012). Bacteria hosts such as *E.coli* often cope with biosynthesis-associated metabolic load by transiently storing the encoded protein as insoluble non-functional aggregates in inclusion bodies (Villaverde and Mar Carrió 2003). Inclusion bodies are highly hydrated dense particles found in bacterial cytoplasm. Recombinant mammalian or chicken CSF1 protein could be recovered *in vitro* from inclusion bodies by preparative refolding steps to gain native conformation. The four helix bundle structure and intrachain disulfide bond of mammalian CSF1 is conserved in chicken CSF1. Avian CSF1 proteins lack an inter-chain disulphide bond, but chicken CSF1 was able to stably exist in the form of non-covalent homodimer structure (Pandit et al., 1992; Garceau et al., 2010).

The chicken CSF1 locus was first identified by a previous student in our group. At the time, CSF1 was not identified in the chicken genome assembly (Garceau et al., 2010) The CSF1 coding sequence was cloned from cDNA of an HH20 chicken embryo RNA into pEF6/V5-His-TOPO vector (Invitrogen) to drive high level expression of a tagged CSF1 protein in transfected HEK293T cells. The expression plasmid construct pEF6-chCSF1 included the biologically active domain of chicken CSF1 encoded by the first five exons of the gene. The CSF1 protein expressed in HEK293 cells was detected in cell suspension and supernatant by western blot and shown to be biologically active on a chicken CSF1-dependent cell line (see below). Accordingly, colleagues from our collaborators at Zoetis (formerly Pfizer Animal Health (PAH), Kalamazoo, MI, USA), generated a synthetic codon-optimized cDNA encoding the active region of chicken CSF1 to insert into the inducible expression vector pET-28(b), referred to as plasmid pTLW54, which directed chicken CSF1 protein expression in inclusion bodies in *E.coli*. Colleagues at PAH also attempted to isolate chicken IL34 with similar bacterial expression approach, but purification was not successful. ChIL34 apparently requires

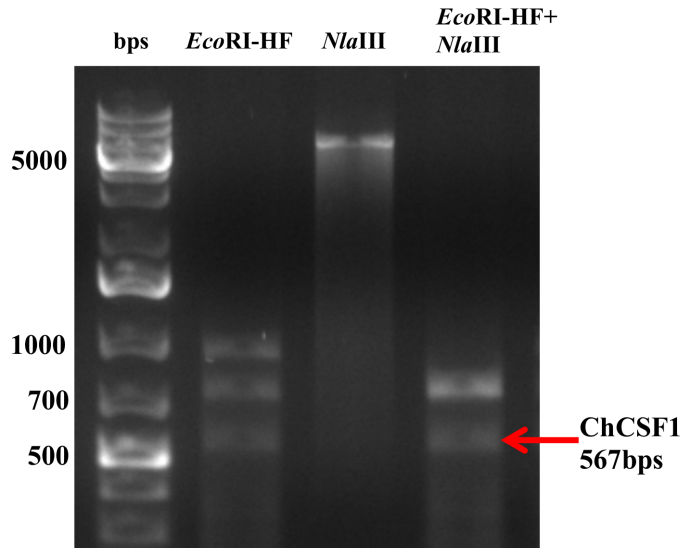
glycosylation for stability in solution (Garceau 2014), the machinery for which is absent in the bacterial system.

To support the current project, initially I needed to have available stocks of active chicken recombinant CSF1 protein for *in vitro* studies. Accordingly, I used the pTLW254 plasmid provided by PAH to isolate large amount of endotoxin free recombinant chicken CSF1 protein. The standardized bacterial protein purification protocol communicated by PAH was developed further to enable isolation of CSF1 protein in-house. This procedure was performed in collaboration with a postdoctoral colleague, Dr. Andreas Alber. The isolated recombinant CSF1 protein was later characterized for its functional activity to enable its use for subsequent cell viability assays.

The results presented from sections 3.2.1 to 3.2.2 describe the isolation, endotoxin free purification, batch quantification and characterization of recombinant chicken CSF1 protein, which was subsequently used in cell viability assays to screen chicken CSF1R kinase inhibitors sections 3.2.3 to 3.2.5

### **3.2.1.1 Transformation verification to identify chicken CSF1 insert**

In order to confirm the presence of the chicken CSF1 insert into the plasmid pTLW54, it was transformed in chemically competent cells BL21 star. The transformants were selected in kanamycin and isolated plasmid pTLW54 was subjected to an analytical restriction digestion reaction to confirm the presence of the chicken CSF1 insert. The active domain of chicken CSF1 (1-189 AAs) was engineered with restriction sites *Bsp*HI and *Eco*RI at 5' and 3' ends respectively and cloned into *E.coli* expression vector pET-28(b) with complementary restriction sites *Nco*I and *Eco*RI. Accordingly, a double digest reaction setup of plasmid pTLW54 with restriction enzymes *Eco*RI-HF and *Nla*III, revealed a band of around 550 bp, corresponding to the expected size of the chicken CSF1 insert (567 bp) (figure 3.1).



**Figure 3-1: Agarose gel electrophoresis identifying chicken CSF1 gene insert fallout from digested plasmid pTLW54, post bacterial transformation**

Plasmid pTLW54 was purified from transformed BL21 star chemically competent cells, in a single cell colony culture containing kanamycin (50 µg/ml). The quantified plasmid was then cleaved as per the engineered restriction sites on pTLW54 with restriction enzymes *EcoRI*-HF and *NlaIII* at 37°C for one hour followed by visualization of cleaved fragments on 1.5% agarose gel electrophoresis. The image shows (from left to right lanes) plasmid DNA cleaved bands obtained with single restriction enzyme digest reaction, *EcoRI*-HF, *NlaIII* and double digest products with both enzymes used reaction. Cloned chicken CSF1 gene is detected at the expected size of 567 bps.

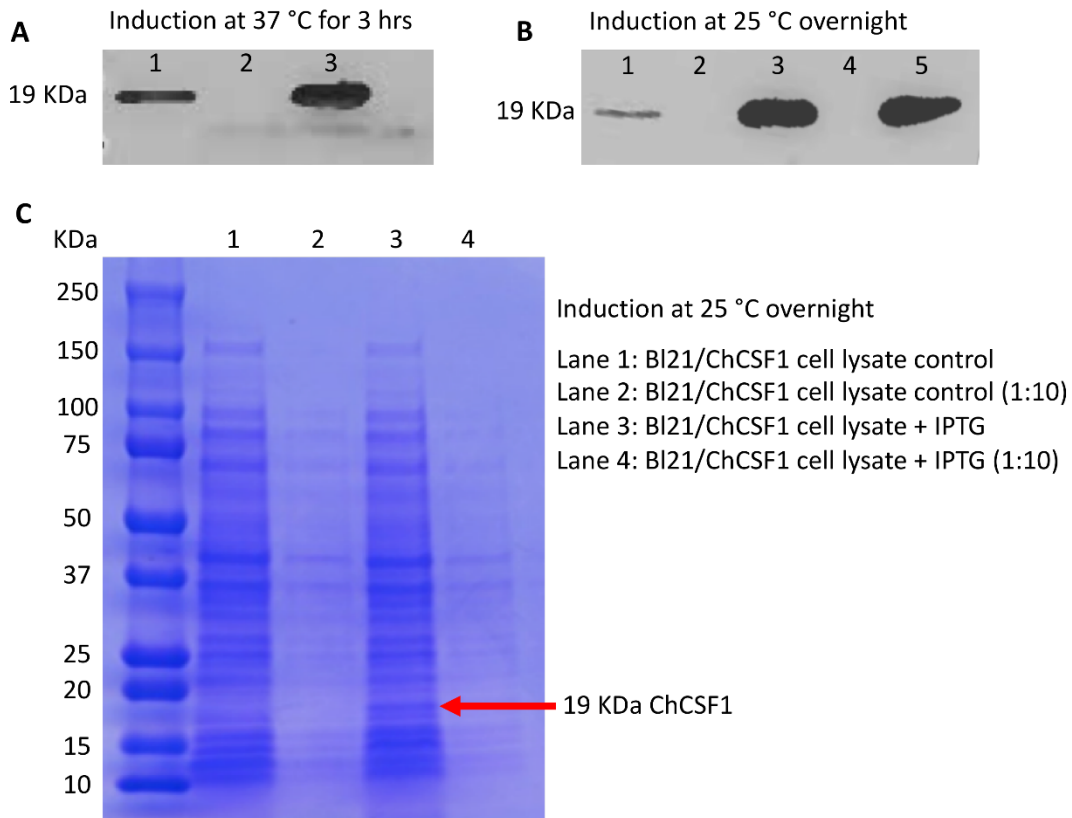
Thus, following confirmation of chicken CSF1 clone in pTLW54 transformed *E.coli* cells, I proceeded with large scale growth of the bacterial culture for purification steps.

### 3.2.1.2 Bacterial culture induction for enhanced CSF1 protein expression

One Shot BL21 star cells transformed with pTLW54 plasmid were grown in medium containing kanamycin, as described in Chapter 2 section 2.1.1. Since recombinant CSF1 protein expression in plasmid pTLW54 is controlled by the lac operon, protein production was induced by adding 0.5 mM IPTG, when the culture reached mid log phase. Bacterial expression of recombinant proteins and inclusion body formation can be influenced by temperature (Villaverde and Mar Carrió 2003). In order to optimize the expression of CSF1, two conditions were tested. Post induction with IPTG, BL21 star cells culture were incubated at 37°C for 3 hrs or 25°C overnight with constant rotation.

### Chapter 3: CSF1R kinase inhibitors

To assess size and purity of the expressed CSF1 protein, bacterial cell culture was harvested, and control/induced cell pellets were processed for immuno-blotting using the novel anti-chicken CSF1 mAb (described in Chapter 4) and SDS PAGE analysis (Chapter 2, section 2.1.4) under reducing conditions. SDS PAGE analysis revealed the presence of a monomeric CSF1 band at the expected size of around 19 KDa. This was more evident in the IPTG induced sample (figure: 3.2C lane 3) compared to the control (figure: 3.2C lane 1). This finding is consistent with the size of recombinant chicken CSF1 expressed in *E.coli* by our previous PAH collaborators (Garceau et al., 2010; Garceau 2014). Comparison of western analysis for control and induced samples at 37°C for 3 hrs (figure: 3.2A lane 3) and at 25°C overnight (figure: 3.2A lanes 3 and 5) showed that the yield of recombinant CSF1 was optimal at 25°C. Therefore, this condition was employed in scaling up the chicken CSF1 protein purification.



**Figure 3-2: Optimizing bacterial growth conditions for the enhanced expression of IPTG induced chicken CSF1 protein**

Plasmid pTLW54 transformed into competent *E.coli* BL21 star cells, were cultured and induced with IPTG as described in Chapter 2, section 2.1.1. CSF1 protein in the induced cell lysate was detected by immuno-blotting. Uninduced and IPTG induced cell lysates after SDS PAGE electrophoresis in the reducing condition and nitro-cellulose membrane transfer, were probed with anti-chicken CSF1 IgG1 mAb (produced in-house). This was followed by blot development with chemiluminescence (A) *E.coli* culture induction at 37°C, grown for 3 hrs; (B) *E.coli* induction at 25°C, for an overnight culture where Lane 1 is an aliquot of commercially purified chicken CSF1, lanes 2 and 4 are uninduced samples, and lanes 3 and 5 are induced samples. A monomeric chicken CSF1 protein band was detected at 19 KDa. (C) Coomassie stain SDS-PAGE gel visualization of uninduced and induced chicken CSF1 protein band at different cell lysate dilution. Gel electrophoresis with optimized 25°C overnight induction culture condition. These data are representative of two independent experiments

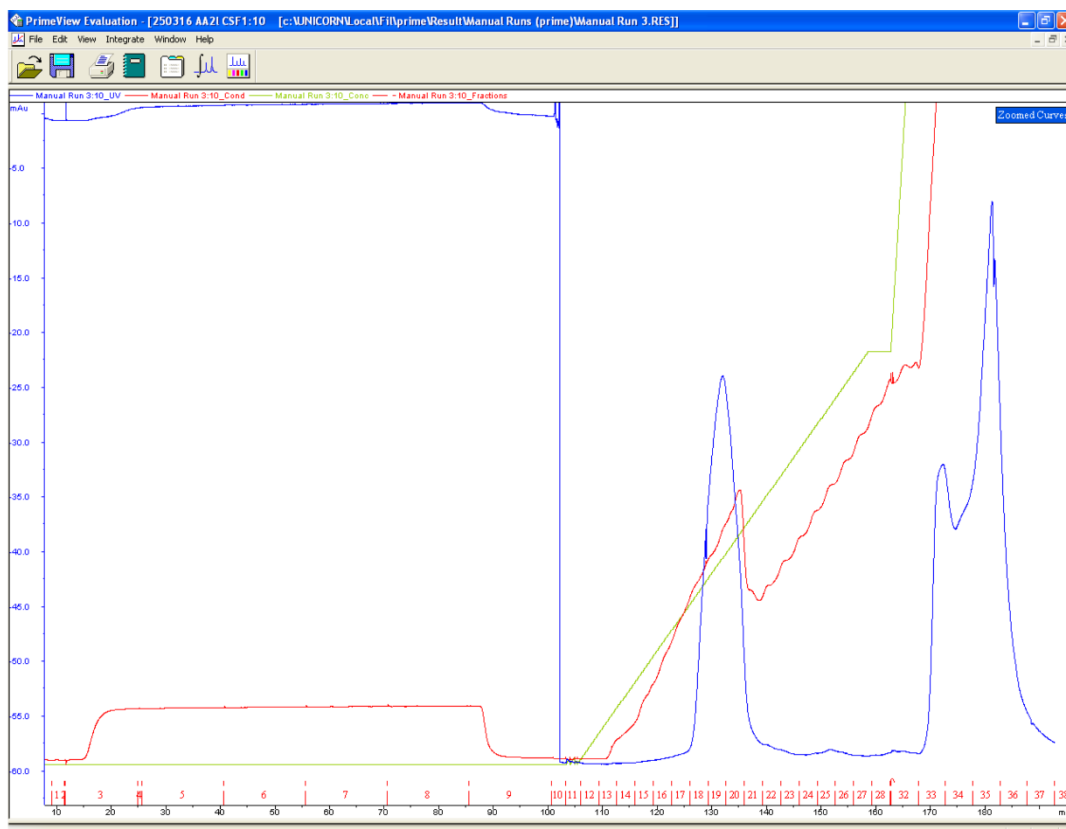
### 3.2.1.3 Isolation and purification of recombinant chicken CSF1 protein

Isolation and purification of recombinant protein from the bacterial system involves three steps: isolation and washing of inclusion bodies, release of recombinant protein from inclusion bodies by solubilisation, and refolding to generate the native or biologically-active form (Villaverde and Mar Carrió 2003). To isolate chicken CSF1 protein from inclusion bodies, bacteria were disrupted by high pressure homogenisation. The inclusion bodies were then solubilised in



buffer containing denaturant guanidine-HCL and reducing agent DTT. These respectively help in disassociating intermolecular interactions and non-native disulfide bond formations, thus promoting complete unfolding of the protein into monomers. Renaturation of denatured CSF1 protein was achieved by slowly (1 ml/min) diluting the solubilized protein in a refold buffer to prevent aggregation. The refold buffer contains redox reagents that promotes oxidation for disulfide bond formation. Since chicken CSF1 lacks interchain disulfide bonds after re-folding it will exist as a non-covalent homodimer (Garceau et al. 2010).

Purification of refolded CSF1 protein solution was achieved by several dialysis steps and by using AKTA ion exchange chromatography system (described in Chapter 2, section 2.1.5). The chromatogram generated from chromatography (figure: 3.3) shows three protein peaks (blue curves) generated by applying an increasing salt gradient concentration to the Q sepharose resin column. Eluted fractions from each peak were analyzed for detecting chicken CSF1 protein with SDS PAGE and western blotting.



**Figure 3-3: A chromatogram generated from prime view software depicting recombinant chicken CSF1 protein curves**

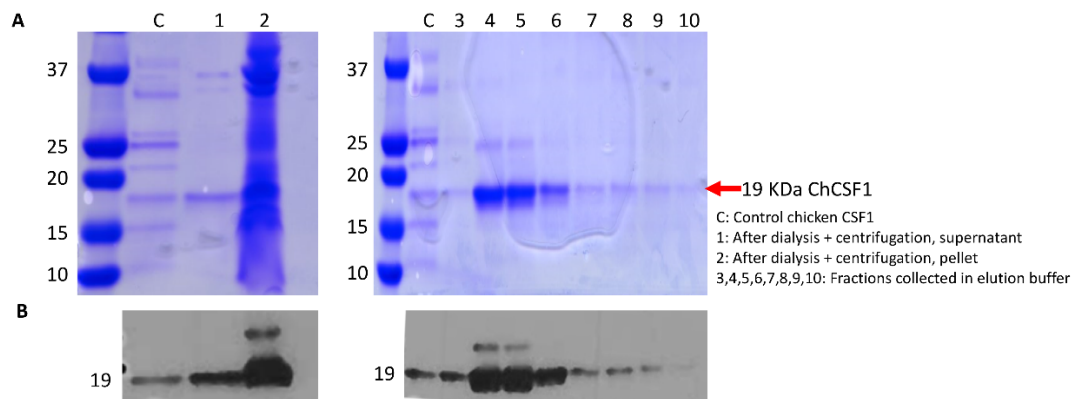
Clarified chicken CSF1 protein solution was purified using the AKTA chromatography system. An XK26 chromatography column containing Q sepharose resins was washed and CSF1 protein solution then loaded at the rate of 3 ml/min. The number of eluted protein fractions are shown in red on the X-axis, and these were subsequently collected and characterized for chicken CSF1 protein expression. The Y-axis represents measurements of Milli-Absorbance Units (mAU) for each fraction eluted from the column. Each fraction contained 10 ml of eluted sample. The signal values obtained during the run are displayed graphically as blue red and green curve. The blue peak represents measurement of the UV absorbance of the protein samples, the red conductivity and the green curve indicates increasing salt gradient concentration of elution buffer QB used in the column to generate linear gradient. The chromatogram is a representative from three independent CSF1 protein purification experiments.

### 3.2.1.4 Confirmation of refolded chicken CSF1 protein in fractions

Refolded protein and peak fraction samples eluted from the chromatography column were run on SDS PAGE gel and stained with Coomassie blue dye as shown in figure: 3.4A. Bands matching to recombinant ChCSF1 protein at the expected size of around 19 KDa was observed in all the samples with different levels of contamination with other proteins. Multiple bands observed in figure: 3.4A lane 2 correspond to the presence of aggregates of protein bands of

various sizes accumulated in the pellet following centrifugation of the refolded protein. A prominent band of around 19 KDa was present in the refolded protein supernatant after centrifugation and also in eluted fractions of lanes 4, 5 and 6 (figure: 3.4A). In addition to chicken CSF1 protein, purified fractions in lanes 4, 5 and 6 also contained an extra faint band at 25 KDa. Chromatography eluted fractions 3, 4, 5, 6, 7, 8, 9 and 10 from SDS PAGE (figure: 3.4A) correspond to the higher UV absorbance peak signals detected in the chromatogram (figure: 3-3).

The identity of the expressed protein was confirmed by western blotting using anti-chicken CSF1 mAb (described in Chapter 4). The antibody bound to a recombinant chicken CSF1 protein band at 19 KDa in lanes 4, 5 and 6, along with the additional band at 25 KDa that may be an incompletely-folded protein (figure: 3.4B). Accordingly, the fractions from lanes 4, 5 and 6 were pooled together for final dialysis against PBS and further downstream analysis.



**Figure 3-4: Evaluation of eluted chromatography fractions for identification of purified chicken CSF1 protein band**

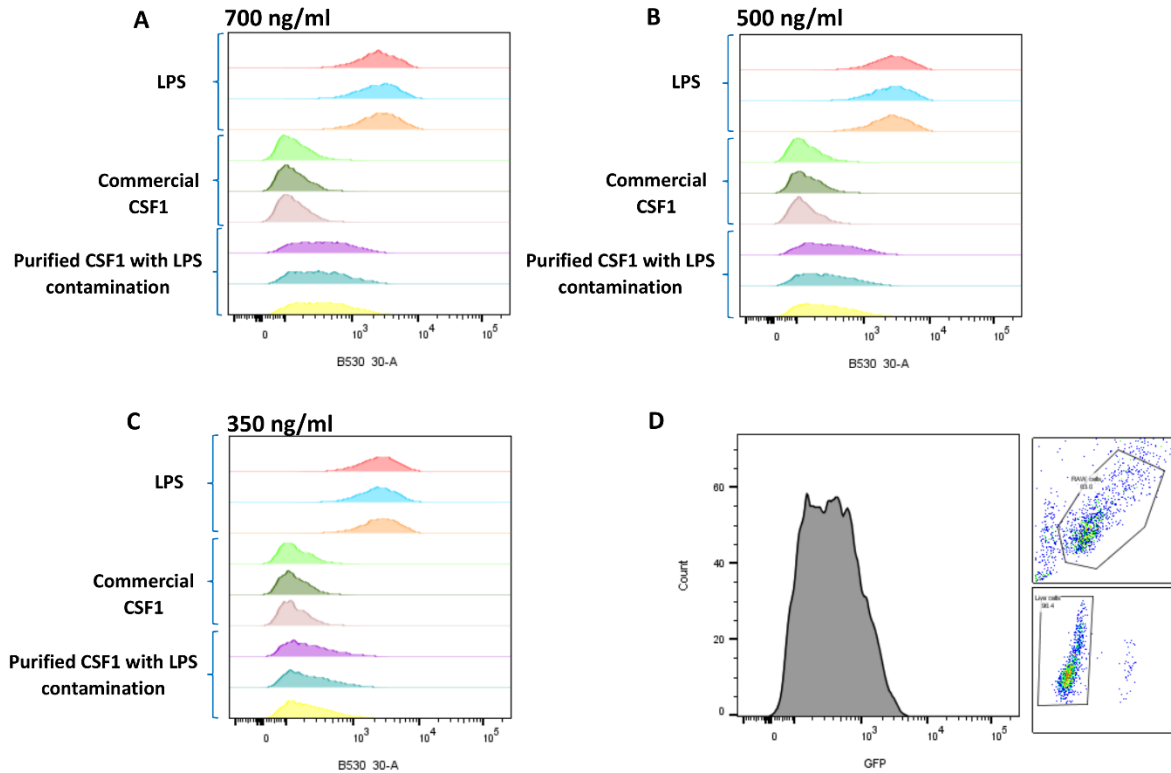
(A) SDS PAGE analysis of aliquots collected in various steps during CSF1 protein purification procedure and (B) Western blot analysis from the same aliquots to detect the expressed chicken CSF1 protein band, probed with anti-chicken CSF1 IgG1 mAb. Lane C: control purified chicken CSF1 protein (0.1 mg/ml). Lanes 1 and 2: refolded protein solution after first dialysis, supernatant and pellet respectively. Lanes 3, 4, 5, 6, 7, 8, 9 and 10: eluted protein fractions collected during column chromatography. The biologically active purified recombinant chicken CSF1 protein band at around 19 KDa is shown with a red arrow. Data are representative from three independent CSF1 protein purification experiments.

### 3.2.1.5 Production of endotoxin free chicken CSF1 protein

Proteins expressed in bacteria are often contaminated with lipopolysaccharide (LPS, endotoxin) which is released upon bacterial lysis and can affect downstream applications. LPS

strongly inhibits CSF1 induced growth of macrophages by effectively downregulating *CSF1R* mRNA and the expression of CSF1R on the cell surface (Sweet and Hume 1996). Accordingly, for use in growing myeloid/macrophages cell lines and screening kinase inhibitors in this project, it was essential to have an endotoxin-free stock of recombinant chicken CSF1 protein. As such endotoxin presence was first detected in the pooled chromatography fractions of chickens CSF1 protein using a macrophage reporter cell line.

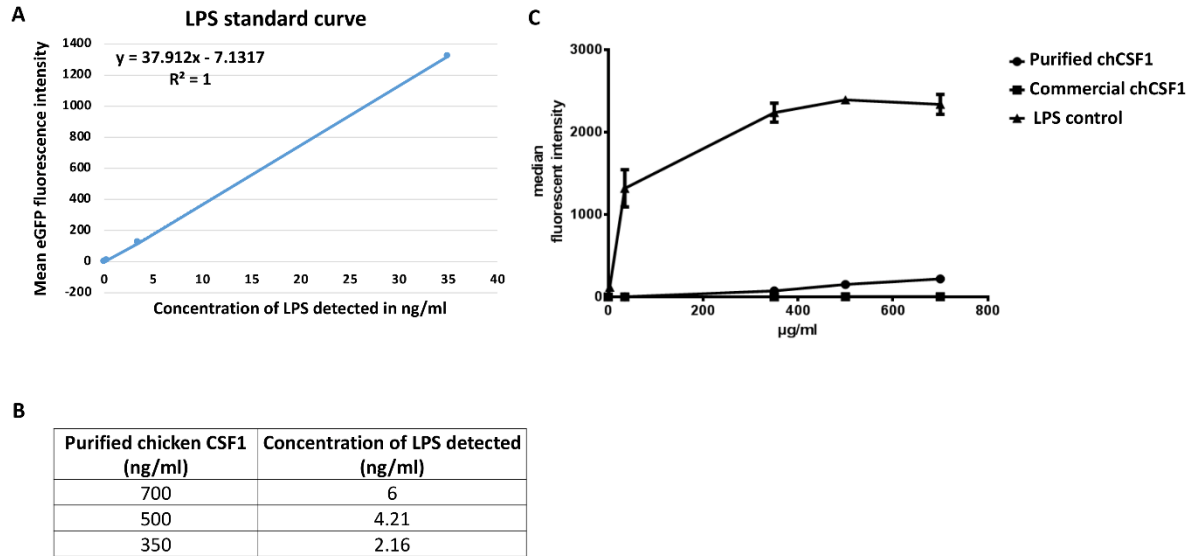
RAW264-ELAM cells is a murine macrophage adherent cell line in which an eGFP reporter gene is controlled by the endothelial leukocyte adhesion molecule (ELAM) promoter that is induced in response to inflammatory stimulant as LPS (Himes et al., 2001). To identify the presence of LPS, cultured RAW264-ELAM cells were incubated for 4 hrs with various dilutions of three batches of chicken CSF1 protein solution, along with pure LPS (as a positive control) and commercially-purified endotoxin-free chicken CSF1 (as a negative control). eGFP reporter gene expression was visualised and fluorescence intensity measured by flow cytometry. Representative half offset histogram profiles comparing median fluorescence intensities (MFI) of commercially purified chicken CSF1 and in-house generated recombinant chicken CSF1 at three dilutions 700 ng/ml, 500 ng/ml and 350 ng/ml are shown in figure: 3.5A, B and C. As seen from the profiles there is presence of LPS contamination at each dilution. It was therefore confirmed that the chicken CSF1 protein solution produced in *E.coli* was contaminated with endotoxins.



**Figure 3-5: Column chromatography purified chicken CSF1 protein was contaminated with endotoxin LPS**

For the endotoxin detection assay, murine macrophage eGFP reporter RAW264-ELAM cell line, was stimulated with pure LPS (as a positive control), commercially purified endotoxin free chicken CSF1 (as a negative control) and the chicken CSF1 protein purified here from the bacterial system at a dilution range of 700 ng/ml, 500 ng/ml, 350 ng/ml, 35 ng/ml, 3.5 ng/ml, 0.35 ng/ml and 0.035 ng/ml in a 96-well plate. The assay was performed in triplicate. Stimulated cells were incubated for 4 hrs in a 37°C incubator. Cells were stained for dead cell marker sytox blue and profiles obtained by flow cytometry. Figure (A) shows half offset histogram of eGFP fluorescence curves detected with RAW264-ELAM cells stimulated with chromatography purified proteins at 700 ng/ml (B) at 500 ng/ml and (C) at 350 ng/ml. Note the profiles of bacterial purified CSF1 protein showing presence of LPS contamination at each dilution. (D) Representative GFP cell count and FSC/SSC flow profiles. Endotoxin assay data, representative from two independent experiments with batch chicken CSF1 purified (1 litre and 2 litre starter culture) is shown.

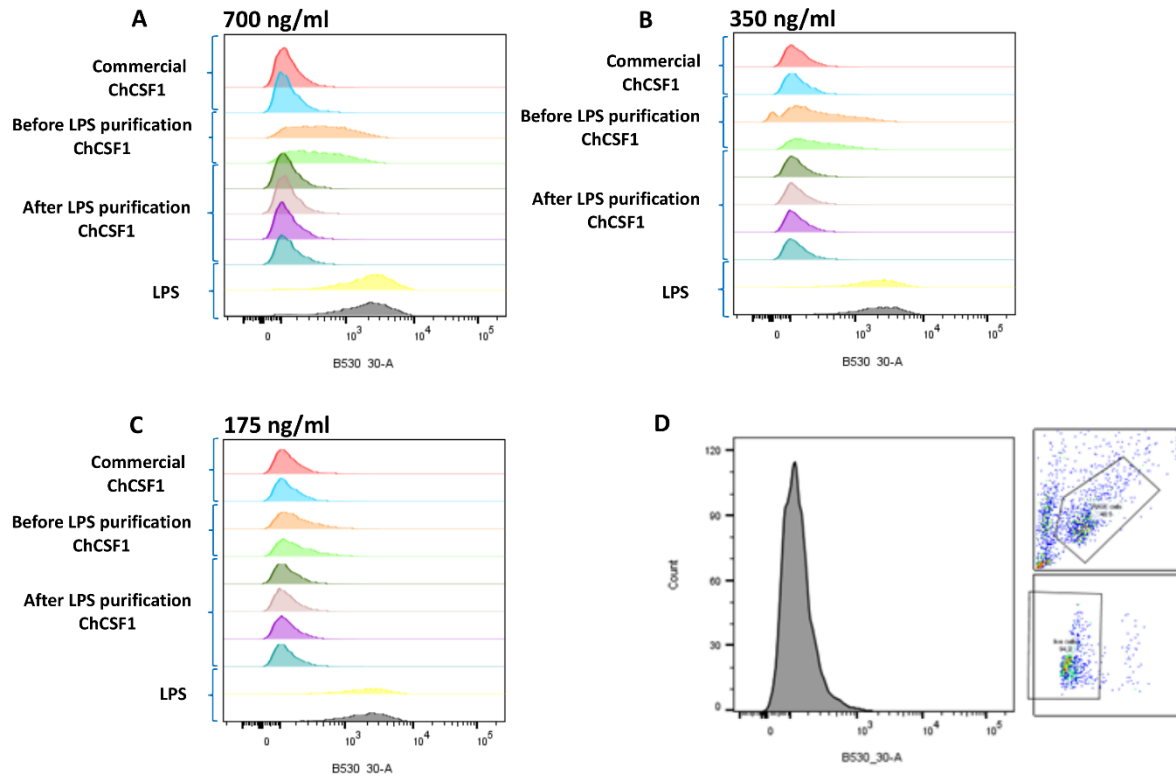
To quantify the amount of LPS levels detected in the purified chicken CSF1 protein solution, a standard LPS dilutions curve (figure: 3.6A) was drawn and accordingly concentration of LPS present in different tested dilutions of purified chicken CSF1 protein was determined. The highest concentration of LPS detected in the CSF1 protein using the RAW264-ELAM assay was 6 ng/ml (figure: 3.6B). Graphical representation comparing MFI of purified chicken CSF1 and commercially purified chicken CSF1 with LPS control values is shown in figure: 3.6C.



**Figure 3-6: Quantification of LPS detected in bacterial purified chicken CSF1 protein**

(A) A standard curve measuring LPS concentrations. (B) Quantification of LPS levels detected in purified chicken CSF1 protein at various dilutions 700 ng/ml, 500 ng/ml and 350 ng/ml. (C) Median fluorescent intensity curve comparing RAW264-ELAM cells stimulated with LPS control, commercially purified chicken CSF1 and the bacterial purified chicken CSF1 protein. Each data point is the average of triplicates +/- SD (standard deviation).

To remove the LPS contamination, the chicken CSF1 protein purified from the first batch of 1 litre pTLW254 plasmid transformed *E.coli* cell culture was processed with endotoxin high affinity poly-L-lysine attached porous cellulose beads columns. A batch method using a spin column was followed as described by manufacturers of the Pierce High-Capacity Endotoxin removal kit. The success of this process was evident from the loss of activity in the RAW264-ELAM assay following endotoxin removal (figure: 3.7 A, B and C).



**Figure 3-7: Endotoxin assay of bacterial purified chicken CSF1 post LPS removal using poly-l-lysine resins column**

eGFP reporter RAW ELAM macrophage cell line, was stimulated with pure LPS (as a positive control), commercially purified endotoxin free chicken CSF1 (as a negative control), bacterial purified chicken CSF1 before and after LPS column purification at dilutions 700 ng/ml, 350 ng/ml and 175 ng/ml in a 96-well plate. The endotoxin assay was performed in triplicate. The reporter cells were incubated for 4 hrs in a 37°C incubator. Dead cells were stained with marker sytox blue. eGFP fluorescence profiles were analysed by flow cytometry. Figure (A) shows half offset histogram of eGFP fluorescence intensity curves detected at 700 ng/ml (B) at 350 ng/ml and (C) at 175 ng/ml. After LPS column removal, profiles of RAW ELAM cells stimulated with the purified bacterial recombinant chicken CSF1 protein show no presence of LPS contamination at any dilution. (D) Representative GFP cell count and FSC/SSC flow profiles. The result is representative of three independent preparations using the endotoxin removal method (1 litre, 2 litre starter culture and 3 litre pooled samples) is shown.

### 3.2.1.6 Batch quantification of chicken CSF1

The yield of purified chicken CSF1 protein was quantified using a bicinchoninic acid colorimetric assay (BCA) (Smith et al., 1985) with bovine serum albumin as a standard and using Nanodrop measurements (Absorbance at 280 nm).

The average yields from the three separate batches of chicken CSF1 prepared are summarised in table 3-4.

**Table 3-4 Quantification of isolated and purified chicken CSF1 protein with and without endotoxin contamination**

Summary of the average nanodrop readings and concentration values obtained from BCA assay for quantifying purified CSF1 protein from different batches.

No.	Starter culture	Before LPS removal (µg/ml)	After LPS removal (µg/ml)	Recovery of CSF1 protein
Batch 1	1 litre	83.4 (30 ml)	64 (24 ml)	77%
Batch 2	2 litre	123.4	60.9 (26 ml)	49%
	pooled 3 litre		55.2 (50 ml)	

Total endotoxin free CSF1 protein purified = 55.2 x 50 ml = 2.760 mg

These results reveal a significant loss of chicken CSF1 protein in the LPS removal step, but this is unavoidable for the protein to be used in subsequent bioassays. In summary, the pooled preparations contained 2.76 mg of chicken CSF1 in a volume of 50 ml. Since CSF1 is active in the ng/ml range, this was more than sufficient to support ongoing studies. In order to find out if purified chicken CSF1 protein is functionally active, it was further biologically characterised in various assays as described below, before it was used for candidate CSF1R kinase inhibitors screening assays.

### 3.2.2 Characterizing biological activity of chicken CSF1 protein

#### 3.2.2.1 Chicken BMDMs culture growth in purified CSF1 protein

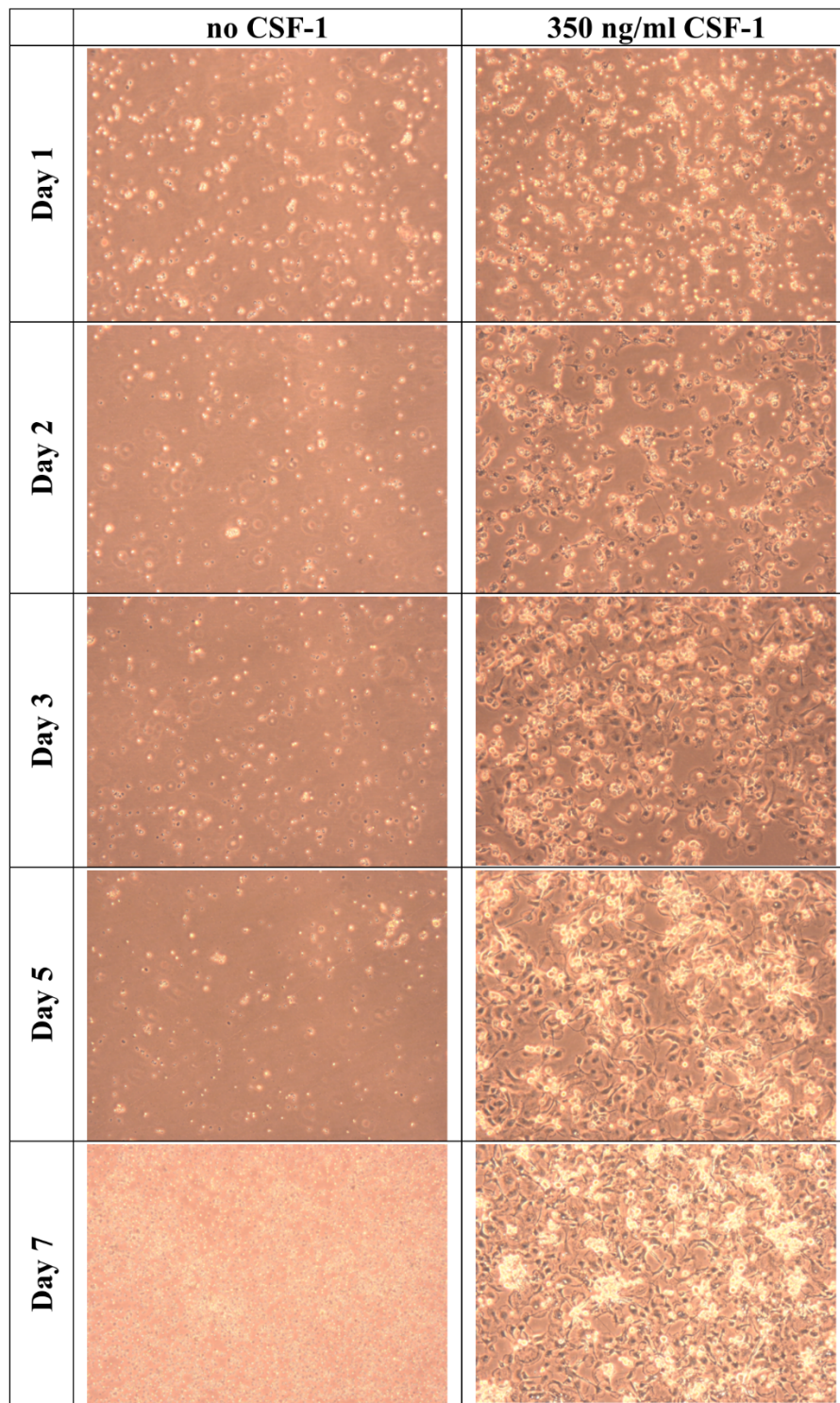
As described in the introduction Chapter 1, *CSF1R* mRNA expression in chickens, as in mammals, is restricted to cells of the mononuclear phagocyte lineage and their progenitors (Garcia-Morales et al., 2013; Balic et al., 2014; Garceau et al., 2015; Hume et al., 2017; Rojo et al., 2017). Production of bone marrow derived macrophages or peripheral blood monocyte derived macrophages, differentiated in the presence of CSF1, has been described before for humans (Irvine et al., 2006), rodents (Hume and Gordon 1983; Trouplin et al., 2013), pigs (Gow et al., 2012; Kapetanovic et al., 2012), dogs (Gow et al., 2012), cats (Gow et al., 2013),



horses (Werners et al., 2004; Young et al., 2018) and chickens (Werners et al., 2004; Garceau et al., 2010; Garcia-Morales et al., 2013; Garceau et al., 2015; Young et al., 2018). In the absence of CSF1, bone marrow macrophage progenitors undergo cell death.

Recombinant chicken CSF1 provided by PAH was used previously to generate pure macrophage cultures *in vitro* for functional studies (Garceau 2014). To confirm that the recombinant CSF1 protein produced above was active, femur derived bone marrow from five-week-old Novagen birds was maintained in culture supplemented with or without 350 ng/ml of chicken CSF1 protein for 7 days. Bright field images were taken on days 1, 2, 3, 5 and 7 (figure: 3.8). Expansion of the cell population was evident with 24 hrs, attachment to the tissue culture dish from day 2/3 and a lawn of adherent large macrophages spread on the substratum by day 7 (figure: 3.8). No viable cells remained by day 7 in the absence of added chicken CSF1. Hence, the purified recombinant chicken CSF1 was biologically active in promoting macrophage differentiation.

Since cultured chicken BMDMs could be cryo-preserved and recovered later in cultures to produce macrophages in response to chicken CSF1 (Garceau 2014), I cryo-preserved these cells for long term storage as described in Chapter 2 section 2.2.3 and used them as resource for culturing chicken BMDMs for further *in vitro* studies in the development of macrophages involving chicken CSF1R inhibitors and antibody to chicken CSF1.



**Figure 3-8: Growth of chicken bone marrow derived macrophage population**

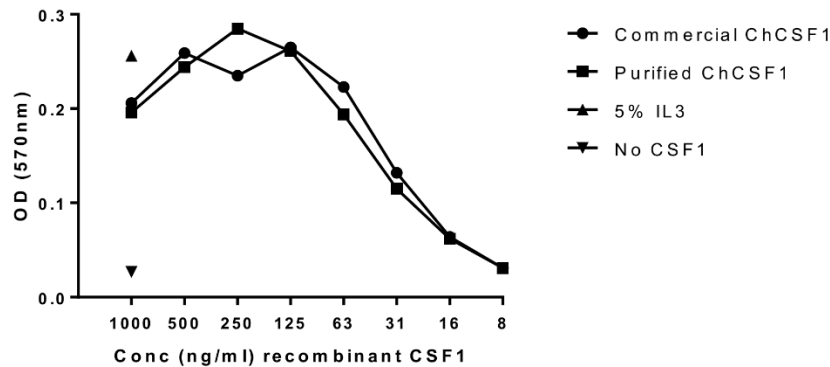
Femoral bone marrow cells were harvested from five-week-old Novagen chickens. Approximately  $2 \times 10^7$  cells in RPMI complete medium, with or without *E.coli* purified chicken CSF1 (350 ng/ml) as indicated were plated onto 6 well plate. Cells were incubated at 37°C and bright field images taken on each day. On day 4 cells were refreshed with media with or without CSF1.

### 3.2.2.2 Purified recombinant CSF1 protein and commercially obtained CSF1 protein have similar metabolic activity

BaF3 is an immortalized bone marrow derived pro-B cell line that is dependent on IL-3 for proliferation and survival, and undergoes cell death in its absence (Palacios and Steinmetz 1985; Ihle and Askew 1989). BaF3 have been widely-used to generate bioassays in which the receptors expressed following stable transfection provide an alternative pro-survival signal. BaF3 cells have been stably-transfected with human or mouse CSF1R (Irvine et al., 2006), pig CSF1R (Gow et al., 2012), feline CSF1R (Gow et al., 2013) and chicken CSF1R to study the biological activity of their respective ligands, CSF1 and IL34, or develop various assays to test binding capacity to CSF1R (Garceau et al., 2010).

BaF3 cells were transfected with PeF6\_ChCSF1R vector and stable clones were selected for their survival and proliferation, initially in IL3 and later substituting with chicken CSF1. BaF3 cells expressing chicken CSF1R (BaF3/ChCSF1R) on the cell surface show proliferative response in the presence of CSF1 (Garceau et al., 2010). An *in vitro* colorimetric MTT cell viability bioassay was developed using these cells. MTT is a water-soluble reagent which, upon reduction by the mitochondrial enzyme succinate dehydrogenase in metabolically active cells generates NADPH and NADH, converting it into insoluble blue formazan crystals (Garceau et al., 2010).

The activity of the above purified endotoxin-free recombinant chicken CSF1 was assessed by dose titration via MTT assay using Ba/F3-ChCSF1R cells. Figure: 3.9 shows a dose response curve comparing PAH chicken CSF1 and the purified recombinant chicken CSF1 generated above. The EC<sub>50</sub> value or half survival response of purified recombinant chicken CSF1 protein and PAH obtained chicken CSF1 protein was almost equivalent 37.37 ng/ml and 31.37 ng/ml respectively. These values are similar to EC<sub>50</sub> values obtained for recombinant porcine CSF1 (29 ng/ml) and recombinant human CSF1 (34 ng/ml) activity on BaF3 cells expressing porcine CSF1R (Gow et al., 2012).



**Figure 3-9: Growth curve of BaF3/ChCSF1R cells with bacterially expressed and purified recombinant chicken CSF1 protein expressed in *E.coli***

$2 \times 10^4$  BaF3/ChCSF1R cells, selected for growth in IL3 (5% X63 supernatant), were washed and seeded in a 96-well plate. Cells were stimulated with appropriate growth factors in 2-fold dilutions for 72 hrs at 37°C and MTT assay performed as described in Chapter 2 section 2.4. Dose response curve of purified endotoxin free recombinant chicken CSF1 protein is compared with commercial chicken CSF1 provided by PAH. Cells cultured in 5% IL3 and no CSF1 were used as positive and negative controls respectively. Each data point shown is the average of three measurements. The experiment was performed two times.

### 3.2.2.3 Optimisation of CSF1 concentration for *in vitro* assays

When CSF1R is expressed on the cell surface, CSF1 is cleared from media by receptor mediated endocytosis and both the ligand and the receptor are degraded. The receptor is continuously resynthesized (Bartocci et al., 1987). In a standard bioassay, CSF1 is continuously consumed, so it is the amount of available CSF1 relative to the starting number of cells, rather than the concentration that determines the dose-response curve. If the cells consume all available CSF1, they will undergo cell death. Since kinase inhibitors do not prevent CSF1 internalisation, a partly-effective inhibitor would be likely to shift the dose-response curve. It was therefore desirable that the ligand CSF1 was not super-saturating. The half-survival metabolic response at 37.37 ng/ml obtained with the purified chicken CSF1 in MTT assay, and maximal survival occurred around 100 ng/ml. Accordingly a concentration of 100 ng/ml of chicken CSF1 protein was used for bioassays.

### 3.2.3 Activity of CSF1R kinase inhibitors on chicken CSF1R signalling

Many novel CSF1R or related kinase specific inhibitors (described in tables 3-1 and 3-2) have been validated for the growth of monocytes and macrophages in *in vitro* cell proliferation

assays using human or mouse CSF1R kinase domains. One of the common approaches is to express the target receptor in a factor-dependent cell. For example, the multi-kinase inhibitor Imatinib (Gleevec) inhibited growth of recombinant human CSF1 stimulated factor dependent cell-Paterson 1 (FDC-P1) expressing CSF1R cells but not cells stimulated with control murine IL3 (Dewar et al., 2005). The BaF3 assay has also been used previously to characterize CSF1R kinase inhibitors. The candidate inhibitor CYC10268 was tested in cells expressing the kinase domains of human CSF1R, FLT3, KIT or chimeric human/mouse CSF1R domains using the MTT bioassay (Irvine et al., 2006). Compound CYC10268 inhibited CSF1 signaling induced survival of BaF3 cells expressing human or mouse CSF1R with equal potency (1  $\mu$ M) but also had some activity against related kinases. The well-characterized inhibitor GW2580, inhibited the survival signal in BaF3 cells expressing human CSF1R, but did not inhibit BaF3 cells ectopically expressing chimeric intracellular related human kinases KIT or FLT3 spliced to extracellular human CSF1R ligand binding domain (Irvine et al., 2009). Apart from GW2580, which does not inhibit primary chicken macrophage proliferation (Garceau 2014), no other compounds have been tested for their inhibitory activity in chickens.

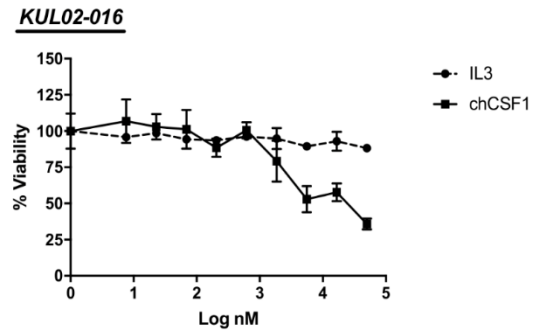
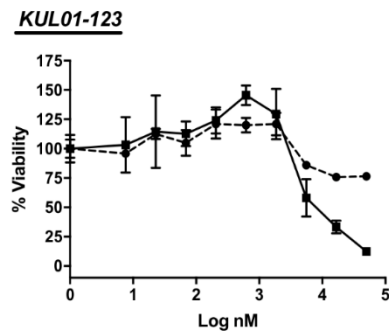
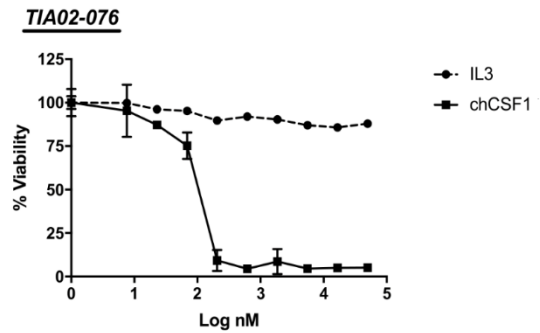
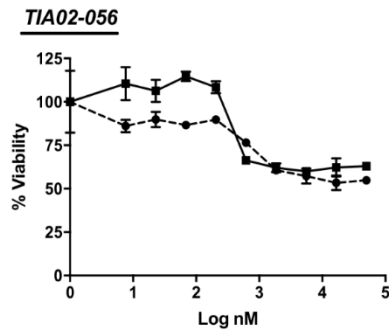
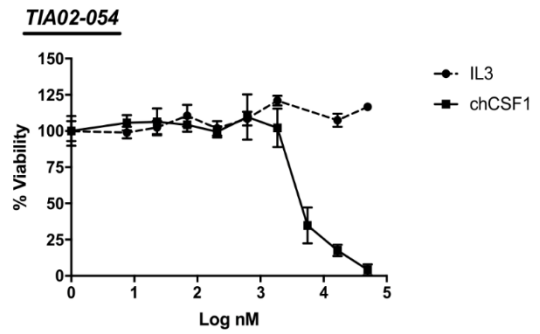
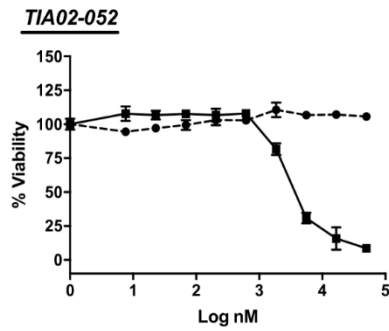
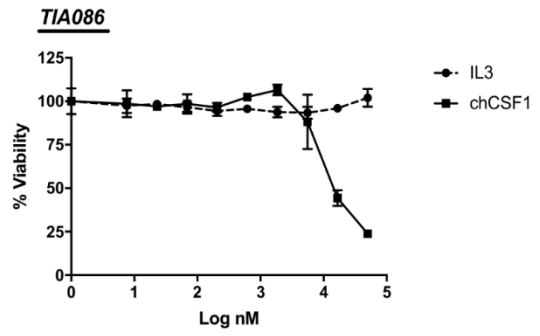
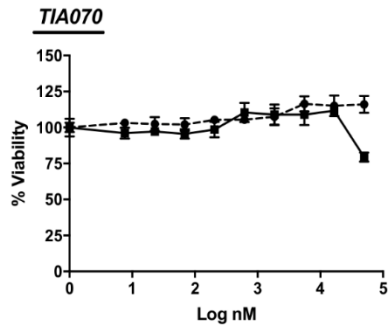
To enable study of chicken macrophages and further assess differences between the chicken and mammalian kinase domains, 10 candidate mammalian CSF1R kinase inhibitors, developed by our collaborators in Norway (Prof. Eirik Sundby, Norwegian University of Science and Technology), were screened for their cell proliferation activity using factor dependent BaF3 cells transformed with chicken CSF1R. As described in sections 3.2.2.2 and 3.2.2.3, BaF3/ChCSF1R cell survival and proliferation is dependent on chicken CSF1 mediated CSF1R signalling, and accordingly cells were cultured in optimised concentration of 100 ng/ml of purified endotoxin free recombinant CSF1 protein. Cells cultivated in IL3 could potentially lose or inactivate chicken CSF1R, since in our earlier studies there was some evidence that CSF1R promotes differentiation. Accordingly, for this series of experiments, I prepared stocks of cryo-preserved BaF3/ChCSF1R cells selected solely on chicken CSF1 protein (Chapter 2 section 2.3.3) and used during the time of inhibitor experiments.

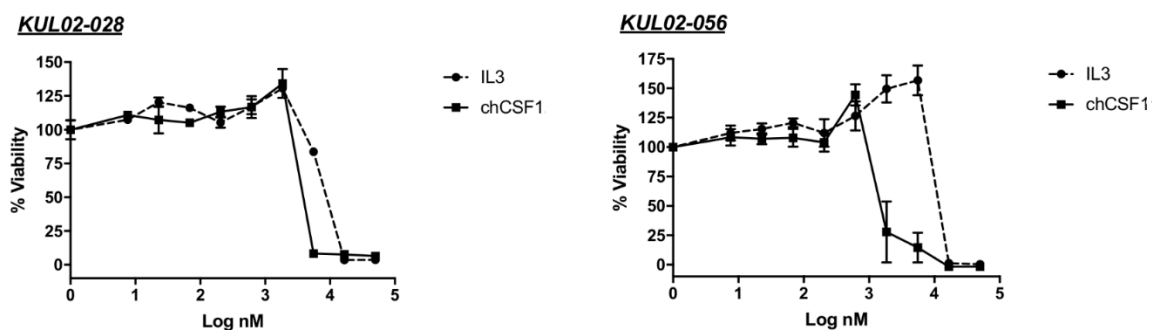
BaF3/ChCSF1R cell cultures were washed three times in plain RPMI media, before seeding cells in 96-well plate, to remove any traces of leftover chicken CSF1 protein for the MTT assay. Cells were induced with murine IL3 or chicken CSF1 in the presence of 10 CSF1R kinase inhibitors for 72 hrs. Ten-point dose response curves for the ten screened inhibitors are shown in figure: 3-10. Of the 10 inhibitors screened, 6 compounds TIA086, TIA02-052, TIA02-054,

TIA02-076, KUL01-123 and KUL02-016 inhibits chicken CSF1 induced CSF1R signalling survival activity in BaF3/ChCSF1R cells and do not inhibit growth of cells stimulated with control murine IL3 cytokine. Inhibitors TIA070 and TIA02-056 in comparison were less effective in inhibiting growth of chicken CSF1 induced BaF3/ChCSF1R cells. BaF3/ChCSF1R cells treated with compounds KUL02-028 and KUL02-056 at higher concentrations, in the presence of IL3 or chicken CSF1 did not survive, displaying cellular toxicity of these compounds.

Thus, evaluation of the 10 candidate mammalian CSF1R kinase inhibitors on cell proliferation activity via chicken CSF1/CSF1R receptor signalling, led to identification of six inhibitors that are potent and selective for chicken CSF1R.

### Chapter 3: CSF1R kinase inhibitors





**Figure 3-10: Effect of potential mammalian CSF1R tyrosine kinase inhibitors on chicken CSF1R**

To validate effects of CSF1R inhibitor compounds on CSF1 induced signalling, BaF3/ChCSF1R cells were tested for their survival *in vitro* with MTT assay. BaF3/ChCSF1R cells selected for growth in purified chicken CSF1 (100ng/ml) were harvested, washed and seeded in 96-well plate. Cells were induced with murine IL3 (5% X63 supernatant) or purified chicken CSF1 protein in the presence of 10 potential CSF1R tyrosine kinase inhibitors. Cells were treated with a 3-fold dilution range of inhibitors from a concentration 50  $\mu$ M for 72 hrs at 37°C. Each data point represents an average of three measurements  $\pm$  SD. The BaF3/ChCSF1R cell percentage viability (normalized response) curve against log dilution values of inhibitors used as TIA070, TIA086, TIA02-052, TIA02-054, TIA02-056, TIA02-076, KUL01-123, KUL02-016, KUL02-028 and KUL02-056 is drawn using GraphPad Prism 7 software. Representative data from three independent experiments is shown.

### 3.2.4 Identification of chicken CSF1R specific, CSF1R kinase inhibitors

The 10-candidate mammalian CSF1R kinase inhibitors activity on chicken CSF1R were evaluated further to compare their cell proliferation activity with human CSF1R signalling. MTT assays with BaF3 cells expressing human CSF1R (BaF3/HuCSF1R) were performed by Dr. Joana Alves in the Hume group. Dose response curves comparing 10 inhibitors activity on human and chicken CSF1R kinase domain are shown in figure: 3-11.

Of the 10 CSF1R inhibitors activities screened, 3 inhibitors TIA086, TIA02-052 and KUL02-016 had almost equivalent half-maximal inhibitory concentration values ( $IC_{50}$ ), on chicken and human CSF1R (table 3-5). This was not unexpected, since the inhibitors were designed to target the human receptor. Inhibitors TIA070 ( $IC_{50}$  Hu 19.45  $\mu$ M - Ch 362  $\mu$ M), TIA02-056 ( $IC_{50}$  Hu 1.26  $\mu$ M - Ch 29.97  $\mu$ M) and KUL01-123 ( $IC_{50}$  Hu 3.97  $\mu$ M - Ch 11.87  $\mu$ M) were more active in inhibiting mammalian CSF1R. In this respect, these inhibitors resemble GW2580 and might conceivably be influenced by the same active site substitution in the chicken CSF1R kinase domain. More unexpected was the finding that compounds TIA02-054 ( $IC_{50}$  Hu 14.22  $\mu$ M –

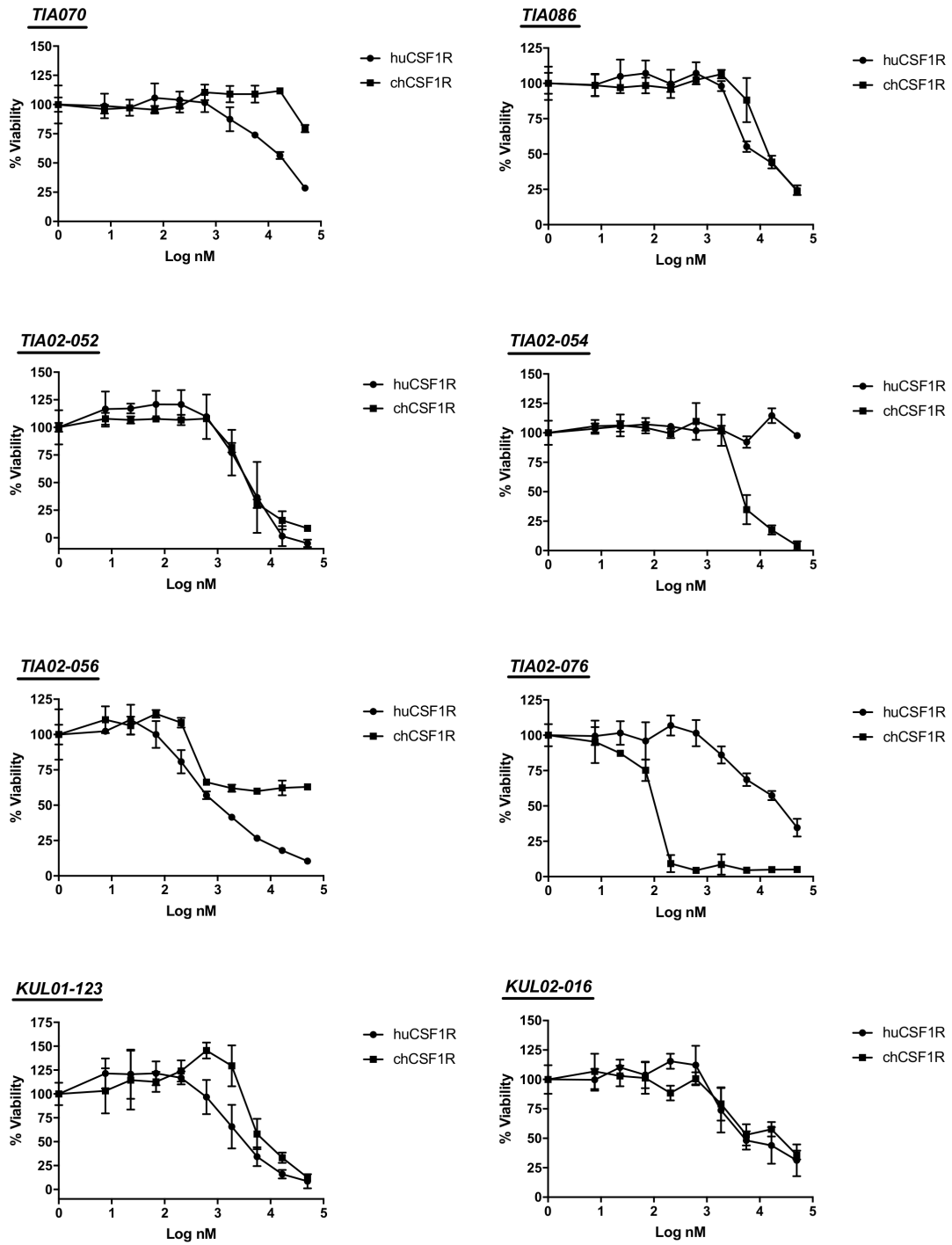


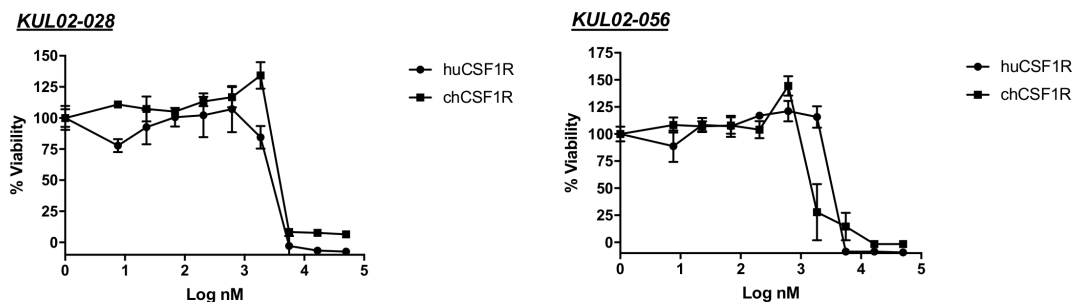
### Chapter 3: CSF1R kinase inhibitors

Ch 5.93  $\mu\text{M}$ ) and TIA02-076 ( $\text{IC}_{50}$  Hu 20.12  $\mu\text{M}$  – Ch 0.095  $\mu\text{M}$ ) were more potent in inhibiting chicken CSF1R cell proliferation activity. Compounds KUL02-028 and KUL02-056 were considered toxic as they also inhibited the survival of BaF3 cells in IL3.

In summary, *in vitro* assessment of the cell survival activity led to the first identification of two inhibitors that are selective for chicken CSF1R kinase, TIA02-054 and TIA02-076.

### Chapter 3: CSF1R kinase inhibitors





**Figure 3-11: Species specific cell proliferation inhibitory activity of CSF1R tyrosine kinase inhibitors**

For comparison of the metabolic activity of CSF1R inhibitor compounds on BaF3 cells expressing chicken CSF1R with BaF3 cells expressing human CSF1R, an MTT assay was performed. BaF3/huCSF1R and BaF3/ChCSF1R cell lines were selected for growth in recombinant human CSF1 protein ( $1 \times 10^4$  U/mL) and purified chicken CSF1 protein (100 ng/ml) respectively. Cells were harvested, washed and seeded in 96-well plate. They were induced with respective CSF1 protein in the presence of 10 CSF1R tyrosine kinase inhibitors. Both cell lines were treated with inhibitors in 3-fold dilution range from 50  $\mu$ M for 72 hrs at 37°C. Each data point represents average of three measurements  $\pm$  SD. Percentage viability (normalized response) curve against log dilution values of inhibitors used as TIA070, TIA086, TIA02-052, TIA02-054, TIA02-056, TIA02-076, KUL01-123, KUL02-016, KUL02-028 and KUL02-056 is drawn using GraphPad Prism 7 software. Representative data from three independent experiments is shown.

**Table 3-5: Summary of cell proliferation ( $IC_{50}$  values) of candidate CSF1R kinase inhibitors towards BaF3/HuCSF1R and BaF3/ChCSF1R cells.**

Inhibitors	$IC_{50}$ Human receptor / $\mu$ M	$IC_{50}$ Chicken receptor / $\mu$ M
TIA070	19.45 (14.90-25.52)	362 (181-2103)
TIA086	12.27 (9.00-16.83)	19.33 (14.87-25.30)
TIA02-052	4.26 (2.44-7.50)	4.66 (3.46-6.32)
TIA02-054	14.22 (7.61-29.41)	5.93 (4.03-8.82)
TIA02-056	1.26 (0.94-1.70)	29.97 (11.78-78.59)
TIA02-076	20.12 (15.40-26.42)	0.095 (0.067-0.13)
KUL01-123	3.97 (2.34-6.85)	11.87 (5.87-24.98)

KUL02-016	9.88 (5.92-16.84)	15.43 (9.51-25.07)
KUL02-028	Toxic	
KUL02-056	Toxic	

### 3.2.5 Effect of chicken CSF1R kinase inhibitors on chicken BMDMs

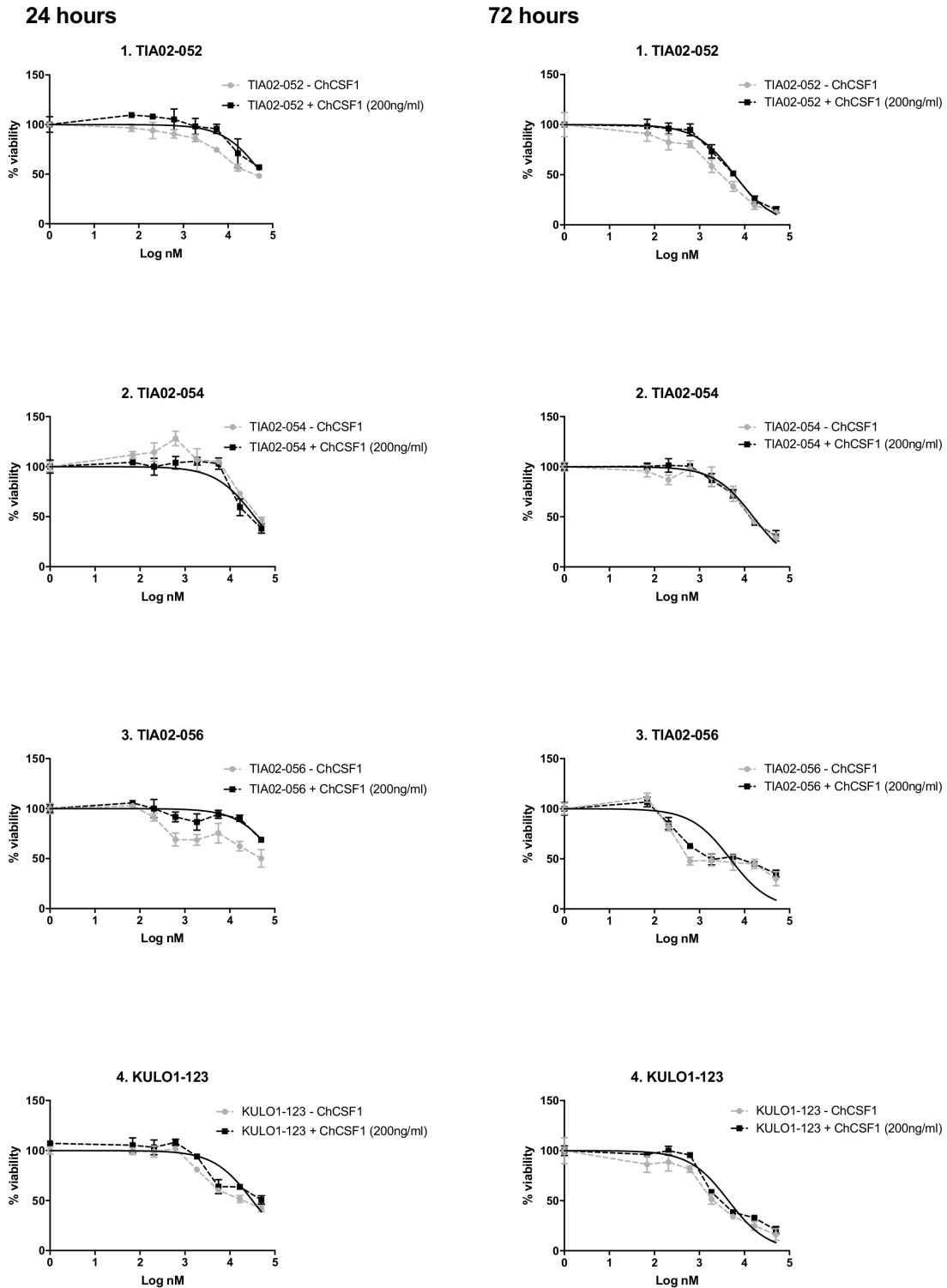
Several CSF1R kinase specific inhibitor compounds have been known to inhibit CSF1 induced growth of mononuclear phagocyte lineage cells. Inhibitor SU11248 which selectively targets class III and V RTKs, reduces human CSF1R phosphorylation, upon stimulation with human CSF1. Upon treatment of mouse bone marrow progenitor cells, SU11248 inhibits CSF1 dependent development of multinucleated osteoclasts (Murray et al., 2003). BLZ945 inhibited CSF1 dependent proliferation of murine BMDMs at IC50 value of 1 nM (Pyonteck et al., 2013). GW2580 inhibits CSF1 driven proliferation of monocytes (at 1  $\mu$ M), mouse tumour cells and CSF1 promoting osteoclasts development, bone degradation in humans and rats (Conway et al., 2005; Conway et al., 2008). PLX3397 is a potent CSF1R and KIT inhibitor which efficiently decreases cell viability of murine BMDMs and human monocyte derived macrophages in the presence of CSF1 (Yan et al., 2017). A novel CSF1R kinase inhibitor Ki20227 suppresses differentiation of osteoclasts *in vitro* and inhibits osteoclasts accumulation and bone destruction *in vivo* (Ohno et al., 2006). Ki20227 treatment has also been effective in suppressing disease severity of autoimmune diseases of the central nervous system and arthritis (Ohno et al., 2008; Uemura et al., 2008). These inhibitors have been widely studied for their potential therapeutic applications in treatment of several autoimmune, bone and tumour associated diseases.

In human and mouse macrophages studies, BMDMs are frequently used to investigate CSF1-induced signalling activity, CSF1R kinase or multi-kinase specific inhibitors activity (tables: 3-1 and 3-2) and gene regulation (Hume and Gordon 1983; Stacey et al., 1995; Irvine et al., 2006; Irvine et al., 2009). Potent and selective inhibitors for CSF1R kinase domains, CYC10268 and GW2580 block the growth and survival of murine BMDMs in the presence of CSF1 (Irvine et al., 2006; Priceman et al., 2010). LPS provides an alternative pro-survival signal to mouse BMDM. Murine BMDMs, stimulated with LPS and incubated in the presence of CYC10268 for 24 hrs were able to survive indicating that this pathway was not dependent

upon autocrine release of CSF1 (Sester et al., 1999; Irvine et al., 2006). A major difference between mouse and chicken BMDM is that once they have differentiated, chicken macrophages themselves produce high levels of *CSF1* mRNA, and no longer require exogenous growth factor. Mouse BMDM, on the other hand, undergo cell death when CSF1 is removed (Irvine et al., 2006). Accordingly, selected candidate CSF1R kinase inhibitors were tested for activity on chicken BMDM survival and proliferation *in vitro*.

Four candidate CSF1R kinase inhibitors from the BaF3/ChCSF1R cells screened inhibitors were selected: TIA02-052 (equal human and chicken CSF1R activity); TIA02-054 (more specificity for chicken CSF1R); TIA02-056 (more specificity for human CSF1R) and KUL01-123 (more specificity for human CSF1R). Cryo-preserved chicken bone marrow cells were thawed and differentiated in the presence of purified recombinant chicken CSF1 protein for seven days. To remove exogenous chicken CSF1, BMDMs were washed three times before seeding into 96-well plate. To test the survival and metabolic proliferation response, chicken BMDMs stimulated with or without purified chicken CSF1 (200 ng/ml) were incubated with the four respective CSF1R kinase inhibitors for short (24 hrs) and longer (72 hrs) durations. The addition of exogenous CSF1 made no difference to the survival of the chicken BMDM as assessed using the MTT assay. All the four CSF1R inhibitors inhibited cell survival/proliferation, regardless of the presence of added CSF1 (figure: 3-12). This finding confirms that chicken BMDMs, unlike their mouse counterparts, are factor-independent, most likely because they produce endogenous CSF1 which provides an autocrine signal that enables them to survive via CSF1R signalling.

Chicken BMDMs MTT assay with inhibitors



**Figure 3-12: CSF1R kinase inhibitors activity on chicken bone marrow derived macrophages survival**

The activity of four chicken CSF1R tyrosine kinase inhibitors (TIA02-052, TIA02-054, TIA02-056 and KULO1-123) was tested for the growth of chicken BMDMs, with or without induction of chicken CSF1. The chicken BMDMs culture was grown in purified

recombinant chicken CSF1 protein (200 ng/ml) for seven days, then harvested, washed two times and seeded at approximate cell density of  $4 \times 10^4$  in a 96-well plate. Cells were stimulated with or without chicken CSF1 (200 ng/ml) in the presence of 3-fold diluted inhibitors from 50  $\mu$ M for 24 hrs (graphs on left column) and 72 hrs (graphs on right column) at 37°C. For measure of cell viability MTT assay was performed. Each data point represents average of three measurements  $\pm$  SD. Percentage viability (normalized response) curve against log dilution values of inhibitors is drawn using GraphPad Prism 7 software.

### 3.3 Conclusion and discussion

To enable the study of CSF1R signalling dependent chicken macrophage biology, this Chapter aimed to generate and validate tools that can affect the survival and proliferation of chicken macrophages *in vitro*. The first half of this Chapter is based on the characterisation and large stock purification of endotoxin-free recombinant chicken CSF1 protein to be used as a source for culturing myeloid and primary chicken macrophages for *in vitro* studies with inhibitors.

Like the mammalian protein, chicken CSF1 is glycosylated, but the protein expressed in bacteria was equally active compared to mammalian-expressed chicken CSF1 in promoting macrophage differentiation (Garceau 2014). Our commercial partners, PAH, did not pursue chicken CSF1 further as a possible immuno-modulator, so the stock available for ongoing studies in our laboratory was limited and it was necessary for me to produce and characterize chicken CSF1. The protocol was relatively straightforward and successful and could be potentially-adapted to larger-scale production or for structure-function studies to determine the importance of individual amino acids in biological activity. Equivalent  $EC_{50}$  values 37.37 ng/ml and 31.37 ng/ml obtained in the dose response curves (figure: 3-9) of the recombinant chicken CSF1 protein and the material provided by commercial partners, PAH, was consistent with the evident purity of the protein (figure: 3-2 and 3-4).

Deposit of bacterial protein aggregates into insoluble inclusion bodies causes a major hindrance for the industrial market, restricting solubility and high yield of proteins (Villaverde and Mar Carrió 2003). However, following rigid treatment of the cell extract and preparative refolding steps, large scale purification of biologically active recombinant protein is possible (Vallejo and Rinas 2004). Depending upon the target protein, optimal incubation time and temperature varies post IPTG induction. Incubation at lower temperatures assist with solubility and folding of the expressed proteins (<https://international.neb.com/Protocols/0001/01/01/fusion-protein-expression-e6901>). Out of the two temperature conditions tested with IPTG induction, 37°C for 3 hrs and 25°C overnight for enhanced expression of chicken CSF1 protein, reduced

temperature of 25°C for longer duration of 16 to 18 hrs significantly improved the expression of chicken CSF1 protein as detected with western blot (figure: 3-2). Thus, this optimum temperature condition was utilised for batch processing. Use of anti-chicken CSF1 IgG1 mAb (ROS-AV183) confirmed the size and quality of the bacterially expressed, refolded chromatography purified chicken CSF1 protein, around 19 KDa size (figure: 3-4).

Chicken BMDM grown in CSF1 respond to LPS with a profound change in gene expression (Garcia-Morales et al., 2015). In mice at least, there is also evidence that low concentrations of LPS produce long-lasting effects on cellular phenotype in macrophages grown in CSF1 (Oppong-Nonterah et al., 2019). The issue of purified recombinant chicken CSF1 protein contamination with LPS was therefore addressed by use of endotoxin high affinity ligand poly- $\epsilon$ -lysine resin columns, which allows for >85% of protein recovery. Endotoxin assay performed to verify the absence of LPS levels using RAW264-ELAM eGFP reporter cell line confirmed the removal of endotoxins from batch-purified recombinant chicken CSF1 protein solution (figure: 3-7). This step came at a cost, since up to 50% of the chicken CSF1 was lost in the filtration step. This loss might be mitigated by optimising the amount of protein applied to the column. The chicken CSF1 protein produced here also contributed to the recently published work from our lab Bush et al., (2018), where it was used to culture chicken BMDMs for RNASeq analysis. This protocol optimized herein was taken over by Roslin Immunological Toolbox facility for mass purification of this chicken reagent. In principle, the bacterial recombinant chicken CSF1 could be applied *in vivo*. However, the protein is relatively small and below the threshold for renal clearance. In mammals, a CSF1-Fc fusion protein was shown to have a much longer circulating half-life and greater efficacy in promoting macrophage proliferation *in vivo* (Gow et al., 2014). A similar chicken CSF1-Fc protein was subsequently produced, again in collaboration with PAH, and shown to promote macrophage proliferation in chicks (Garceau et al., 2015). I was also involved in the initial preparations for the production of recombinant chicken CSF1-Fc fusion protein using a CHO cell line, which was then carried forward by Post Doc colleague Dr. Lucy Freem.

The analysis of candidate mammalian CSF1R kinase domain inhibitor compounds obtained from Norwegian drug development group in collaboration identified several promising molecules. Leaving aside compounds KUL02-028 and KUL02-056, which proved to be toxic, 8 compounds (TIA070, TIA086, TIA02-052, TIA02-054, TIA02-056, TIA02-076, KUL01-123, KUL02-016) exhibited variable efficacy on chicken and human CSF1R kinase domains



(figure: 3-11). The intra-cellular tyrosine kinase domain of CSF1R (AAs 540 to 977) is extremely conserved in all mammalian species and birds (Garceau et al., 2010). One key active site amino acid substitution, cysteine (C665) in mammals compared to arginine in birds has been proposed as an explanation for differential activity of GW2580. The R665 substitution is found in all avian CSF1R sequences (DA Hume, personal communication). Docking inhibitors to the homology model of chicken CSF1R, revealed amino acids glycine (G663) and aspartate (D664) to be more twisted when compared with the corresponding amino acids in human CSF1R, indicating internal differences in the inhibitors activity on chicken CSF1R (DA Hume, personal communication). Structural analyses of the auto-inhibited CSF1R kinase domain also implicate C665 in the binding of the more generic kinase inhibitor, Gleevec, and of a quinolone inhibitor series to the human CSF1R (Schubert et al., 2007; Walter et al., 2007). One other interesting difference between mammalian and avian CSF1R lies immediately adjacent to the activation loop. The DFG sequence is conserved in all kinases, and the aspartate (D796 in human, D792 in chicken) is the catalytic base in the phosphotransferase reaction (Schubert et al., 2007; Walter et al., 2007). The activation loop (D796 to P814 in human) is conserved in chicken (Garceau et al., 2010) but the immediately adjacent glycine (G795 in mammals) is substituted with cysteine (C791) in chicken and all birds (DA Hume, personal communication). Interaction with D796 is key to actions of CSF1R kinase inhibitors (Schubert et al., 2007; Walter et al., 2007). Each of these subtle amino acid changes could explain the differential activity of both compounds that are selective for inhibition of mammalian CSF1R, and those that are selective for avian CSF1R.

In conclusion, I have successfully reproduced the commercial methodology of purifying recombinant protein from bacterial expression system, by purifying recombinant chicken CSF1 protein. Several chicken CSF1R kinase specific inhibitor compounds were identified. They provide promising tools for *in vivo* studies of chicken macrophage biology, especially in combination with macrophage reporter transgenes.

## Chapter 4 *In vivo* effects of neutralising antibody to chicken CSF1

### 4.1 Introduction

As described in detail in Chapter 1, the macrophage growth factor CSF1 stimulates the survival, proliferation and differentiation of progenitor cells into mature mononuclear phagocyte lineage cells by signaling activity via the receptor CSF1R. A second ligand, IL34, can also signal through CSF1R. Biology of CSF1/IL34/CSF1R system is functionally conserved in birds (Garceau et al., 2010). Chicken is a widely used experimental model organism in studying developmental biology and development of the mononuclear phagocyte system (Garceau et al., 2010; Balic et al., 2014; Garceau et al., 2015; Hu et al., 2019), but the role of CSF1 has not been directly demonstrated. The overall project objective is to study the function of CSF1 in developmental biology of monocytes and macrophages in chickens by manipulating the expression or function of CSF1 and CSF1R *in vitro* and *in vivo*. Chapter 3 was based on identifying several inhibitor compounds targeting specifically chicken CSF1R kinase activity, with potential utility *in vivo*. Potent inhibitors of chicken CSF1R kinase TIA02-054 and TIA02-076 were identified. This Chapter takes advantage of the second tool; a novel chicken CSF1 specific neutralising monoclonal antibody ROS-AV183, that targets and blocks chicken CSF1R signalling activity.

In mice, a small proportion of CSF1 is normally filtered in the kidneys. (Tushinski et al., 1982; Bartocci et al., 1987). Most CSF1 is rapidly and selectively cleared from circulation under normal physiological conditions by mature macrophages via CSF1R receptor mediated endocytosis and intracellular degradation (Bartocci et al., 1987). When macrophages are depleted, or the CSF1 receptor is blocked or mutated, there is an increase in the concentration as well as half-life of CSF1 in circulation (Bartocci et al., 1987; Dai et al., 2002; MacDonald et al., 2010). As a consequence, tissue macrophages negatively regulate monocyte production from the bone marrow by controlling the circulating CSF1 concentration (Davies et al., 2013; Jenkins et al., 2013; Jenkins and Hume 2014; Hume et al., 2019) Therefore, elevation in circulating CSF1 levels for example by administration of exogenous CSF1 is saturable (Hume et al., 1988).

Trophic role of tissue macrophages is evident from studies on macrophage deficient mice and rats. *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice demonstrate severe deficiency in mononuclear phagocytes and osteoclasts along with other abnormalities in skeletal muscles, low body weight, absence of teeth eruption, male and female fertility, mammary gland development, neural development, dermal thickness, haematopoiesis and short life span (reviewed in (Pollard 2009; Jenkins and Hume 2014; Chitu and Stanley 2017)). A subset of the defects in *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice were rescued completely or partially by restoring circulating CSF1 levels (Wiktor-Jedrzejczak et al., 1990), by injection of human recombinant CSF1 in neonatal mice for longer duration of 3 months (Cecchini et al., 1994) or by use of full length CSF1 encoding transgene (Ryan et al., 2001; Dai et al., 2004). CSF1 signalling has also been known to play an important role in maintaining immunity, in cancer and in various other inflammatory disorders reviewed in (Chitu and Stanley 2006; Hume and MacDonald 2012). The abnormalities are predicted to be due to reduction in the trophic and/or scavenger functions of tissue macrophages regulated by CSF1, secondary to CSF1 concentration reduction in tissues (Cecchini et al., 1994; Stanley et al., 1997).

Several phenotypes of *Csf1r<sup>-/-</sup>* mice including abnormalities in development are more severe than *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice, attributed in part to the alternate signaling activity of the second ligand of CSF1R, IL34. Some of the pleiotropic phenotypes exhibited by CSF1 deficient *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice were reproduced in neonate mice subjected to regular subcutaneous injection of a neutralising anti-mouse CSF1 antibody until levels were elevated relative to adult levels (Wei et al., 2005). These include reduced growth rate; adult body weight; reduced bone marrow osteoclasts number inducing osteopetrosis; and reduced tissue macrophage density in liver, kidney, synovial membrane and dermis. The anti-mouse CSF1 antibody characterised in this study was effective in blocking all the three isoforms of CSF1 *in vivo*, namely secreted proteoglycan, glycoprotein found in circulation and cell surface glycoprotein and efficiently reduced tissue macrophages and osteoclasts (Wei et al., 2005). Alternatively, treatment of adult mice for three weeks with a blocking antibody against mouse CSF1R, reduced circulating monocyte precursor cells of resident tissue macrophages, preventing their replacement or local self-renewal and reducing a large proportion of tissue macrophages (excluding lung and reproductive tissues). Macrophage depletion in adults with anti-CSF1R did not mimic defects seen in CSF1 and CSF1R deficient mice (MacDonald et al., 2010; Sauter et al., 2014), suggesting that these are primarily associated with postnatal development. The gross

phenotypes observed in the *Csf1<sup>op</sup>/Csf1<sup>op</sup>* and *Csf1r<sup>-/-</sup>* mice (due to deficiency in mononuclear phagocytes and osteoclasts) significantly improved with age despite the lack of CSF1 (Begg et al., 1993; Dai et al., 2002), suggesting other growth factors can compensate in part for the deficiency.

One major difference between mice and chicken is that in mice, there is a substantial supply of CSF1 from the mother via the placenta. In mice, CSF1 locally regulates the differentiation of macrophages in the reproductive tract and placental growth (Arceci et al., 1989). CSF1 is expressed locally in the epithelium cells of the uterus in pregnant females throughout the gestation period. Also, *CSF1R* mRNA is exceptionally expressed by trophoblast cells that later develops into definitive layers of placenta, maintaining local maternal immunity against pathogens in placenta (Guleria and Pollard 2000). Ironically, a study involving the use of blocking anti-mouse CSF1R mAb demonstrated that macrophages of the reproductive tissue function independently of CSF1R signalling (MacDonald et al., 2010). In comparison, chicken embryo develops *in ovo*, presumably devoid of maternal CSF1 supply (although the presence of CSF1 in egg protein has not been excluded).

A monoclonal antibody against chicken CSF1R that recognises the expression of cell surface CSF1 receptor, as a definitive cell marker for bone marrow progenitor cells, circulating blood monocytes and tissue macrophages, labelled as ROS-AV170 was characterised earlier by colleagues from our laboratory (Garcia-Morales et al., 2013). However, when tested *in vitro* in a competitive BaF3/ChCSF1R cell survival assay this antibody failed to block chicken CSF1 mediated signalling. To further enable study of CSF1/CSF1R signalling mediated biology of mononuclear phagocyte cells in birds, a monoclonal antibody against CSF1R receptor ligand chicken CSF1 labelled ROS-AV183 was generated by immunising mice with conjugated CSF1-Fc protein and its biological activity characterised (Wu *et al*, manuscript in progress). Preliminary data indicated that anti-chicken CSF1 mAb (hereby referred as anti-ChCSF1 mAb) could block chicken CSF1 mediated signalling based on an *in vitro* BaF3/ChCSF1R cell viability assay, described in more detail in Chapter 3 section 3.2.2.2. However, the question remains: how will this neutralising anti-ChCSF1 mAb impact chicken macrophages *in vivo*? A capture ELISA assay developed to measure circulating CSF1 levels in chickens, indicated that CSF1 is expressed at an increasing level in the layer chickens around the day of hatch, within the first 24 to 48 hrs, with subsequent variability thereafter up until day 7 in post-hatch birds (Wu *et al*, manuscript in progress). Accordingly, based upon these preliminary data, in this

project it was decided to test anti-ChCSF1 mAb *in vivo* in hatchling chickens. As mentioned previously in the introduction Chapter 1 (section 1.10), *CSF1R*-eGFP and *CSF1R*-mAPPLE reporter transgenic chicken lines that have been widely used to study the development and functions of macrophages and their subsets (Balic et al., 2014; Garceau et al., 2015; Sutton et al., 2018; Hu et al., 2019) will be utilised in this project to examine the *in vivo* effects of the neutralizing anti-ChCSF1 mAb on immune cells and lymphoid organs of chicken. Accordingly, the objectives of this Chapter are,

- To purify and generate a large stock of anti-ChCSF1 mAb and isotype control mAb reagents
- To determine the ability of anti-ChCSF1 mAb to block CSF1 signalling *in vitro*
- To determine the impact of neutralizing anti-ChCSF1 mAb *in vivo* on macrophage numbers, immune cells, development and overall survival on post-hatch *CSF1R*-eGFP/*CSF1R*-mAPPLE reporter transgenic chicken lines
- To detect expression of chicken CSF1 on lymphoid organs using anti-ChCSF1 mAb

## 4.2 Results

### 4.2.1 Batch purification of anti-CSF1 and control antibody

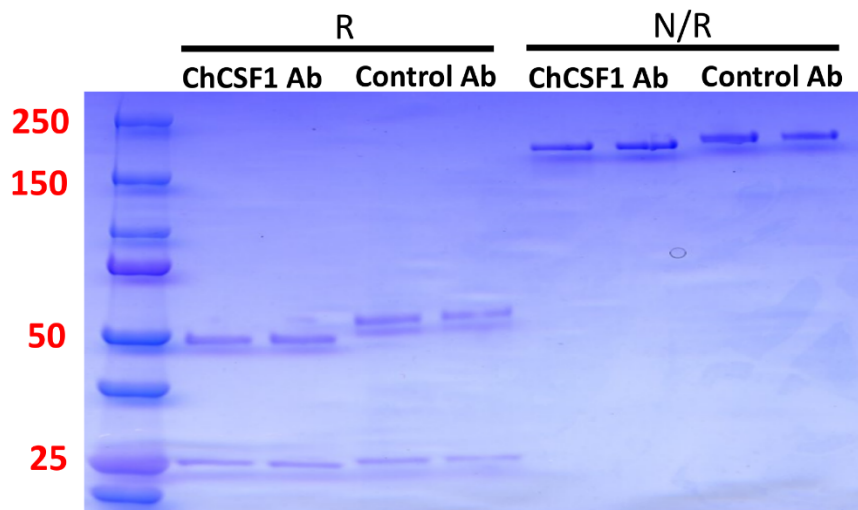
Production of chicken chimeric protein CSF1-Fc formed by the fusion of active chicken CSF1 domain and the Fc region of the immunoglobulin IgY constant heavy chains domains 3-4, have been described earlier (Garceau et al., 2015). The purpose was to increase the size of the CSF1 protein just above the renal clearance threshold, thereby increasing its circulating half-life, and retaining macrophage growth-promoting activity for *in vivo* injection experiments. A similar construct was also generated for pig CSF1 protein (Gow et al., 2014). To analyse the function of CSF1-dependent macrophages in birds, it was essential to generate a non-inflammatory chicken-specific novel tool to help gain knowledge about the chicken immune system function. The obvious next step after successful purification and validation of chicken CSF1-Fc protein on embryonic macrophages and hatchling birds (Garceau et al., 2015) by colleagues from our laboratory, was expansion of this study by generating an antibody against chicken CSF1 that

could potentially elevate macrophage numbers *in vivo*. Accordingly, anti-ChCSF1 mAb (clone ROS-AV183) was produced using chicken CSF1-Fc as an immunogen in mice (Wu *et al*, manuscript in progress), and utilised in this project to examine the biology of chicken mononuclear phagocytes and immune cells.

Anti-ChCSF1 mAb was characterised recently by western blot analysis, immuno-fluorescence, FACS and chicken macrophages cell survival assay (Wu *et al*, manuscript in progress). For the purpose of this thesis, in order to determine the biological activity of ROS-AV183 on the development of chicken macrophages both *in vitro* and *in vivo*, it was necessary to first produce a large stock of functionally active anti-ChCSF1 mAb as well as isotype control mAb. Accordingly, I made use of cryo-preserved mouse anti-ChCSF1 mAb (clone, ROS-AV183) (which was identified as an IgG1 antibody) and a control IgG1 mAb (clone, mouse anti-ovine CD335 (GR13.1)) hybridoma cells to culture large preparations of respective culture supernatants using standard cell culture procedures. Purification of monoclonal antibodies was performed using a HiTrap protein G column by affinity chromatography as described in Chapter 2 section 2.6.2. The column contains protein G sepharose beads that are able to capture broad range of IgG antibodies from most eukaryotic species with higher affinity (<https://www.gelifesciences.com/en/us/shop/chromatography/prepacked-columns/affinity-specific-groups/hitrapprotein-g-hp-antibody-purification-columns-p-03524>). Monoclonal antibodies were purified from eight separate preparations under sterile conditions, quantified using nanodrop measurements and pooled together after assessing purity and size with SDS PAGE from each batch (data not shown). Batch hybridoma culture supernatant processing and purification, yielded a total of 56 mg of anti-ChCSF1 mAb and 27 mg of isotype control mAb. Antibodies were further concentrated to prepare a stock solution of 5 mg/ml for the *in vivo* subcutaneous injection experiment (see below section 4.2.3).

Figure: 4-1 shows SDS PAGE gel demonstrating the purity and integrity of the pooled antibody preparations under reducing (R) and non-reducing conditions (N/R). Denaturation of anti-ChCSF1 mAb in reducing condition revealed two sharp bands of heavy chain 50 KDa and light chain 25 KDa, consistent with the structure of a typical IgG molecule (Janeway et al., 2001). Denaturation of the control mAb in reducing condition suggested a somewhat larger heavy chain of around 60 KDa and light chain of 25 KDa. Antibodies in non-reducing conditions revealed molecular weight of around 150 KDa for anti-ChCSF1 mAb and around 170 KDa for isotype control mAb, consistent with the molecular weight combinations of their two heavy

and two light chains. The difference in molecular weight suggests that the control is not precisely the same isotype, but the nature of the difference is unclear. Since the mouse Fc region is unlikely to signal in chickens, the GR13.1 antibody is an appropriate control Ig.



**Figure 4-1: Characterisation of purified mouse anti-ChCSF1mAb and isotype control IgG1 mAb by SDS PAGE**

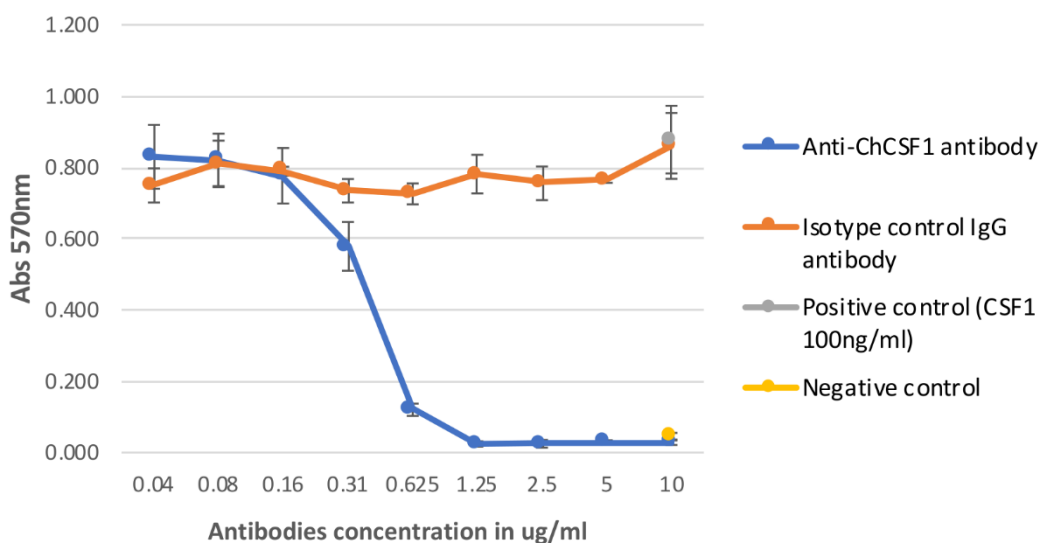
Quality and integrity of pooled aliquots of anti-ChCSF1 mAb and isotype control mAb was assessed by SDS PAGE gel electrophoresis. 10 µg aliquots of anti-ChCSF1 mAb and isotype control mAb were separated in reducing (R) and non-reducing conditions (N/R) as indicated.

#### 4.2.2 Anti-CSF1 antibody blocks CSF1 signalling *in vitro*

Monoclonal antibody against CSF1 has only been reported in humans. An anti-human CSF1 mAb inhibited the growth of CSF1R reporter murine myeloid cell line in response to recombinant bacterial or mammalian expressed CSF1 (Halenbeck et al., 1989; Nakoinz et al., 1990). The anti-mouse CSF1 antibody that binds and blocks CSF1 *in vivo* was a purified hetero-antiserum, stabilised with polyethylene glycol (PEG) (Wei et al., 2005). In chickens, a mAb against chicken CSF1R that binds blood monocytes and chicken macrophages was developed previously to enable studies of chicken macrophages (Garcia-Morales et al., 2013) but this antibody did not block CSF1 signalling in *in vitro* bioassay. Preliminary data with the recently generated novel mAb to chicken CSF1 (ROS-AV183) indicated that preincubation of chicken CSF1 with ROS-AV183 neutralized the ability of CSF1 (Wu *et al*, manuscript in progress) to promote the growth and proliferation of BaF3 cells ectopically expressing chicken CSF1R (BaF3/ChCSF1R) (Garceau et al., 2010). In order to confirm that the purified stock of

anti-ChCSF1 mAb produced for studying the growth and development of CSF1 dependent chicken macrophages in subsequent *in vitro* and *in vivo* experiments, is able to neutralize and block CSF1 mediated CSF1R signaling activity, I performed a cell viability assay using IL-3 dependent BaF3/ChCSF1R cells to measure cell survival activity (described in Chapter 3, section 3.2.2.2).

BaF3/ChCSF1R cells were cultured in the optimised concentration of 100 ng/ml of endotoxin-free purified recombinant chicken CSF1 protein (purification steps and functional characterisation is described in Chapter 3, section 3.2.1) and incubated with anti-ChCSF1 or isotype control mAb for 72 hrs. Anti-ChCSF1 mAb was able to completely block the CSF1-dependent growth of BaF3/ChCSF1R cells at a concentration of around 1.25 µg/ml (figure: 4-2). The relative stoichiometry, 1.25 µg/ml of anti-ChCSF1 mAb blocking 100 ng of growth factor chicken CSF1 suggested it would be possible to achieve an inhibitory concentration *in vivo*.



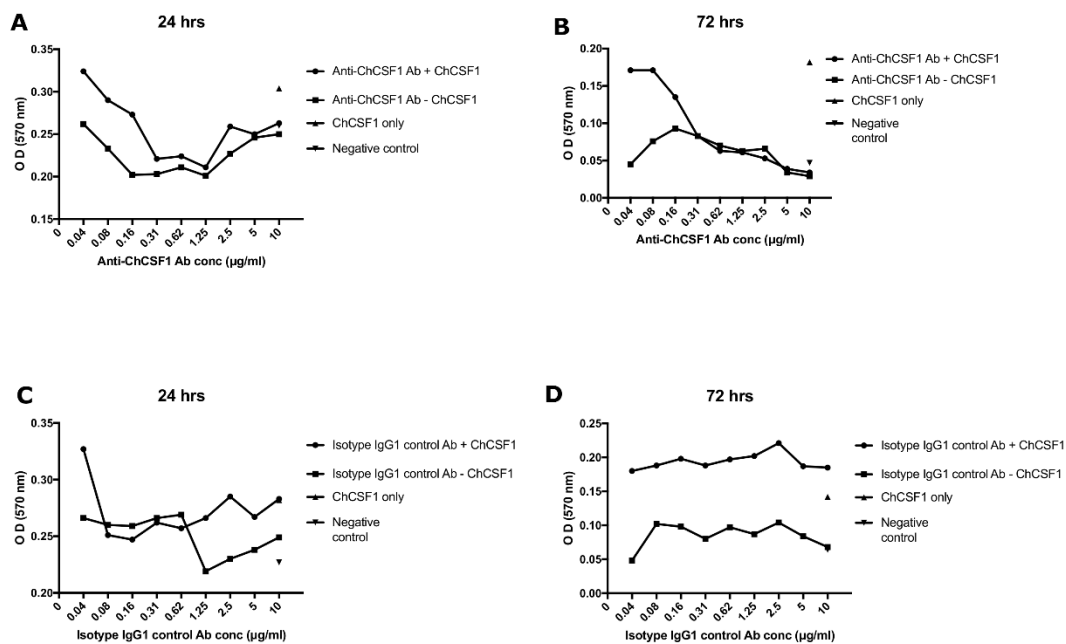
**Figure 4-2: Effect of anti-ChCSF1 mAb on chicken CSF1/CSF1R signalling**

BaF3/ChCSF1R cells culture grown in chicken CSF1 were harvested washed and seeded into 96-well plate at cell density of  $4 \times 10^4$ . Cells were then incubated with purified chicken CSF1 (100 ng/ml) and two-fold serially diluted purified anti-ChCSF1 or isotype control mAb from 10 µg/ml for 72 hrs at 37°C. Wells containing cells with chicken CSF1 was positive control and wells without cells, but media only as negative control. MTT assay was performed as described in Chapter 2, section 2.4. Each data point represents average of three measurements +/- SD. Blank subtracted absorbance readings at 570 nm is plotted against antibodies concentration.



#### Chapter 4: *In vivo* effects anti-CSF1 antibody

To extend the findings to primary chicken macrophages, a 7-day culture of chicken BMDMs grown in recombinant chicken CSF1 protein was treated with or without chicken CSF1 at optimised concentration in the presence of anti-ChCSF1 or isotype control mAb. The MTT assay was performed at two time points of shorter (24 hrs) or longer duration (72 hrs). At both the time points anti-ChCSF1 mAb inhibited survival of chicken BMDMs at concentration of around 0.31  $\mu\text{g/ml}$ , irrespective of the presence or absence of growth factor chicken CSF1 (figure: 4-3A 24 hrs, 4-3B 72 hrs). However, by 72 hrs anti-ChCSF1 mAb led to complete inhibition of chicken BMDMs survival. Treatment with control mAb with or without the presence of CSF1 had no effect on BMDMs survival at either 24 hrs (figure: 4-3C) or 72 hrs (figure: 4-3D). Note that chicken BMDM, unlike mouse, also survived without CSF1 for at least 3 days in the MTT assay (figure: 4-3D) consistent with the presence of an autocrine CSF1 signal. This result supports the findings from Chapter 3 section 3.2.5 regarding autocrine CSF1 signalling, factor independent growth of chicken BMDMs.



**Figure 4-3: Effect of anti-ChCSF1 mAb on the survival of chicken bone marrow derived primary macrophages**

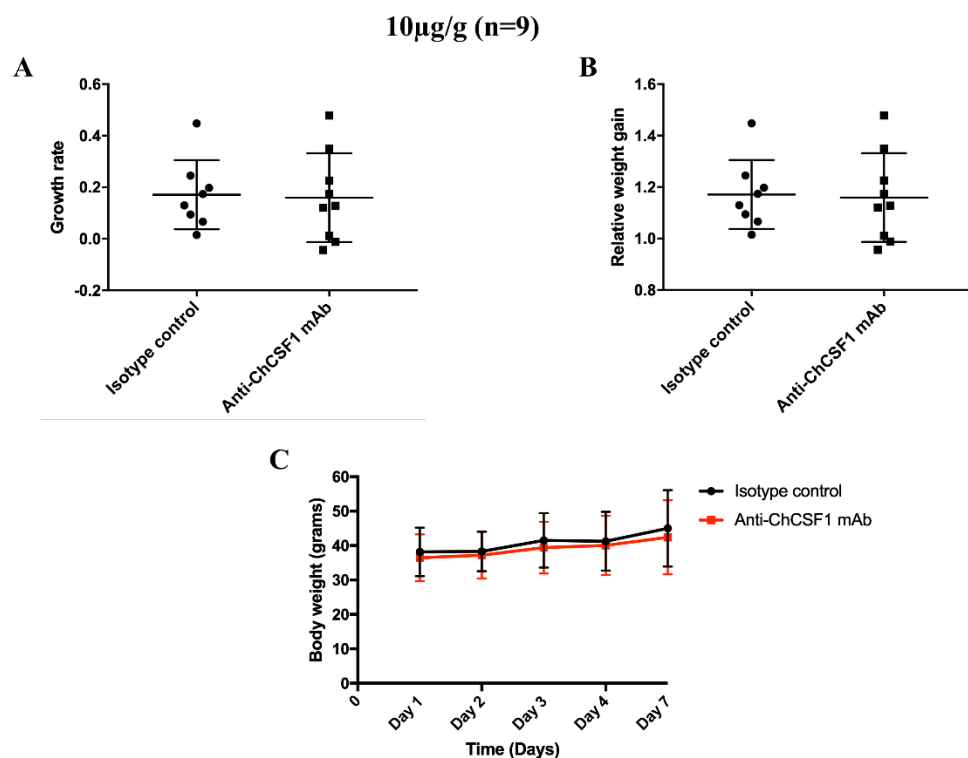
Batch protein G column purified stock of anti-ChCSF1 mAb was tested for survival of bone marrow derived primary macrophages by MTT assay at two time points-24 hrs (A, C) and 72 hrs (B, D). Seven-day culture of chicken BMDMs, grown in purified recombinant chicken CSF1 at 200 ng/ml was harvested, washed two times and seeded at an approximate cell density of  $4 \times 10^4$  in 96-well plate. Cells were stimulated with two-fold dilution concentrations from 10 µg/ml of purified anti-ChCSF1 mAb or isotype control mAb in the presence or absence of chicken CSF1 (200 ng/ml) at 37°C. Cells stimulated with chicken CSF1 and cells grown without CSF1 were used as positive control and negative controls respectively. MTT assay was performed as described in Chapter 2, section 2.4. Each data point represents an average of three measurements. Blank subtracted absorbance readings at 570 nm are plotted against the antibody concentrations.

To test the effect of anti-CSF1 *in vivo* on post-hatch development, newly hatched *CSF1R*-eGFP reporter transgenic birds (Balic et al., 2014) were weight matched and subcutaneously injected with 500 µg (calculated as 10 mg/kg = 10 µg/g) of anti-ChCSF1 mAb (ROS-AV183) or isotype control mAb on days 1, 2, 3 and 4 of age. The amount injected is arbitrary, but if the bird was considered as entirely extracellular space with a density of 1 mg/ml (the density of water), the amount injected would give a concentration of at least 10 µg/ml. Birds were monitored closely after every hour post-injection for a few hours and then each day for any signs of discomfort up until the last day (day 7) before sacrifice. Some birds experienced transient discomfort, causing swelling or haematoma at the puncture site, but overall no adverse effects on health

was observed throughout the term of this experiment. No treated bird failed to consume food or drink or became unconscious post-injection. The birds developed normally.

### 4.2.3 Effect of anti-CSF1 mAb on growth of hatchling chickens

3-week old transgenic mice with ubiquitous KO homozygous *Csf1* gene demonstrate significantly delayed growth rate as well as decreased body weight (Harris et al., 2012). *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice and mice injected with neutralizing anti-mouse CSF1 Ab during the post-natal period demonstrate similar significant reduced growth rate and adult body weight phenotype (Wei et al., 2005; Jenkins and Hume 2014). Rats homozygous for the CSF1R mutation by 11-weeks of age are smaller in body size compared to wild type and CSF1R heterozygous littermates (Pridans et al., 2018). To study the effect of anti-ChCSF1 mAb on growth rate, the body weights of all MacGreen hatchling birds were determined immediately before anti-ChCSF1 mAb injections. Anti-ChCSF1 mAb injections were performed on days 1, 2, 3, 4 and on the last 7<sup>th</sup> day before cull. Anti-ChCSF1 mAb did not have any effect on the average growth rate (figure: 4-4A) nor relative weight gain compared to birds injected with control antibody (figure: 4-4B, 4-4C).



**Figure 4-4: Effect of anti-ChCSF1 mAb on growth of hatchling chickens**

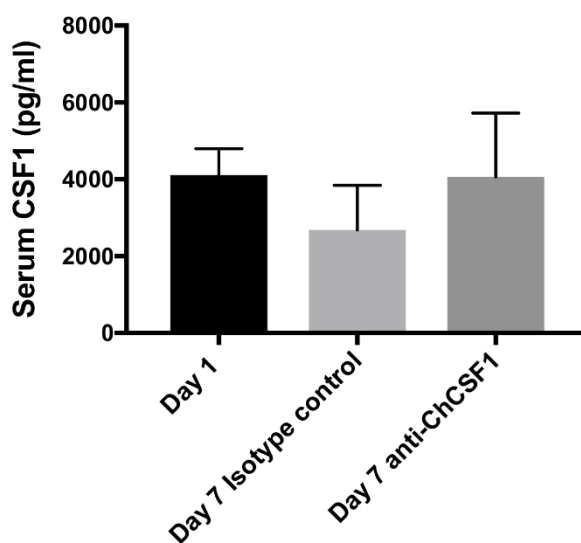
*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500  $\mu$ g dose (10  $\mu$ g/g) of either isotype control mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Body weight was recorded prior to each injection and on the last 7th day of the experiment. Graphs show: (A) each individual hatchling growth rate, mean growth rate  $\pm$  SEM and relative weight gain (normalized to day 1); (B) mean relative weight gain  $\pm$  SEM. A non-parametric Mann Whitney test was performed to assess significance between the two groups. (C) Shows the mean body weight change  $\pm$  SEM during all the days of injection experiment.

#### 4.2.4 Effect of anti-CSF1 mAb on circulating CSF1 levels

In humans, the serum CSF1 levels at birth was found to be three times higher than in adults and ranged from 12-15 ng/ml (Roth and Stanley 1995). In mice, foetal and early post-natal serum CSF1 levels was found to be highest, ranging from 30-40 ng/ml (Roth and Stanley 1996). In addition, this foetal and neonatal serum CSF1 level significantly declines with advancing age in both humans and mice. In chickens, expression of circulating CSF1 measured by ELISA in post hatch layer bird's serum was found to be in the range of 3-5 ng/ml up until day 14 of age (Wu *et al*, manuscript in progress). In order to determine whether the neutralising anti-ChCSF1 mAb treatment can elevate or have any impact on the concentration of circulating CSF1, a capture ELISA was performed on the serum samples collected on day 1 without any

treatment and on day 7 post anti-ChCSF1 or isotype control mAb injections. The capture ELISA was kindly performed by Dr. Zhiguang Wu.

There is a large increase in serum CSF1 concentration following anti-mice CSF1R injection in 3-weeks old mice (MacDonald et al., 2010) and also in *Csf1r* knockout mice and rats (Dai et al., 2002; Pridans et al., 2018) due to the loss of receptor-mediated clearance. Treatment with anti-CSF1 could increase (if there was compensatory production or prevention of receptor-mediated clearance), decrease (if antibody promoted clearance or interfered with the assay) or have no effect on circulating CSF1. Based upon the ability of the anti-CSF1 to prevent receptor stimulation, treatment might mimic the effect of blocking anti-CSF1R antibody. However, the concentration of circulating CSF1 on day 7 ( $4.06 \pm 1.66$  ng/ml) was identical to the circulating concentration on day 1 ( $4.11 \pm 0.68$  ng/ml) prior to treatment (figure: 4-5). The level of circulating CSF1 in hatchling birds treated with isotype control mAb was not significantly different ( $2.68 \pm 1.16$  ng/ml). Thus, surprisingly there was no detectable effect of anti-ChCSF1 mAb on the levels of circulating chicken CSF1 post treatment in hatchling birds.



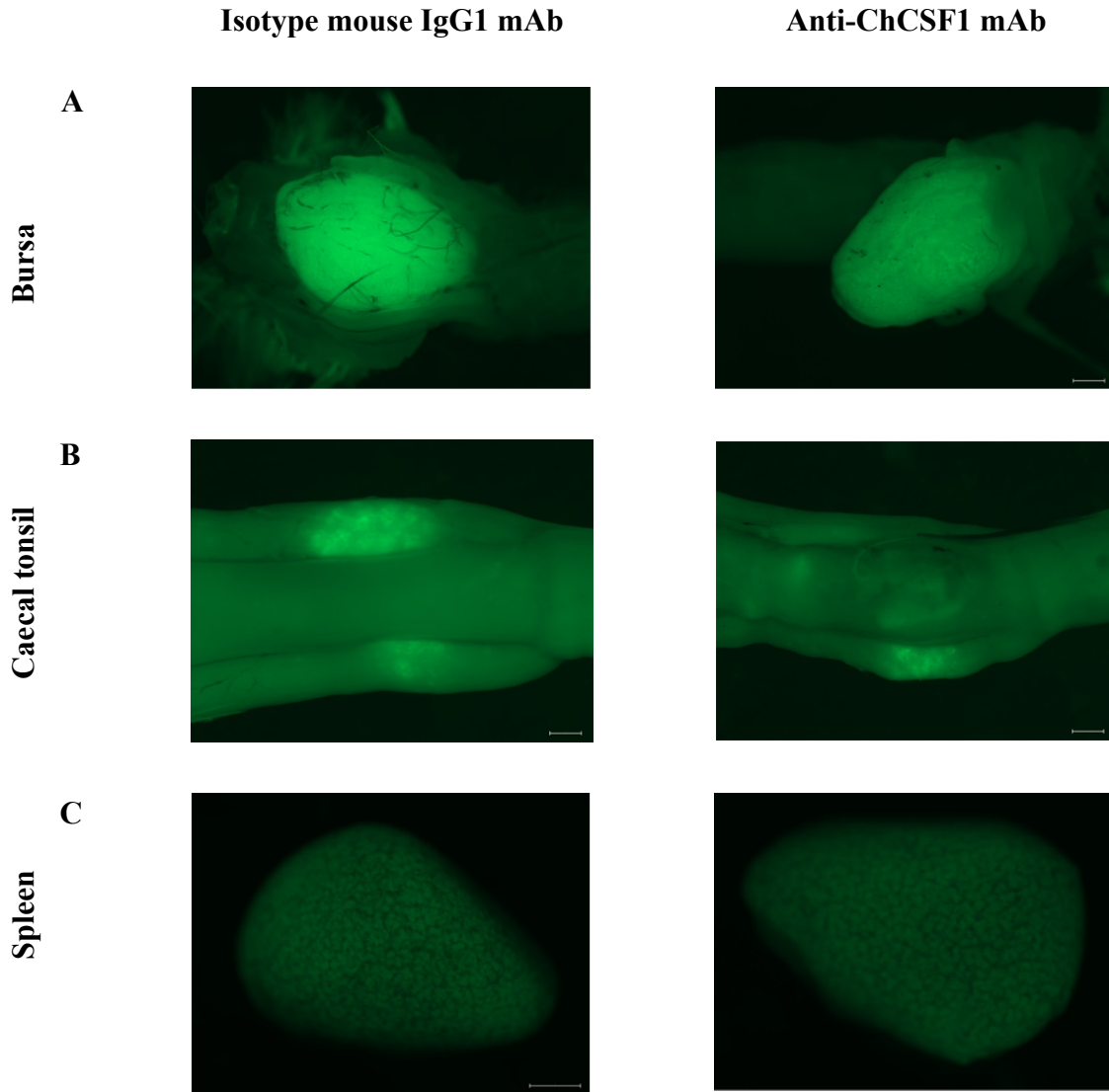
**Figure 4-5: Effect of anti-ChCSF1 mAb on blood serum CSF1 concentrations**

*CSF1R*-mAPPLE transgenic reporter hatchling chicken were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control mAb (n = 6) or anti-ChCSF1 mAb (n = 6) on days 1, 2, 3, 4 of age and humanely culled on day 7. On day 1, eight hatchlings were culled, and blood sera collected to define baseline serum CSF1 concentration. On day 7 blood from two groups of birds was collected in heparin coated tubes post sacrifice. Blood serum was separated and stored at -20°C until capture ELISA was performed, as described in Chapter 2, section 2.7.4. CSF1 concentration for each hatchling chicken was measured in triplicates.

Individual value plot showing CSF1 levels (pg/ml) as indicated was drawn using MiniTAB software.

### 4.2.5 Effect of anti-CSF1 mAb on lymphoid organs

To investigate the actions of anti-ChCSF1 mAb on tissues, major chicken lymphoid organs were examined for distribution of the *CSF1R*-eGFP transgene reporter expressing cells (Balic et al., 2014). Representative whole mount images comparing treated and untreated bursa, caecal tonsils and spleen are shown in figure: 4-6. *CSF1R*-eGFP transgene fluorescence intensity was clearly reduced in bursa (figure: 4-6A) and caecal tonsil (figure: 4-6B) of anti-ChCSF1 treated birds compared to the tissue of birds receiving the control mAb. There was no difference in the expression of *CSF1R*-eGFP transgene fluorescence intensity in spleen (figure: 4-6C). There were no signs of inflammatory reactions on these tissues post anti-ChCSF1 mAb treatment.

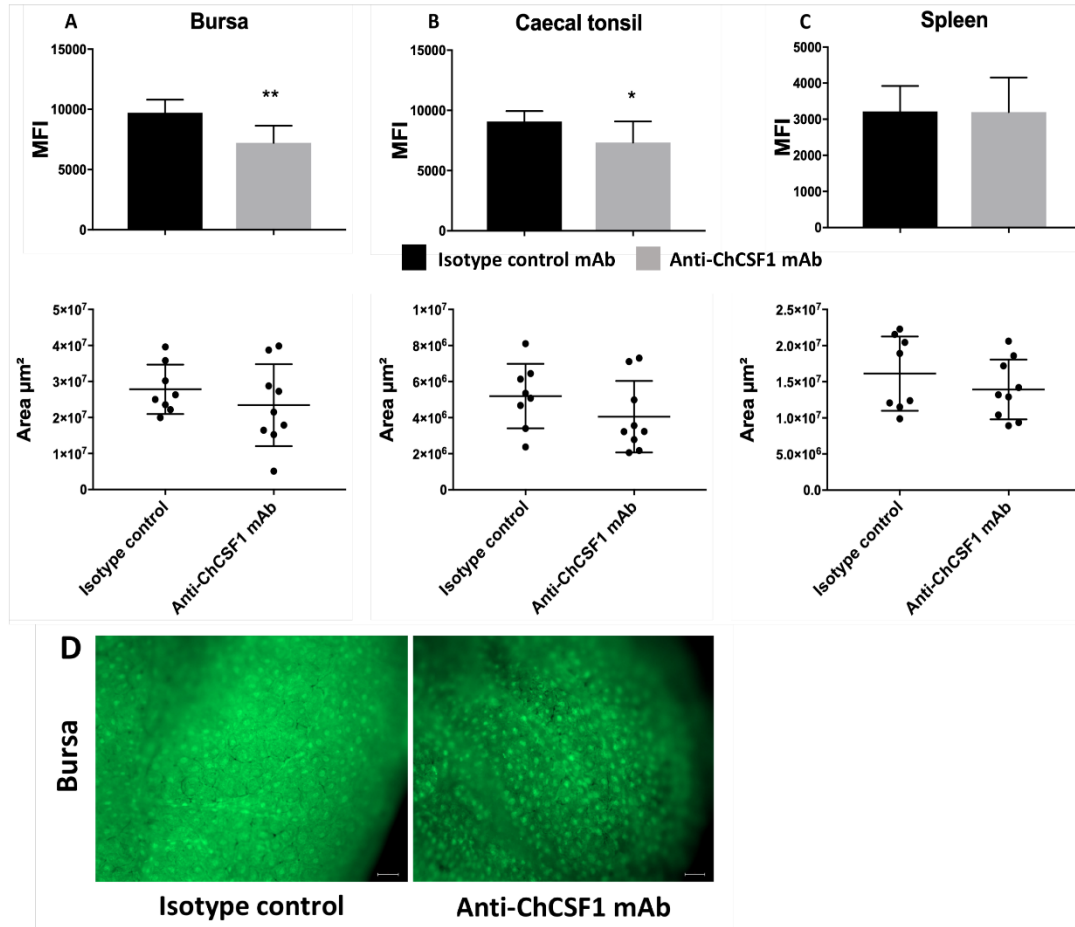


**Figure 4-6: Effect of anti-ChCSF1 mAb on lymphoid organs**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Lymphoid tissues were collected immediately in ice cold PBS and placed in petri dish containing PBS for imaging under UV light using a green fluorescence filter for an appropriate constant amount of time. Images were captured by Dr. Adam Balic using Zeiss Axio Cam HR microscope. Representative whole mount images of (A) Bursa, (B) Caecal tonsil, (C) Spleen are shown. Scale bar = 1mm

Surface area and mean fluorescence intensity (MFI) values of transgene expression was obtained from whole mount images analysis using Zeiss blue software. Control spleen surface area was very strongly correlated to body weight (Spearman rho value  $r = 0.905$ ) and strongly ( $r = 0.714$ ) and moderately ( $r = 0.524$ ) correlated to caecal tonsil and bursa respectively (data

not shown). There was no significant difference in the MFI or the surface area of spleen (figure: 4-7C) but a significant decrease in the MFI of transgene expression was detected in both caecal tonsil (figure: 4-7B) and bursa (figure: 4-7C) after anti-ChCSF1 mAb injection. The surface area of bursa and caecal tonsil tissue remained unaffected. The decrease in the *CSF1R*-eGFP transgene expression in bursa in anti-ChCSF1-treated birds is shown in figure: 4-7D at higher magnification.



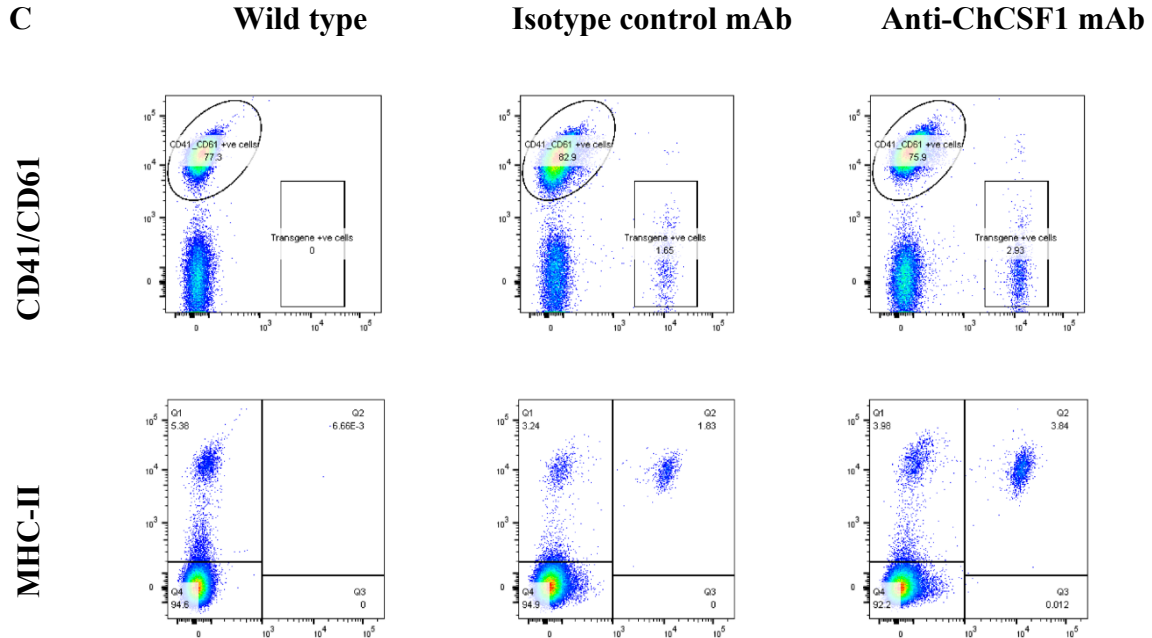
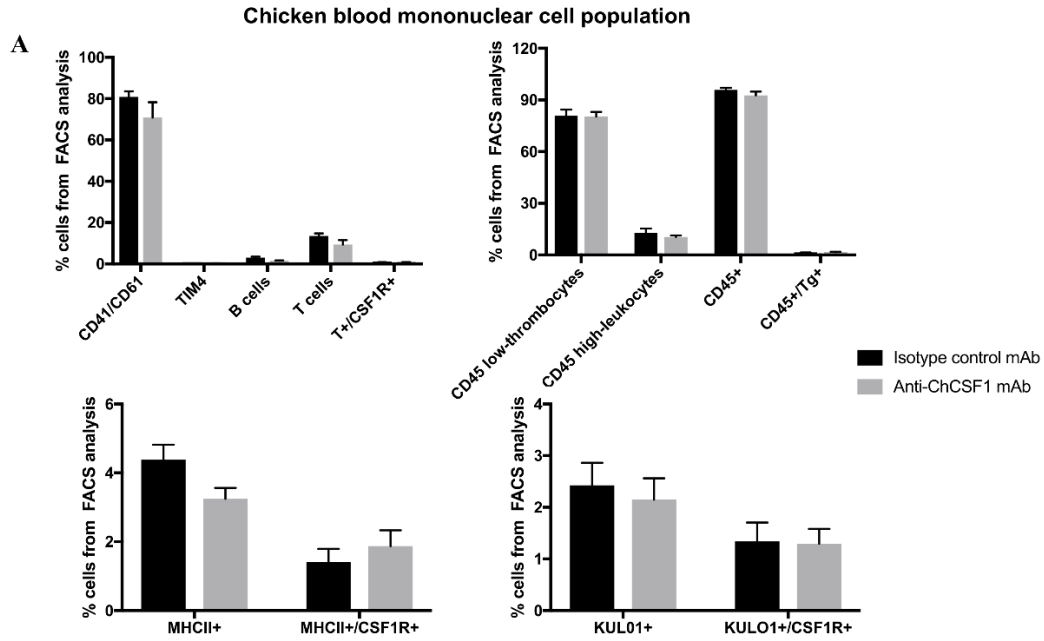
**Figure 4-7: Effect of anti-ChCSF1 mAb on *CSF1R*-eGFP transgene expression**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with a 500  $\mu\text{g}$  dose (10  $\mu\text{g}/\text{g}$ ) of either isotype control mAb ( $n = 8$ ) or anti-ChCSF1 mAb ( $n = 9$ ) on days 1, 2, 3, 4 of age and humanely culled on day 7. *CSF1R*-eGFP expression on lymphoid tissues was quantified from the whole mount images of (A) Bursa, (B) Caecal tonsil and (C) Spleen. Images were processed using Zen blue 2012 software measuring MFI and surface area. Graphs were plotted on GraphPad Prism 7 software as the mean  $\pm$  SEM. Mann Whitney test was performed to assess significance between the two groups. Significance is indicated as \* $P < 0.05$ , \*\* $P < 0.01$ . (D) Shows comparison of *CSF1R*-eGFP transgene expression in bursa between the two groups at higher magnification 0.2 mm.



#### 4.2.6 Effect of anti-CSF1 mAb treatment on blood PBMC

In chicken hatchling birds, injection with CSF1-Fc induced leucocytosis, increasing white blood cells population CD45<sup>hi</sup>, B cells and T cells number. Blood monocytes were increased in proportion to corresponding loss of thrombocytes (Hume et al., 1988; Garceau et al., 2015). In MacGreen chickens, *CSF1R*-eGFP reporter transgene is co-expressed with the surface CSF1R on blood monocytes, expressed in low levels in thrombocytes, and is absent in lymphocytes (T and B cells) (Balic et al., 2014). Figure: 4-8C shows the profiles of PBMC from wild type chicken and *CSF1R*-eGFP birds treated with anti-ChCSF1 or isotype control mAb. Blood cells were stained for chicken thrombocyte marker CD41/61, monocyte markers MHC-II, KUL01, TIM4, leucocytes marker CD45 and lymphocytes marker BU-1, CD3. The proportions of the different cell populations were variable between birds, regardless of treatment. There was no impact of the anti-ChCSF1 mAb on the thrombocyte population, which in chickens are equivalent to mammalian blood coagulating platelets (Ferdous et al., 2016). In contrast to rise in the leukocyte CD45<sup>+</sup> and lymphocytes B cell (BU-1<sup>+</sup>), T cell (CD3<sup>+</sup>) numbers in chicken CSF1-Fc injected hatchling birds (Garceau et al., 2015), injections with anti-ChCSF1 mAb in post-natal chickens had no significant effect on CD45<sup>low</sup> (or thrombocytes), CD45<sup>high</sup> (or leukocytes), total CD45<sup>+</sup> population and T cell lymphocytes, as indicated in figure: 4-8A. The ratio of CSF1R transgene expression (mean monocyte number) to CD45<sup>high</sup> and CD3<sup>+</sup> T cells was elevated indicating a relative decrease in these leukocyte population post anti-ChCSF1 mAb treatment (figure: 4-8B). There was marginal reduction in the blood monocyte population MHC-II<sup>+</sup> (which labels both B cells and monocytes), BU-1<sup>+</sup> and chicken monocyte restricted marker KUL01<sup>+</sup> cells (Mast et al., 1998) (figure: 4-8A). There was no significant effect of anti-ChCSF1 mAb on MHC-II<sup>+</sup>/CSF1R<sup>+</sup> and KUL01<sup>+</sup>/CSF1R<sup>+</sup> monocytes (figure: 4-8A). In summary, anti-ChCSF1 mAb had little influence on blood haematopoiesis in the first seven days following hatch.



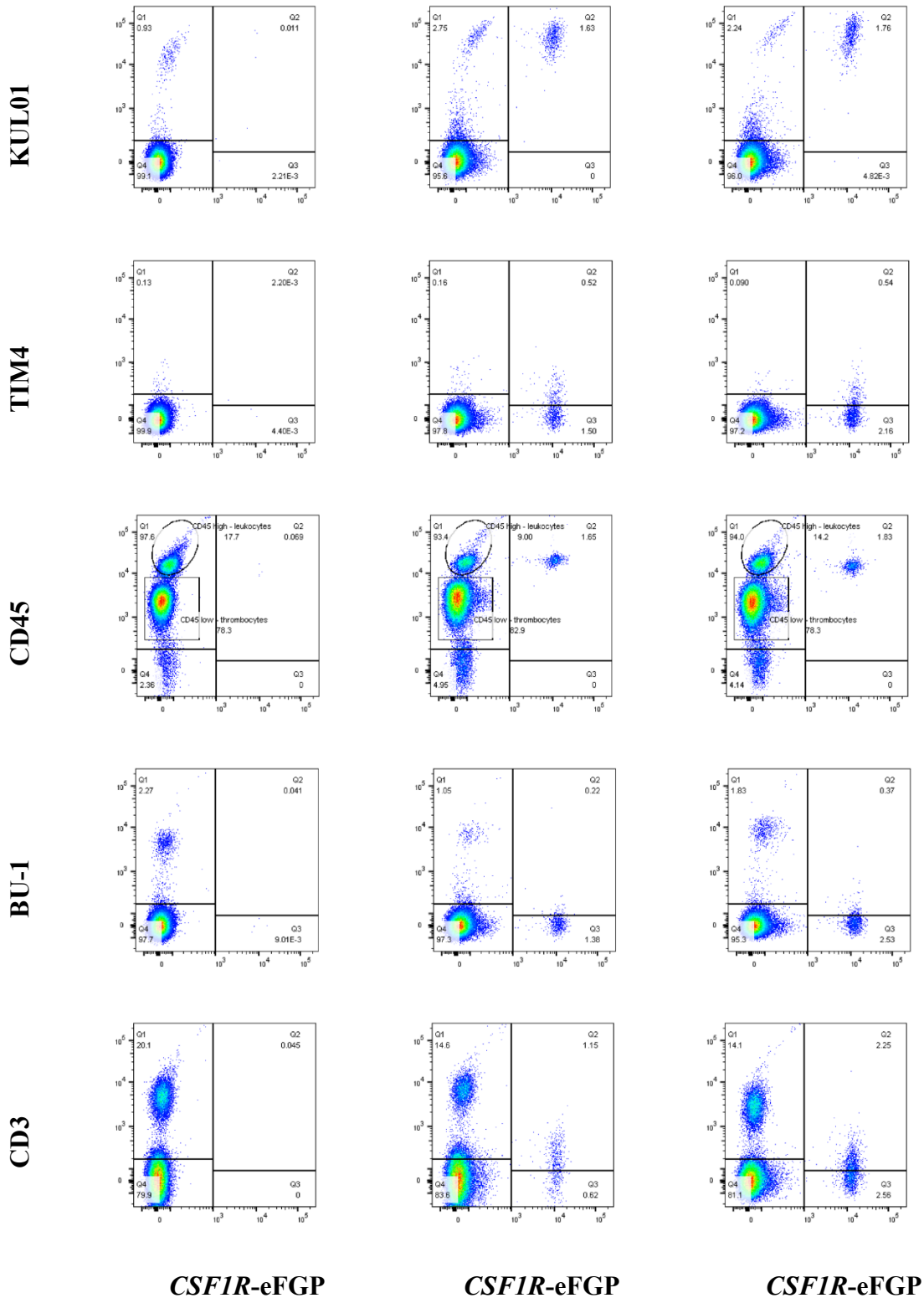


Figure 4-8: Flow cytometry analysis of PBMCs from MacGreen hatchlings treated with anti-ChCSF1 mAb

CSF1R-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500  $\mu$ g dose (10  $\mu$ g/g) of either isotype control IgG1 mAb (n = 7) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Blood was harvested using

anti-coagulant and PBMC separated by density gradient centrifugation. PBMC were stained with anti-CD41/CD61, anti-MHC-II, anti-KUL01, anti-TIM4, anti-CD45, anti-BU1 and anti-CD3 mAbs followed by data analysis via flow cytometry. (A) Effect of anti-ChCSF1 mAb on expression of markers as indicated by day 7. The graphs show mean percentage of cells +/- SEM (B) CSF1R<sup>+</sup> transgene expressing cells, mean monocyte number, from both the groups was compared to mean thrombocyte number (CD41/CD61<sup>+</sup>), mean monocyte/macrophages number (MHC-II<sup>+</sup>), mean thrombocyte number (CD45<sup>low</sup>), mean leukocyte number (CD45<sup>high</sup>), mean thrombocyte + leukocyte number (CD45<sup>+</sup>) and mean T cell number (CD3<sup>+</sup>). (C) Representative FACS profile of stained markers used for calculating the values in B.

### 4.2.7 Effect of anti-CSF1 mAb treatment on Splenocytes

For FACS analysis of the myeloid and lymphoid lineage parameters, spleen tissue from anti-ChCSF1 and isotype control mAb injected birds were disaggregated to form single cell suspension and utilised for immuno-staining. Figure: 4-9C shows the profiles of splenocytes stained with chicken thrombocyte marker CD41/61, monocyte marker MHC-II, KUL01, TIM4, leucocytes marker CD45 and lymphocytes marker BU-1, CD3.

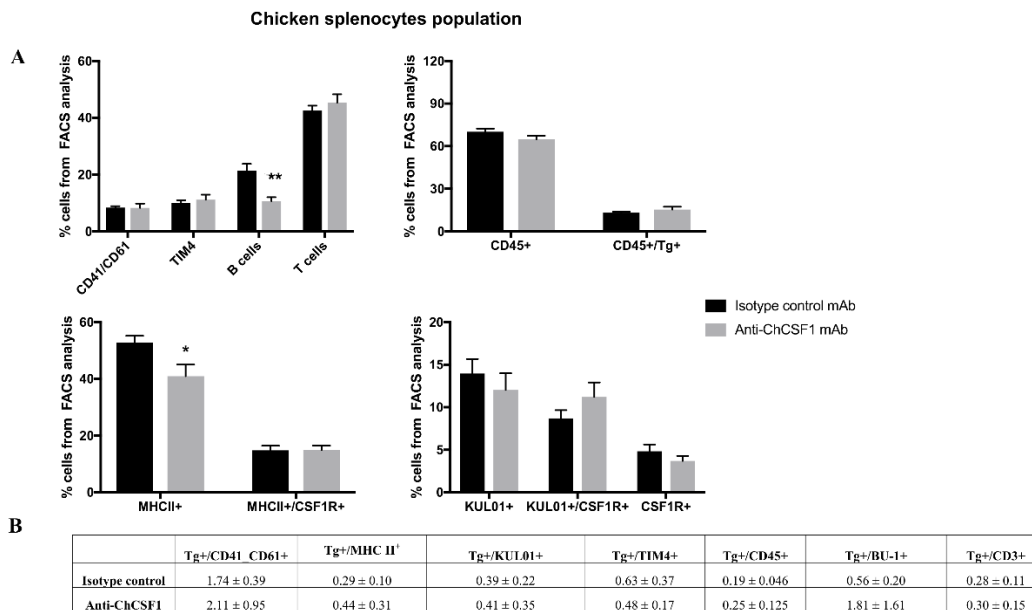
There was no change in the percentage of cells of spleen thrombocytes (CD41/CD61<sup>+</sup>) post anti-ChCSF1 mAb treatment (figure: 4-9A), although comparison of CSF1R transgene expression (mean monocyte number) to CD41/CD61<sup>+</sup> ratio, indicated a slight decline in the thrombocyte population (figure: 4-9B). All transgene expressing cells were uniformly positive for leukocyte marker CD45 and their ratio comparison indicated a small decrease in the leukocyte population probably corresponding to the significant decrease in the B cells expressing surface BU-1 ( $p=0.0012$ ) (figure: 4-9A). The apparent reduction in B cell population is also reflected in the large elevation of transgene and B cell number ratio (figure: 4-9B). There was no significant change in T lymphocytes number. Anti-ChCSF1 mAb effectively reduced the tissue macrophage population MHC-II<sup>+</sup> cells ( $p=0.0292$ ), which were also uniformly positive for CSF1R transgene in spleen and as reflected in the ratio comparing transgene to MHC-II<sup>+</sup> cells and BU-1<sup>+</sup> cells (figure: 4-9A, 4-9B). Anti-ChCSF1 mAb treatment had no significant impact on the other chicken macrophage subsets MHC-II<sup>+</sup>/CSF1R<sup>+</sup>, KUL01<sup>+</sup>, KUL01<sup>+</sup>/CSF1R<sup>+</sup> or transgene expressing (CSF1R<sup>+</sup>) cells (figure: 4-9A) in spleen. Staining of splenocytes with the recently characterised subset of chicken macrophages (TIM4<sup>+</sup> cells) that are involved in the recognition and elimination of apoptotic cells (Hu et al., 2019), indicated that there was no effect of anti-ChCSF1 mAb treatment on this subpopulation of cells in spleen (figure: 4-9A). The FACS profile of the anti-ChCSF1 and isotype control mAb treated

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spleens revealed around 9% TIM4<sup>+</sup> cells also co-expressing CSF1R-eGFP transgene (figure: 4-9C).

Despite reduction in few resident myeloid and lymphoid cell population in chicken spleen, post anti-ChCSF1 mAb treatment, there were no signs of pathology as visualised by whole mount imaging. In summary, although the neutralising anti-ChCSF1 mAb did not cause a drastic change in the expression of *CSF1R*-eGFP transgene in spleen tissue, it did affect CSF1-dependent resident macrophages of spleen.

Efforts to confirm the apparent loss of CSF1R<sup>+</sup> cells in bursa and cecal tonsil observed in whole mounts were unsuccessful, since standard disaggregation protocols applied to the control produced minimal numbers of positive cells

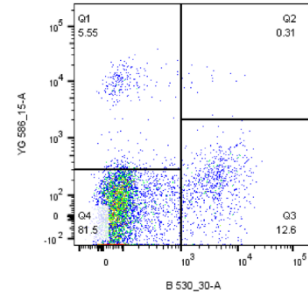
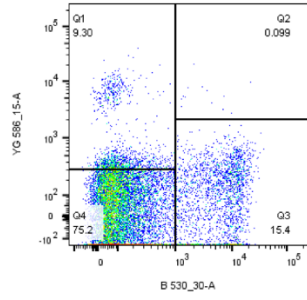


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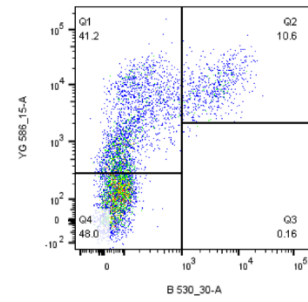
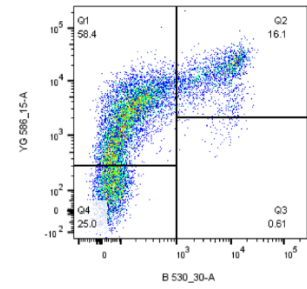
Isotype control mAb

Anti-ChCSF1 mAb

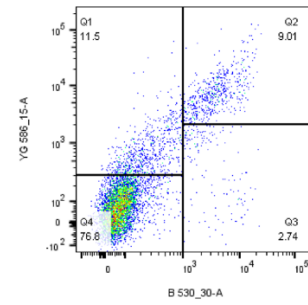
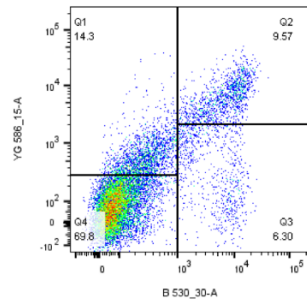
CD41/CD61



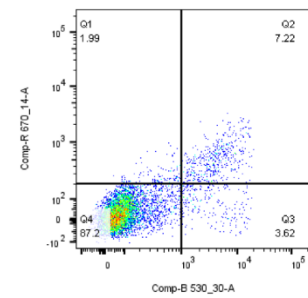
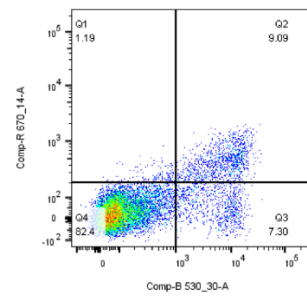
MHC-II

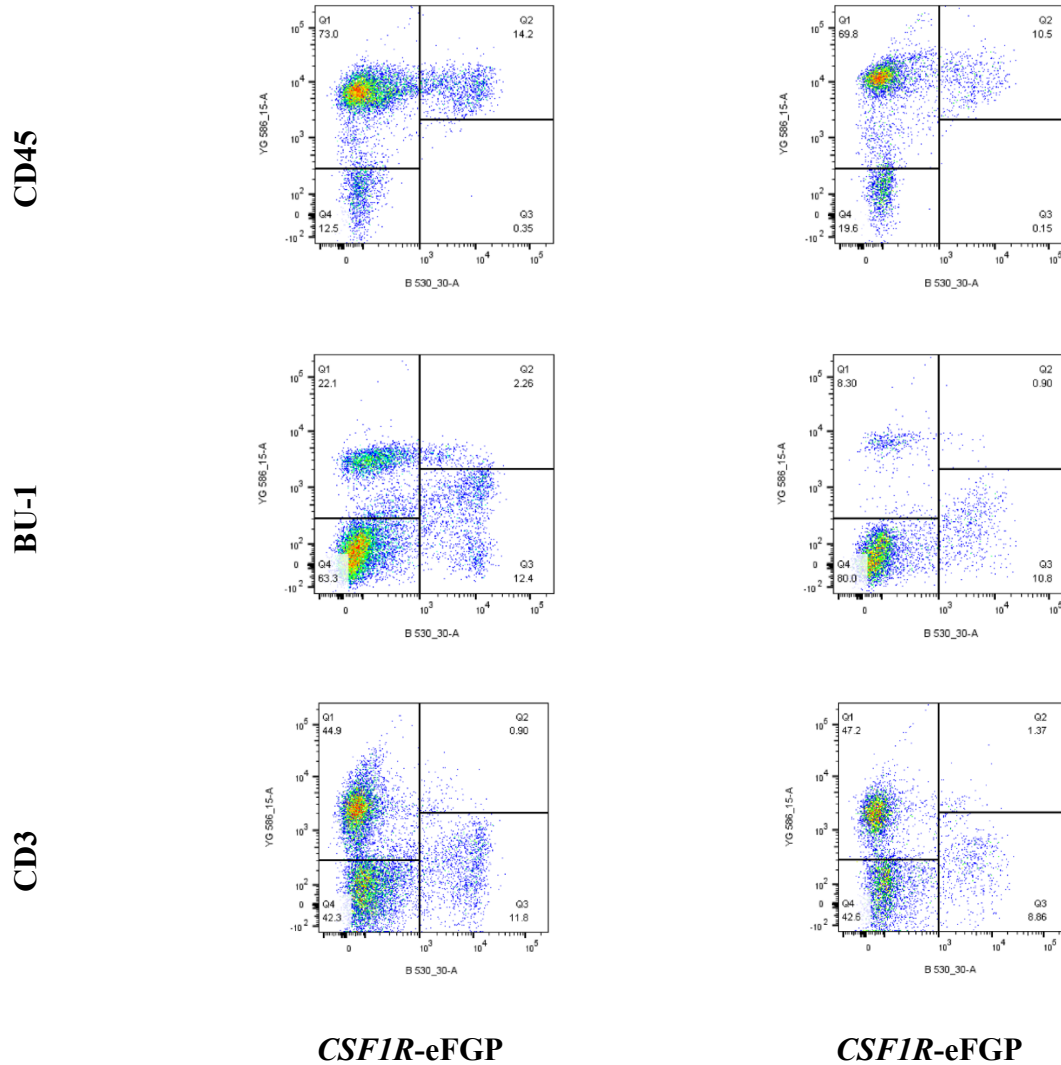


KUL01



TIM4





**Figure 4-9: Flow cytometry analysis of splenocytes from MacGreen hatchlings treated with anti-ChCSF1 mAb**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control IgG1 mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Single cell suspension of spleen tissue was isolated as described in Chapter 2 section 2.7.7 and stained with mAbs, anti-CD41/CD61, anti-MHC-II, anti-KUL01, anti-TIM4, anti-CD45, anti-BU1 and anti-CD3 for analysis by flow cytometry. (A) Shows effect of anti-ChCSF1 mAb on expression of markers as indicated. The graphs show mean percentage of cells +/- SEM (B) *CSF1R*<sup>+</sup> transgene expressing cells (mean monocyte number) from both the groups was compared to mean thrombocyte number (CD41/CD61<sup>+</sup>), mean monocyte/macrophages number (MHC-II<sup>+</sup> KUL01<sup>+</sup>), mean Kupffer cell marker (TIM4<sup>+</sup>), mean thrombocyte + leukocyte number (CD45<sup>+</sup>), mean B cell number and mean T cell number (CD3<sup>+</sup>). (C) Representative FACS profile of stained markers used for calculating the values in B. Significance is indicated as \**P* < 0.05, \*\**P* < 0.01

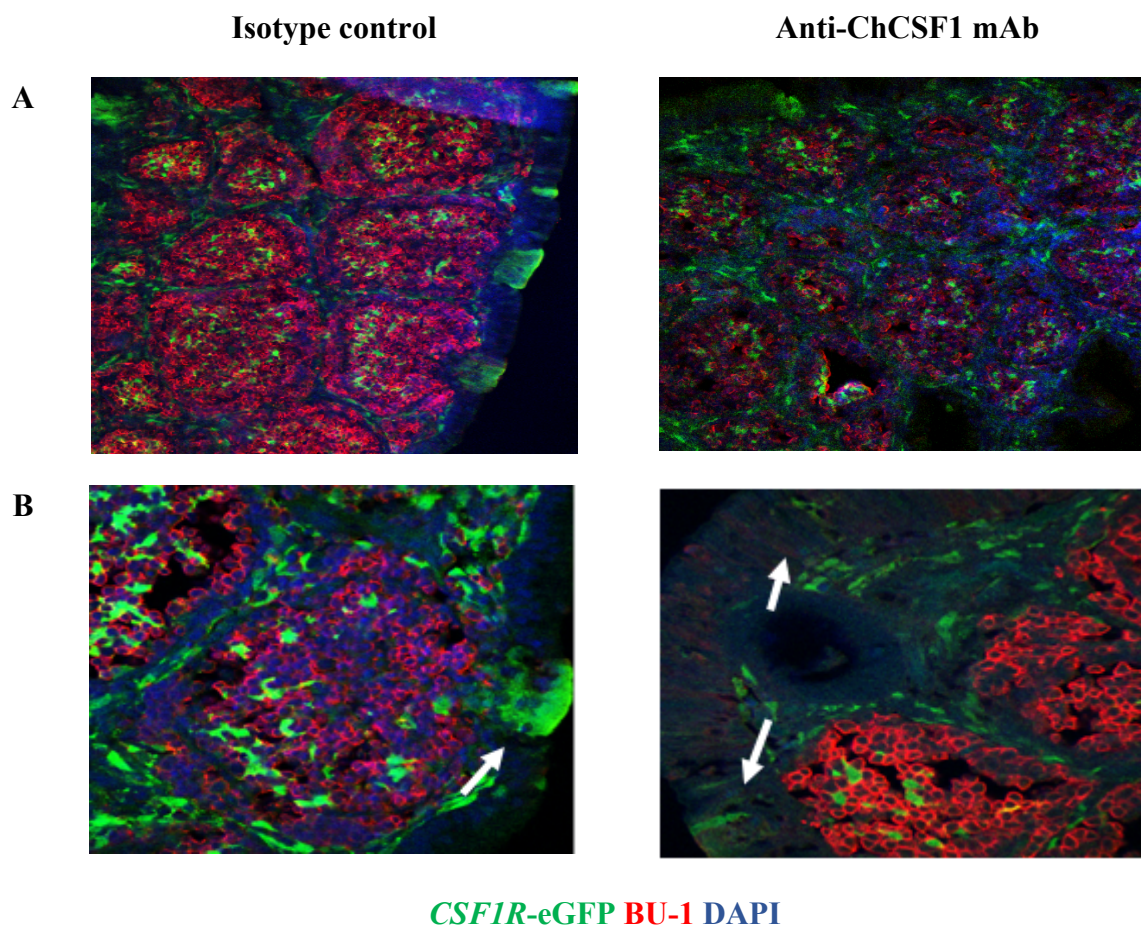
Treatment of anti-CSF1 antibody in neonatal mice for two months decreased resident macrophage density in tissues like bone marrow, liver, dermis, synovium and kidney (Wei et

al., 2005). In this study, depletion in macrophages was detected by tissue sections staining with mature macrophages marker F4/80 (Hume et al., 1983). To fully examine the effects of anti-ChCSF1 mAb treatment in MacGreen hatchling chickens, confocal microscopy of lymphoid organs was performed. Use of *CSF1R*-eGFP transgenic hatchlings in the current study is a valuable tool that permits the analysis of transgene positive macrophages or lymphocytes distribution in chicken tissues. Primary lymphoid organ bursa and other lymphoid tissues such as caecal tonsil, spleen and liver from anti-ChCSF1 and isotype control mAb treated birds were processed as described in Chapter 2 section 2.7.9 and sections imaged by confocal microscopy. Images of immune cells and chicken macrophages from these tissues is also shown in Chapter 8 (section 8.2, appendix B).

### **4.2.8 Effect of anti-CSF1 mAb on follicle associated epithelium (FAE) cells in bursa**

In bursa of Fabricius, the *CSF1R* transgene is highly expressed in the medulla of the B cell follicles in cells identified as bursal secretory dendritic cells (BSDCs) (Oláh et al., 2014) and in the inter-follicular tissues (Balic et al., 2014). *CSF1R*-mAPPLE hatchling chickens treated with CSF1-Fc demonstrate an increase in the number as well as expression brightness of the transgene positive cells in the bursa, particularly in the macrophages of the connective tissue between the B cell follicles (Garceau et al., 2015). In comparison, there was slight decrease in the brightness of the *CSF1R* transgene expressing cells (green) of bursa post anti-ChCSF1 mAb treatment in the hatchling birds (figure: 4-10). FAE lie on the surface epithelium of each follicle in bursa. These cells serve as an attachment point to the bacterial antigens and are devoid of lymphocytes, but are penetrated by macrophages (Oláh et al., 2014). Imaging bursa sections of control MacGreen birds revealed that *CSF1R*-eGFP transgene is expressed on the FAE cells (figure: 4-10A) and that the treatment with anti-ChCSF1 mAb particularly altered the epithelial cell proliferation and survival as determined by the difference in the brightness of transgene expression on FAE cells (figure: 4-10A). Figure: 4-10B highlights this change in the transgene expression in FAE cells (white arrow) post anti-ChCSF1 mAb treatment to MacGreen birds at higher magnification. Although, since the sample size per group was 4, inclusion of more bursa sections post anti-ChCSF1 treatment for histological analysis would be desirable.





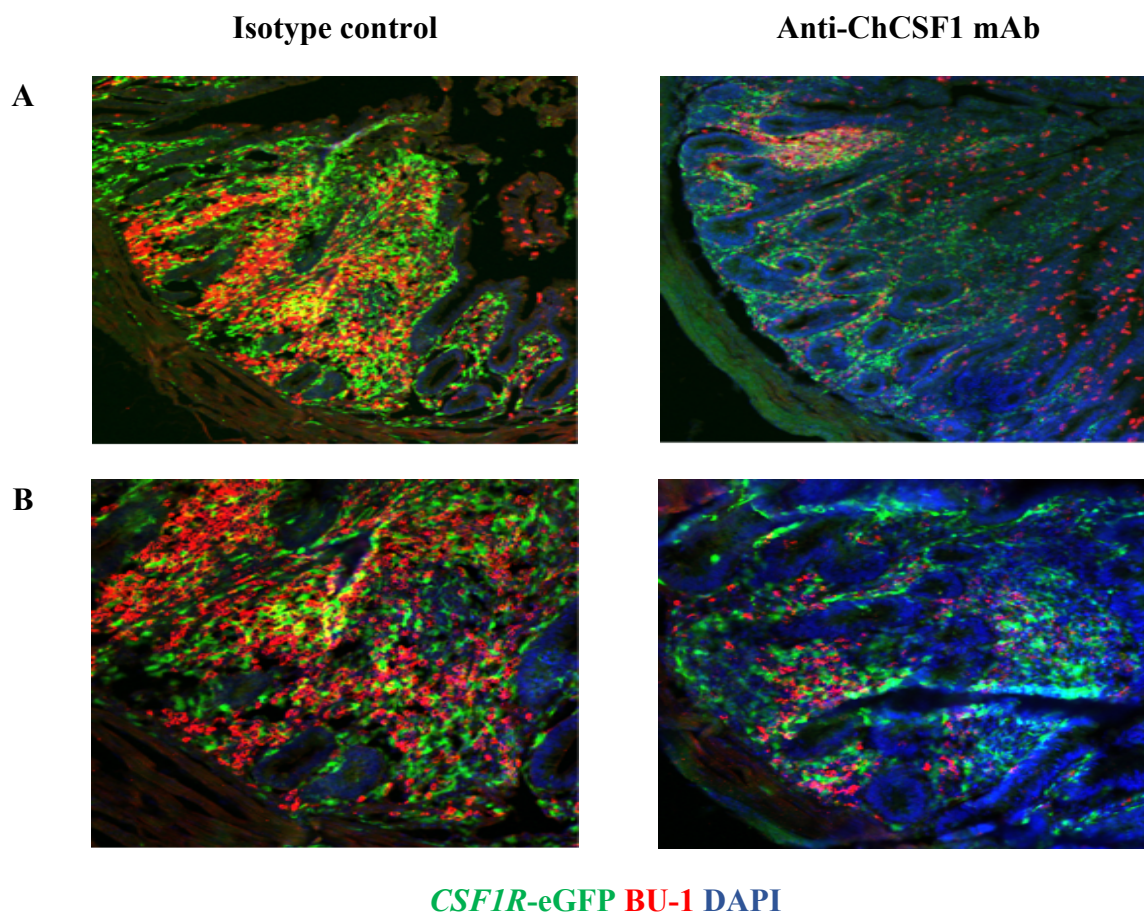
**Figure 4-10: Histological analysis of the effects of anti-ChCSF1 mAb in bursa**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500  $\mu\text{g}$  dose (10  $\mu\text{g}/\text{g}$ ) of either isotype control IgG1 mAb ( $n = 8$ ) or anti-ChCSF1 mAb ( $n = 9$ ) on days 1, 2, 3, 4 of age and humanely culled on day 7. Bursa was collected, fixed in 4% paraformaldehyde followed by cryo-embedding and sectioning. Sections were stained with primary antibody (mouse anti-chicken BU-1), secondary antibodies (donkey anti-mouse AF647, rabbit anti-GFP AF488) and DAPI for staining nuclei as described in Chapter 2 section 2.7.9. (A) Shows representative 20X magnification image of bursa from anti-ChCSF1 treated and control birds. Immunofluorescence detection was performed on four bursa samples from each group. Green indicates CSF1R transgene expression, red is BU-1<sup>+</sup> B cells and blue is nuclei. (B) Shows representative bursa image from 5 images taken per sample each group at 40X magnification. White arrows indicate follicle associated epithelium cells.

#### 4.2.9 Effect of anti-CSF1 mAb on B cells in caecal tonsil tissue

In chicken gut tissue, CSF1R-transgene expressing cells in caecal tonsils tissue are a dense network of follicular dendritic cells (FDCs) and is highly expressed in the medulla region of the B cell dominated germinal center. Scattered cells are also present in the surrounding T cell

rich area (Balic et al., 2014). In chickens, the number of lymphocytes in the caecal tonsil tissue increases during the first week of hatch (Oláh et al., 2014) and the macrophage-enriched germinal centers develop from the second week of age with subsequent increase in their numbers with age (Oláh et al., 2014). In *CSF1R*-eGFP hatchling chickens treated with anti-ChCSF1 mAb there was a marginal decrease in CSF1R transgene positive cells present in the medulla region of the germinal center (or FDCs) and scattered cells throughout the caecal tonsil tissue (figure: 4-11). By contrast, there was substantial depletion in the BU-1<sup>+</sup> B lymphocytes present in the medulla region of the germinal center in the anti-ChCSF1 mAb treated caecal tonsil tissue which was evident from the confocal imaging of sections (figure: 4-11). This observation suggests that anti-ChCSF1 treatment compromises B cell development in the caecal tonsil.



**Figure 4-11: Histological analysis of the effects of anti-ChCSF1 mAb in caecal tonsil tissue**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control IgG1 mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Caeca from gut tissue was

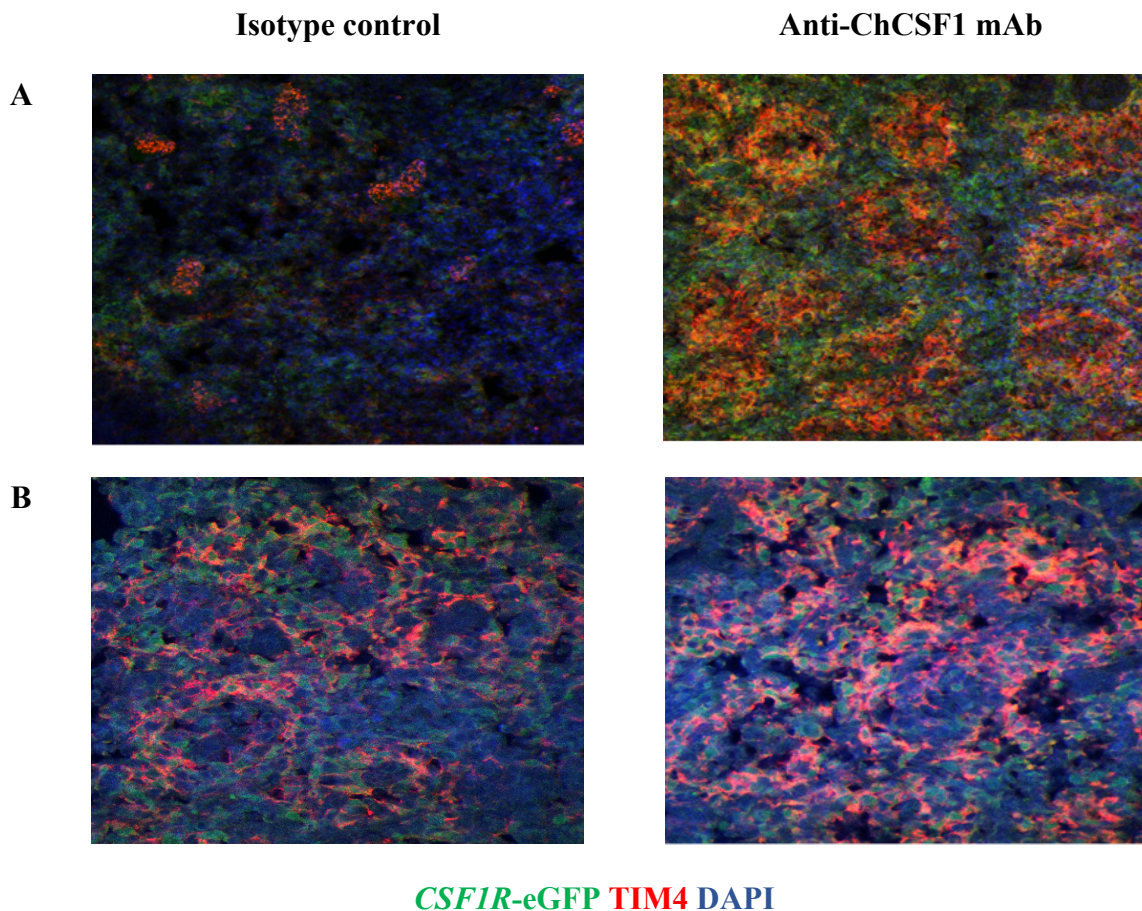
collected, fixed in 4% paraformaldehyde followed by cryo-embedding and sectioning. Sections were stained with primary antibody (mouse anti-chicken BU-1), secondary antibodies (donkey anti-mouse AF647, rabbit anti-GFP AF 488) and DAPI for staining nuclei as described in Chapter 2, section 2.7.9. (A) Shows representative image of caecal tonsil tissue from anti-ChCSF1 treated and control birds at 10X magnification. Green indicates CSF1R transgene expression, red is B cells and blue is nuclei. (B) Shows representative caecal tonsil image from each group at 20X magnification. Data representative of two experiments.

### 4.2.10 Effect of anti-CSF1 mAb on TIM4<sup>+</sup> cells in spleen

In chickens *CSF1R*-eGFP transgene in spleen is expressed predominantly in subsets of macrophages and interdigitating dendritic cells in the white pulp (Balic et al., 2014; Oláh et al., 2014). In *CSF1R*-mAPPLE hatchling chickens treated with chicken CSF1-Fc the number of transgene expressing cells increased in the white pulp area (Garceau et al., 2015).

In chickens, a subset of mononuclear phagocytes expresses the TIM4 surface marker which functions in the recognition of apoptotic cells (Hu et al., 2016; Hu et al., 2019). In chicken spleen TIM4<sup>+</sup> macrophages are highly expressed at the junction between peri ellipsoidal white pulp and red pulp area, and within the B cell dominated germinal center (Hu et al., 2019). These cells do not actually express the *CSF1R* reporter transgene although they do express *CSF1R* mRNA. In the control hatchling birds, the spleen *CSF1R*-eGFP positive cells (green) and TIM4<sup>+</sup> macrophages (red) were readily distinguishable (figure: 4-12A), TIM4<sup>+</sup> cells being distributed at the interface between white pulp and red pulp area. In birds injected with anti-ChCSF1 mAb, there appeared to be aggregation of TIM4<sup>+</sup> and *CSF1R*-eGFP expressing cells at the interface between peri ellipsoidal white pulp and red pulp area (figure: 4-12B). This result is ambiguous. It could be that anti-ChCSF1 mAb actually induced apoptosis of a population of cells in the spleen and TIM4<sup>+</sup> cells are involved in the recognition and phagocytic uptake of apoptotic cells. In mice there is evidence that despite high expression level of TIM4<sup>+</sup> cells in the marginal zone in spleen, these cells are not essential for recognition and clearance of apoptotic cells (Wong et al., 2010).





**Figure 4-12: Histological analysis of the effects of anti-ChCSF1 mAb in spleen**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control IgG1 mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Spleen tissue was collected, fixed in 4% paraformaldehyde followed by cryo-embedding and sectioning. Sections were stained with primary antibody (anti-chicken TIM4, JH9), secondary antibodies (donkey anti-mouse AF647, rabbit anti-GFP AF488) and DAPI for staining nuclei as described in Chapter 2, section 2.7.9 (A) Shows representative image of spleen from anti-ChCSF1 treated and control birds at 20X magnification. Green indicates CSF1R transgene expression, red is TIM4<sup>+</sup> cells and blue are nuclei. Immunofluorescence detection was performed on four spleen samples from control group and five samples from treated group. (B) Shows representative spleen image from each group at 40X magnification.

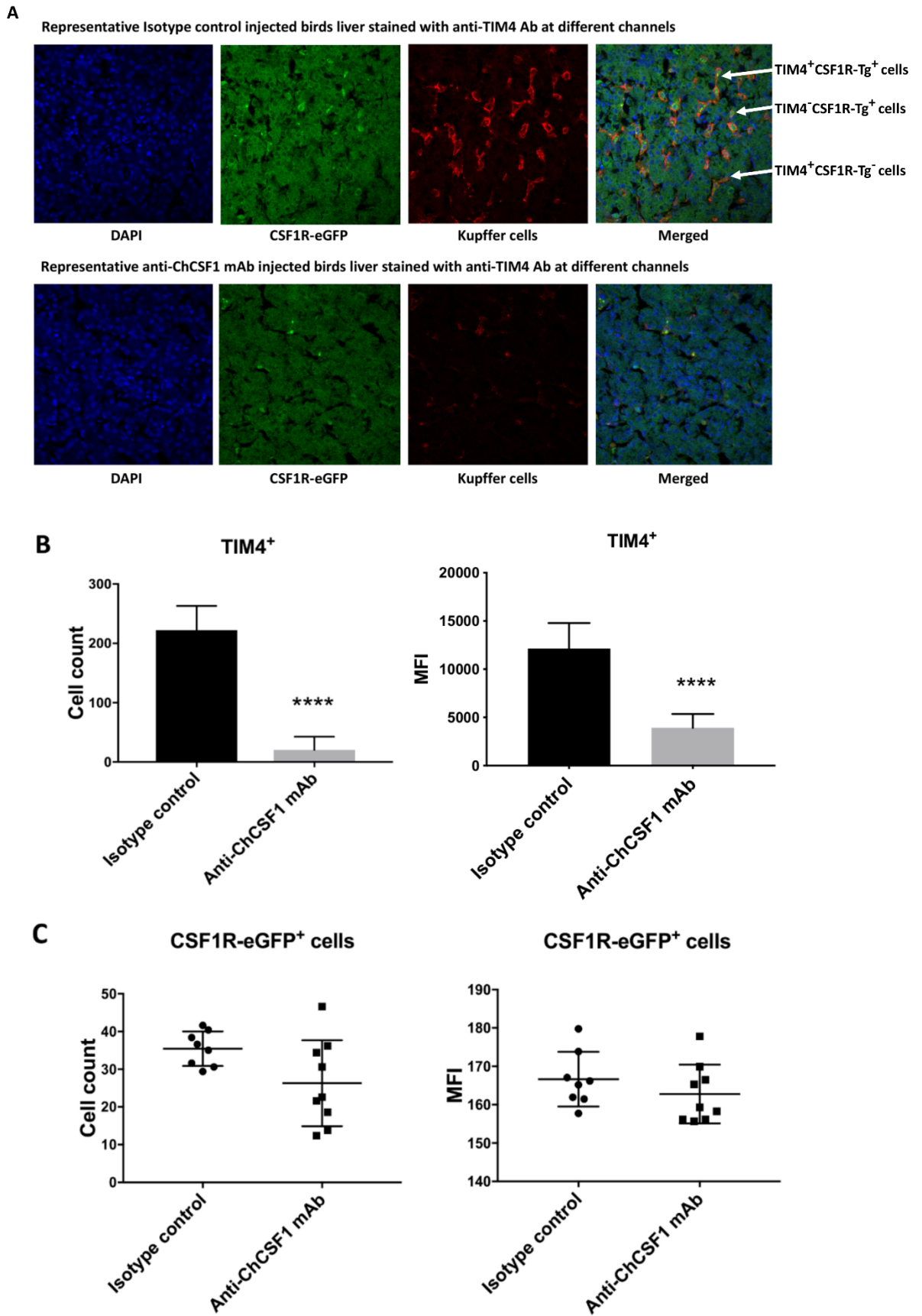
#### 4.2.11 Effect of anti-CSF1 mAb on TIM4<sup>+</sup> cells in liver

In mouse liver, *Csf1r*-eGFP transgene is specifically expressed on sinusoidal macrophages or Kupffer cells (Sasmono et al., 2003) and treatment with blocking anti-mouse CSF1R Ab for three weeks or six weeks significantly deplete these cells along with reduction in the absolute weight of the liver (MacDonald et al., 2010; Sauter et al., 2014). Subcutaneous injection of

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mice at neonatal stage with the neutralising anti-mouse CSF1 antibody for a period of two months also reduced Kupffer cells numbers (Wei et al., 2005). In chicken liver *CSF1R*-eGFP transgene and apoptotic cell marker TIM4 distinguish two population of cells, Kupffer cells and dendritic cell like cells. Kupffer cells are phagocytic, express high levels of TIM4 marker, but like the macrophages of the bursa they do not express *CSF1R*-eGFP transgene protein ( $TIM4^+CSF1R-Tg^-$ ) despite the presence of *CSF1R* mRNA. The second population resembles mammalian conventional dendritic cells. They are not phagocytic and express low levels of TIM4 marker but high levels of *CSF1R*-eGFP transgene protein ( $TIM4^-CSF1R-Tg^+$ ). A subset of chicken liver cells expressed both TIM4 as well as *CSF1R*-eGFP transgene protein ( $TIM4^+CSF1R-Tg^+$ ), thus identifying three populations of mononuclear phagocytes in chicken liver (Hu et al., 2019). These three cell types are seen in the control chicken liver samples by immunohistochemistry (figure: 4-13A). Administration of anti-ChCSF1 mAb to *CSF1R*-eGFP transgene expressing reporter hatchling birds completely ablated  $TIM4^+$  (red) Kupffer cells ( $TIM4^+CSF1R-Tg^-$ ,  $TIM4^+CSF1R-Tg^+$ ) as shown in figure: 4-13A. Figure: 4-13A shows representative image of liver samples (in single channels for *CSF1R*-eGFP, TIM4 and DAPI stained as wells as merged) from eight isotype control mAb injected and nine anti-ChCSF1 mAb injected birds. Quantification of  $TIM4^+$  cell number (red) as well as mean fluorescence intensity (MFI) by ImageJ indicated that there was significant reduction in both of these parameters, ( $TIM4^+$  cell number,  $p < 0.0001$ ; MFI,  $p < 0.0001$ ) (figure: 4-13B).

By contrast, anti-ChCSF1 mAb treatment did not alter the abundance or the eGFP fluorescence of the TIM4-negative, eGFP-positive liver dendritic like cells in liver ( $TIM4^-CSF1R-Tg^+$ ) ( $p = 0.062$  for cell count and  $p = 0.276$  for MFI) (figure: 4-13C).



### **Figure 4-13: Histological analysis of the effects of anti-ChCSF1 mAb in liver tissue**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 µg dose (10 µg/g) of either isotype control mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Liver tissue was collected, fixed in 4% paraformaldehyde followed by cryo-embedding and sectioning. Sections were stained with primary antibody (anti-chicken TIM4, JH9), secondary antibodies (donkey anti-mouse AF647, rabbit anti-GFP AF488) and DAPI for staining nuclei as described in Chapter 2 section 2.7.9 (A) shows representative image of liver from the two groups at 40X magnification split in single channels. Green indicates *CSF1R* transgene expression, red is TIM4<sup>+</sup> cells and blue are nuclei. The amount of fluorescence for TIM4<sup>+</sup> Kupffer cells (B) and *CSF1R*-eGFP transgene expressing dendritic cells (C) quantified using Image J by counting the cell number and the mean fluorescence intensity (MFI) from 5 different images from 5 different regions of liver sample per bird at 40X magnification is shown. Cell count number and MFI value graphs shows mean +/- SEM. Significance is indicated by \*\*\*\**P* < 0.0001 using a Mann–Whitney test.

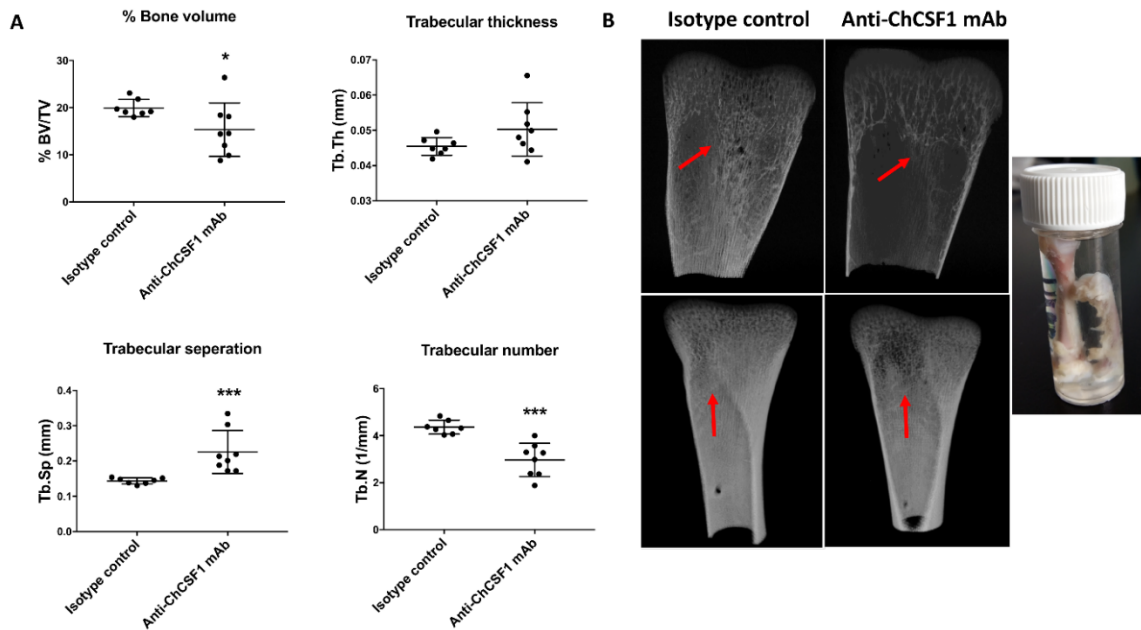
In summary, anti-ChCSF1 mAb was effective in depleting a subset of myeloid and lymphoid tissue resident macrophages in major lymphoid organs in post hatch chickens. The treatment did not induce any overt pathology in any organ.

### **4.2.12 Effect of anti-CSF1 mAb on bone architecture**

Western blot analysis revealed a high level of CSF1 protein expression in bone marrow cells of chicken (Wu *et al*, manuscript in progress). *CSF1R*-mAPPLE transgene expressing hatchling chickens injected with CSF1-Fc on four consecutive days demonstrated an unexpected increase in femur bone volume, trabecular thickness and trabecular number as of 5<sup>th</sup> day of age (Garceau *et al.*, 2015). To determine if anti-ChCSF1 mAb administration to chicken hatchling birds has any impact on bones, femur bones were collected, fixed and processed to be sent to Dr. Robert Wallace, at University of Edinburgh campus for microCT scan analysis as described in Chapter 2, section 2.7.11.

Quantification of 3D volumetric microCT bone parameters measured in a defined volume of interest indicated significant reduction in the % bone volume and trabecular number in femurs of birds treated with anti-ChCSF1 mAb compared to controls (figure: 4-14A). Accordingly, trabecular separation was increased (figure: 4-14A). The apparent marginal increase in the trabecular thickness on 7<sup>th</sup> day was not significant (figure: 14A). Representative two-dimensional (2D) and three dimensional (3D) microCT scan images from both the groups highlight reduction in the femoral growth plate (figure: 4-14B). Staining of femur sections from isotype control and anti-ChCSF1 mAb injected birds for bone osteoclasts showed that their number was greatly reduced (data not shown) post anti-CSF1 treatment. Analysis of *CSF1R*

transgene expression in bone marrow cells by FACS revealed no change in the expression amongst the two group (data not shown).



**Figure 4-14: microCT scan analysis of the effects of anti-ChCSF1 mAb on bone density**

*CSF1R*-eGFP transgenic reporter hatchling chickens were subcutaneously injected in the breast region with 500 μg dose (10 μg/g) of either isotype control IgG1 mAb (n = 8) or anti-ChCSF1 mAb (n = 9) on days 1, 2, 3, 4 of age and humanely culled on day 7. Femurs were collected post-mortem from all birds, fixed in 4% paraformaldehyde for 24 hrs and stored in 70% ethanol. Bones after embedding in agarose gel were analysed by microCT scanning. (A) Average values were calculated for parameters as bone volume, trabecular thickness, trabecular number and trabecular separation and graphed as mean  $\pm$  SEM for each individual bird bone measurement. Significance is indicated by \* $P < 0.05$  and \*\*\* $P < 0.001$  using a Mann–Whitney test. (B) 2D images (upper) and 3D images (lower) of microCT scan from both the groups highlight the growth plate of femur bones (red arrows). Scale bar = 5 μm.

#### 4.2.13 Localisation of chicken CSF1 in lymphoid organs

An attempt to locate the expression of CSF1 in the immune tissues was carried out using the fluorochrome labelled anti-ChCSF1 mAb by immunofluorescence. Several organs (caecal tonsil, liver and lung) from a 4-week-old layer chicken were removed, washed, sectioned and fixed in methanol and stained with anti-ChCSF1 AF647 mAb or isotype control AF647 mAb.

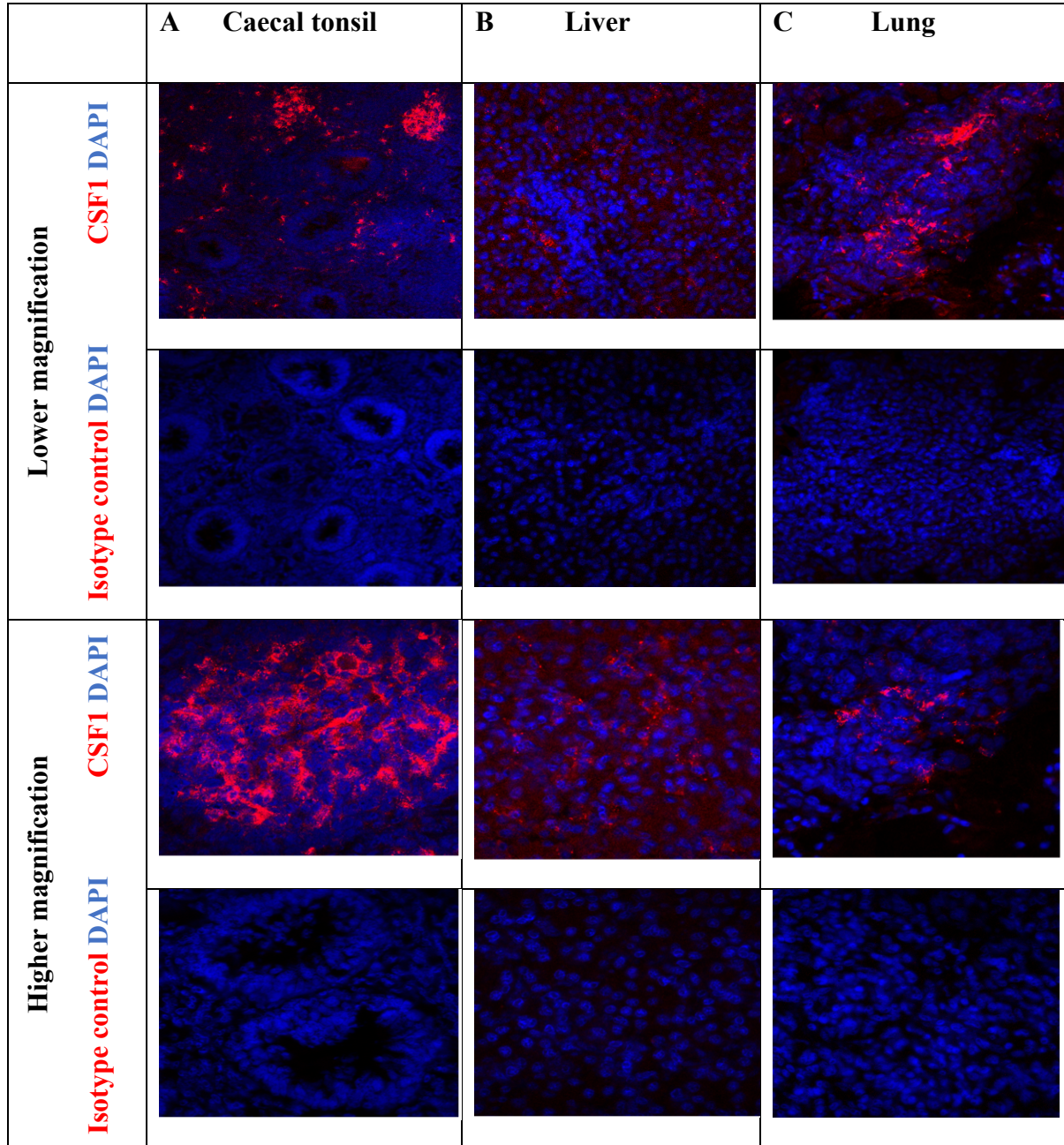
As discussed in section 4.2.2 of this chapter, chicken macrophages themselves express *CSF1R* mRNA and appear autocrine for CSF1 signalling. Caecal tonsil tissue in chickens comprises



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epithelial zone, lamina propria, germinal centre and interfollicular areas. Anti-ChCSF1 mAb located the presence of CSF1, scattered throughout the lamina propria of caecal tonsil tissue as well as concentrated in the B cell dominated germinal centers (figure: 4-15A). The location is consistent with the high expression of mononuclear phagocytes scattered in the lamina propria and their dense network in the medulla region of B cell follicles (Balic et al., 2014; Oláh et al., 2014).

As mentioned above *CSF1R*-eGFP transgene expression distinguished conventional dendritic like cells in chicken liver (Hu et al., 2019). Characterization of lung tissue in chickens recently revealed distribution of CSF1R expressing transgene over the entire mucosa of primary bronchi and as dense aggregates in germinal centers (Sutton et al., 2018). Staining with anti-ChCSF1 mAb revealed presence of CSF1 protein abundantly in liver tissue (figure: 4-15B) and throughout the para-bronchial wall in lung tissue (figure: 4-15C). However, without double staining, it is not possible to determine whether the growth factor is expressed by resident macrophages themselves or simply concentrated in areas where macrophages are abundant. Double staining of chicken lymphoid organs with anti-ChCSF1 Ab chicken lymphoid organs and markers of monocyte/macrophage and B lymphocytes and staining of isolated cells by FACS may provide additional insight.



*Figure 4-15: Localisation of chicken CSF1 in lymphoid organs*

CSF1 (Red) and DAPI (blue). Lymphoid organs from a 4-week-old chicken were sectioned, fixed in methanol and stained with anti-ChCSF1 AF647 mAb or isotype control IgG1 AF647 mAb and DAPI for staining nucleus. Figure shows location of chicken CSF1 in caecal tonsil tissue (A), liver (B) and lung (C) at lower magnifications (20X, 40X above) and higher magnification (63X below). Scale bar = 50  $\mu$ m (20X), 20  $\mu$ m (40X) and 10  $\mu$ m (63X).

### 4.3 Conclusion and discussion

This Chapter is based on studying the biology of chicken CSF1, a crucial growth factor involved in the development of mononuclear phagocytes. The data presented here determines

the significance of CSF1 signalling in macrophages and immune cells development in post hatch chickens. Most of the knowledge about CSF1 biological activity and role in macrophage homeostasis comes from extensive studies performed in rodents, either by exogenous CSF1 injections, due to induced or natural mutations in CSF1/CSF1R or antibody to CSF1/CSF1R injections (Hume et al., 1988; Ulich et al., 1990; Pollard 2009; Hume and MacDonald 2012; Chitu and Stanley 2017; Pridans et al., 2018). The limited studies performed in chickens demonstrated that CSF1 is the regulator for development of mononuclear phagocytes in both embryo and hatchling birds (Garceau et al., 2015). To further understand post-natal CSF1 regulation of monocytes, macrophages and other immune cells *in vivo* in chickens I made use of the recently characterised novel neutralising antibody to chicken CSF1 (Wu *et al*, manuscript in progress).

Batch purified anti-ChCSF1 mAb was able to successfully inhibit chicken CSF1 induced activation of CSF1R in BaF3/ChCSF1R cells at a concentration of 1.25 µg/ml (figure: 4-2). On the other hand, a concentration of 0.31 µg/ml was required to inhibit chicken CSF1 induced growth of primary chicken macrophages (figure: 4-3A, B). Their continued survival in the absence of exogenous CSF1 confirms that they secrete some form of endogenous CSF1 probably at low levels that bind competitively with anti-ChCSF1 mAb (figure: 4-3B). Western blot analysis of the chicken serum and tissue extracts revealed that this CSF1 protein is highly expressed in bone marrow, along with other major lymphoid organs as thymus, bursa and spleen, which is 13-15 KDa in size, similar to the size of CSF1 protein detected in blood serum (Wu *et al*, manuscript in progress). Anti-ChCSF1 mAb was able to detect surface CSF1 on chicken BMDMs by FACS analysis, confirming its expression. The level was further regulated by exogenous CSF1. The autocrine CSF1 signalling action of chicken macrophages is also supported by RNAseq studies of chicken BMDMs. RNAseq studies indicated that chicken BMDMs express high levels of *CSF1* mRNA amongst all cells and tissues, and that it is the integral membrane form encoded by the longer *CSF1* mRNA transcript ([www.biogps.org/chickenatlas](http://www.biogps.org/chickenatlas)) (Bush et al., 2018).

CSF1 in mice is widely expressed *in vivo* by endothelial cells, bone marrow osteoblasts, epithelial cells of uterus, ovary, fibroblasts, marginal zone and red pulp area of spleen, outer cortical regions of the lymph node, within cortex of thymus, mammary gland, Paneth cells of the small intestine, interstitial cells of testis and many different cell types (Ryan et al., 2001; Pixley and Stanley 2004; Chitu and Stanley 2006). Mouse BMDMs do not express *CSF1*

mRNA and require exogenous CSF1 for their survival (Sester et al., 1999). A transgenic mouse with autocrine release of CSF1 regulating CSF1R expressing cells develops a rapid lethal inflammatory phenotype including osteoporosis and tissue macrophage accumulation (Wei et al., 2006). By contrast in pigs (<http://biogps.org>) (Freeman et al., 2012) humans, (Consortium 2014) sheep (Clark et al., 2017) and even rats (DAH. C.Pridans, personal communication, *Csfl* mRNA is most highly-expressed by mature macrophages. A recent study located CSF1 in mouse lymph nodes to lymphatic endothelial cells and confirmed the function in maintenance of the subcapsular sinus macrophage population (Mondor et al., 2019).

Two anti-CSF1R antibodies (clones M279 and AFS98) have been shown to block CSF1 signaling *in vitro* and deplete tissue macrophages and prevent monocyte infiltration *in vivo*, in various mouse disease models. Subtle differences in their relative activity are likely to be due to different model systems used, including mouse strain and sex (MacDonald et al., 2010; Hume and MacDonald 2012; Sauter et al., 2014). In contrast to mouse anti-CSF1R antibodies, characterized pig and chicken anti-CSF1R antibodies, recognizes CSF1R expressing mononuclear phagocytes but does not block CSF1 signaling (Chihara et al., 2010; MacDonald et al., 2010; Garcia-Morales et al., 2013; Moffat et al., 2014).

Previous studies from our laboratory had demonstrated that administration of exogenous CSF1-Fc protein for four consecutive days had little effect on circulating blood monocytes but induced a global leucocytosis and a massive expansion of *CSF1R* transgene expressing tissue macrophages in embryonic and post hatch birds (Garceau et al., 2015). Interestingly, CSF1-Fc of neonatal rats also produced an increase in tissue macrophages without increasing blood monocytes (Pridans et al., 2018). Neutralizing anti-CSF1 mAb depletes CSF1 dependent macrophages *in vivo* in mice and mimics some effects of the *Csfl<sup>op</sup>/Csfl<sup>op</sup>* mutation (Wei et al., 2005), but this has never been studied in birds before. Accordingly, in this project, neutralising anti-ChCSF1 mAb was injected *in vivo* in *CSF1R*-eGFP reporter transgene expressing hatchling birds to determine whether CSF1 was essential for the development and /or maintenance of tissue macrophages. CSF1 signalling activity proved to be limiting in chickens for the proliferation and differentiation of specific tissue macrophage populations, similar to the findings in mammals (Alikhan et al., 2011; Gow et al., 2014; Garceau et al., 2015; Sauter et al., 2016). In order to maintain high level of circulating anti-ChCSF1 mAb in the steady state and also based on previous anti-CSF1 Ab injection studies in mice (Wei et al., 2005) it was decided to inject 500 µg (10 mg/kg) dose per bird per day for four consecutive

days and analyse the impact on 7<sup>th</sup> day of age. In chickens, the half-life of circulating CSF1 protein is short (less than one hour) (Garceau 2014). Multiple dose of anti-ChCSF1 mAb ensured sustained elevation long enough to prevent mononuclear phagocyte progenitors entering cell cycle (Ulich et al., 1990). Depletion of immune tissues and cells could potentially make hatchlings susceptible to infection, accordingly increased biosecurity measures were taken.

By contrast to the reduced growth rate and lower adult weight observed in *Csf1<sup>op</sup>/Csf1<sup>op</sup>*, *Csf1<sup>-</sup>/Csf1<sup>-</sup>* and *Csf1r<sup>-/-</sup>* and anti-CSF1 Ab injected mice (Dai et al., 2002; Wei et al., 2005; Jenkins and Hume 2014), anti-ChCSF1 mAb treatment in MacGreen hatchling birds had no effect on the average growth rate or relative weight gain (figure: 4-4A, B, C). CSF1-Fc treatment of hatchling chickens also had no effect on the average body weight during the first five days after birth (Garceau et al., 2015). CSF1 plays an important role in normal postnatal growth development and organogenesis in rodents by regulating the release of predominant growth factor, insulin growth factor-1 (IGF-1) by macrophages (Gow et al., 2010; Alikhan et al., 2011; Jones and Ricardo 2013). One possible explanation for the lack of impact of anti-CSF1 in birds is that BMDM grown in CSF1, unlike those of mice and rats, express no detectable *IGF1* mRNA whereas it is highly-expressed in liver as it is in mammals ((Bush et al., 2018) see [www.biogps.org/chickenatlas](http://www.biogps.org/chickenatlas)).

Because it is cleared by receptor-mediated endocytosis, the circulating CSF1 concentration is increased markedly in *Csf1r* knockout rodents and in response to anti-CSF1R treatment (Dai et al., 2002; Pridans et al., 2018). Anti-ChCSF1 mAb treatment on MacGreen birds did not increase the detectable circulating CSF1 concentration as measured during the first and the last (7<sup>th</sup>) day of the experiment (figure: 4-5). This is a complex observation. Anti-CSF1 might have protected CSF1 from clearance by the CSF1 receptor but on the other hand, the anti-CSF1 antibody present in the serum could have interfered with the assay.

Bone marrow monocytes developed postnatally up until 3 months of age do not depend on circulating CSF1 for their survival. In *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice after their development they fail to differentiate into macrophages due to deficiency of CSF1, but CSF1 is not required for monocyte survival (Cecchini et al., 1994). In mouse and pig treatment with exogenous CSF1 increased blood monocyte numbers (Hume et al., 1988; Gow et al., 2014; Sauter et al., 2016), but administration of anti-CSF1R Ab for 3 weeks in adult mice did not deplete total blood

monocyte number. However it did selectively deplete monocyte differentiation into resident subset population (MacDonald et al., 2010). In contrast to increased leukocytes and lymphocytes number upon CSF1-Fc treatment of hatchling birds (Garceau et al., 2015), anti-ChCSF1 mAb did not alter the white blood cell, thrombocyte or T lymphocyte cell number as measured by FACS analysis (figure: 4-8). There was marginal reduction in the expression of the available chicken blood monocyte markers MHC-II and KUL01, with a proportional decrease in the B lymphocytes but other monocyte subsets as MHC-II<sup>+</sup>/CSF1R<sup>+</sup> and KUL01<sup>+</sup>/CSF1R<sup>+</sup> remained unaffected (figure: 4-8). In contrast, in *Csf1r*<sup>-/-</sup> rats, monocytes were almost undetectable (Pridans et al., 2018). At present there is no evidence for functional heterogeneity of chicken monocytes, but presumably they do not or partially depend on circulating CSF1 for their production and survival.

In the spleen of *Csf1r*-eGFP mice the transgene is expressed abundantly in the macrophages of marginal zone of the lymphoid follicles and within the red pulp area (RP) (Sasmono et al., 2003). Treatment of these mice with anti-mouse CSF1R Ab for three weeks depleted the transgene positive cells of marginal zone surrounding the lymphoid follicles (the white pulp, WP) leaving macrophages of the RP unaffected (MacDonald et al., 2010). Red pulp macrophages are only partly-depleted by the *Csf1r* mutation in rats where the marginal zone is entirely ablated (Pridans et al., 2018). Extended anti-CSF1R Ab treatment of mice for six weeks completely depleted the *CSF1R*-eGFP transgene positive macrophages of spleen correspondingly reducing the overall organ weight (Sauter et al., 2014). Administration of chicken CSF1-Fc in hatchling chickens increased *CSF1R*-mAPPLE transgene expression in spleen tissue (Garceau et al., 2015). However, anti-ChCSF1 mAb had no effect on macrophage populations in chicken spleen (figure: 4-6C, 4-9). Macrophage subsets co-expressing *CSF1R* transgene MHC-II<sup>+</sup>, KUL01<sup>+</sup> and TIM4<sup>+</sup> did not change (figure: 4-9). The selective loss of MHCII<sup>+</sup> cells that lacked the monocyte markers was attributed to the loss of B cells which could be secondary to the impacts on the bursa. The absence of an effect on macrophages in spleen is difficult to interpret, because it may be influenced by whether the antibody effectively penetrates the tissue and/or the local CSF1 concentration. It may also be that there is no functional equivalent of the marginal zone macrophages in chicken spleen.

Analysis of the distribution of *CSF1R*-eGFP reporter transgene expression on lymphoid tissues of anti-ChCSF1 mAb treated birds revealed a significant decrease in the transgene expression as well as fluorescence intensity in bursa (figure: 4-6A, 4-7A) and caecal tonsil tissue (figure:

4-6B, 4-7B). This is the reciprocal finding to the ability of CSF1-Fc treatment to increase the number and brightness of transgene expressing cells in hatchling bursa and caecal tonsil tissue (Garceau et al., 2015). In bursa, anti-ChCSF1 mAb treatment altered the survival and the brightness in expression of *CSF1R* transgene co-expressed on FAE cells that are present on the epithelium surface of bursal follicles (figure: 4-10). Transgene expressing cells in the medulla and cortical region remained unaffected. Western analysis of tissue lysates in chicken revealed high level of CSF1 expression in bursa (Wu *et al*, manuscript in progress). In caecal tonsil tissue upon anti-ChCSF1 mAb treatment, there was a partial loss in the overall expression of *CSF1R* transgene scattered throughout the tissue, including transgene positive cells co-expressed on FDCs in the medulla region of germinal centre. B lymphocytes from B cell dominated germinal centre were also substantially reduced (figure: 4-11).

Whereas there was a limited impact on the abundance of tissue macrophages, anti-ChCSF1 mAb clearly affected B cell lymphopoiesis in hatchling chickens. Anti-ChCSF1 mAb effectively decreased B lymphocyte numbers in blood PBMCs (figure: 4-8A), caecal tonsil (figure: 4-11) and spleen tissue (figure: 4-9A) whereas T cells were unaffected (and increased in relative terms).

There is some evidence for a role for CSF1 in control of B cell development in mammals. In *Csf1r*<sup>-/-</sup> rats, B and T lymphocyte populations were unchanged in the blood but expression profiling of spleen indicated selective loss of B cell markers and increased relative expression of T cell markers (Pridans et al., 2018). Based upon studies in *Csf1<sup>op</sup>/Csf1<sup>op</sup>* mice there is evidence that circulating or local CSF1 regulates the survival of IL7-dependent precursor B cells in the bone marrow micro-environment (Tagaya et al., 2000; Lu and Osmond 2001). A selective defect in B cell lymphopoiesis was also reported in analysis of 3-week old homozygous *Csf1* knockout mice, wherein there was significant reduction in the B cell numbers of bone marrow, peripheral blood and spleen (Harris et al., 2012). In transgenic mice (2 months age) that expressed secreted proteoglycan form of *Csf1* on *Csf1<sup>op</sup>/Csf1<sup>op</sup>* background, B lymphocyte numbers in haematopoietic organs bone marrow, blood and spleen were restored (Nandi et al., 2006). There is no evidence that CSF1R is expressed in chicken B cells, either based upon the *CSF1R* transgene, or direct staining with anti-CSF1R antibody (Balic et al., 2014). Accordingly, it can be predicted that CSF1-dependent macrophages in the bursa and spleen provide trophic factors that can influence the development or survival of B cells. One macrophage-dependent candidate regulator of bursal B cell development is B cell activating



factor (BAFF, TNFSF13B). It is highly expressed by avian monocytic cells within bursal follicles and upregulated during the growth and maturation of B cells (Koskela et al., 2004). In the chicken expression atlas ([www.biogps.org/chickenatlas](http://www.biogps.org/chickenatlas)), *TNFSF13B* mRNA is enriched in bursa, expressed by BMDM, and strongly induced following stimulation with LPS.

In spleen tissue there appeared to be an aggregation of cells expressing TIM4 surface marker at the junction between peri ellipsoidal WP and RP area upon anti-ChCSF1 mAb treatment (figure: 4-12), perhaps resulting from the recognition of apoptotic cells induced due to antibody treatment. To gain clarity on this result, it would be desirable to co-stain the spleen sections with chicken monocyte/macrophage markers like MHC-II, KUL01 and lymphocyte markers as BU-1 (B cells), CD3 (T cells). In mammals, circulating CSF1 exclusively regulates mature macrophages F4/80<sup>+</sup> cells of liver during postnatal development (Cecchini et al., 1994). In the liver tissue, administration of anti-ChCSF1 mAb to MacGreen chickens completely removed highly phagocytic apoptotic cell marker expressing TIM4<sup>+</sup> Kupffer cells (figure: 4-13). On the other hand, *CSF1R*-eGFP transgene expressing dendritic like cells in liver remained unaffected. In the recent study on chicken mononuclear phagocyte subsets, these cells in liver were identified to be non-phagocytic, highly co-expressing other markers as *FLT3*, *MHCII*, *XCRI* that associated them with conventional dendritic cells of mice (Hu et al., 2019)

Anti-ChCSF1 mAb treatment significantly depleted the number of bone-resorbing osteoclasts. microCT analysis of femur bones revealed significant reduction in the percentage bone volume and trabecular number with the corresponding increase in trabecular separation (figure: 4-14A). This result is the reciprocal of the effect of CSF1-Fc treatment of hatchling birds (Garceau et al., 2015). In mice, CSF1R is expressed in osteoclasts and administration of CSF1-Fc increases the number of osteoclasts in femur bones epiphyseal plate inducing osteoclastogenesis but paradoxically increasing bone density (Gow et al., 2014). Prolonged treatment with anti-mouse CSF1R Ab ablates osteoclasts number but also increases bone density and trabecular volume (Sauter et al., 2014). Similarly, anti-CSF1 treatment in mice from early post-natal period up until two months of age, decreased osteoclast number, but increased trabecular bone density (Wei et al., 2005). This apparent conflict probably arises because ossification mediated by osteoblasts depends upon a novel macrophage populations that is also CSF1-dependent (Batoon et al., 2017). This study in chicken is conducted in juvenile birds with rapid bone growth to which CSF1 may contribute predominantly in regulating skeletal growth.



#### Chapter 4: *In vivo* effects anti-CSF1 antibody

In conclusion, anti-ChCSF1 proved to be useful reagents to study the role of CSF1 in postnatal immune development of chickens. *In vitro* studies with anti-ChCSF1 mAb revealed that primary chicken macrophages are autocrine for CSF1 signalling. The antibody was successful in localising CSF1 protein expression in lymphoid tissues in chickens, although more knowledge regarding its localisation could be obtained by double staining with tissue macrophage markers. *In vivo* effects of anti-ChCSF1 Ab demonstrated here support the significance of using CSF1R reporter transgenic chicken lines for visualising the development of monocytes and macrophages (Garceau et al., 2015). Neutralising anti-ChCSF1 mAb provides a new tool to study the roles of CSF1 in both innate and acquired immunity in birds. In mice, constant high levels of anti-CSF1 antibody had to be maintained in the circulation to suppress its functions due to short half-life of the ligand (10 mins) (Wei et al., 2005). Injecting this antibody in postnatal chickens for longer duration or into fully developed adult chicken in the steady state will help gain more insight in understanding the biology of mononuclear phagocyte lineage cells which depends upon CSF1R signaling for their survival, differentiation and proliferation.

# Chapter 5 Generation of chicken CSF1R knockout model using genome editing CRISPR/Cas9 tools

## 5.1 Introduction

The introduction to this thesis described three approaches that will enable the study of CSF1 in function and development of the chicken mononuclear phagocyte system (Chapter 1, section 1.11). Chapter 3 demonstrated that chicken macrophages secrete endogenous CSF1 that signal through autocrine activity, enabling survival and proliferation without the addition of exogenous CSF1. A set of high activity chicken CSF1R kinase-specific inhibitor compounds were identified. Chapter 4 described the use of neutralizing anti-CSF1 antibody to dissect the significance of CSF1/CSF1R signaling leading to evidence for a role in the development of B cell lymphopoiesis in post hatch chickens. This Chapter is based on the application of a third approach that aims to generate *CSF1R* knockout chickens using CRISPR Cas9 genome editing tools.

Before the advent of gene editing technology allowed direct genome manipulation in fertilized oocytes, most knockouts in mice were generated by mutating embryonic stem cells, which could then be reintegrated into early embryos. The equivalent approach in chickens used is primordial germ cells (PGCs) as described in the introduction Chapter 1. During the early stages of egg incubation, endogenous PGCs arise from the extra-embryonic region germinal crescent and migrate in embryo through circulation into the genital ridge to produce functional sperm and oocytes (van de Lavoie et al., 2006; Macdonald et al., 2010). PGCs can be isolated, cultured *in vitro* for long term while maintaining lineage specificity and germline competency post re-introduction into recipient embryos (van de Lavoie et al., 2006; Macdonald et al., 2010; Macdonald et al., 2012). Cryopreservation of PGCs using conventional techniques without compromising their germline competency has also been described (van de Lavoie et al., 2006; Naito et al., 2015). Transmission of the modified PGC's DNA through germline chimeras generates transgenic birds (Sang 2006; Davey et al., 2018). Female PGCs form functional oocytes in female recipients and male PGCs form sperm in male recipients. Therefore, cultured PGCs which remain germline competent and can be modified and propagated *in vitro* are most commonly used to produce genetically modified chickens. Thus, chicken PGCs serve as valuable resource for genome editing and transgenesis.

## Chapter 5: CSF1R knockout chicken model

Several genome modification technologies have been successfully used to modify DNA in chicken PGCs. These include programmable engineered nucleases known for their specificity to alter a precise allele in a genome *in vitro* and *in vivo*. They are transcription activator-like effector nucleases (TALENs) (Park et al., 2014; Taylor et al., 2017), Zinc finger nucleases (ZFNs) and CRISPR/Cas9 (Dimitrov et al., 2016; Oishi et al., 2016).

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) nuclease are components of bacterial immune system (Jinek et al., 2012). CRISPR guide RNA is a custom made 20 nucleotide sequence that recognizes a complementary target sequence of interest and guides Cas9 nuclease to the target site to generate double strand breaks. The only requirement for the Cas9 nuclease from *Streptococcus pyogenes*, directed by guide RNA to any genomic locus, is that the target site is preceded by a 5'-NGG protospacer adjacent motif (PAM). The induced break promotes gene editing repair either by a non-homologous end joining (NHEJ) mechanism which leads to small insertions and/or deletions (error prone) or by a homology directed repair (HDR) mechanism which generates defined mutations. (Ran et al., 2013). NHEJ mediated INDELS that occur within a coding exon of a gene can result in frameshifts leading to premature stop codon mutation resulting in a gene knockout.

Compared to ZFNs and TALENS, the CRISPR/Cas9 system benefits from ease of customization and higher targeting efficiencies (Lee et al., 2017). The convenience of CRISPR/Cas9 vector constructions makes them a popular tool for genome editing. However, intensive experimental procedures involved in optimizing upstream and downstream procedures such as obtaining PGCs, maintaining their viability, proper culture conditions, delivery of Cas9/gRNA vector, isolating clonal mutant populations, selection and low mutation efficiencies limits its facile application in chickens.

TALENs were used to knockout the *ovalbumin* gene in chicken PGCs *in vitro* as well as in the genome edited progeny generated. The mutation frequency in PGCs was found to be 33.3%, whereas the germline transmission rate from *ovalbumin* gene mutant chimeric chicken was on average 8% (Park et al., 2014). TALENs in combination with a vector was used to target germ cell specific *DDX4* gene, located on Z-chromosome in chicken PGCs to generate heterozygous knockout G1 offspring. The germline transmission efficiency obtained from the founder chimeric birds was 6% (Taylor et al., 2017).

CRISPR/Cas9 system was also used to target the ovalbumin and ovomucoid gene in chicken PGCs by transfection and the use of selective antibiotic markers. Transplantation of mutant ovomucoid PGCs into chicken embryos generated mutant ovomucoid heterozygous and homozygous offspring. The mutation frequency of *ovalbumin* gene targeting in chicken PGCs *in vitro* was 100% and PGC derived heterozygous G1 offspring was 50%, an improvement over other methods using this approach (Oishi et al., 2016). This was the first study that reported the generation of knockout chicken using the CRISPR/Cas9 system. Subsequently, the chicken immunoglobulin heavy chain (IgH) locus was targeted by CRISPR induced HDR in PGCs by transfection and drug resistant clonal selection. This approach generated IgH heterozygous transgenic progeny that hatched and developed normally. Germline transmission rate of G1 progeny varied between 0 and 100% (Dimitrov et al., 2016).

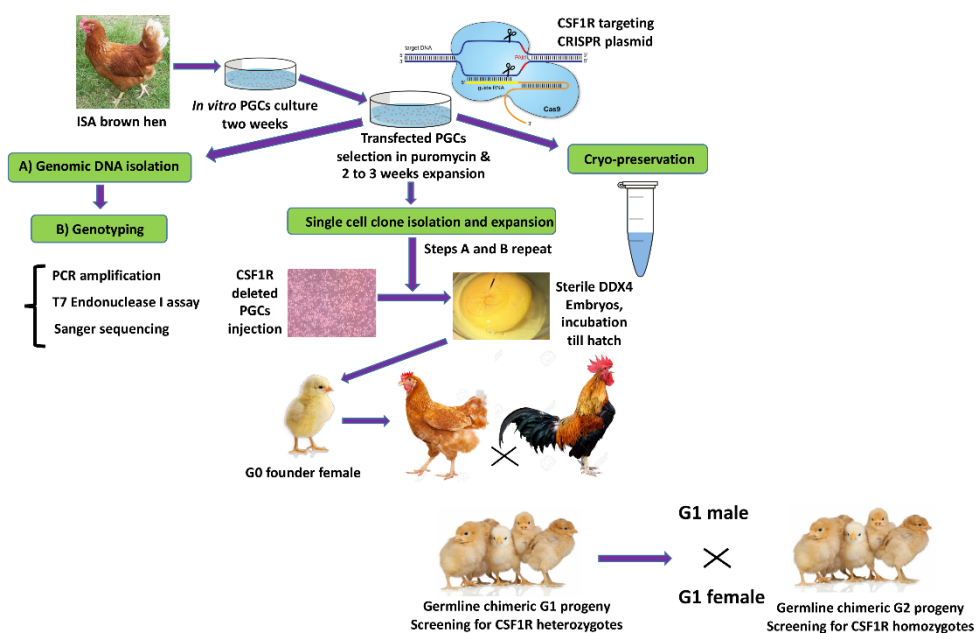
With relevance to industry applications, genetically modified chickens with reduced abdominal fat deposition and altered blood fatty acid composition have been produced using CRISPR/Cas9 tools and germline transmission via modified donor PGCs (Park et al., 2018). Also, using CRISPR/Cas9 tools, critical avian leukosis virus (ALV) entry attachment residues of a receptor was mutation in DF-1 chicken fibroblast cells, which led to resistance of the most commonly infected retrovirus ALV-subgroup J in chicken line (Lee et al., 2017). Similarly, knockout of tumor virus locus B gene encoding receptor for ALV subgroup B attachment, using CRISPR/Cas9 induced NHEJ in DF-1 cells led to resistance against ALV subgroup B infection (Lee et al., 2017). The same approach was used to induce frameshift mutations into genes encoding receptors for the remaining subgroups of ALV infection A, C, and J in DF-1 cells. All three knockout receptor loci resulted in resistance to the respective ALV subgroups in clones of chicken DF-1 cell (Koslová et al., 2018). These studies highlighted the significance of using CRISPR/Cas9 system to generate virus resistant chickens.

In a recent study, an adenoviral CRISPR Cas9 vector was injected directly into quail blastoderm that generated homozygous and heterozygous offspring with a targeted mutation in a gene (melanophilin) involved in feather pigmentation (Lee et al., 2019). This enabled the recognition of mutants based on feather colour of offspring. In another study (Lee et al., 2019), a GFP gene cassette was integrated into the chicken sex Z chromosome using CRISPR/Cas9 vector tools in PGCs. This led to the generation of chickens expressing green fluorescent protein from the Z chromosome which conveniently distinguished male (ZZ) from female progeny (ZW) during embryogenesis

## Chapter 5: CSF1R knockout chicken model

As described in the introduction section of Chapter 4, targeted disruption of *Csf1r* loci in homozygous mice and rats does affect their viability. They demonstrate postnatal growth retardation, loss of blood monocytes, osteoclasts deficiency, lack tooth eruption and exhibit severe loss of few tissue macrophages (Dai et al., 2002; Li et al., 2006; Pridans et al., 2018). On the other hand, heterozygous *Csf1r* knockout mice and rats are viable and fertile. As opposed to *Csf1r*<sup>-/-</sup> mice, *Csf1r*<sup>-/-</sup> rats survive till adulthood but are infertile (Li et al., 2006; Pridans et al., 2018). Because the biology of major organs systems is different in birds and *CSF1R* receptor expression is not completely similar, it is interesting to study the phenotype of *CSF1R* knockout chickens.

To generate *CSF1R* knockouts in chickens, CRISPR Cas9 tools were designed to enable targeted gene disruption of the gene followed by isolation of clonal populations of PGCs with the subsequent objective of using these PGC to generate germ-line mutations. An overview of the workflow for this Chapter is shown in figure: 5-1.



**Figure 5-1: Schematic representation of study design and experimental procedures**

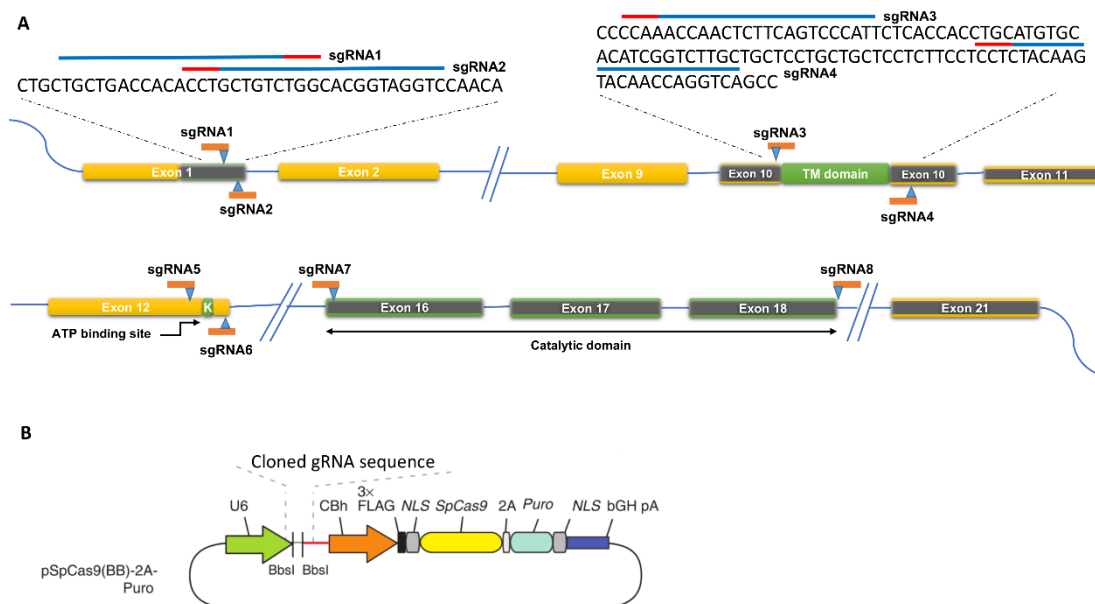
### 5.2 Results

#### 5.2.1 Preparation of target gRNA oligos for genome editing

For targeted gene disruption of chicken *CSF1R*, the annotated gene sequence was obtained from Ensembl. As described in the introduction Chapter 1, *CSF1R* is characterized by five extracellular immunoglobulin domains, a single transmembrane domain (TM) and a cytosolic domain which is comprised of a regulatory juxta-membrane (JM) domain followed by a protein tyrosine kinase domain (PTK) containing a kinase insert domain (KID) in an auto-inhibited state. Seven conserved tyrosine residues are located within the JM, PTK and KID (catalytic domain) (Walter et al., 2007). Four different targeting sites from the coding regions; exon 1, transmembrane domain (TM) (exon 10), ATP binding site exon 12 (K611 in chickens) and catalytic domain (exons 16, 17, 18) were selected for INDEL mutations. Accordingly, 8 guide RNAs (gRNAs) were designed in total (two for each target sequence) surrounding the regions of interest to direct double strand cutting of the genome (listed in table 2-2 of Chapter 2 and shown as in figure: 5-2A). gRNAs with predicted less off-target genomic sites, were selected. All gRNA cloning oligos were synthesized in sense and anti-sense directions.

To prepare gRNAs for delivery into PGCs culture, all gRNAs were cloned separately into the SpCas9 nuclease encoding vector, pSpCas9(BB)-2A-Puro or (PX459) V2.0 as described previously in (Ran et al., 2013). This vector encodes Cas9 nuclease derived from *Streptococcus pyogenes* also known as wild type Cas9 nuclease and contains puromycin resistance gene for selection of gRNA transfected cells. Cloned gRNA site into PX459 vector, driven by U6 promoter is shown in figure: 5-2B. All 8 gRNAs oligos were cloned into the PX459 vector using a *BbsI* restriction site as described in Chapter 2 section 2.9.1, herein referred to as CRISPR plasmid construct for the respective gRNA. As a negative control a PX459 vector only ligation reaction was also performed.

To validate gRNAs orientation into cloned CRISPR plasmid constructs, plasmids were Sanger sequenced and referenced against sequence of PX459 vector (<https://www.addgene.org/62988/sequences/>). Plasmids confirming the alignment of the 20-nucleotide gRNA sequence between the U6 promoter and the gRNA scaffold of PX459 vector (Ran et al., 2013) were used to prepare plasmid maxiprep stock. Sequence alignment analysis revealed that all the designed 8 gRNA targeting sequences aligned with the cloning site of PX459 vector (data not shown) and thereby utilised further for functional validation as below.



**Figure 5-2: Schematic representation of gRNAs targeting different regions of CSF1R loci and CRISPR Cas9 vector used**

(A) Depiction of exon-intron assembly of chicken CSF1R loci, consisting of 21 coding exons. Eight guide RNAs designed to target different regions of interest (exon 1, transmembrane domain (TM), ATP binding site and catalytic domain) are shown. Target sequences selected for functional validation with gRNA1, 2, 3 and 4 are marked with blue bar above the nucleotide sequences and adjacent protospacer adjacent motif (PAM) sequences are highlighted in red. (B) pSpCas9(BB)-2A-Puro (PX459) V2.0 vector construct encoding for the cloned gRNA sequence and Cas9 protein driven by U6 promoter and CAG promoter respectively is shown. Puromycin resistance marker (PuroR) for selection of transfected colonies is indicated. Adapted from (Ran, Hsu et al. 2013).

## 5.2.2 PGC culture and characterization of gRNAs encoding CRISPR plasmid constructs NHEJ mediated genome editing in chicken PGCs

### 5.2.2.1 Functional validation of gRNA 1 and 2 targeting exon 1 of CSF1R

To find out if CSF1R can be modified *in vitro* in chicken PGCs, genome editing using the CRISPR/Cas9 tools via NHEJ break repair, was performed. A chicken PGC line derived from a female ISA brown layer embryo in culture was provided by Dr. Mike McGrew, at The Roslin Institute. Chicken PGCs were maintained in culture as described in (Whyte et al., 2015) for two weeks in 24 well plate. When the cell number reached  $2 \times 10^5$  cells/well (~80% confluent), they were harvested for lipofectamine transfection. PGCs were co-transfected with two

## Chapter 5: CSF1R knockout chicken model

CRISPR/Cas9 plasmid construct expressing guides target sites in exon 1 (gRNA1, 2). Transfection protocols were previously optimized by a colleague, Dr. Alewo Idoko-Akoh. Selection of transiently transfected cells was based upon puromycin resistance gene in the Crispr/Cas9 plasmid. 99% of control PGCs and cells not transfected with CRISPR plasmid construct were gradually killed in the subsequent 5 days post-transfection. Clumps of living PGCs that had outgrown dead cells appeared eventually over two weeks for cells transfected with gRNA1, gRNA2 and gRNA1+2. Cells were bigger and identifiable compared to the smaller granular dead cells against the background. These cells demonstrating transient puromycin resistance and proliferation were expanded further for two-three weeks (as described in Chapter 2 section 2.11) to obtain enough cells for genomic DNA isolation and genotyping.

PGCs were assayed for INDELS by PCR. Primers were designed to amplify the guide target site of *CSF1R* exon 1 within a 879 bp product. Both gRNAs 1 and 2 were designed to direct double strand cutting by Cas9 at approximately the same site 1122 bp downstream of the translation initiation site of the *CSF1R*. Amplification of gRNA1 transfected PGCs DNA revealed visible DNA fragment of about the expected size (879 bp) on the DNA gel (figure: 5-3A). Band of similar size was also visible for PGCs transfected with gRNA2 (figure: 5-3A). To detect the knock-out efficiency of each gRNA post-transfection, PCR amplified products were digested with T7 endonuclease I (T7EI) enzyme, identifying single base mismatches by cleavage at the site detected on agarose gel in the form of cleaved products. The T7EI assay revealed additional cleaved products at sizes approximately 530 and 350 bps, along with the expected DNA fragment of size 879 bp, for PGCs transfected with gRNA1 and gRNA2 (figure: 5-3B). To determine on-target genome editing efficiency, DNA fragment's relative band intensities were quantified using ImageJ. Guide RNAs 1 and 2 targeting exon 1 were effective in mutating receptor *CSF1R* in PGCs culture *in vitro* with targeting efficiency of 36.2% and 34.2% respectively (figure: 5-3B).

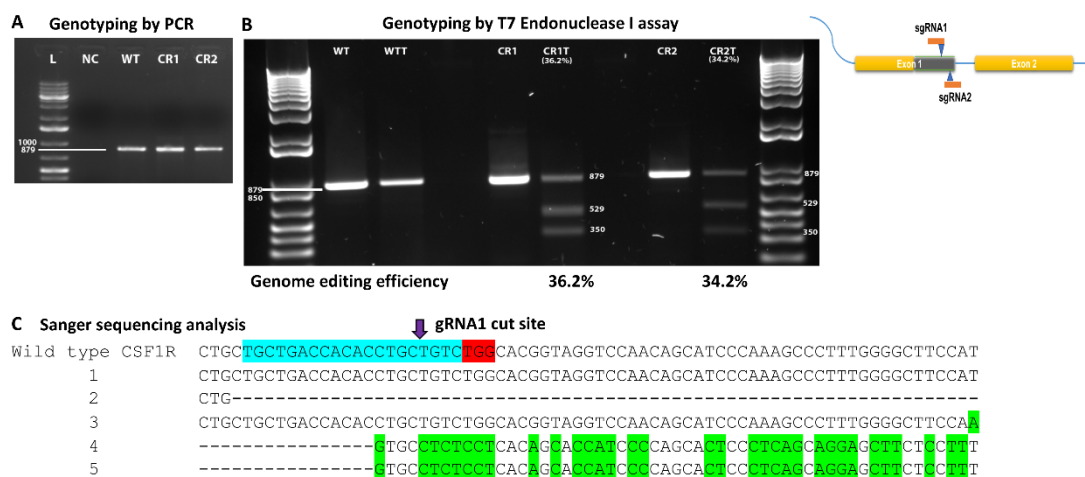
To verify the INDEL mutations, amplified gRNA 1 and 2 target sites were cloned, and Sanger sequenced. Analysis of sequences from individual subcloned PCR reactions from the sequencing of a mixed population of cells generated with gRNA1 transfection showed inserted sequences in 2 (number 4 and 5) out of 5 sequencing reactions near Cas9 cleavage site. There was large deletion of nucleotide sequences in sequencing reaction 2. No INDEL mutations were obtained in sequence reaction number 1 and 3 (figure: 5-3C). Analysis of sequences from

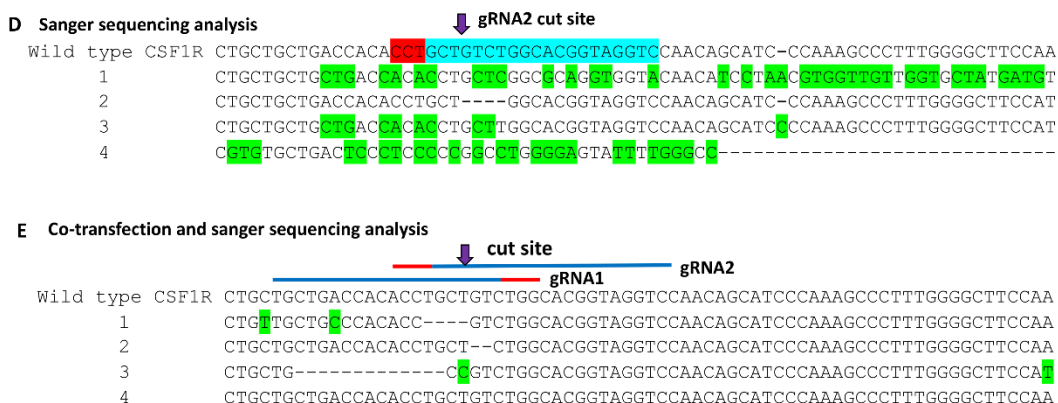


## Chapter 5: CSF1R knockout chicken model

individual subcloned PCR reactions generated with gRNA2 transfection showed inserted sequences in 3 (number 1, 3 and 4) out of 4 sequencing reactions near Cas9 cleavage site. There was a deletion of 4 bp nucleotide at the predicted Cas9 cleavage site in sequence reaction number 2 (figure: 5-3D). Analysis of sequences from individual subcloned PCR reactions generated with co-transfection of gRNAs 1 and 2 showed INDEL mutations in 3 (number 1, 2 and 3) out of 4 sequencing reaction. No INDEL mutations were obtained in sequence reaction number 4 (figure: 5-3E).

Thus, designed gRNAs targeting exon 1 of receptor CSF1R were successful in inducing NHEJ mediated INDEL mutation in chicken PGCs.





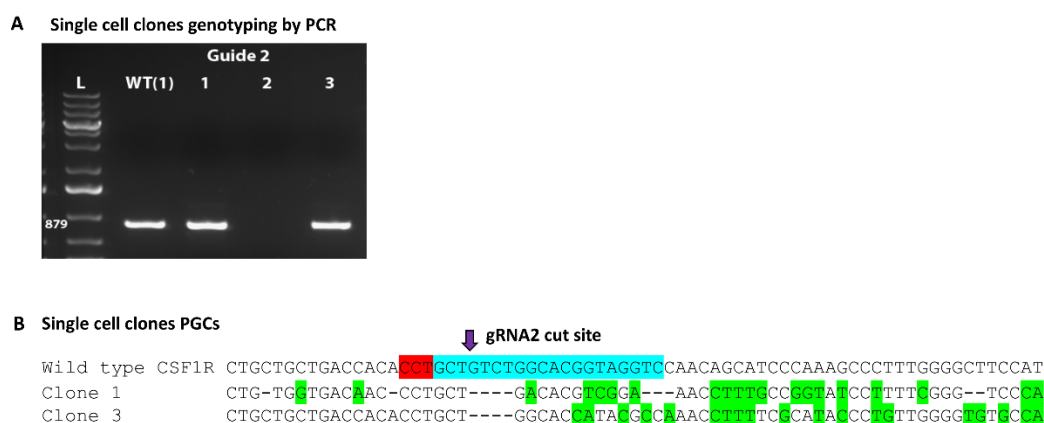
**Figure 5-3: Screening for gRNA1 & 2 CRISPR plasmid induced mutation in exon 1 of chicken CSF1R in transfected non clonal population of PGCs by genotyping and Sanger sequencing**

(A) Genomic DNA amplification by PCR of a mixed population of PGCs transfected with gRNAs1 (CR1) and 2 (CR2). Primers utilised for amplification of corresponding gRNAs (CR1 and CR2) target region are mentioned in table 2-2 of Chapter 2. NC is negative control with no PGCs genomic DNA and WT is wild type un-transfected female PGC line’s genomic DNA. Amplicon at predicted location post transfection is indicated (879 bps). (B) Agarose gel visualisation of genomic DNA INDEL mutations (cleaved PCR substrates) with gRNA1 and 2 transfected PGCs digested with or without T7 endonuclease I. T denotes enzyme treatment. Genome editing efficiency (calculated using ImageJ) obtained with each gRNA is indicated below. (C) Sanger sequencing alignments of target DNA sequences from PGCs transfected with gRNA 1 (C), 2 (D) and co-transfected with gRNA 1 and 2 (E) in a mixed PGC population is shown. Target sequence of the CRISPR guide is indicated in light blue and PAM with red. Arrows indicate cleavage site of CRISPR/Cas9 vector. Insertions are shown in green and deletions with dashes. The number of sequencing reactions is indicated on the left along with a reference wild type CSF1R sequence at the top for comparison.

After stable genome edited clones of PGCs were identified by both PCR and Sanger sequencing post-transfection with CRISPR Cas9 plasmid construct encoding gRNA 1 and 2, the mixed cell population was manually sorted to obtain single cell clonal populations. A minimum of 10 single cell clones per gRNA transfected were cultured for expansion (as described in Chapter 2, section 2.11.5), isolation of genomic DNA, and genotyping. 4 out of 10 cultured single cell clones for gRNA1 transfected cells survived and proliferated, while 3 out of 10 cultured single cell clones for gRNA2 transfected cells survived and proliferated. All single cell clones were cryopreserved for long term storage at cell density of  $2 \times 10^5$  cells/0.5ml media. As described above for the mixed cell population, single cell clones of gRNA2 transfected PGCs were genotyped to identify INDEL mutations at the target site exon 1. PCR amplification of gRNA2 transfected PGCs DNA revealed visible DNA fragment at the expected size 879 bp for single

cell clone 1 and clone 3 (figure: 5-4A). Cell density of single cell clone 2 was insufficient to obtain a sufficient amount of genomic DNA for PCR amplification, hence there was absence of DNA fragment in single cell clone 2 (figure: 5-4A).

Sanger sequencing alignment of direct sequencing reactions from clones 1 and 3 revealed insertions and 4 bp nucleotide deletion near the Cas9 cleavage site (figure: 5-4B) leading to frame shift mutation. Thus, exon 1 mutation in chicken CSF1R induced by gRNA2 in PGCs *in vitro* is out of frame.



**Figure 5-4: Screening for gRNA2 CRISPR plasmid induced mutation in exon 1 of chicken CSF1R in single cell clones of PGCs by PCR and Sanger sequencing**

(A) Genomic DNA amplification by PCR of isolated and expanded single cell clones of PGCs transfected with gRNA2 (CR2). WT is wild type un-transfected female PGC line’s genomic DNA. Amplicon at predicted location post transfection is indicated (879 bps). (B) Sanger sequencing alignment of target DNA sequences from PGCs transfected with gRNA2 in two single cell PGC clones is shown. Target sequence for CRISPR/Cas9 vector is indicated in light blue and PAM with red. Arrows indicate cleavage site of CRISPR/Cas9 vector. Insertions are shown in green and deletions with dashes. The number of clones is indicated on the left along with a reference wild type CSF1R sequence at the top for comparison.

### 5.2.2.2 Production of donor PGC line with bi-allelic deletion of transmembrane domain of CSF1R

The next target site of CSF1R that was functionally validated *in vitro* in chicken PGCs is exon 10 encoding for transmembrane domain (TM) of the receptor. Accordingly, CRISPR/Cas9 mediated NHEJ genome editing was implemented. As the intention was to perform targeted deletion of the TM domain of the receptor, the two gRNAs 3 and 4 were cloned separately in CRISPR plasmid construct as described above in section 5.2.1 and co-transfected in the female

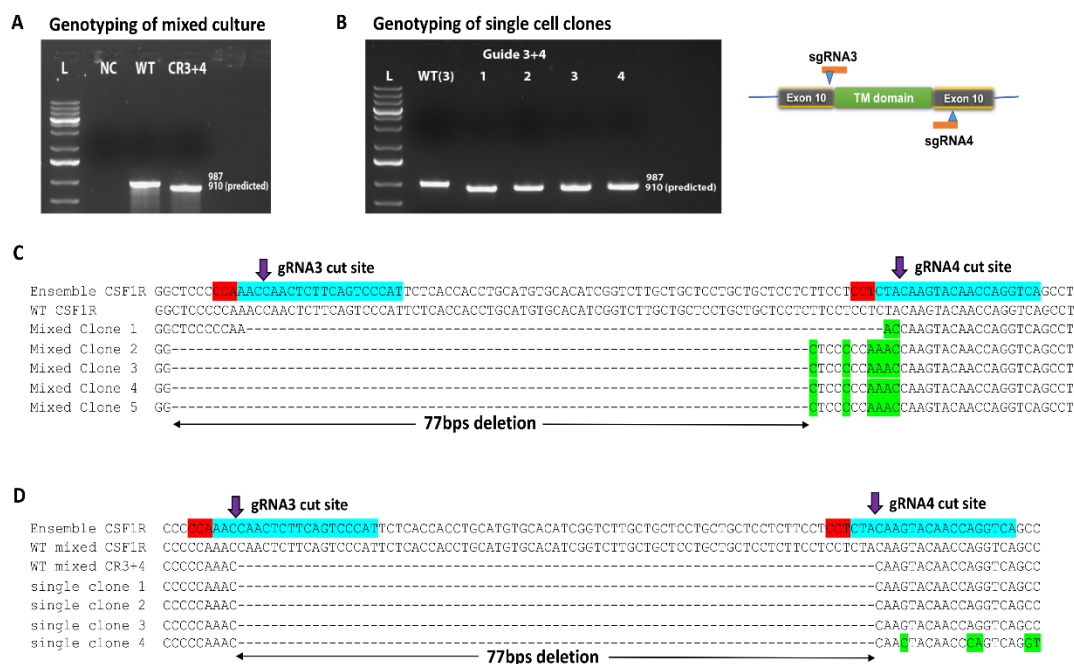
PGC culture. Cells surviving puromycin resistance appeared in two weeks. They were further expanded for two to three weeks for genomic DNA isolation and genotyping, similar to PGCs transfected with gRNA 1 and 2 above.

For assessing DSB repair by PCR, primers were designed to amplify *CSF1R* exon 10 target site to obtain 987 bp product. Amplification of co-transfected mixed PGCs DNA interestingly revealed one visible DNA fragment smaller than the un-transfected PGCs DNA size of around 910 bp on the DNA gel (figure: 5-5A). Difference in the amplified DNA fragment between un-transfected and co-transfected PGCs genomic DNA suggested a bi-allelic deletion of the target site TM of *CSF1R*. Predicted Cas9 cleavage by designed gRNA3 and gRNA4 made a deletion of 77 bp of TM domain, which was also equivalent to the difference in size between the two DNA fragments around 77 bp (987-910 bp).

To confirm deletion of the TM domain region of *CSF1R*, mixed PGC DNA co-transfected with gRNA3 and 4 was amplified, cloned, and Sanger sequenced. Analysis of sequences from individual PCR subcloned colonies generated from the mixed cell population showed deletion of 77 bps in all of the five products sequenced (figure: 5-5C). This validated the deletion of the target site TM domain by Cas9 cleavage with gRNA3 and 4, obtaining 100% efficiency *in vitro*. This deletion induced an out of frame mutation thereby confirming bi-allelic knockout of chicken *CSF1R* receptor in PGCs. In order to know the likely expression of *CSF1R* protein in germline chimeras post TM domain deletion, it was decided to use these bi-allelic TM domain deleted PGCs as donor cells for germline transmission.

In order to transplant pure donor PGCs in recipient chicken embryos, TM domain deleted single cell clonal population was obtained. Accordingly, gRNA 3 and 4 co-transfected mixed cell populations were manually sorted to obtain single cell clonal populations. 10 single cell clones were cultured for expansion (as described in Chapter 2 section 2.11.5), isolation of genomic DNA and genotyping. 4 out of 10 cultured single cell clones survived and proliferated. These were cryopreserved for long term storage until chicken embryo injection experiments to produce GE chicken. To validate TM domain deletion mutation in single cell clones, clones were similarly genotyped by PCR and Sanger sequencing. PCR amplification of gRNA3 and 4 co-transfected PGCs DNA revealed visible DNA fragment at the expected size 910 bp for all single cell clones 1, 2, 3 and 4 (figure: 5-5B), smaller than the un-transfected PGCs DNA (987 bp). Sanger sequencing alignment of single cell subcloned sequencing reactions 1, 2, 3, 4,

including a mixed cell population control, revealed deletion of 77 bps in all of the five sequencing reactions (figure: 5-5D). This result confirmed deletion of the target site TM domain by Cas9 cleavage with gRNA3 and 4 in single cell clones of chicken PGCs with 100% mutation efficiency. The putative translated amino acid protein product size post CSF1R deletion is 595AAs, while the wild type CSF1R protein size is 967AAs. Translated cDNA sequences of CSF1R before and after deletion is shown in Chapter 8, section 8.4, Appendix D. Thus, TM domain deleted bi-allelic chicken *CSF1R* receptor gene knockout was generated in PGCs using genome editing CRISPR/ Cas9 tools.



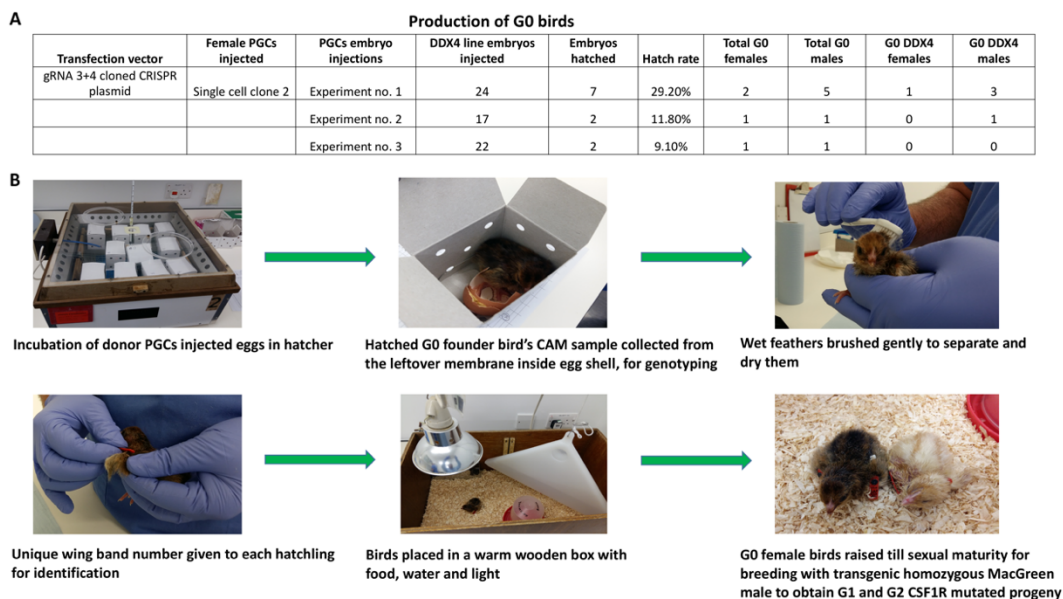
**Figure 5-5: Screening for gRNA2 & 3 CRISPR plasmid induced mutation in transmembrane domain of chicken CSF1R in mixed and single cell clones of PGCs by PCR and Sanger sequencing**

Genomic DNA amplification by PCR of mixed (A) and single cell clones (B) of PGCs co-transfected with gRNAs 3 and 4. NC is negative control with no PGCs genomic DNA and WT is wild type un-transfected female PGC line genomic DNA. Amplicon at predicted location with WT DNA and post transfection is indicated. Sanger sequencing alignments of target DNA sequences from PGCs co-transfected with gRNAs 3 and 4 in mixed (C) and single cell (D) PGCs population is shown. Target sequence for CRISPR guide is indicated in light blue and PAM with red. Arrows indicate cleavage site of CRISPR/Cas9 vector. Insertions are shown in green and deletions with dashes. The number of sequencing reaction is indicated on the left along with a reference wild type CSF1R sequence at the top for comparison.

### 5.2.3 Chicken *CSF1R* edited PGCs embryonic injection and generation of G0 founder birds

The female PGC line with bi-allelic TM domain deletion of *CSF1R* gene was assessed for their ability to generate *CSF1R* deficient chickens via germline transmission. Accordingly, all four cryopreserved single cell clonal line of PGCs carrying confirmed homozygous deletion of TM domain of *CSF1R* gene were thawed, cultured and expanded for 2 to 3 weeks as per the procedures described in Chapter 2, section 2.11.5. Out of 4 single cell clones cultured, viability of clone 2 was highest with most cells and less visible cell death. Therefore, clone 2 was selected as donor PGCs to inject in developing recipient chicken embryos. A previous knockout of *DDX4* gene revealed its role for oogenesis in birds. Fertile eggs from the germ cell-deficient *DDX4* transgenic chicken line as described in (Taylor et al., 2017) were utilised for injection of *CSF1R*-edited PGCs. Donor PGCs were injected at cell density of 8000 PGCs/ $\mu$ l at approximately stage 16HH into the dorsal aorta (McGrew et al., 2004; Taylor et al., 2017) and as described in Chapter 2, section 2.12. Three independent PGCs injection experiments were performed and embryos incubated until hatch to generate G0 founder birds. Dr. Mike McGrew performed embryo injections as per the licensed animal procedures.

Out of the 24 total embryos injected in experiment 1, 7 survived to hatch (29.2%). 2 embryos out of 17 survived in experiment 2 (11.8%) and 2 embryos out of 22 survived in experiment 3 (9.1%) (Figure: 5-6A). The difference in egg incubation conditions was accountable for the difference in the hatch rate. Eggs incubated in the wooden hatcher had a better hatch rate than those incubated into the ova easy cabinet. The sex of hatched birds was determined by W-PCR analysis using genomic DNA from both chorioallantoic membrane (CAM) (post hatch) and blood (taken at 3 weeks age) samples (Clinton et al., 2001). A summary of the hatched G0 bird's male and female numbers obtained from all the three PGCs injection experiment is shown in (figure: 5-6A). To identify germ cell deficient *DDX4* knockout sterile G0 birds, PCR for the GFP transgene was performed as these chimeric chickens carry GFP transgene (Taylor et al., 2017). Only one female *DDX4* knockout sterile G0 founder chicken was hatched from all the three PGCs injections experiments combined (figure: 5-6A). A picture of it is shown along with the representative steps for obtaining hatched chickens (figure: 5-6B). It was raised to sexual maturity for breeding along with other non-sterile wild type females hatched from the three experiments.



**Figure 5-6: Injection of CSF1R edited donor PGCs in sterile DDX4 embryos and generation of G0 founder birds**

Recipient embryos at stage 16HH from sterile DDX4 heterozygote males mated with wildtype hens were injected with verified culture of donor PGCs single cell clone with bi-allelic transmembrane domain deleted region of chicken CSF1R. 1  $\mu$ l of donor PGCs with a cell density of 8000 PGCs/ $\mu$ l was injected as per licensed procedures and embryos incubated to hatch (A) shows the hatch rate obtained from three independent donor PGCs injection experiment. Subsequent sexing and screening for eGFP CAM samples from the hatched off-springs identified the number of female G0 DDX4 negative founder bird carrying CSF1R edited donor PGCs. (B) demonstrates an overview of the steps involved in hatching chicks in general in this study. The G0 founder birds hatched chicks are shown.

## 5.2.4 Successful transmission of chicken CSF1R knockout allele in G1 and G2 progeny

### 5.2.4.1 Generation and screening of G1 CSF1R heterozygous mutant chickens

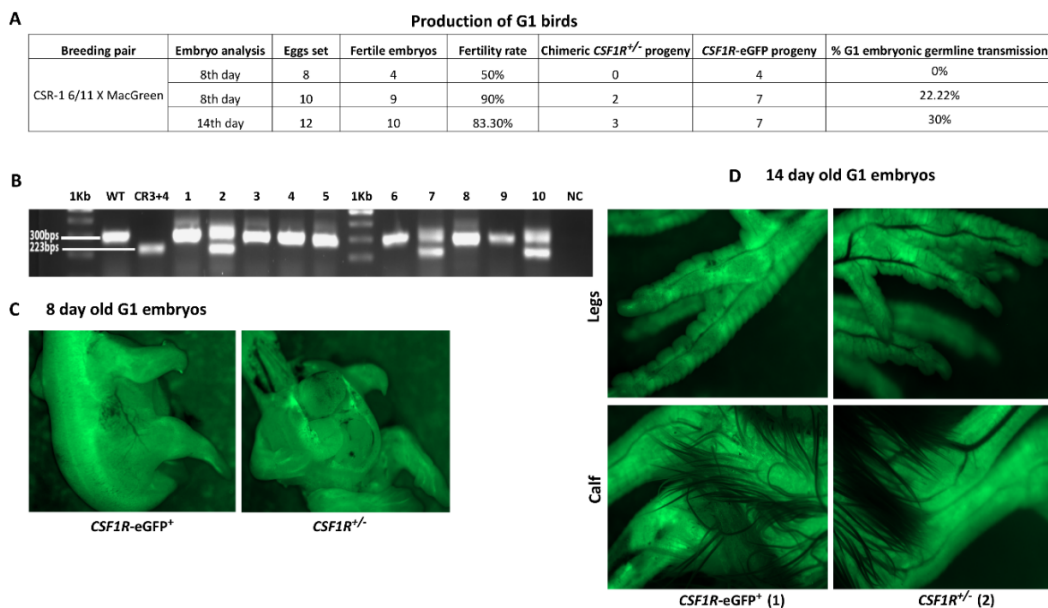
To find out if CSF1R knockout allele carried by PGCs injected into G0 founder females can be germline transmitted to progeny, the sexually mature G0 founder females CSR1-6 (wild type chicken) and CSR1-11 (DDX4 knockout sterile) were mated to a sexually mature homozygous CSF1R-eGFP (MacGreen) transgenic (Balic et al., 2014) male chicken by artificial insemination. Initially, to assess potential abnormalities in CSF1R mutant heterozygous G1 progeny, impact on embryonic development *in ovo* was examined. Accordingly, G1 embryos were analysed *in ovo* at two time points, day 8 and day 14 of incubation.

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Day 8 embryos were examined in two batches of egg sets. In first batch, 4 out of 8 eggs were fertile (50% fertility rate) and in second batch, 9 out of 10 eggs were fertile (90% fertility rate) (figure: 5-7A). Screening of G1 embryos for the *CSF1R* targeted deletion of embryonic genomic DNA by PCR from batch 1 eggs did not detect *CSF1R* heterozygous embryos. Screening from batch 2 eggs, 2 chimeric *CSF1R* heterozygous embryos were detected (22.22% germline transmission rate) (figure: 5-7A). Next, examining G1 embryos on the day 14 of incubation, out of 12 eggs set, 10 eggs were fertile (83.30%) and 3 embryos carried the *CSF1R* INDEL heterozygous allele (figure: 5-7A). Genotyping by PCR amplification of the target site for these 10 fertile embryos using genomic DNA is shown in figure: 5-7B. The three G1 *CSF1R* deleted heterozygous day 14 embryos, numbered 2, 7 and 10 are shown in the gel in comparison to the *CSF1R* wild type genomic DNA.

All G1 embryos from day 8 and 14 of incubation were examined by whole mount imaging for the expression of *CSF1R*-eGFP transgene. There was no impact of *CSF1R* deletion on the expression of *CSF1R*-eGFP transgene at the 8<sup>th</sup> and 14<sup>th</sup> day in *CSF1R* heterozygous G1 embryos. Representative day 8 whole embryo GFP fluorescence comparison between *CSF1R*-eGFP and *CSF1R*<sup>+/-</sup> G1 progeny is shown in figure: 5-7C. 14 day old limb comparison between *CSF1R*-eGFP<sup>+/+</sup> and *CSF1R*<sup>+/-</sup> G1 progeny is shown in figure: 5-7D. *CSF1R* heterozygous G1 birds depicted no obvious abnormalities or observed phenotypes compared to wild type hatch mates and developed normally. G1 progeny was then subsequently hatched to analyse phenotype of adult *CSF1R* heterozygous chickens.





**Figure 5-7: Production and screening of chimeric *CSF1R* heterozygous G1 embryos at different time points**

A sexually mature G0 female DDX4 knockout hen carrying *CSF1R* edited donor PGCs was bred with transgenic homozygous *CSF1R*-eGFP male by artificial insemination and G1 embryos screened for *CSF1R* heterozygous genotype by PCR of CAM samples (A) shows the fertility rate obtained from analysis of embryos at two time points (day 8 and 14 of incubation). Number of *CSF1R* heterozygous embryos obtained on each day is indicated along with the percentage of germline transmission. (B) demonstrates genotyping of embryonic samples by PCR for screening *CSF1R* heterozygous embryos. Numbers represent embryo number analysed. (C) GFP fluorescence of 8-day old G1 embryos comparison between *CSF1R*-eGFP<sup>+/+</sup> and *CSF1R* heterozygotes via whole mount imaging. Scale bar 1000  $\mu$ m (D) shows the limbs and calf comparison between the two groups of 14-day old G1 embryos. Scale bars for all images 1000  $\mu$ m.

#### 5.2.4.2 *CSF1R* heterozygous knockout adult chickens have no observed phenotypes

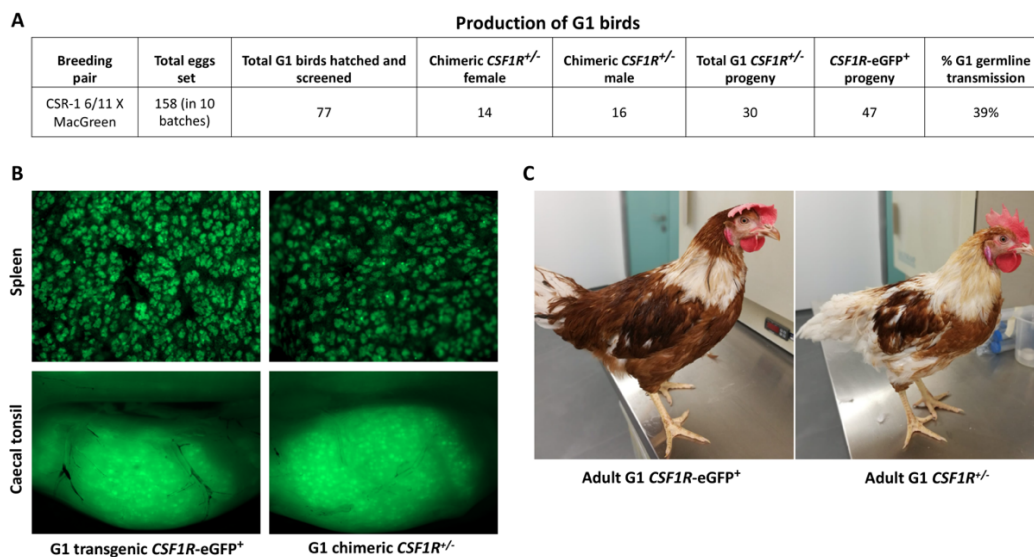
There are no apparent impacts of a *Csf1r* heterozygous knockout mutation in adult mice or rats (Dai et al., 2002; Pridans et al., 2018). To analyse the phenotype of *CSF1R* deleted heterozygous G1 birds, eggs were collected from the mating of G0 founder females CSR1-6 and CSR1-11 to *CSF1R*-eGFP (MacGreen) transgenic chickens and incubated to hatching. In total 158 eggs were set for incubation in 10 batches, out of which 77 chicks hatched (48.73% hatch rate) (figure: 5-8A). The lower hatch rate of G1 birds could be due to fertility of DDX4 females containing clonally derived genome modified PGCs or the fertility of the semen from the MacGreen chicken used to inseminate the G0 hens. Sexing by W-PCR and screening of G1 chimeric progeny for *CSF1R* target site deletion established in total 30 *CSF1R* heterozygous

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birds (39% germline transmission efficiency), of which 14 were females and 16 were males (figure: 5-8A). 47 of the 77 birds contained the *CSF1R*-eGFP transgene. All *CSF1R* heterozygous birds were healthy and developed normally into adulthood similar to *CSF1R*-eGFP chickens (wild type hatch mates). Similar to *Csf1r* heterozygous adult mice and rats, developmental growth of *CSF1R* heterozygous chickens was normal. Generated chimeric adult *CSF1R*-eGFP chicken and *CSF1R* deleted heterozygous adult G1 chicken is shown in (figure: 5-8C). All G1 heterozygous birds were raised to sexual maturity.

To examine if the heterozygous deletion of *CSF1R* had any impact on the expression of *CSF1R*-eGFP transgene in lymphoid organs of adult G1 *CSF1R* heterozygous birds, spleen and caecal tonsil tissue was collected in PBS and imaged for transgene expression in *CSF1R*-eGFP (n=4) and *CSF1R* heterozygous (n=3) chickens after culling. There was no difference in *CSF1R*-eGFP transgene expressing macrophages in any of the *CSF1R* heterozygous bird's spleen and caecal tonsil tissue compared to *CSF1R*-eGFP chickens (figure: 5-8B).

These results indicated successful transmission of TM domain deleted *CSF1R* knockout allele in G1 progeny with no obvious pathology or phenotype.



**Figure 5-8: Production and screening of chimeric  $CSF1R$  heterozygous G1 chicken line**

A sexually mature G0 female DDX4 knockout hen carrying  $CSF1R$  edited donor PGCs was inseminated with transgenic homozygous  $CSF1R$ -eGFP male semen and G1 birds were maintained until adulthood for subsequent breeding. CAM and blood DNA samples (obtained after 3 to 4 weeks) were screened for sex and genotyping of G1 birds as given in (A). Percentage germline transmission of donor  $CSF1R$  mutated PGCs in G1 birds is calculated from the total  $CSF1R$  heterozygote birds obtained. (B) demonstrates comparison of lymphoid organs spleen and caecal tonsil tissue of G1 transgenic  $CSF1R$ -eGFP chicken (n=4) and  $CSF1R$  heterozygous (n=3) chicken by whole mount imaging. Scale bar for all images 500  $\mu$ m (C) shows the adult G1 birds.

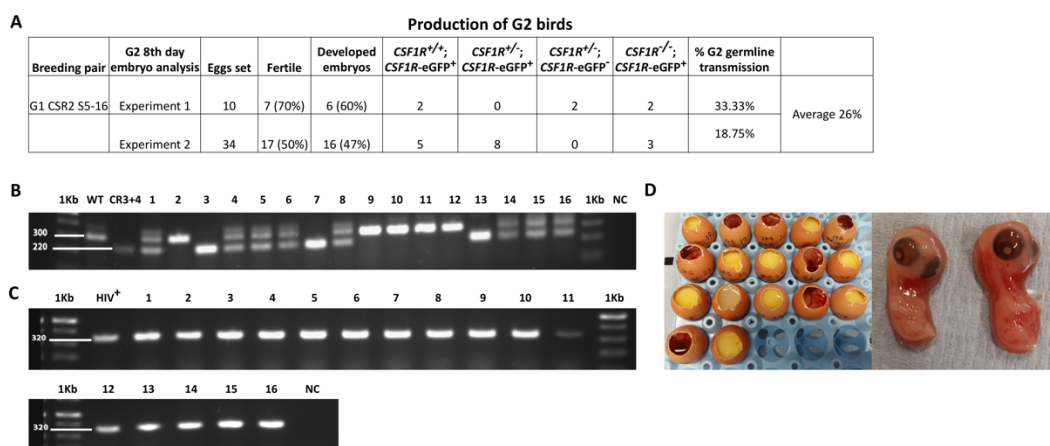
#### 5.2.4.3 Generation and screening of G2 $CSF1R$ homozygous mutant embryos

To find out whether homozygous  $CSF1R$  deleted mutated chickens can be generated, sexually matured male (CSR2-S5) and female (CSR2-S16) carrying  $CSF1R$  heterozygous knockout allele were bred to obtain G2 progeny. For initial survival and developmental analysis, G2 embryos were examined *in ovo*. G2 eggs obtained from the above breeding pair were set for incubation for analysis on day 8 of embryonic development. From the two independent experiments performed, 7 fertile embryos were obtained from 10 eggs set in experiment 1, 6 of which were fully developed embryos. From the second experiment, 17 out of 34 fertile embryos were obtained, 16 of which were fully developed (figure: 5-9A). Screening of chimeric G2 embryos for  $CSF1R$  targeted deletion and presence of  $CSF1R$ -eGFP transgene (Balic, Garcia-Morales et al. 2014) by HIV PCR of the CAM samples genomic DNA delivered four genotypes  $CSF1R^{+/+}/CSF1R$ -eGFP<sup>+</sup>,  $CSF1R^{-/-}/CSF1R$ -eGFP<sup>+</sup>,  $CSF1R^{+/-}/CSF1R$ -eGFP<sup>+</sup> and  $CSF1R^{+/-}/CSF1R$ -eGFP<sup>-</sup>. HIV PCR enable to identify  $CSF1R$ -eGFP transgene expressing

chickens, by amplifying lentiviral transduced GFP protein carried by *CSF1R*-eGFP chickens (Balic et al., 2014). Summary of the numbers obtained from each genotype from the two experiments is shown in figure: 5-9A.

The *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> genotype germline transmission frequency was 26% in G2 embryos. Figure: 5-9B shows PCR genotyping gel depicting amplification of *CSF1R* target site from all the 16 G2 embryos from experiment 2. *CSF1R* wild type (embryos 2, 9, 10, 11 and 12), heterozygotes (embryos 1, 4, 5, 6, 8, 14, 15 and 16), and homozygotes (embryos 3, 7 and 13) from G2 progeny are clearly differentiated. All G2 chimeric embryos from experiment 2 egg sets were positive for *CSF1R*-eGFP transgene as identified by HIV PCR (figure: 5-9C).

Thus, there was successful generation of TM domain deleted *CSF1R* knockout G2 embryos.



**Figure 5-9: Production and screening of chimeric *CSF1R* homozygous G2 embryos**

Sexually mature male and female G1 birds heterozygous for *CSF1R* were bred to obtain *CSF1R* homozygous G2 embryos. (A) shows the analysis of G2 embryos screened on 8<sup>th</sup> day of egg incubation from two independent experiments. Fertility and genotype frequencies of G2 embryos are indicated. The four genotypes obtained from screening of CAM samples are shown, including the donor PGCs transmission frequency in G2 birds. (B) shows CAM samples screening of G2 embryos for genotypes (wild types, homozygous and heterozygous alleles of *CSF1R*) as indicated by PCR amplification of donor PGCs target *CSF1R* region. (C) shows screening for *CSF1R*-eGFP transgene positive birds from CAM samples using HIV primers by PCR. Numbers above are embryo numbers. (D) shows a representative picture of incubated eggs for screening 8-day old G2 embryos.

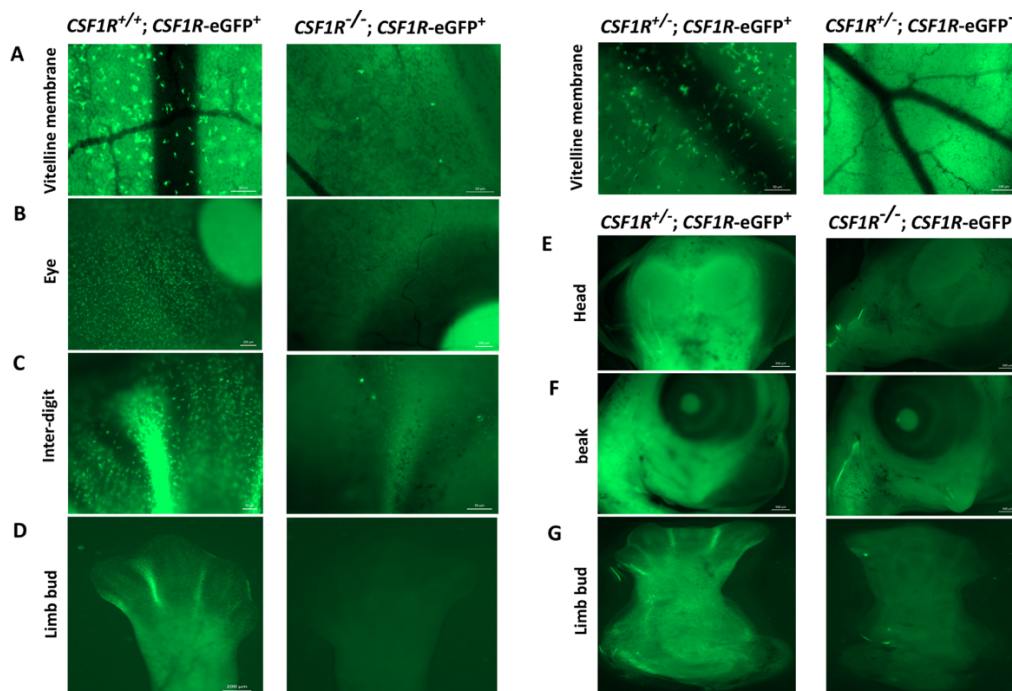
### 5.2.4.4 *CSF1R* homozygous mutant embryos are devoid of *CSF1R*-eGFP transgene expressed in macrophages

In *CSF1R*-eGFP reporter chickens the *CSF1R* regulatory sequences direct the expression of eGFP protein in the cytoplasm of macrophages (Balic et al., 2014). Embryonic macrophages in developing embryos play an active role in phagocytosing areas of programmed cell death like apoptosis of cells during remodelling of interdigital regions of mouse footplate (Hopkinson-Woolley et al., 1994). In chickens, examination of embryonic macrophages with *CSF1R*-eGFP reporter lines revealed that these macrophages are phagocytic and able to proliferate locally in tissues. They are concentrated in areas of programmed cell death, are able to recognise and phagocytose pathogens but are not recruited to wound site (Balic et al., 2014). In contrast, mouse embryonic macrophages are recruited to excisional wounds and play a role in wound healing when embryos are post E14.5 of development, but are not recruited younger than this stage (Hopkinson-Woolley et al., 1994). The earliest yolk sac derived macrophages appearing in chicken embryo at stage HH13 are confined to the lumen of primitive blood vessels. *CSF1R*-transgene expressing macrophages are widely distributed in developing embryos in ramified *CSF1R*<sup>+</sup> cell population appearing in speckled pattern (Garceau et al., 2010; Balic et al., 2014).

In order to analyze the phenotype of screened G2 chimeric embryos above, embryos were dissected by microsurgical procedures and examined by whole mount imaging. Analysis of 8-day old *CSF1R* homozygous mutant embryos revealed deficiency in the expression of the GFP protein encoded by *CSF1R*-eGFP transgene in embryonic mononuclear phagocyte population in the majority of the tissues (figure: 5-10). Representative images comparison of *CSF1R*-eGFP transgene expressing cells in vitelline membrane between the four genotypes *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup>, *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup>, *CSF1R*<sup>+/-</sup>/*CSF1R*-eGFP<sup>+</sup> and *CSF1R*<sup>+/-</sup>/*CSF1R*-eGFP<sup>-</sup> are shown in figure: 5-10A. In chickens, vitelline macrophages are highly mobile and found within and around blood vessels. They can crawl within the vitelline blood vessels as single cells or in clusters (Balic, Garcia-Morales et al. 2014). As opposed to *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup> (wild type) and *CSF1R*<sup>+/-</sup>/*CSF1R*-eGFP<sup>+</sup> (heterozygous) embryos, G2 *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> (homozygous) embryos were completely deficient in perivascular vitelline GFP<sup>+</sup> macrophages figure: 5-10A. *CSF1R*-eGFP transgene expression is concentrated in the interdigit region of stage HH33 embryo leg buds, an area of programmed cell death (Balic, Garcia-Morales et al. 2014). Consistent with this finding *CSF1R*-eGFP transgene was concentrated in the interdigit region of the limb bud of stage HH34 (8 day old) (Viktor and L.

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1992) *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup> embryo, but there was complete absence of transgene expression in *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> embryo's interdigit and limb bud (figure: 5-10C, D). *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> embryos lacked transgene expression in the eye primordium (figure: 5-10B). Representative images comparing G2 *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup> and *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> embryos head area, beak and limb bud is shown in (figure: 5-10E, F, G). In all the three tissues, *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> embryos lacked *CSF1R*-eGFP transgene expression.



**Figure 5-10: Analysis of germline transmitted chimeric homozygous *CSF1R* knockout G2 embryos**

8 day of incubation G2 embryos were genotyped for *CSF1R* target region and *CSF1R*-eGFP transgene and different body parts analysed for embryo development via whole mount imaging for the expression of *CSF1R*-eGFP transgene. (A) shows comparison of mononuclear phagocytes in vitelline membrane (scale bar 50  $\mu\text{m}$ ) between the four G2 genotypes, *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup>, *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup>, *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>-</sup>, *CSF1R*<sup>+/-</sup>/*CSF1R*-eGFP<sup>-</sup>. Comparison of mononuclear phagocytes between *CSF1R*<sup>+/+</sup>/*CSF1R*-eGFP<sup>+</sup> and *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> genotypes are shown for the eye region (B) scale bar 100  $\mu\text{m}$ , inter-digit area (C) scale bar 50  $\mu\text{m}$  and forelimbs (D) scale bar 200  $\mu\text{m}$ . Comparison of mononuclear phagocytes expression in the genotypes *CSF1R*<sup>+/-</sup>/*CSF1R*-eGFP<sup>+</sup> and *CSF1R*<sup>-/-</sup>/*CSF1R*-eGFP<sup>+</sup> is shown for the head region (E) scale bar 500  $\mu\text{m}$ , beak (F) scale bar 500  $\mu\text{m}$  and limb bud (G) scale bar 500  $\mu\text{m}$ .

Overall, the development of homozygous *CSF1R* knockout G2 embryos appeared to be relatively normal. CRISPR/Cas9 induced loss of function in chicken *CSF1R* gene allele by NHEJ was successfully transmitted to G2 embryos depicting MPS cell deficient phenotype. Stable knockout of expression of the *CSF1R* transgene was observed in multiple tissue types in G2 embryos. Analysis of the phenotype of homozygous *CSF1R* mutant birds and loss of endogenous protein is ongoing.

### 5.3 Conclusion and discussion

This Chapter aimed to generate CSF1R knockout chickens that will enable studying the specific role of macrophages in early embryonic chicken development. At present this is the first study demonstrating the generation of mononuclear phagocyte deficient birds produced using CRISPR/Cas9 genome editing tools. The myeloid lineage cell surface marker CSF1R was genetically modified *in vitro* in female chicken PGCs which upon transplantation in recipient embryos produced *CSF1R* heterozygous G1 and homozygous knockout G2 chickens. *CSF1R* reporter transgenic chickens provided the earliest evidence in the production of macrophages in chicken embryo. CSF1R expression first appeared in chicken embryonic yolk sac at stage HH13 restrained to the lumen of primitive blood vessels and expression was missing from the knockout embryos (Balic et al., 2014).

Unlike mammals, generating genetically modified birds has been difficult due to their unique reproduction system. Complex structure of the chicken zygote and the difficulty in gaining access to it at one cell stage in oviduct of hen make it challenging to inject constructs or apply somatic cell nuclear transfer (SCNT) approach to produce transgenic chickens (Sid and Schusser 2018). In this study, chimeric *CSF1R* knockout chickens were generated using CRISPR/Cas9 machinery in PGCs, indicating the benefit of this approach in producing gene specific knockout in birds. Compared to homologous recombination and TALEN based methods, CRISPR Cas9 tools are easy to synthesize and efficient in genome modification (Ran et al., 2013; Lee et al., 2017), as also validated from results in this study. The only limitations of applying this approach in chickens involve a long waiting period of approximately six months for them to reach sexual maturity for breeding, and maintenance of knockout birds during this period and after their generation.

Transgenic chickens homozygous for immunoglobulin (Ig) heavy chain J segment and light chain VJCL segment have been generated by homologous recombination in PGCs, shedding light in the development of B cell population (Schusser et al., 2013; Schusser et al., 2016). Homozygous Ig heavy chain knockout chickens lack in peripheral B cell population and have no effect on other immune cell types. Upon immunization these chickens however lack antibody response. Development of B cell precursors into mature B cells is compromised, along with their migration pathway from bursa (Schusser et al., 2013). In contrast, in homozygous Ig light chain knockout chickens B cell development in bursa is not completely compromised and there is migration of B lymphocytes in the periphery (Schusser et al., 2016).



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Guide RNAs targeting four different regions of interest of the receptor CSF1R were designed and cloned in Cas9 encoding vector PX459. Guide RNAs were designed with the expectation to generate frame shift mutations resulting in truncation of majority of the CSF1R protein when targeting exon 1 and exon 10. The expected out of frame mutation by deleting the TM domain should also lead to a null phenotype. The TM domain plays an important role in the dimerization of cell surface RTKs, essential for signal transduction (Li and Hristova 2006). It was anticipated that if the deletion of TM domain results in in frame mutation, it can result in proteolytic cleavage and secrete the extracellular Ig domain into serum exhibiting a dominant inhibitory allele of CSF1R mutation. In mammals, the extracellular domain can be produced as a proteolytic cleavage product and is present in the circulation. However, the outcome of *CSF1R* mutation would not have been known, before functionally validating them *in vitro* in chicken PGCs. It was therefore decided to initially test the gRNAs targeting exon 1 and TM domain of CSF1R for INDEL mutations by transfection with CRISPR plasmid construct. The other two target sites for which gRNAs were designed included ATP binding site and catalytic domain. ATP binding site tyrosine residue is also a part of intracellular catalytic domain. As phosphorylation of several tyrosine residues in the catalytic domain, by donor ATP molecule results in functional change of the target protein (Lemmon and Schlessinger 2010), gRNAs for ATP binding site and catalytic domain were cloned in PX459 to be used in case the desired *CSF1R* mutation was not obtained using earlier gRNA target sites.

Transient selection of gRNAs encoding CRISPR plasmid transfected PGCs clonal population was based upon puromycin resistance. Puromycin resistant clonal selection of CRISPR/Cas9 mediated gene knockouts in DF-1 chicken fibroblast cells have been shown to significantly improve mutation frequency in genetically modified cells (Bai et al., 2016). A single CRISPR plasmid transfection with gRNA1 and 2 and transient antibiotic selection in PGCs *in vitro* resulted in inducing NHEJ mediated INDEL mutation of exon 1 with an efficiency of 36.2% and 34.2% respectively (figure: 5-3B). On the other hand, co-transfection with CRISPR plasmid encoding gRNA3 and 4, targeting exon 10 resulted in 77 bps deletion of TM domain in mixed population PGCs (figure: 5-5C) and clonal PGCs (figure: 5-5D) both, with 100% targeting efficiency. Previous studies have reported that to efficiently generate a defined genomic deletion, use of two gRNAs for each locus inducing double stranded breaks via NHEJ repair is favored, as in certain circumstances use of single gRNA might not work for unknown reasons (Ran et al., 2013; Canver et al., 2014). It has also been proposed that there exists an

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inverse relationship between the size of the deletion and mutation efficiency (Canver et al., 2014). Clonal PGC line with bi-allelic TM domain deletion of *CSF1R* by use of two gRNAs and high targeting efficiency obtained for small deletion (77 bp) in this study does support these findings. Sanger sequencing alignment analysis of individual PGCs clones with targeted exon 1 and exon 10 Cas9 cleavage revealed out of frame mutations for both the target sites. After bi-allelic deletion of *CSF1R* exon 10, the putative protein translates to 595AAs in size, while the wild type *CSF1R* is 967AAs in size. Thus, it can be implied that genome editing by CRISPR/Cas9 led to knockout of *CSF1R* protein, leading to truncation by pre-mature stop codon post germline transmission.

In order to transmit the *CSF1R* mutation, a single cell clonal population of female PGCs line with bi-allelic TM domain deletion of chicken *CSF1R* gene were transplanted into surrogate host, *DDX4* knockout sterile chicken embryos. As described earlier in this chapter, a female transgenic chicken line with a loss of function of the chicken vasa homologue, *DDX4* loci revealed its role in the regulation of PGC formation (Taylor et al., 2017). *DDX4* mutant G1 female chicks are hemizygous knockouts and have no yellow or white follicles in the ovaries and therefore do not lay eggs. In these females there is initial formation of PGCs but during development of ovary PGCs are lost at the meiosis stage which leads to sterility in adult females. As a result, the genome edited donor PGCs injected into these sterile female recipient embryos will carry the mutated germ cells contributing to germ cell lineage with higher efficiency and thus have potential to be used for the generation of genome modified chickens. It was therefore decided to use fertile eggs from *DDX4* heterozygote cockerels mated to wildtype females for injection of *in vitro* propagated single cell clone of PGCs with validated biallelic *CSF1R* gene deletion.

*CSF1R* modified PGCs when transplanted into stage 16HH germ cell deficient *DDX4* embryos, were able to migrate into developing gonads differentiating into functional gametes and transmitted the donor PGCs in G0 founder chickens. Apart from PGCs, CRISPR/Cas9 have also been successfully used to modify avian somatic cells (Veron et al., 2015; Abu-Bonsrah et al., 2016) and embryonic stem cells (ESCs) (Zuo et al., 2017) with high efficiency. In developing embryo, CRISPR/Cas9 combined with electroporation induced loss of function mutation of transcription factor *PAX7* in tissues (Veron et al., 2015). In chicken ESCs, CRISPR/Cas9 induced knockout of *CIEIS* gene inhibited the differentiation of ESCs into male germ cells (Zuo et al., 2017).

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All the male G0 progeny was culled as birds carrying *CSF1R* edited donor female PGCs will be functional only in female G0 founder birds (Macdonald et al., 2010). The one *DDX4* sterile G0 founder female obtained was successful in transmitting the *CSF1R* mutation to G1 offspring with 39% germline transmission efficiency. The fact that *CSF1R* monoallelic knockout male and female G1 chickens hatched and survived indicated that CRISPR/Cas9 mediated genome editing of PGCs did not have any impact upon chromosomal distribution at meiosis stage or gamete differentiation as well as on *in ovo* embryo development. Just like *Csf1r* heterozygous mice and rats, G1 *CSF1R* heterozygous chickens displayed no developmental abnormalities or phenotypes compared to wildtype hatch mates, and were viable and fertile (Dai et al., 2002; Li et al., 2006; Pridans et al., 2018). Consistently, there was no change in *CSF1R*-eGFP transgene expression in the lymphoid organs, spleen and caecal tonsil tissue analyzed (figure: 5-8B) between the two groups.

Finally, the loss of function *CSF1R* allele was successfully transmitted in G2 embryos at an average 26% germline transmission efficiency. The frequency obtained in the two batches of embryo analysis 33.33% and 18.75%, varied from the expected 25% *CSF1R* homozygous transmission in G2 embryos (figure: 5-9A). 8-day old *CSF1R* homozygous G2 embryos lacked *CSF1R*-eGFP transgene expressing mononuclear phagocytes in most tissue parts but developed normally (figure: 5-10). This result implies that primitive myeloid progenitor cells failed to differentiate into embryonic macrophages due to CSF1R deficiency. CSF1R is expressed in macrophages and osteoclasts in birds as it is in mammals (Garceau et al., 2015; Rojo et al., 2017). At present, analysis of the G2 *CSF1R* homozygous chickens is ongoing. It can be anticipated that the phenotype will be similar to the knockout in outbred rats and mice, which are viable but infertile, and there is reduced somatic growth and osteopetrosis (Dai et al., 2002; Pridans et al., 2018).

Mice homozygous for *Csf1r* mutation was generated by homologous recombination using embryonic stem cells containing stop codon in *Csf1r* gene exon 3 followed by injection in blastocyst (Dai et al., 2002). Recently, *Csf1r* knockout rats were generated with detailed phenotypic analysis of CSF1R signaling and macrophage development and homeostasis. They were produced by homologous recombination using embryonic stem cells in which coding region exon 1 was disrupted and injected into blastocyst (Pridans et al., 2018). Although development of embryonic macrophages was not examined in this study, post-natal *Csf1r*<sup>-/-</sup> rats lacked CSF1R signaling dependent mononuclear phagocyte population including blood

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monocytes, Langerhans cells, brain microglia, osteoclasts, splenic marginal zone macrophages and peritoneal macrophages. The study highlighted redundant and non-redundant impacts of *Csf1r* mutation on macrophage population in rats and distinguished differences corresponding to the loss of macrophages in *Csf1r*<sup>-/-</sup> mice (Dai et al., 2002; Pridans et al., 2018).

Monoallelic mutation of *CSF1R* in humans is associated with autosomal dominant adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) neurological disease (Pridans et al., 2013; Konno et al., 2017). Recently, patients carrying bi-allelic *CSF1R* mutation has been reported, exhibiting brain malformations and skeletal phenotype, in addition to neurological pathologies (Guo et al., 2019). In another study, bi-allelic *CSF1R* mutation was detected in individuals in their childhood demonstrating several brain anomalies and affecting their growth. Post mortem analysis revealed complete absence of microglia within their brain. The study highlighted significance of CSF1R signaling in brain development (Oosterhof et al., 2019). Further examination of the knockout chicken should be informative.

In conclusion, in this study a methodology for generating knockout chickens using PGCs and genome engineering was demonstrated. Designed gRNAs targeting exon 1 and TM domain encoding exon 10 in combination with CRISPR/Cas9 plasmid were effective in inducing out of frame mutation *in vitro* in clonal population of PGCs. Receptor *CSF1R* mutation in donor PGCs was successfully transmitted to chimeric G1 and G2 generation birds, producing *CSF1R* heterozygous and homozygous knockout mutants in chickens respectively.

## Chapter 6 Conclusions and future perspectives

The primary aim of this thesis was to study the biology of CSF1/CSF1R signalling and its impact on mononuclear phagocytes and the overall development of chickens. The three approaches designed to vary the levels of CSF1R signalling enabled to gain further insight into the biology of chicken macrophages. Importantly, the novel tools generated and characterised in this project has shed light into various functional regulatory roles of chicken macrophages. These include viability and proliferation; CSF1 dependency of blood monocytes and tissue macrophages in postnatal birds; and the developmental phenotypes associated with CSF1R mutation. Identification and activity of chicken CSF1R kinase specific inhibitors on primary chicken macrophages along with the use of neutralising anti-ChCSF1 mAb demonstrated that as in mammals the biological actions of CSF1 is mediated through the receptor CSF1R, thereby supporting the earlier findings (Garceau et al., 2010). Chicken macrophages *in vitro* can survive without the addition of exogenous growth factor exhibiting autocrine CSF1/CSF1R signalling function behaviour. Several questions posed at the start of this project (Chapter 1, section 1.10) regarding the significance of CSF1/CSF1R signalling in the development of chickens can now be addressed from the findings of this study as discussed below.

### **Is CSF1 required for normal postnatal development in birds as it is in mammals?**

Chicken CSF1 was found to be required for the physiological, immunological and normal postnatal development in birds similar to its known requirement for the development and maintenance of tissue macrophages in mammals (Pixley and Stanley 2004; Wei et al., 2005; Alikhan et al., 2011; Pridans et al., 2018). Treatment with anti-ChCSF1 mAb did not impact the body weights of postnatal chickens, but the antibody compromised the development of subset of MPS population in the major lymphoid organs in chickens. These include substantial reduction in the *CSF1R*-eGFP transgene<sup>+</sup> and FAE cell numbers in the bursa of Fabricius; *CSF1R*-eGFP transgene<sup>+</sup> and B lymphocytes in caecal tonsil tissue; tissue resident MHC-II<sup>+</sup> and B lymphocytes in spleen; Kupffer cell macrophages of liver and osteoclast number in bone leading to decrease in the bone density. Administration of anti-ChCSF1 mAb to hatchling birds revealed that the CSF1-dependent chicken macrophages exhibit trophic functions that also impacts the development of B cells, thereby regulating the adaptive immune response at steady state in chickens. The lack of impact of anti-chCSF1 on somatic growth (where similar but

more prolonged treatment in mice was inhibitory (Wei et al., 2005)) and on blood monocytes and other tissue macrophages cannot be interpreted for three reasons. Firstly, it is not clear that the antibody can generate complete neutralisation of CSF1 at the concentration injected. Secondly, the treatment is not quite equivalent, as a hatchling bird is more developed than a new-born mouse. And finally, there is limited information about the relative importance and expression of IL34 in birds.

**Given the severe pleiotropic effects of CSF1R mutations in rats, mice and humans (Hume, Caruso et al. 2019) are CSF1R-dependent macrophages also critical for development in birds?**

This question is best addressed by the generation of CSF1R mutated transgenic chicken model to examine the developmental phenotypes associated with the CSF1R mutation *in ovo* as well as in adult birds. For this purpose, specific region of chicken *CSF1R* loci was targeted for deletion using genome editing tools CRISPR/Cas9 in PGCs culture, which was followed by transplantation of edited donor PGCs in reporter embryos for germline transmission (Chapter 5). The *CSF1R* heterozygous mutant embryos and adult birds were viable and fertile (detailed in Chapter 5), consistent with the absence of heterozygous phenotypes of null mutations in rodents and more recently described in humans. Examination of the 8-day old *CSF1R* homozygous embryos indicated complete absence of the CSF1R protein expression throughout embryo. The results implied absence of the embryonic mononuclear phagocyte population. Ongoing studies in the laboratory on the *CSF1R* homozygous post hatch birds revealed severe growth retardation and compromised viability (A. Balic, D. Hume, personal communication) similar to observations in mice and rats (Dai et al., 2002; Pridans et al., 2018).

There are a number of studies which could be further performed from this project to study avian macrophages role in immunity and development, especially after the generation of novel tools, chicken CSF1R specific kinase domain inhibitors, anti-ChCSF1 mAb and CSF1R-deficient transgenic chicken line. The immediate extension of the study using two identified chicken CSF1R kinase inhibitors (TIA02-054, TIA02-076) and anti-ChCSF1 mAb is to perform *in ovo* injections in embryonic vessels and determine morphology or macrophage function associated phenotypes. Both of these experiments were planned based upon the availability of these reagents and MacGreen embryos, to be injected on day 10 from egg incubation followed by imaging on day 14. After standardising the inhibitors *in ovo* dosage concentration to inject (10µg/gm for anti-ChCSF1 mAb and IC<sub>50</sub> values for CSF1R kinase

compounds), I underwent training to perform *in ovo* injections in embryonic blood vessel. Unfortunately, due to time constraints, these experiments could not be completed.

Another experimental analysis that could be performed using anti-ChCSF1 mAb would be to extend the subcutaneous injections days in *CSF1R*-eGFP post hatch birds for a more prolonged period (e.g. 8 weeks (Wei et al., 2005)). A more effective tool would be a blocking anti-chicken CSF1R antibody. Anti-chicken CSF1R antibodies have been generated by our group (Garcia-Morales et al., 2013) but they did not block CSF1 action on the factor-dependent BaF3 cell line. A deliberate screen for such antibodies could be undertaken. Such studies have also been performed in *Csf1r*-eGFP mice using rat anti-mouse CSF1R mAb (M279) for three weeks or six weeks duration (three times weekly), in postnatal or adult mice and the impact or the requirement of CSF1R signalling in various MPS cells population and the overall development have been analysed in detail (MacDonald et al., 2010; Sauter et al., 2014).

Some of the experiments using anti-ChCSF1 mAb injections experiment in post hatch birds (Chapter 4) clearly need to be repeated with greater numbers of birds and more focussed analysis. When the experiment was harvested at day 7 of age the small sized bursa tissue was further cut in half to be used for immune-histochemistry and the other half for FACS analysis. Although sectioning, staining and imaging the bursa was straightforward, the cell number obtained from the other half tissue disaggregation was insufficient for the FACS cell population analysis.

In mammals, CSF1 is implicated in various chronic inflammatory, cancer and autoimmune diseases. One obvious extension of the study would be to analyse the role of CSF1 and CSF1R in pathogenic diseases of birds including Marek's disease virus (MDV), infectious bursal disease virus, bronchitis virus or influenza virus. For this purpose, it would be possible to test the effects of anti-ChCSF1 mAb and the various CSF1R inhibitors. CSF1 could have protective or pathological roles. Initially at the start of this project, a fourth approach that was considered in the study of CSF1/CSF1R biology in chickens involved injection of birds with a novel and *in vivo* stable protein chicken CSF1-Fc, at different stages of development including *in ovo*, post hatch and at sexual maturation. The detailed phenotypic analysis of chicken macrophages at these stages of development was to be an extension of previous study in our laboratory by a student that characterised the chicken MPS (Garceau et al., 2015). The protein CSF1-Fc for this experiment needed to be purified in sufficient amount, which given the timeline of PhD studies did not seem feasible. Another alternative approach in this study involves application

of cell transplantation methods *in ovo* and in hatchling birds to determine the origin of different subsets of MPS tissues in adult birds via lineage tracing studies (Garceau et al., 2015). Using fluorescent ligand CSF1 and single or double CSF1R transgene reporter mice, expression of CSF1R *in vivo* in different MPS cell subsets was studied recently in a mice model (Hawley et al., 2018). The same approach to understanding CSF1R expression dynamics is now feasible in the chicken model, especially with the availability of chicken specific purified CSF1 protein, CSF1-Fc protein, anti-ChCSF1 mAb, anti-ChCSF1R mAb and the CSF1R reporter chickens.

The *CSF1R* homozygous chickens generated in this study provide another source for further detailed phenotypic analysis of mononuclear phagocytes and immune cells in different lymphoid organs. Studies demonstrating pleiotropic impacts of CSF1R mutation in rodents have been only very recently published (Pridans et al., 2018; Rojo et al., 2019), but no such detailed macrophage behavioural and functional analysis have been performed in the chicken model for vertebrate development. As noted above, the homozygous mutation affects growth and viability, but the reasons need to be studied. Both mouse (Bennett et al., 2018) and rat (Hume-D, personal communication) *Csf1r* mutations can be rescued by bone marrow transplantation. It will be interesting to see whether this can also be achieved in the chick. Furthermore, using genome editing tools targeted mutation in CSF1R receptor ligands, CSF1 and IL34 loci could be introduced in chicken PGCs and respective mutated transgenic chicken lines could be studied for understanding macrophage ontogeny and functions.

With the generation and availability of chicken specific novel reagents and chicken lines, it appears that studies revolving around chicken immune system and lymphoid tissues have only just started. Many of the commercial chicken vaccines are delivered *in ovo*. Therefore, developmental studies of the innate and adaptive immunity in chickens become important. Additional research is required to study the role of CSF1 in avian myelopoiesis and postnatal growth in this economically important species.



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## Chapter 8 Appendices

### 8.1 Appendix A: Reagents and antibodies list

#### General reagents

- Coding sequence of chicken CSF1 cloned in pET-28(b) vector (plasmid pTLW54) was obtained from Zoetis (formerly Pfizer Animal Health PAH) (Kalamazoo, MI, USA)
- Kanamycin sulphate, (Life Technologies, cat no. 11815024), stock 5gm, used 50µg/ml
- One Shot® BL21 Star™ (DE3) chemically competent *E. coli* (Thermo Fisher scientific, cat no. C6010-03)
- Restriction enzyme *EcoRI*-HF 20,000units/ml (New England biolabs, cat no. R3101S)
- Restriction enzyme *NlaIII* 10,000units/ml (New England biolabs, cat no. R0125S)
- CutSmart buffer 10x (New England biolabs, cat no. B7204S)
- Isopropyl β-D-thiogalactopyranoside or IPTG (Sigma Aldrich, cat no. 1284), stock 200mM, used 0.5mM
- 2x Laemmli sample buffer (BioRad cat no. 161-0737)
- RPMI-1640 medium (Sigma Aldrich, cat no. R5886EB, 500ml)
- Heat inactivated Fetal bovine serum (PAA, cat no. A15-104, 500ml) 10% working
- Penicillin 10,000units/ml, streptomycin 10,000µg/ml antibiotic (Gibco, cat no. 15140122, 100ml, 100x)
- Glutamax (Thermo Fisher Scientific, cat no. 35050087, 100x)
- Thiazolyl Blue Tetrazolium Bromide or MTT solution (Sigma Aldrich, cat no. M5655)
- IL3 source, 5% supernatant from X63 Ag8-653 myeloma cells containing an expression vector for IL-3
- LPS from Salmonella Minnesota strain Re 595 (Sigma Aldrich, cat no. L9764) 1mg/ml stock
- Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Sigma Aldrich, cat no. D5796, 500ml)
- Fetal bovine serum (FBS) heat inactivated (10%) (PAA Cell Culture Company, cat no. A15-104)
- Sodium pyruvate 100mM (Gibco®, cat no. C-11360039EB) 1mM working
- MEM NEAA 100x (Gibco®, cat no. C-11140035EB)
- Glutamax 100x (Gibco®, cat no. 35050-087)

## **Composition of avian KO DMEM FAOT medium used for culturing chicken PGCs**

Stock avian KO DMEM medium comprised of,

- B27 supplement 1x (Thermo Fisher Scientific, cat. no. 17504044)
- Glutamax 2.0 mM (Thermo Fisher Scientific, cat. no. 35050038)
- Non-essential amino acids (NEAA) 1x (Thermo Fisher Scientific, cat. no. 11140035)
- Embryo-max nucleosides 1x (Merck Millipore, cat. no. ES-008-D)
- Pyruvate 1.2 mM (Thermo Fisher Scientific, cat. no. 11360039)
- 2-mercaptoethanol 0.1 mM (Thermo Fisher Scientific, cat. no. 31350010)
- Calcium chloride 0.15 mM
- Ovalbumin 0.2% (Sigma Aldrich, cat. no. A5503-5G)
- Sodium heparin 0.01% (Sigma Aldrich, cat. no. H3149-25KU)
- Avian KO DMEM basal medium (255mOsm/kg, 12.0mM glucose, and calcium chloride free, Thermo Fisher Scientific, a custom modification of KO DMEM).

Stock medium was filtered using 0.25 $\mu$  filter and stored at 4°C. The growth factor supplements,

- Human FGF2 4ng/ml (R&D Systems, cat. no. 234-FSE-025)
- Human activins A and B 25ng/ml (PeproTech, cat. no. 120-14-10)
- Ovotransferrin 5 $\mu$ g/ml (Sigma Aldrich, cat. no. C7786-100MG)

Herein referred to as FAOT were added freshly to the stock avian KO DMEM medium while culturing PGCs.

## **Antibodies and stains**

Antibodies and dilutions used for FACS or Immuno-fluorescence were as follows:

- Mouse anti-chicken Bu-1-RPE (clone AV20, Southern biotech, cat no. 8395-09), 1:1000 for FACS
- Mouse anti-chicken BU1 unlabelled (clone AV20, Southern Biotech, cat no. 8395-01), 1:300 for immunohisto-fluorescence
- Mouse anti-chicken CD3-RPE (clone CT3, Southern biotech, cat no. 8200-09), 1:125 for FACS
- Mouse anti-chicken Monocyte/Macrophage-RPE (clone KUL01, Southern biotech, cat no. 8420-09 for FACS

- Mouse anti-chicken CD41/CD61-RPE (clone 11C3, Bio-Rad, cat no. MCA2240PE) 1:10 for FACS
- Mouse anti-chicken MHC II-RPE (clone 2G11, Southern biotech, cat no. 8350-09) 1:1000 for FACS
- Mouse anti-chicken CD45-RPE (clone LT40, Southern biotech, cat no. 8270-09) 1:125 for FACS
- Mouse anti-chicken TIM4 alexa fluor 647 (clone JH9, (Hu, Wu et al. 2016, Hu, Wu et al. 2019)) 1:500 for FACS, unlabelled 1:1000 for immunohisto-fluorescence
- Anti-ChCSF1 alexa fluor 647 mAb (batch produced mAb labelled with fluorochrome by colleague Dr. Anna Raper) 1:500 for immunohisto-fluorescence
- Isotype control IgG1 alexa fluor 647 mAb (batch produced mAb labelled with fluorochrome by Dr. Anna Raper) 1:500 for immunohisto-fluorescence
- Mouse anti-cattle IgG1 alexa fluor 647 Ab (made in lab) 1:500 for FACS
- Mouse Isotype IgG1 RPE (made in lab) 1:500 for FACS
- Donkey anti-mouse IgG (H+L) (Invitrogen, cat no. A31571) 1:300 for immunohisto-fluorescence
- Rabbit anti-GFP alexa fluor 488 (LifeTech, cat no. A21311) 1:300 for immunohisto-fluorescence
- Chicken CSF1-Fc alexa fluor 647 (Recombinant CSF1-Fc protein labelled with fluorochrome by colleague Dr. Anna Rapper in our lab) 1:200 for FACS
- Nucleic acid stain 4',6-Diamidino-2-phenylindole, dilactate or DAPI (Sigma Aldrich, cat no. D9564) 1:1000, 10mg stock
- Sytox blue dead cell stain (Invitrogen, cat no. S34857) 1.0 $\mu$ M working concentration



## 8.2 Appendix B: Imaging major lymphoid organs

### Chicken macrophages and immune cells

	Spleen	Bursa	Caecal tonsil	Liver
BU-1				
TIM4				
MHC-II				
CD11				

### Distribution of immune cells in chicken bursa of Fabricius

BU-1	MHC-II	TIM4

## 8.3 Appendix C: Chicken CSF1R protein sequences

### Wild type genomic DNA sequence of CSF1R

```
CCCCCAAACCAACTCTTCAGTCCCATTCTCACCACCTGCATGTGCACATCGGTCTTGCTG
505 P F N Q L F S P I L T T C M C T S V L L
CTCTTGCTGCTCCTCTTCTCCTCTACAAGTACAACCAGGTCAGCCTCCCTGTTGCACAG
L L L L L F L L Y K Y N Q V S L P V A Q
CAAGGGTTGCAAGGCCTGCCAGCTTCAGAGGCCACACTGCCTCAGTGGCAGCCACAGGC
Q G L Q G L P S F R G H T A S V A A T G
AAAGCCCATGCACCGAAACCAAAATTAATAACCCCTCCTTCACACGCCACACTGCCTC
K A H A P K P K L N N P L L H T P H C L
AGTGTCAACCACAGGCAAAGTCCATGCACCACAACCAAAATAAAATAAAGACC-----
S V N H R Q S P C T T T K I N K D 967 AAs (WT)
```

### Mutated CSF1R putative sequence (post TM domain deletion)

```
CCCCAAAC--77bps deleted--CAAGTACAACCAGGTCAGCCTCCCTGTTGCACA
505 P F N Q V Q P G Q P P C C T
GCAAGGGTTGCAAGGCCTGCCAGCTTCAGAGGCCACACTGCCTCAGTGGCAGCCACAGG
A R V A R P A Q L Q R P H C L S G S H R
CAAAGCCCATGCACCAAAACCAAAATAACCCCGGCCTGCCCTCCTTCGCCGGCCTGCCTG
Q S P C T K T K I T P A C P P S P A C L
CCTCGGTGGACCCACAAAGCAAAGCCATACAACAAAAA*
P R W T H K A K P I Q T K * 595 AAs (del)
```

Where,

Amino acids highlighted in blue: Similar in both the sequence types

Amino acids highlighted in black: deleted post CRISPR/Cas9 genome editing

Amino acids highlighted in green: Stop codon

Note the dissimilarities in the amino acids post deletion mutation of CSF1R.